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REGULAR ARTICLE

Soil diffusive fluxes constitute the bottleneck to tree nitrogen nutrition in a Scots pine forest

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Abstract
Background and aims In nutrient poor environments, plant nitrogen (N) acquisition is governed by soil diffusive fluxes and root uptake capacities. However, the relationship between these two processes is not well understood. We explored a way of comparing the processes, enabling identification of the limiting factor for tree N acquisition.
Methods The study comprised N-fertilized and N-limited Scots pine stands, and measurements of uptake capacities of detached tree roots and of induced soil diffusive fluxes (through in-situ microdialysis) done at the onset and the end of the growing season.
Results Soil N fluxes were higher at the onset than at the end of the growing season and amino acids comprised a larger fraction of N than inorganic N. N fertilization reduced root uptake capacities of NH_4^+, glycine and NO_3^- but not of arginine. For all N compounds except NO_3^-, diffusive fluxes were significantly lower than root N uptake capacities.
Conclusions Our results suggest that soil N supply in both, N-fertilized and N-limited forest stands, is dominated by amino acids, thus being the major component of plant-available N. Uptake of N appears more constrained by the diffusive fluxes of N compounds rather than root uptake capacity, except for NO_3^-.

Keywords Amino acids · Microdialysis · Nutrient uptake · Plant nutrition · Stable isotopes

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Introduction

The supply of soil nitrogen (N) for plant uptake involves complex biological and chemical processes in soil and especially at the plant-soil interphase. These processes result in the movement of soil N to the roots and their subsequent uptake by roots and mycorrhizal hyphae (hereafter referred to as roots) (cf. Chapin et al. 2011). The movement of soil N towards plant roots involves two main processes: diffusion and mass flow (Lambers et al. 2008a; Jungk and Claassen 1997; Tinker and Nye 2000; Nye and Marriott 1969). Diffusion results from the creation of concentration gradients between root surfaces and the surrounding soil driven by the uptake of N compounds, while mass flow is propelled by plant transpiration, resulting in mass transport of water and

nutrients from the bulk soil to the roots. Traditionally, the share of N delivered via diffusion has been estimated as the difference between total plant N uptake over a period of time and N delivered by mass flow (Jungk and Claassen 1997; Lambers et al. 2008a). This approach is indirect and suffers from uncertainties in the assessment of both accumulated N uptake and mass flow. To overcome this obstacle, a novel tool based on microdialysis has been developed allowing direct estimation of diffusion and mass flow of soil N (Inselsbacher et al. 2011, 2014; Inselsbacher and Näsholm 2012; Oyewole et al. 2014). In this study, we focused on diffusion because it is believed to be the dominant process delivering soil N to plant roots in nutrient poor ecosystems (Comerford 2005; Smethurst 2000; Barber 1995; Nye 1979) such as many boreal forests. Diffusion is particularly important for N forms that are relatively immobile such as NH_4^+ and amino acids (Smethurst 2000), which are often the main constituents of plant-available N pools in boreal forests (Inselsbacher and Näsholm 2012). Microdialysis induces diffusive fluxes of soil N over a semi-permeable membrane driven by the concentration gradient between the perfusate solution (distilled water) on the inside of the membrane and the soil solution on the outside (Kehr 1993; Torto et al. 2001; Seethapathy et al. 2008; Inselsbacher et al. 2014). At high perfusate flow rates, this concentration gradient is kept high and close to maximum diffusive fluxes of external N compounds can be obtained (Inselsbacher et al. 2011). Since a similar pattern can be found at root surfaces during active N uptake, we propose that monitoring soil N fluxes using microdialysis is a reliable measure of N supply for plant uptake. One other shortcoming of previous study efforts was the inability to directly compare soil N supply rates and root N uptake capacities directly in the field (cf. Näsholm et al. 2009).

The soil solution typically contains a large variety of N forms, including inorganic N and amino acids (Näsholm et al. 2009), and a large range of other organic N compounds of varying molecular size (Paungfoo-Lonhienne et al. 2008, 2012; Warren 2013) suggesting that plants have access to a diverse pool of N for their nutrition. Plants are able to take up and use this variety of N forms for their growth, and the responsible uptake mechanisms have been studied extensively in the past. Root N uptake is mediated by high- and low-affinity transporters (cf. Näsholm et al. 2009; Nacry et al. 2013), with different transporters involved in uptake of NH_4^+, NO_3^-, neutral/acidic amino acids, and basic amino acids, respectively (Frommer et al. 1993; Hirner et al. 2006; Svennerstam et al. 2007, 2008; Lee et al. 2007; Lehmann et al. 2011). Due to this diversity of transporters, different plant species also have different capacities for uptake of organic and inorganic N compounds (Öhlund and Näsholm 2004; Metcalfe et al. 2011; Pfautsch et al. 2009; Jones and Darrah 1993; Kielland 1994; Thornton and Robinson 2005; Sauheitl et al. 2009). For instance, uptake of NH_4^+-N was higher than NO_3^--N uptake in some conifer species (Kammingavan Wijk and Prins 1993; Kronzucker et al. 1997; Stoelken et al. 2010), while the uptake of amino acids by Scots pine (*Pinus sylvestris*) was found to be similar to NH_4^+-N uptake (Persson et al. 2006; Öhlund and Näsholm 2004). Further, the uptake of different N compounds may be affected by the internal N status of the plant, external soil N concentrations and the simultaneous presence of different N compounds either in soils or in experimental incubation solutions (Persson and Näsholm 2002). Recently, Gruffman et al. (2014) studied N uptake by Scots pine seedlings incubating roots with mixtures of different N compounds at low and high concentrations. Consistent with results from earlier studies, the seedlings showed similar uptake capacities for NH_4^+-N and arginine-N, which were about ten times higher than NO_3^--N uptake capacities. In addition, NO_3^- uptake decreased in the presence of NH_4^+ and the uptake of N compounds was generally down-regulated in response to high internal N status of seedlings. These findings indicate that variations in external N supply (e.g., through fertilization) may affect both soil N availabilities and root uptake capacities, but the relative effect on these processes is still unknown.

Here we hypothesized that long-term fertilization leads to increased availabilities of both inorganic and organic N, but reduced potential N uptake capacities of mature Scots pine trees. Further, we hypothesized that soil N fluxes and plant N uptake would be higher at the beginning than at the end of the growing season. To test these hypotheses we measured soil N fluxes using in-situ microdialysis in control and fertilized boreal forest soils at the onset and end of the growing season. Simultaneously, we estimated uptake rates of NH_4^+, NO_3^-, glycine (neutral amino acid) and arginine (basic amino acid) of roots of mature Scots pine trees excised from the same sites. This allowed us to calculate maximum N uptake per unit root surface area per hour, and therefore allowing direct comparison between induced soil fluxes (calculated per unit microdialysis-probe surface area and time) and maximum root uptake flux capacities for inorganic and organic N (per unit root surface

area and time). With this novel approach, we aimed to discern the relationship between soil N delivery via diffusion and the roots' ability to acquire N, and to determine which of these is more limiting to plant N nutrition in boreal forests.

Materials and methods

Site description

Root sample collection and microdialysis experiments were performed in a 75-year-old naturally regenerated Scots pine heath forest site at the Rosinedal Research area near Umeå, Sweden (64°10′20″ N, 19°44′30″ E). Annual average precipitation is 590 mm and annual mean air temperature 1.9 °C. The forest soil is nutrient poor and classified as a sandy glacial till Haplic podzol (FAO 2006) with 20 g kg^{-1} silt, 970 g kg^{-1} sand and 10 g kg^{-1} gravel. The organic layer is 5–10 cm deep, has a pH (H_2O) of 5.2 and a C/N ratio of 39. Total wet and dry N deposition in the study area is approximately 2 kg N ha^{-1} yr^{-1} (Gundale et al. 2011). The tree layer is dominated by Scots pine and the understory vegetation by ericaceous shrubs (*Vaccinium myrtillus*, *Vaccinium vitis-idaea*), mosses (*Pleurozium schreberi*, *Hylocomium splendens*) and lichens (*Cladonia spp.*) (Hasselquist et al. 2012).

The research area was established in 2006 as an eddy-covariance measurement area for quantifying the effect of N fertilization on net ecosystem carbon exchange. Because the study was conducted in the footprints of eddy-covariance flux measurement towers, control and fertilized subplots were nested within single control or fertilization plots. The treatment plots (15 ha) received either no fertilizer input (Control plot) or were fertilized with NH_4NO_3 (100 kg $ha^{-1}yr^{-1}$ from 2006 through 2011, and 50 kg $ha^{-1}yr^{-1}$ in 2012) (Fertilized plot). Fertilization was administered in June each year reaching a total of 650 kg ha^{-1} by 2012 at the time of this investigation, 2 weeks before the first experiments were performed. Within each of the two 15 ha treatment plots, 6 trees were selected randomly for the collection of roots for uptake capacity measurements and for estimation of soil diffusion flux measurements.

Microdialysis system and set up

The microdialysis system was set up according to Inselsbacher et al. (2011). Briefly, it consisted of two syringe infusion pumps (CMA 400), equipped with eight gas-tight glass syringes (5 ml, Hamilton, Bonaduz, Switzerland) which provided the perfusate solution. Each syringe was connected to a microdialysis probe (CMA 20) with a polyarylethersulphone membrane (10 mm long; 500 μm outer and 400 μm inner diameter; molecular weight cut-off of 20 kDa). The probes were perfused with high-purity distilled (MilliQ) water and effluxes from the probes (dialysates) were collected with two refrigerated microfraction collectors (CMA 470) in 300 μl glass vials. Microfraction collector temperature was kept at 6 °C throughout the experiments. All equipment is commercially available at CMA Microdialysis AB (Solna, Sweden).

Uniform performance of all probes was ensured throughout the experiments by calibrating each microdialysis probe before and after each sampling event according to the general calibration method (Bungay et al. 1990; Torto et al. 2001; Nandi and Lunte 2009), and as described for low molecular weight N compounds by Inselsbacher et al. (2011). Briefly, microdialysis probes were submerged in a standard solution containing NH_4^+, NO_3^- and 17 amino acids (AAS 18 standard solution, plus additional glutamine and asparagine; Sigma Aldrich). The standard solution was kept at a constant temperature of 22 °C and 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). The probes were perfused with MilliQ water at a constant flow rate of 5.0 μl min^{-1} for 2.5 h. Dialysates were collected continuously at 30 min intervals and were immediately prepared for chemical analysis as described below. The performance of each membrane was checked through analysis of relative recoveries of the individual N compounds as given in Eq. (1):

$$\text{Relative Recovery}(\%) = C_{dial}/C_{std}*100 \quad (1)$$

where C_{dial} is the concentration of the measured N compound in the dialysate and C_{std} is the concentration of the compound in the standard solution.

The induced diffusive fluxes (diffusion due to the creation of concentration gradients between the perfusate (MilliQ water) on the inside and soil solution on the outside of the membranes) of N compounds during each sampling time were calculated based on (Inselsbacher and Näsholm 2012; Inselsbacher et al. 2014), Eq. (2):

$$\text{Diffusive flux}\left(\text{nmol m}^{-2}\text{s}^{-1}\right) = \left(C_{dial}*V_{pump}\right)/\left(A_{probe}*t\right) \quad (2)$$

where V_{pump} is the volume provided at each individual pump flow rate (1), A_{probe} is the membrane surface area ($15.9 * 10^{-6}$ m^2) and t is the sampling time (s).

Estimation of soil diffusive N fluxes using microdialysis

Microdialysis field samplings were conducted in early June and early September 2012 to estimate the induced diffusive fluxes of individual N compounds from the soil. Diffusive fluxes for early June have been recalculated from previously presented data (Inselsbacher et al. 2014). Eight microdialysis probes were inserted into the organic soil layer in each of the treatment plots (control and fertilized). Understory vegetation and the moss layer were carefully lifted, and a guiding channel was prepared by vertically inserting a steel needle (0.5 mm outer diameter) to a depth of 5 cm into the organic mor-layer. The probes were carefully inserted into the channel using a specially designed split tubing introducer (CMA Microdialysis AB, Solna, Sweden) to protect the membrane surface. At the time of sampling, soil water contents ranged between 47 and 57 % and were high enough to guarantee diffusion of N compounds through soil towards the microdialysis membranes. The soil surrounding the probe was then slightly compressed to ensure full contact of the membrane surface with the surrounding soil layer. The probes were perfused with MilliQ water at a flow rate of 5 μl min^{-1} and were left in the soil for a 100 min sampling period. Dialysates were collected continuously at 20 min intervals and stored at −20 °C until chemical analyses (within 2 weeks from sampling) as described below.

Collection of root samples

Fine roots from six randomly chosen Scots pine trees were collected in early June and early September 2012 in each treatment plot (control and fertilized). Small roots were manually collected by digging into the uppermost organic soil layer of control and fertilized plots using hand trowel. Fine roots (<1 mm) with ectomycorrhiza were carefully detached from the small roots. Root samples were wrapped in moist tissue papers, covered with aluminum foil and kept at *c.* 4 °C (to prevent desiccation of the roots and to keep the roots active until further processing). In the laboratory, root samples were washed with tap water to remove adhered soil particles. The roots were arranged in bundles of 6 roots (each 5–7 cm long). One root bundle from each of the sampled six trees was prepared for each root uptake treatment and stored in the dark at a temperature of 4 °C overnight prior to the uptake experiment. Six additional root samples from the sampled trees from each treatment plot were scanned and the total surface areas of roots were determined by analysis of the scanned images using the WinRhizo software (Regent Instruments Inc., Quebec, Canada).

Uptake experiment and stable isotope analysis

The root uptake experiment was performed according to Jämtgård et al. (2008) and Gruffman et al. (2014). Isotopically labeled N compounds were provided as U-[$^{13}C_6$], [$^{15}N_4$]-Arginine, [^{15}N]-Glycine, $K^{15}NO_3$ and $^{15}NH_4Cl$ (Cambridge Isotopes Laboratories, Andover, MA; all at labeling rate of 96–98 atom%). The incubation solutions contained four combinations of one labeled and three unlabeled N compounds (Table 1), which were either: ^{13}C,^{15}N-Arginine + (Glycine, NH_4^+ and NO_3^-) or ^{15}N-Glycine + (Arginine, NH_4^+, NO_3^-) or $^{15}NH_4^+$ + (Arginine, Glycine, NO_3^-) or $^{15}NO_3^-$ + (Arginine, Glycine, NH_4^+). Each of the incubation solution combinations was prepared in two concentrations: 50 or 500 μM of each compound. Root bundles were dried gently with tissue paper, washed in three aliquots of 0.5 mM $CaCl_2$ to remove charged compounds from the surface, and again dried with tissue paper. Each bundle was transferred into a 50 ml falcon tube containing 40 ml of either 50 or 500 μM incubation solutions. Six replicates of each incubation solution and concentration were performed. Incubation solutions were aerated using an aquarium pump through a 0.8 mm needle via a sterile

Table 1 The chemical compositions of isotopically labeled N compounds used in 50 and 500 μM incubation solutions for estimation of uptake of labeled N compounds by roots from control (0 kg N ha^{-1} yr^{-1}) and fertilized (100 kg N ha^{-1} yr^{-1}) boreal forest soils

Labeled compound	Composition
Arginine	^{13}C, ^{15}N-Arg, Gly, NH_4^+, NO_3^-
Glycine	Arg, ^{15}N-Gly, NH_4^+, NO_3^-
NH_4^+	Arg, Gly, $^{15}NH_4^+$, NO_3^-
NO_3^-	Arg, Gly, NH_4^+, $^{15}NO_3^-$

Incubation solutions contained four different combinations of three unlabeled and one labeled N compounds. *Abbreviations*: *Arginine* U-[$^{13}C_6$], [$^{15}N_4$]-Arginine, *Glycine* ^{15}N-Glycine, *NH_4^+* $^{15}NH_4Cl$, *NO_3^-* $K^{15}NO_3$

filter (0.22 μm) during the 2 h uptake experiment. At the end of the experiment, root bundles were dried gently with tissue paper, washed with 0.5 mM $CaCl_2$ to remove tracer adsorbed on the root surface, dried with tissue paper and oven-dried at 65 °C for 72 h. Dried roots were weighed and milled into fine powder in a bead mill. The ^{15}N and ^{13}C contents of the roots were analyzed with an isotope ratio mass spectrometer (DeltaV, Thermo Fisher Scientific, Bremen, Germany) interfaced to an element analyzer (Flash EA 2000).

Chemical analyses

Amino acids, NH_4^+ and NO_3^- in microdialysis samples and incubation solutions were analyzed according to Inselsbacher et al. (2011). Briefly, NH_4^+ and amino acids were analyzed by reversed-phase liquid chromatography using a Waters (Milford, USA) Ultra High Performance Liquid Chromatography (UPLC) system with a Waters Tunable UV (TUV) detector. Aliquots of sample (20 μl) were derivatized with a Waters AccQ-Tag™ Ultra Derivatization kit for amino acid analyses. Individual amino acids were separated on an AccQ-Tag™ Ultra column by elution with a mixture of 0.1 % formic acid (solution A) and 10 % acetonitrile (solution B) using the following gradient: 0–5.74 min isocratic 99.9 % solution A, declining to 90.9 % solution A from 5.74 to 7.74 min, to 78.8 % solution A at 8.24 min and then to 40.4 % solution A at 8.74 min, before re-equilibration with 99.9 % solution A from 8.74 to 9.54 min. The flow rate was 0.6 ml min^{-1} and the column temperature was 55 °C. Nitrate was analyzed by the Vanadium (III) chloride (VCl_3) and Griess method as described by Hood-Novotny et al. (2010) based on the technique described by Miranda et al. (2001).

Calculations and statistical analyses

Spots sampled by each microdialysis probe can be seen as independent replicates since the impact area of each probe is extremely small (< 5 cm) and in no way interferes with each other. For each treatment plot (control and fertilized), eight probes were used (n=8).

Atom % excess of ^{15}N and ^{13}C in the roots was calculated by subtracting the average natural abundances of non-labeled root samples (n=12, 6 from each treatment plot) of the heavier isotopes from the atom % from each labeled sample (n=6, from each treatment plot and each incubation solution concentration). The atom % excess was converted to μmol g^{-1} root dw h^{-1}, and based on average total root surface areas and incubation times also converted to nmol m^{-2} s^{-1}. Uptake of intact arginine was verified by comparing the relationship between excess ^{13}C and excess ^{15}N in root samples to that of labeled arginine (i.e., 1.5; Supplementary Figure 1).

Statistical analyses were conducted using one-way and two-way ANOVA followed by Tukey's honestly significant difference post-hoc test using SAS 9.3 for Windows (SAS Institute Inc, Cary, NC, USA). When necessary, data were either square-root or log10-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. Differences were considered statistically significant at $P<0.05$.

Results

Induced soil diffusive fluxes

Induced diffusive fluxes of N in both control and fertilized soils were dominated by amino acids, accounting for 47–80 % of total amino acids and inorganic N (herein referred to as AA+IN) in both seasons. The most abundant amino acids at the onset of the growing season were histidine, glycine, serine and alanine. At the end of the growing season, glycine, aspartate, glutamate and alanine were the most abundant amino acids (Supplementary Table 1). Fluxes of arginine were below detection limits in both seasons (Fig. 1and b; Supplementary Table 1). The contributions of glycine, NH_4^+ and NO_3^- to AA+IN in both soils were similar during both seasons, with the largest contribution from NH_4^+ followed by glycine and NO_3^- (15–41, 5–12 and 1.5–6 % respectively), except in the fertilized soil at the end of growing season where NO_3^- contributed most (27 %; Fig. 1a and b; Supplementary Table 1).

Fertilization resulted in higher fluxes of NH_4^+ compared to control soil at both sampling time points (Fig. 1a and b; Supplementary Table 2). Fertilization also increased fluxes of NO_3^-, but only at the end of the growing season (Fig. 1b; Supplementary Table 2). We observed higher induced diffusive fluxes of AA+IN in both soils at the onset than at the end of growing season (Fig. 1a and b). Fluxes of NH_4^+ in control and fertilized plots were about 5 times higher at the onset than at the

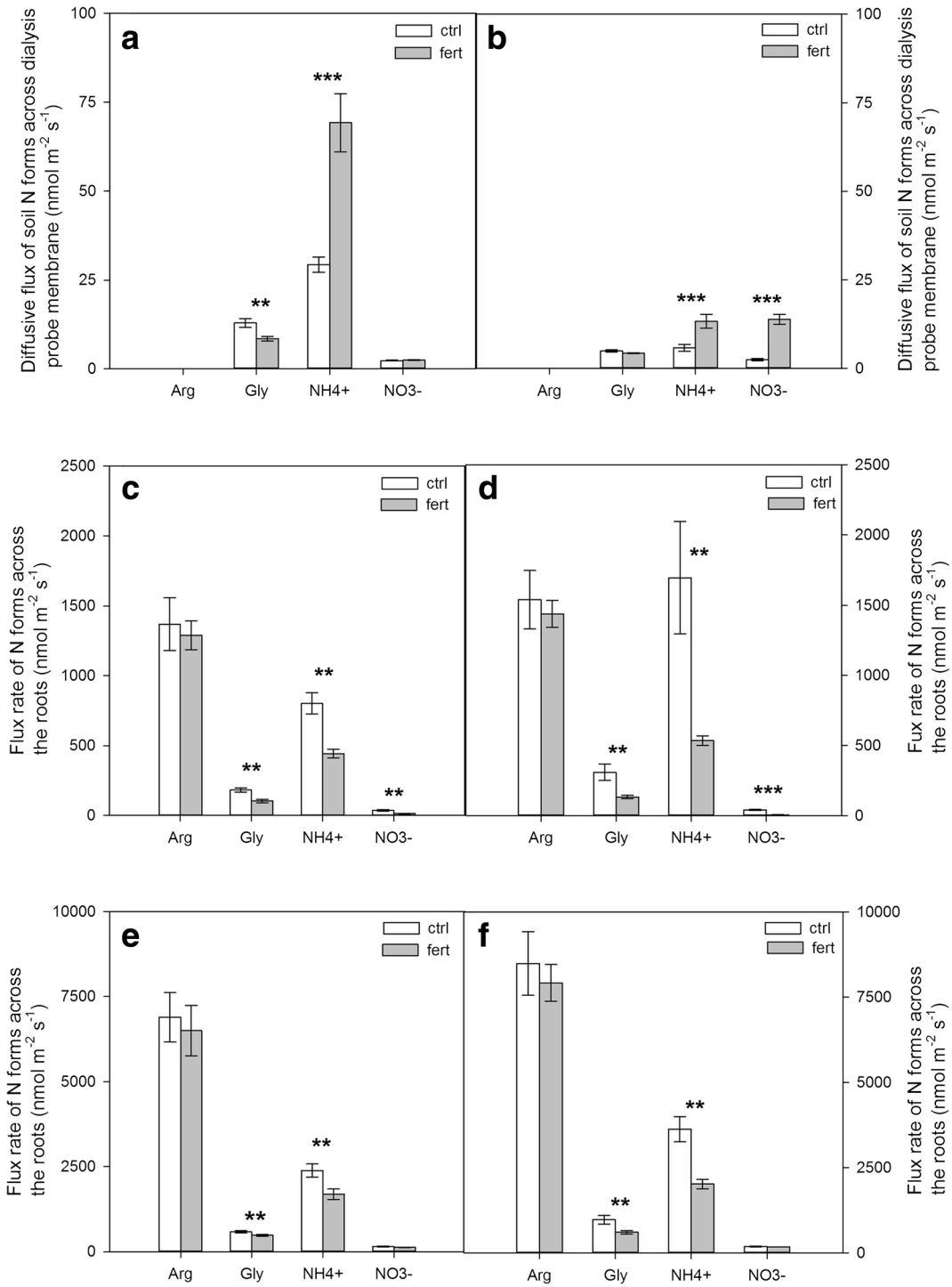

Fig. 1 Induced diffusive fluxes of soil arginine, glycine, NH_4^+ and NO_3^- in control (0 kg N ha^{-1} yr^{-1}) and fertilized (100 kg N ha^{-1} yr^{-1}) Scots pine forest soil at **a** the onset of growing season 2012, and **b** the end of growing season 2012. *Bars* represent mean±SE (nmol m^{-2} s^{-1}). n=8. Uptake capacity for dual labeled arginine, glycine, NH_4^+ and NO_3^- by roots from the above soils in **c** 50 μM incubation solution at the onset of growing season 2012. **d** 50 μM incubation solution at the end of growing season 2012. **e** 500 μM incubation solution at the onset of growing season 2012. **f** 500 μM incubation solution at the end of growing season 2012. *Bars* represent means±SE (nmol m^{-2} s^{-1}). n=6. *Asterisks* indicate significant differences between control and fertilized soils. ***P<0.001, **P<0.01, P<0.05

end of the growing season (Fig. 1a and b; Supplementary Table 1); and glycine fluxes in control and fertilized plots were also two to three times higher at the onset than at the end of the growing season (Fig. 1a and b; Supplementary Table 1). Diffusive flux of NO_3^- in fertilized soil was about 6 times higher at the end than at the onset of the growing season (Fig. 1b; Supplementary Table 2). Interactions were significant between fertilization and season on soil fluxes of glycine, NH_4^+ and NO_3^- (P=0.025 for glycine, P=0.002 for NH_4^+ and P=0.009 for NO_3^-; Supplementary Table 2).

Root uptake capacities

Root uptake rates (expressed per unit root surface area) were highest for arginine, followed by NH_4^+, glycine and NO_3^- in both seasons and both treatments (Fig. 1c and d). On a root dry weight basis (μmol g^{-1} root dw h^{-1}), arginine uptake by roots harvested from control and fertilized soils was more than 100 times higher than NO_3^- uptake, 4–16 times higher than NH_4^+ uptake, and 20–53 times higher than glycine uptake. Ammonium uptake rates were higher than glycine uptake rates which in turn were higher than NO_3^- uptake rates, which were generally very low (Table 2). These patterns were observed both at the onset and end of the growing season and for uptake assessments both using 50 and 500 μM incubation solutions. The isotope labeling indicated that whole molecules of arginine were taken up, rather than the molecules broken down followed by ammonium uptake. The relationship between excess of ^{13}C and excess of ^{15}N from U-[$^{13}C_6$], [$^{15}N_4$]-Arginine in roots matched the expected slope of 1.5, indicating >95 % of the N from arginine was taken up in the amino acid form (Supplementary Figure 1 and 2).

Scots pine roots from fertilized soil took up less glycine from both incubation solutions (50 and 500 μM) compared to roots from the control soil in both seasons (Fig. 1c–f; Supplementary Table 3). Likewise, fertilization reduced root uptake rates of NH_4^+ from both incubation solutions in both seasons (Fig. 1c–f; Supplementary Table 3). Uptake rates of NO_3^- by roots from control soil were significantly higher than those by roots from fertilized soil in both seasons from the 50 μM incubation solution, but not from the 500 μM incubation solution (Fig. 1c and d; Supplementary Table 3). We also observed that roots from both soils had higher uptake rates of glycine (p<0.05) and NO_3^- (p<0.001) at the end of the growing season compared to the onset of the growing season from the 500 μM, but not the 50 μM incubation solution (Fig. 1e and f; Supplementary Table 3). There was no interaction effect of fertilization and season on root uptake rates of arginine, glycine, NH_4^+ and NO_3^- (Supplementary Table 3).

Comparison of root uptake rates with in situ assessment of induced diffusive fluxes

We compared the measured induced diffusive fluxes with root uptake capacities measured at the concentration chosen to represent the high affinity transporter concentration range (50 μM). Root uptake rates of glycine and NH_4^+ from the 50 μM incubation solution were

Table 2 Estimated uptake of arginine, glycine, NH_4^+, and NO_3^- by fine roots from control (0 kg N ha^{-1} yr^{-1}) and fertilized (100 kg N ha^{-1} yr^{-1}) boreal forest soils from 50 to 500 μM mixed incubation solution at the onset and end of growing season in 2012

N compounds	Conc (μM)	June			September		
		Control	Fertilized	P	Control	Fertilized	P
Arginine	50	1.01±0.14	0.95±0.08	0.716	1.05±0.14	0.99±0.07	0.672
	500	5.10±0.54	4.80±0.55	0.712	5.80±0.64	5.42±0.37	0.612
Glycine	50	0.13±0.01	0.08±0.01	**0.002**	0.21±0.04	0.09±0.01	**0.013**
	500	0.43±0.02	0.36±0.02	**0.035**	0.67±0.09	0.42±0.04	**0.022**
NH_4^+	50	0.59±0.06	0.33±0.02	**0.001**	1.16±0.27	0.37±0.02	**0.016**
	500	1.76±0.14	1.25±0.11	**0.018**	2.48±0.25	1.38±0.09	**0.002**
NO_3^-	50	0.03±0.00	0.01±0.00	**0.001**	0.03±0.00	0.003±0.00	**< 0.001**
	500	0.11±0.01	0.09±0.00	0.062	0.13±0.01	0.12±0.00	0.202

Values represent mean±se (μmol g^{-1} root dw h^{-1}). n=6. Bold texts indicate significant differences between control and fertilized soils. P<0.05.

6–290 times higher than their corresponding induced diffusive fluxes in both soils both at the onset and the end of the growing season (Fig. 1; Table 3). Further, root uptake rates of arginine from the incubation solution were higher than the induced diffusive fluxes in control and fertilized soils at the onset and the end of the growing season (Fig. 1; Table 3). The diffusive fluxes of NO_3^- and its uptake rates from 50 μM incubation solution by roots from control and fertilized soils were very low in both seasons (Fig. 1). Root uptake rates of NO_3^- were *c.* 6–16 times higher than induced diffusive fluxes, except at the end of the growing season when fluxes in fertilized soil exceeded uptake rates by roots from fertilized soil by *c.* 3 times (Fig. 1b and d; Table 3).

Discussion

In this study, we set out to relate in situ diffusive fluxes of N in soils to potential root N uptake rates. Plant growth and biomass production strongly depend on the availability of N at the root surface, but reliable determination of which N forms are available for, and ultimately taken up by plants, remains a challenging task. Destructive soil sampling and subsequent handling procedures disrupt the soil matrix and the natural equilibrium of soil N introducing artefacts such that the quantity and quality of estimated N deviate from those in situ (Inselsbacher 2014). Further, soil N fluxes and thus continuous N supply rates are better indicators of N availability than soil solution N concentrations (Nye 1979; Clarkson and Hanson 1980; Shaver and Chapin 1991; Leadley et al. 1997; Tinker and Nye 2000; Comerford 2005; Lambers et al. 2008a). In nutrient poor ecosystems, such as boreal forests, diffusion is regarded as the dominant process supplying soil N to plant roots (Nye 1979; Barber 1995; Smethurst 2000; Comerford 2005). However, a major shortcoming of these studies was that results were based on indirect estimations of diffusive N fluxes in soils. Recently, a novel tool based on microdialysis has been introduced for direct estimation of diffusion in soils in situ and was used in this study for identifying the limiting process for tree N acquisition in boreal forests (Inselsbacher et al. 2011, 2014; Inselsbacher and Näsholm 2012). Plant N preferences can be determined with excised or intact roots (Weigelt et al. 2005; Falkengren-Grerup et al. 2000), even though experiments using excised roots may underestimate uptake of N that is supplied by mass flow (Falkengren-Grerup et al. 2000; Hoagland and Broyer 1936). In this study, root N uptake capacities were quantified under conditions of reduced microbial competition, so the results represent the maximum possible N acquisition rate of roots. By contrast, in situ microdialysis probes acquire N in competition with soil microbes, representing realistic rates of N supplied for root uptake. Microdialysis probes share several apparent similarities with plant roots (cf. Inselsbacher et al. 2014): First, this technique allows estimation of diffusion rates in virtually undisturbed soil, a significant advantage over other sampling techniques. Second, the form and dimensions of microdialysis membranes are similar to fine roots and act at scales relevant for plant roots. Third, microdialysis continuously creates a diffusional gradient between bulk soil and the membrane surface, similar to active roots. Clearly, many other features of active plant N uptake cannot be simulated by microdialysis (cf. Inselsbacher et al. 2014), but it allows reliable estimation of N arriving at root surfaces after competition with microbes.

Here we estimated diffusive fluxes of N through soil towards and across microdialysis membranes in situ (Inselsbacher et al. 2011) in spring and autumn in unfertilized and fertilized boreal forest sites. We found that soil diffusive N fluxes were higher at the onset than at the end of the growing season. This was expected since spring is period of frequent freeze-thaw cycles affecting soil N turnover (Ivarson and Sowden 1966; Edwards and Cresser 1992; Lipson and Monson 1998) and a similar pattern has been observed previously in boreal forest sites (Inselsbacher and Näsholm 2012; Inselsbacher et al. 2014).

Root uptake rates of all N compounds except NH_4^+, on the other hand, were not affected by season. Root uptake capacities of arginine did not exhibit a significant variation depending on time of the season and fertilization.

Earlier studies have shown that arginine is present in salt extracts of boreal forest soils, but it was below

Table 3 Ratios of roots uptake rates of organic and inorganic nitrogen compounds to soil fluxes of each N compound

N compounds	50 μM			
	June		September	
	Control	Fertilized	Control	Fertilized
Arginine	∞	∞	∞	∞
Glycine	14	12	63	31
NH_4^+	27	6	290	40
NO_3^-	16	6	16	0.3

n=6 for roots uptake rate and *n*=8 for soil fluxes

detection limit in either water extracts or dialysates (e.g., Inselsbacher and Näsholm 2012). The present study shows that although arginine was not detected in the dialysates, it's root uptake capacities were the highest among the four tested N compounds (except for NH_4^+ in control soils in autumn; Fig. 1). Obviously, the microdialysis technique, while simulating root acquisition of N compounds, failed to mimic the processes through which arginine is acquired by roots. Further developments of the technique, mainly through inclusion of cation exchange capacity of microdialysis membranes may enable better estimations of arginine fluxes in soils in the future.

In contrast to arginine, fertilization reduced glycine uptake capacities. Earlier studies on Scots pine seedlings have demonstrated that root uptake capacities for organic N compounds are similar to or higher than NH_4^+ uptake, while NO_3^- uptake is comparatively low (Öhlund and Näsholm 2004; Persson et al. 2006; Miller and Hawkins 2007; Metcalfe et al. 2011; Gruffman et al. 2014). We found a similar pattern for mature trees, where roots displayed highest uptake rates for arginine followed by NH_4^+, glycine and NO_3^-, although the uptake rates recorded in the current study were lower than those recorded by Gruffman et al. (2014) for NH_4^+ and NO_3^- (up to 10 times lower) whereas for arginine the uptake rates were in the same range. This difference could be due to the use of intact seedlings in Gruffman et al. (2014) and the additional potential uptake of N delivered by mass flow (Falkengren-Grerup et al. 2000; Hoagland and Broyer 1936). Further, in the present study we studied the simultaneous uptake of four N compounds, while Gruffman et al. (2014) aimed at investigating the effect of the presence of NO_3^- on either arginine or NH_4^+ uptake capacities. Still, root uptake rates of all compounds were much higher (6–290 times) than soil diffusive fluxes except for NO_3^- at the end of the growing season when soil diffusive fluxes of this compound were higher than root uptake in fertilized soil (Table 3). To our knowledge we have for the first time direct measurements showing that in boreal forests diffusion, not plant uptake rates is the limiting process for tree N acquisition. This confirms what has been suggested previously by indirect measurements of diffusion in nutrient limiting ecosystems like the boreal forest (Chapin 1980) and studies using plant nutrient uptake models (Raynaud and Leadley 2004). Depolymerization of high molecular weight organic N is generally considered the rate-limiting step of amino acid availability in soil (Schimel and Bennett 2004). Our study shows that not only depolymerization, but also the supply rate of N monomers for root uptake via diffusion can be rate limiting. Generally, diffusion is limiting the supply of immobile ions (Lambers et al. 2008b) but in the boreal forest studied here it also limits inorganic N uptake, with the exception of NO_3^- in the end of the growing season in the fertilized soil.

Fertilization increased diffusive fluxes of inorganic N in the soil but also decreased root uptake rates (Fig. 1). This was especially prominent for NH_4^+ at the onset of the growing season whereas diffusive fluxes of NO_3^- were higher than the control in the fertilized soil only at the end of the growing season. One reason for this pattern most likely is that NH_4^+ is relatively immobile and remains longer in the top soil after application. Nitrate, on the other hand, is mobile in soils and prone to losses through leaching to deeper soil layers and the groundwater. Accordingly, application of ^{15}N-labeled NO_3^- at the same site led to a short period (3 days) of increased $^{15}NO_3^-$ fluxes in soils but could not be detected in soils anymore after 2 weeks (Inselsbacher et al. 2014). Despite the application of NO_3^- the root uptake capacities of NO_3^- remained very low (Fig. 1). The lower uptake capacities of glycine, NH_4^+ and NO_3^- by roots from fertilized than from the control plots may be linked to the higher internal N status of the roots (Table 4), which might have led to down-regulation of high-affinity NH_4^+ and NO_3^- transporters (Rawat et al. 1999; Vidmar et al. 2000; Nazoa et al. 2003) and neutral/acidic amino acids transporters. In agreement with Gruffman et al. (2014) we observed no effect of internal N status of roots on root uptake rates of arginine.

For NO_3^-, root uptake capacities trailed soil diffusive fluxes at the end of the growing season. However, NO_3^- diffusive flux remained quite low, as amino acids dominated the diffusive fluxes, and high root uptake capacities exceeded the diffusive flux rates. This suggests that

Table 4 Total nitrogen content of plant fine roots from control (0 kg N ha^{-1} yr^{-1}) and fertilized (100 kg N ha^{-1} yr^{-1}) boreal forest soils after uptake experiments in 50 and 500 μM mixed incubation solutions at the onset and end of growing season in 2012

	Conc (μM)	Control	Fertilized	*P*
Summer	50	0.57±0.01	0.83±0.04	< 0.001
Autumn	50	0.60±0.02	1.06±0.04	< 0.001
Summer	500	0.62±0.02	0.85±0.04	< 0.001
Autumn	500	0.66±0.02	1.14±0.04	< 0.001

Mean±se (%). n=24

more amino acid N than inorganic N is available for uptake by Scots pine roots under both low N (control) and high N (fertilized) conditions. Similar to previous studies, amino acids accounted for the majority of measured diffusive fluxes in both stands (control and fertilized) and at both the onset and the end of the growing season (Inselsbacher et al. 2014).

Conclusions

The current study explores the combination of measurements of soil diffusive fluxes and root uptake capacities for N, aiming at identifying the limiting process for tree N acquisition in boreal forests with different fertility. Our data suggest that for both N limited and N fertilized stands, amino acids exhibit a higher share of the induced soil diffusive N fluxes than inorganic N. The data also suggest soil diffusive fluxes, and not root uptake capacities exert the limiting step to tree N nutrition. A potential exception to this conclusion was NO_3^-, for which the low root uptake capacity, in particular in fertilized stands may increase the risk of N losses from the soil by leaching.

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Compliance with Ethical Standards

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