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# Nitrogen dynamics in boreal forests

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# $N_2$ Fixation in Feather Mosses is a Sensitive Indicator of N Deposition in Boreal Forests

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## ABSTRACT

Nitrogen (N) fixation in the feather moss–cyanobacteria association represents a major N source in boreal forests which experience low levels of N deposition; however, little is known about the effects of anthropogenic N inputs on the rate of fixation of atmospheric $N_2$ in mosses and the succeeding effects on soil nutrient concentrations and microbial community composition. We collected soil samples and moss shoots of *Pleurozium schreberi* at six distances along busy and remote roads in northern Sweden to assess the influence of road-derived N inputs on $N_2$ fixation in moss, soil nutrient concentrations and microbial communities. Soil nutrients were similar between busy and remote roads; $N_2$ fixation was higher in mosses along the remote roads than along the busy roads and increased with increasing distance from busy roads up to rates of $N_2$ fixation similar to remote roads. Throughfall N was higher in sites adjacent to the busy roads but showed no distance effect. Soil microbial phospholipid fatty acid (PLFA) composition exhibited a weak pattern regarding road type. Concentrations of bacterial and total PLFAs decreased with increasing distance from busy roads, whereas fungal PLFAs showed no distance effect. Our results show that $N_2$ fixation in feather mosses is highly affected by N deposition, here derived from roads in northern Sweden. Moreover, as other measured factors showed only weak differences between the road types, atmospheric $N_2$ fixation in feather mosses represents a highly sensitive indicator for increased N loads to natural systems.

**Key words:** acid deposition; bryophytes; acetylene reduction; microbial biomarker; nitrogen fertilization; anthropogenic global change.

## INTRODUCTION

Boreal forests are considered to be nitrogen (N)-limited ecosystems (Tamm 1991) due to the accumulation of recalcitrant litter with high carbon (C)-to-N or high lignin-to-N ratios resulting in immobilization of inorganic N and therefore minimal net N mineralization (Keeney 1980; Scott and Binkley 1997). Low concentrations of available N in boreal forest soils are the result of a tight internal N cycle where immobilization processes dominate

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(Giesler and others 1998). Total N capital in boreal ecosystems is accumulated slowly over time with one of the primary sources of N originating from the reduction of atmospheric $N_2$ by cyanobacteria (biological N fixation) (Cleveland and others 1999; DeLuca and others 2002), which colonize bryophytes (for example, feather mosses) (Houle and others 2006; DeLuca and others 2002) and fix substantial amounts of atmospheric N in pristine, unpolluted environments (DeLuca and others 2002; Zackrisson and others 2004). Feather mosses can account for over 80% of the groundcover in boreal forests; they provide a stable and favorable environment for cyanobacteria, promoting $N_2$ fixation by maintaining relatively constant abiotic factors and a high water retention capacity (Dickson 2000), therefore representing a large source of N for nutrient-limited systems (DeLuca and others 2002).

$N_2$ fixation rates in cyanobacteria associated with feather mosses are positively correlated with numbers of cyanobacterial cells in the feather mosses (DeLuca and others 2007) and as a result, bacterial density on moss leaves can be used as an indicator for the reduction of atmospheric $N_2$. Four genera of cyanobacteria have been identified living epiphytically on the feather moss *Pleurozium schreberi* (Brid.) Mitt., one of the dominant mosses in boreal forests (Gentili and others 2005; Ininbergs and others 2011). In bryophyte symbioses, cyanobacteria receive shelter and protection and in return transfer N to its host (Steinberg and Meeks 1991; Zielke and others 2005). It has been estimated that only 20% of the N fixed by cyanobacteria is commonly retained, the remaining fraction is transferred as $NH_4^+$ to the plant host (Adams 2002; Meeks and Elhai 2002). Cyanobacterial $N_2$ fixation in feather mosses contributes approximately 2 kg N $ha^{-1}$ $y^{-1}$ to the N pool in mature forest ecosystems (DeLuca and others 2002), which may exceed atmospheric N deposition in the boreal biome (1–2 kg N $ha^{-1}$ $y^{-1}$) (for example, Gundale and others 2011). In addition, mosses alter soil C and phosphorus (P) pools by releasing dissolved organic C, N, and P after drying–rewetting cycles (Carleton and Read 1991). Therefore, mosses could represent a nutrient reservoir and N source for microbes and plants in N-limited ecosystems, leading to higher microbial biomass under moss carpets (Biasi and others 2005).

Mosses are of particular relevance in northern biomes (Longton 1997) due to their ubiquitous nature, often dense surface cover, high biomass production and broad contribution to habitat heterogeneity (Longton 1988). Mosses exert a strong control over the hydrology, temperature, moisture, and chemistry of boreal forest soils (Cornelissen and others 2007). However, mosses are highly sensitive to increased N deposition (Press and others 1986; Dirkse and Dobben 1989; Nordin and others 1998; Pitcairn and others 2003). Fertilization experiments in boreal forests have shown that enhanced N inputs result in decreased biomass of abundant and dominant moss species (Kellner and Mårshagen 1991; Mäkipää 1995; Solga and others 2005; Nordin and others 2006). Further, increased N loads lead to a reduction or total exclusion of $N_2$ fixation in cyanobacteria–moss associations (Zackrisson and others 2004; DeLuca and others 2008). In contrast, with decreasing N availability, $N_2$ fixation in mosses increases (Zackrisson and others 2004).

Roads represent a major source of disturbance and pollution in natural habitats (Gadsdon and Power 2009). Road-associated N pollutants ($NO_x$, $NH_3$) have the potential to alter ecosystem function (Boxman and others 1995; Green 2005), plant growth (Bignal and others 2008), C uptake and tissue concentration (Liu and others 2010), and N tissue concentrations (Caporn and others 2000; Sheppard and Leith 2002). Bryophytes in particular are known to be sensitive to $NO_2$ emissions (Bell and others 1992). Bignal and others (2008) specifically demonstrated that $NO_2$ emissions and N deposition along roadways increases shoot elongation and tissue N concentration in bryophytes, including *P. schreberi*. In contrast, Bell and others (1992) found an inhibitory effect of $NO_2$ on shoot growth in the moss *Polytrichum formosum*.

Road traffic is also accompanied with enhanced levels of dust, heavy metals, and salts (Spellerberg 1998). Various studies have found that road dust leads to reduced photosynthesis in plants due to blocked stomata, reduced diffusive resistance, and an increase in leaf temperature (for example, Flückiger and others 1977, 1978). Further, heavy metal (for example, Pb, Zn) concentration in tissue of various moss species has been shown to be positively correlated with traffic exposure (Pearson and others 2000).

We hypothesize that road traffic has an inhibitory effect on $N_2$ fixation in feather mosses due to elevated N inputs close to busy roads. To test this, we measured $N_2$ fixation in the feather moss *P. schreberi* at various distances from busy and remote roads in northern Fennoscandia where atmospheric N deposition is low. In addition, we measured soil nutrient and metal concentrations at varied distances from the roads to identify the source of inhibition of $N_2$ fixation in the moss.

Further, we analyzed soil microbial communities along the roads, which are governed by N loads (Demoling and others 2008) and therefore, can be used as indicators for N gradients. By combining data on $N_2$ fixation with data on soil nutrients and microbial community composition, we aimed to identify the effects of road-derived N deposition on nutrient cycling in N-limited systems, focussing on the influence on $N_2$ fixation in the feather moss *P. schreberi*. To our knowledge, this is the first study that combines responses to road-derived nutrient inputs in a pristine environment of soil nutrient cycling processes and of an essential ecosystem service, $N_2$ fixation in feather mosses.

## Materials and Methods

### Study Sites

Study sites were located in northern Sweden between latitude 64–66°N, longitude 18–19°E and between 230 and 540 m above sea level. Mean annual temperature and precipitation are 1°C and 570 mm, respectively. Our study included four busy, paved, road segments [Borup (65.0060°N, 19.2509°E); Nyvall (65.2308°N, 19.2632°E); Strömsforsheden (65.0813°N, 18.5179°E); Vilan (64.3068°N, 18.5266°E)], on busy highways in northern Sweden (see traffic information below), and four remote, unpaved, roads [Dötternoive (65.4375°N, 18.4440°E); Jegge (65.4762°N, 18.4319°E); Reivo (65.4595°N, 19.0847°E); Tjädness (65.4749°N, 18.4379°E)] (rarely used gravel roads with a large road prism). All study sites along busy and remote roads are forest reserves that receive no fertilization or timber harvest. The annual average daily traffic in numbers of motor vehicles for the busy roads is: 640 with ±30% uncertainty, 930 ± 24, 860 ± 17, and 880 ± 23% for Borup, Nyvall, Strömsforsheden, and Vilan, respectively (personal communication, Trafikverket, Sweden, 2010). The estimated daily average traffic along the remote roads is: fewer than five cars at Dötternoive and Reivo and one or less car per day at Jegge and Tjädness, with no automobile traffic during winter (roads are closed and impassable in winter). The vegetation at all sites was dominated by Scots pine (*Pinus sylvestris* L.), Norway spruce [*Picea abies* L. (Karst)], feather moss carpets composed of *P. schreberi* (Brid.) Mitt. and *Hylocomium splendens* (Hedw.), shrubs like *Vaccinium vitis-idea* (L.), *V. myrtillus* (L.), and *Empetrum hermaphroditum* (Hagerup). The soils along all the road sites were classified as Typic Haplocryods. All forest stands were of mid- to late-successional status.

### Sampling and Soil Nutrient Analyses

Soil samples were collected in June 2010 at six distances (0, 5, 10, 50, 100, 150 m) from each road with three subsamples per distance using a 2.5-cm diameter stainless steel soil core to a depth of about 10 cm, and separating out only the O-layer. Samples from 0 m distance were collected as close as possible to the roads where the clear forest vegetation emerged. Soil samples were directly returned to the laboratory and stored at 5°C until analyses. Moss shoots of *P. schreberi* were collected at each distance along each road for $N_2$ fixation analyses. We used an ion exchange system adapted from the ''resin lysimeters'' described by Susfalk and Johnson (2002) to monitor the accumulated throughfall N over a 1-year period (June 2010–June 2011). These throughfall collectors were placed with the opening at moss height at each distance from the road with three replicates for each distance. Throughfall collectors were constructed by placing the resin capsule between 5 mm of clean, nutrient-free mineral wool in open-bottomed conical polycarbonate tubes, measuring 2.5 cm in diameter at the surface opening, 5 cm in depth, and with a 0.8 cm bottom opening (DeLuca and others 2008). The ionic resins in the collectors were mixed bed, anion–cation exchange resins contained in a polyester mesh capsule, holding 1 g of resin (Unibest, Bozeman, MT). The collectors were placed into the moss bottom layer so that the open throughfall collectors were exposed to the atmosphere, receiving N from wet and dry deposition as well as from canopy and understory throughfall and leachate from fine litter deposition.

Stand characteristics, understory vegetation composition, and humus depth data were collected from all eight sites. Percentage ground cover of field and bottom layer vegetation was estimated at the species level in six replicated 50 × 50 cm frames and compared against previous assessments on these same sites (DeLuca and others 2002). All vegetation plots were established and assessed during the summer of 2010.

Soil pH was determined in the lab on field-moist soil (1:1 w/w soil:distilled water). Soil temperature was measured in situ with a probe integrating over 0–3 cm depth. Soil moisture content was estimated gravimetrically, by measuring the moisture loss after drying for 24 h at 80°C.

To assess extractable ammonium ($NH_4^+$), nitrate ($NO_3^-$), and net N mineralization and nitrification potential, we conducted 28-day aerobic incubations. In brief, 5 g of fresh soil was extracted with 20 ml 0.5 M $K_2SO_4$, shaken for 30 min on a reciprocating shaker, followed by centrifugation for 15 min at 4000 rpm and subsequent filtration through Whatman 42 filters. The extracts were analyzed by microplate-colorimetric technique using the salicylate–nitroprusside method of Mulvaney (1996) for $NH_4^+$-N and the vanadium method for $NO_3^-$-N (Miranda and others 2001). A second soil sample (5 g fresh weight) was incubated in 50 ml polycarbonate tubes at 25°C for 28 days in a growth chamber with 24 h light/dark cycles; followed by extraction and analyses for $NH_4^+$-N and $NO_3^-$-N as described above. Net nitrification was then calculated as $NO_3^-$-N at day 28 minus $NO_3^-$-N at time zero; net ammonification was calculated as $NH_4^+$-N at 28 days minus $NH_4^+$-N at time zero; and net mineralization was calculated as total inorganic N ($NH_4^+$-N plus $NO_3^-$-N) at day 28 minus total inorganic N at time zero. Samples from the first extraction were used for analyses of dissolved organic N (DON) and dissolved organic C (DOC) using a Shimadzu TIVC-TNM1 analyzer (Shimadzu Corp., Kyoto, Japan). $K_2SO_4$ extracts were tenfold diluted with deionized water prior to analysis. Total C (TC) and total N (TN) in soil samples were analyzed by oxidative combustion using an elemental analyzer interfaced to a continuous flow isotope ratio mass spectrometer (IRMS) (Sercon Ltd., Cheshire, UK) to detect C and N.

## $N_2$ Fixation Analyses

$N_2$ fixation analyses were performed using a calibrated acetylene reduction assay (Schöllhorn and Burris 1967) as described by Zackrisson and others (2004). In brief, 30 moss shoots from each site were placed in a 50 ml tube, sealed, and 10% of the headspace was replaced with acetylene. Moss samples were incubated for 24 h at room temperature. Ethylene generated in the headspace by the cyanobacterial nitrogenase enzyme was measured by gas chromatography equipped with a flame ionization detector (Varian, Santa Clara, USA). Former studies assessed the reduction of acetylene at a ratio of 3 mol of ethylene per mol N in *Pleurozium*–cyanobacteria association (DeLuca and others 2002; Zackrisson and others 2004), which we used herein to calculate the amount of $N_2$ fixed by cyanobacteria. Data are represented as acetylene reduced to ethylene in $\mu$mol $m^{-2}$ $d^{-1}$; where 10 moss shoots of *P. schreberi* represent an area of 2.8 $cm^2$.

## Heavy Metals

Trace elemental analyses of dried and ground soil and moss samples were performed using the principle of total reflection X-ray fluorescence spectroscopy (Bruker AXS Inc., Madison, USA). In brief, 20 mg of ground sample was weighed into a 1.5 ml centrifuge tube and suspended in 1 ml 1% Triton X-100 after which 10 µl of a 1000 µg $ml^{-1}$ as standard was added. Five microliters of the suspension was subsequently used for analyses.

## Resin Analyses

Resins were extracted with 10 ml of 0.5 M $K_2SO_4$, shaken for 30 min, and the solution phase decanted to new tubes. This procedure was repeated three times to get an end volume of 30 ml. The extracts were then subjected to $NH_4^+$, $NO_3^-$, DOC, and DON analyses (see above).

## Phospholipid fatty acids (PLFA) analyses

PLFA were determined using 1 g of soil according to Frostegård and others (1993) with modifications (Nilsson and others 2007). The PLFAs chosen to indicate bacterial biomass were i15:0, a15:0, i16:0 16:1$\omega$9, 16:1$\omega$7t, i17:0, cy17:0, 17:0, 18:1$\omega$7, and cy19:0, whereas 18:2$\omega$6,9 was used to indicate fungi (Frostegård and Bååth 1996). The quantities of the fatty acids were obtained using 19:0 as an internal standard.

## Statistical Analyses

Differences in nutrient concentrations, transformations, and abiotic factors (pH, soil temperature) in soil samples as well as differences in $N_2$ fixation rates in the mosses and throughfall N via resins between the road types and between distances were analyzed using analysis of co-variance (ANCOVA) approaches. Relationships between distance, road types, soil nutrients, $N_2$ fixation, throughfall N, and PLFA concentrations were subjected to linear regressions analyses. Data were tested *a priori* if they met the assumptions of ANCOVA; appropriate transformations were applied when the assumptions were violated. The relative amounts (mol%) of individual PLFAs were subjected to a principal component analyses (PCA) after standardizing to unit variance. The resulting factor scores of the first and second principal components (PC1, PC2) were tested for correlation with distance to the roads and with various soil variables. Fungal and bacterial PLFA concentrations were also subjected to ANCOVA and linear regression analyses. All analyses were performed using R 2.13 (R Development Core Team 2011).

# RESULTS

## $N_2$ Fixation in *Pleurozium schreberi*

$N_2$ fixation rates (acetylene reduction) were higher in remote roads than in busy roads ($F = 75.1$, $P < 0.001$) and increased with increasing distance from busy roads ($r^2 = 0.48$, $P < 0.001$, degree of freedom (DF) = 23) (Figures 1, 2). At 100 m distance from busy roads, $N_2$ fixation reached values of $N_2$ fixation in mosses similar to that along remote roads (Figure 1). The values ranged from 4.9 μmol $m^{-2}$ $d^{-1}$ at 0 m to 495.0 μmol $m^{-2}$ $d^{-1}$ at 150 m away from busy roads and between 70.6 and 753 μmol $m^{-2}$ $d^{-1}$ near remote roads with no systematic pattern. No significant relationship was found between $N_2$ fixation rates and N trapped in throughfall collectors (see below).

## Soil pH, Nutrients, and Ground Cover of *Pleurozium schreberi*

Soil pH was similar in both road types, ranging between 3.5 and 5.2 in busy and 3.3 and 5.5 in remote roads (Table 1), and on average at 4.25 ± 0.15 [mean ± standard error (SE)] and 4.27 ± 0.11, in busy and remote roads, respectively. Ground cover of *P. schreberi* along the busy roads ranged from 50 to 70% closest to the road (0 m) to 35–70% farthest away from the road (150 m) and hence, did not change significantly with distance from the roads. TC and TN did not differ significantly between road types nor were they correlated with distance from the roads (Table 1). C/N ratios in soils ranged between 35 and 56 in busy road sites with no clear distance pattern from the roads. Similarly, there was no clear pattern between distance from remote roads and C/N, which varied between 26 and 68 (Table 1). DON concentrations were significantly higher in busy roads than in remote roads ($F = 7.05$, $P = 0.01$), with an average of 0.06 ± 0.01 mg $g^{-1}$ soil dw in busy roads and 0.03 ± 0.01 mg $g^{-1}$ soil dw in remote roads, but showed no correlation with distance (Table 1; Figure 3B). We found a negative relationship between DOC and distance from busy roads ($F = 4.33$, $P = 0.04$, DF = 23) (Table 1; Figure 3A).

No distance effect was found for soil $NH_4^+$-N, $NO_3^-$-N concentrations, and mineralization rates, nor was a significant difference between the road types detectable (Table 1; Figure 3C, D). Average mineralization rates in soils along the busy roads were 6.38 ± 1.32 mg N $kg^{-1}$ $d^{-1}$ and along the remote roads 4.22 ± 1.16 mg N $kg^{-1}$ $d^{-1}$.

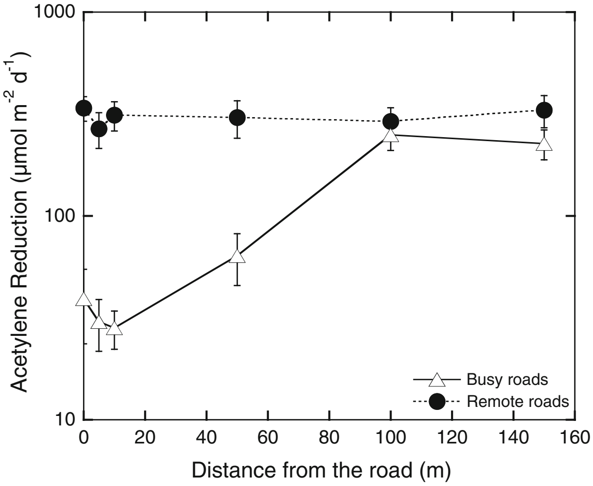

**Figure 1.** Acetylene reduction in μmol $m^{-2}$ $d^{-1}$ as an indicator of $N_2$ fixation in *P. schreberi* along remote (*filled circles*) and busy (*open triangles*) roads in northern Sweden. Values represent mean ± SE ($n = 12$). Note the logarithmic scale of the *y*-axis.

## Heavy Metals in Soil

Zinc (Zn) and lead (Pb) concentrations in soil samples along the busy roads ranged from 44.0 to 63.7 and from 34.3 to 58.2 μg $g^{-1}$, respectively. In the remote road soil samples, zinc concentrations varied between 40.4 and 76.2 μg $g^{-1}$ and lead concentrations ranged between 25.3 and 37.5 μg $g^{-1}$. Zinc and Pb concentrations were higher closer to busy roads (64 ± 0.005 μg Zn $g^{-1}$ (mean ± SE); 54 ± 0.005 μg Pb $g^{-1}$) and decreased with increasing distance ($F = 24.35$, $P = 0.008$, DF = 23 and $F = 10.19$, $P = 0.033$, DF = 23, respectively) from the roads (Figure 4). Concentrations of Pb in soils from busy roads were significantly higher than in remote road soils ($t = 3.47$, $P = 0.001$). However, heavy metals in moss tissue showed no consistent pattern with distance from busy roads.

## Nitrogen Accumulated in Throughfall Collectors

Throughfall of $NO_3^-$, DOC, and DON accumulated in the resins of throughfall collectors were higher in busy road samples than in remote road samples (Table 2). Throughfall $NH_4^+$-N and $NO_3^-$-N were significantly correlated with distance from the roads for several sites, indicating a distance effect for individual roads (Table 2; Figure 5: resin-sorbed $NH_4^+$-N; Borup, Vilan ($r^2 = 0.4$, $P = 0.003$, $r^2 = 0.23$, $P = 0.045$, respectively; both DF = 6)).

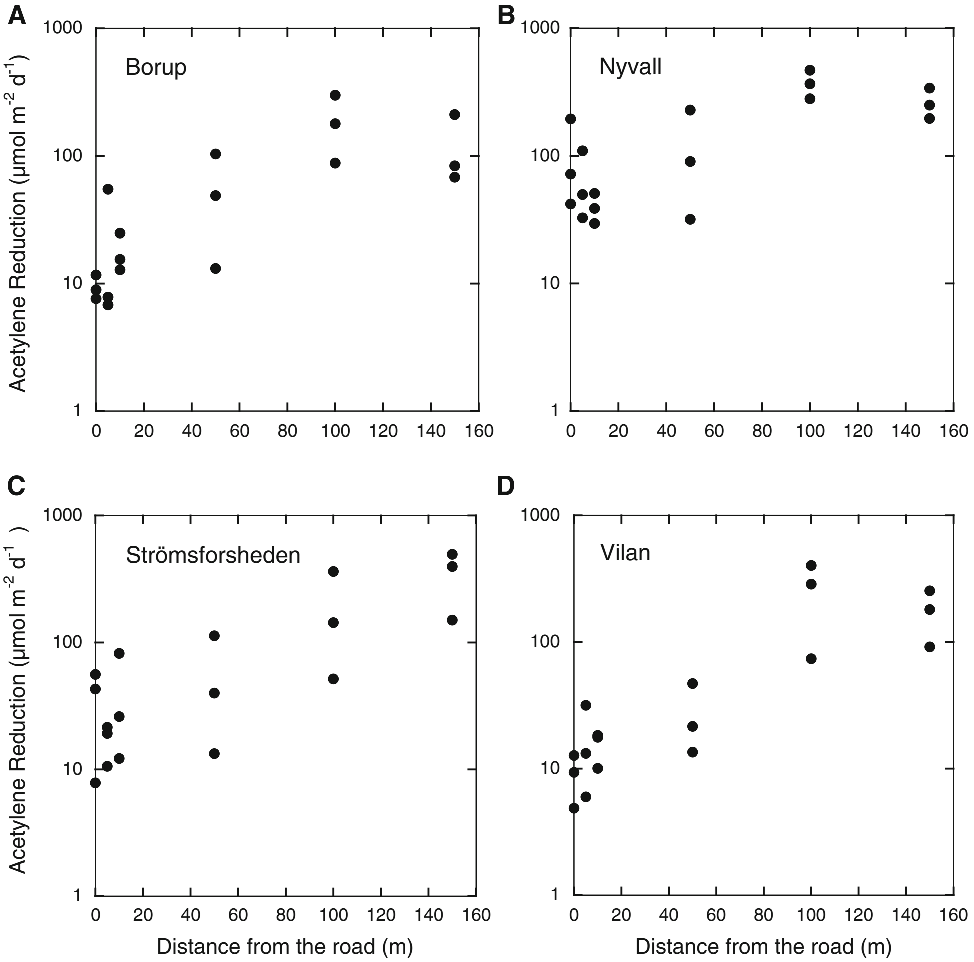

**Figure 2.** Acetylene reduction (µmol $m^{-2}$ $d^{-1}$) in *P. schreberi* at six distances from the four busy roads in northern Sweden. Three replicated measurements of acetylene reduction per distance are shown.

## Microbial Community Composition

Higher fungal ($F = 18.92$, $P < 0.001$) and bacterial ($F = 7.67$, $P = 0.008$) PLFA concentrations were found in busy than in remote road soil samples. Total PLFA and bacterial PLFA concentrations decreased with increasing distance from busy roads ($F = 4.34$, $P = 0.049$; $F = 5.35$, $P = 0.03$, DF = 23, respectively) whereas no distance relationships could be found in remote roads. Bacterial PLFA concentrations showed a significant correlation with soil DON in busy road samples ($F = 4.26$, $P = 0.05$, DF = 23), with resin-DON ($F = 11.43$, $P = 0.002$, DF = 23) and resin-$NH_4^+$ ($F = 3.91$, $P = 0.05$, DF = 23). Total PLFA concentration was positively correlated with resin-DON ($F = 4.44$, $P = 0.04$, DF = 23). The PCA analysis of the overall PLFA composition revealed a separation between the composition of busy roads, clustered toward lower PC1 values, and the PLFA composition of remote roads clustered toward higher PC1 values ($F = 4.76$; $P = 0.04$) (Figure 6A). PLFAs related to fungi (18:2ω6,9 and 18:1ω9) were found to the left of PC1, indicating that fungi are relatively more abundant in the busy road samples, and PLFAs related to bacteria (18:1ω7, 16:1ω7c, 16:1ω5, 16:1ω9, cy19:0) were found to the right of PC1, indicating that bacteria are relatively more abundant in the remote roads (Figure 6B). PC2 (accounting for 16.9% of the variation) did not separate samples from the different road types. PC1 showed a positive correlation with soil DON ($r^2 = 0.08$, $P = 0.03$, DF = 45), PC2 with C/N ratio ($r^2 = 0.08$, $P = 0.04$, DF = 45).

## DISCUSSION

We found that $N_2$ fixation in the feather moss *P. schreberi* is highly sensitive to road-derived N inputs, suggesting that mosses can be used as effective and sensitive indicators for N inputs to natural ecosystems. $N_2$ fixation rates in *P. schreberi* along the remote roads were high and constant along the various distances and the different forest stands. This stands in sharp contrast to the low

**Table 1.** Mean Values ($n = 4$) ± SE of the Main Soil Nutrients: Total Carbon (TC) (mg C $g^{-1}$ soil dw), Total Nitrogen (TN) (mg N $g^{-1}$ soil dw), C/N, Dissolved Organic Nitrogen (DON) (mg $g^{-1}$ soil dw), Ammonium ($NH_4^+$-N), and Nitrate ($NO_3^-$-N) (mg $g^{-1}$ soil dw) in Soil Samples Collected at Six Distances (m) along the Busy and Remote Roads

| Distance | Busy roads | | | | | | Remote roads | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | TC | TN | C/N | DON | $NH_4^+$-N | $NO_3^-$-N | TC | TN | C/N | DON | $NH_4^+$-N | $NO_3^-$-N |
| 0 | 254 ± 35 | 6 ± 0.75 | 42.8 ± 2.81 | 0.03 ± 0.02 | 0.0006 ± 8 × $10^{-5}$ | 0.001 ± 0.0002 | 224 ± 61 | 5.9 ± 1.15 | 36.1 ± 3.72 | 0.02 ± 0.02 | 0.0005 ± 0.0001 | 0.001 ± 0.0002 |
| 5 | 308 ± 28.2 | 6.9 ± 0.52 | 45.1 ± 3.19 | 0.05 ± 0.02 | 0.0007 ± 0.0001 | 0.002 ± 0.0003 | 353 ± 61.4 | 8.5 ± 0.9 | 41 ± 5.55 | 0.1 ± 0.04 | 0.0008 ± 0.0002 | 0.002 ± 0.0002 |
| 10 | 262 ± 43.3 | 6.4 ± 0.89 | 40.5 ± 1.5 | 0.08 ± 0.04 | 0.0007 ± 0.0003 | 0.001 ± 0.0001 | 312 ± 37 | 7.7 ± 0.37 | 40.4 ± 3.34 | 0.03 ± 0.03 | 0.0007 ± 0.0001 | 0.002 ± 0.0001 |
| 50 | 328 ± 68.2 | 7.2 ± 1.42 | 45 ± 1.62 | 0.09 ± 0.04 | 0.001 ± 0.0007 | 0.002 ± 0.0005 | 345 ± 23.7 | 8.2 ± 0.26 | 42.2 ± 3.4 | 0.005 ± 0.01 | 0.0009 ± 0.0002 | 0.002 ± 9 × $10^{-5}$ |
| 100 | 312 ± 63.3 | 6.7 ± 0.98 | 45.6 ± 4.38 | 0.08 ± 0.02 | 0.0008 ± 0.0001 | 0.002 ± 0.0006 | 359.5 ± 39.2 | 8.2 ± 0.55 | 43.4 ± 3.08 | −0.01 ± 0.02 | 0.001 ± 0.0002 | 0.002 ± 7 × $10^{-5}$ |
| 150 | 316 ± 59.8 | 7.1 ± 1.08 | 43.5 ± 2.71 | 0.03 ± 0.01 | 0.0003 ± 9 × $10^{-5}$ | 0.001 ± 0.0004 | 413.2 ± 10.7 | 9.7 ± 0.14 | 41.9 ± 0.41 | 0.03 ± 0.05 | 0.002 ± 0.001 | 0.002 ± 0.0002 |

$N_2$ fixation rates close to busy roads with a gradual and significant increase with increasing distance from the roads, reaching the values of the remote roads at 100 m away from the roads. In contrast, estimates of soil nutrient concentrations in our study system were not responsive to road pollutants: only soil DON was significantly different between busy and remote roads, and neither soil C nor inorganic soil N ($NH_4^+$, $NO_3^-$) showed clear differences between the road types. However, soil inorganic N concentrations in boreal forest soils are known to be comparably low and therefore a difference between the sites could be difficult to detect. Also, feather mosses intercept atmospheric pollutants and thus attenuate the influence of these inputs on soil and soil processes (Lindo 2010; Turetsky and others 2010; Gundale and others 2011), this is also indicated by the lack of a consistent pattern in the nutrient limitation of bacterial growth (Demoling and others 2008) estimated in the same soil samples (K. Ackermann, J. Rousk, T.H. DeLuca, unpublished data).

We observed higher Pb concentrations in soil samples along the busy roads and decreased Zn and Pb concentrations with increasing distance from busy roads, suggesting the pollution originates from the roads, probably due to tire wear and vehicle exhaust (Bakirdere and Yaman 2008). Higher metal concentration in busy roads is a result of higher traffic along these roads as well as higher salt inputs in winter, which increase heavy metal mobility (Grolimund and others 1996; Norrström and Jacks 1998). As an additional analogy, Zn and Pb have been found to be elevated in urban runoff (Maltby and others 1995). However, we did not detect a distance effect for Zn and Pb in moss tissue, which suggests no significant short-term effect on the aboveground plant biomass, rather, the metals slowly accumulate in the bryophyte litter and associated humus over time. Also, our sampling took place in summer, when heavy metal pollution may be lower than in winter.

The lack of a distance effect of heavy metals in the moss layers does not exclude the possibility that the aboveground biomass is stressed by heavy metal inputs. Overall plant growth could be reduced due to heavy metal input (Tyler and others 1989). However, as several studies showed, mosses are highly sensitive to N loads (for example, DeLuca and others 2008; Gundale and others 2011), which is potentially more inhibitory for biomass production and $N_2$ fixation than metals. Furthermore, comparing $N_2$ fixation rates with heavy metal concentrations along our study sites, $N_2$ fixation in moss followed a steep increase with dis-

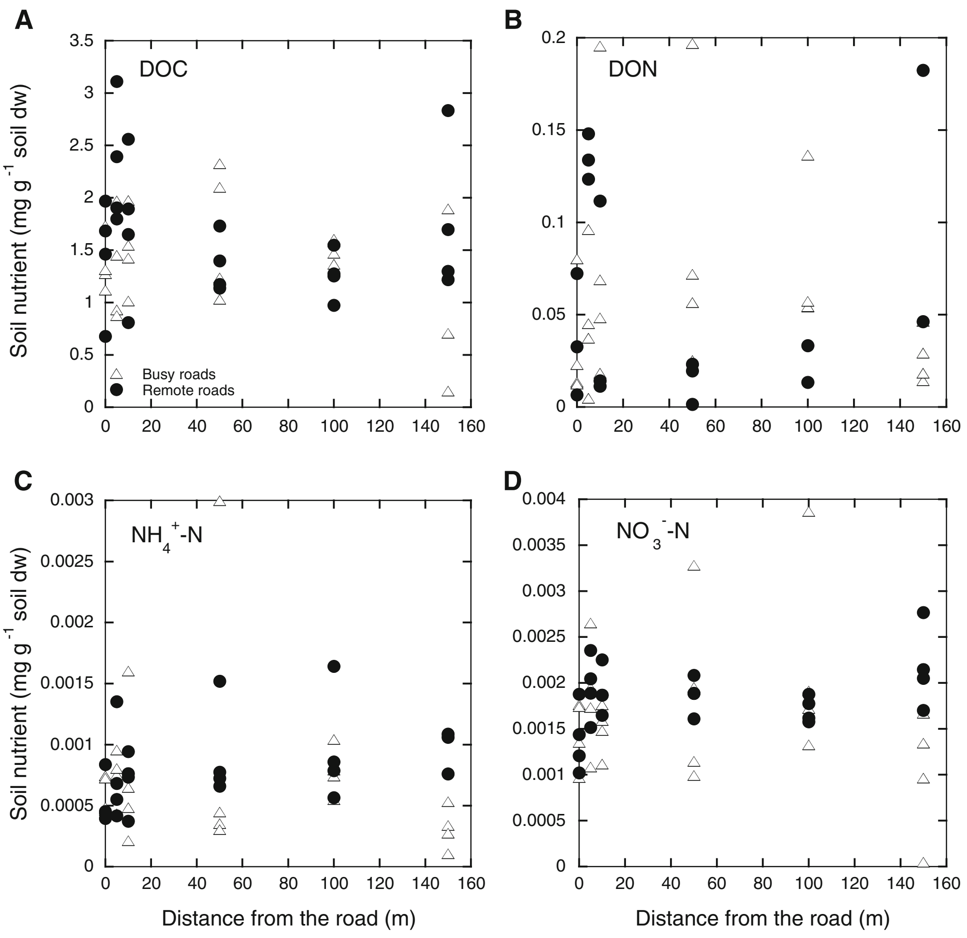

**Figure 3.** Extractable soil nutrients (**A** DOC, **B** DON, **C** $NH_4^+$-N, **D** $NO_3^-$-N in mg g$^{-1}$ soil dw) at 0–150 m distance from busy (*triangles*) and remote (*circles*) roads in northern Sweden.

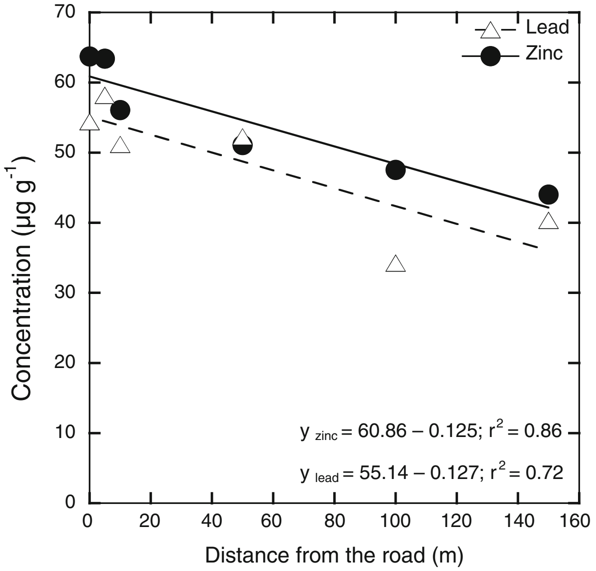

**Figure 4.** Zinc and lead concentrations (µg g$^{-1}$) in soil samples along busy roads in Northern Sweden. *Filled circles* represent zinc and *open triangles* represent lead concentrations. Regression equations, regression lines and $r^2$ values are given. Values represent means ($n = 4$) ± SE. *Error bars* are hidden by symbols.

tance, whereas heavy metal concentrations did not decrease in a similar distinctive way with distance from the busy roads (Figures 1, 4). In addition, Zn concentrations were not significantly higher in soil samples from the busy roads. We therefore assume that $N_2$ fixation is inhibited by road-derived N rather than by metal pollution originating from the roads.

Throughfall N was higher along busy than along remote roads, but we were not able to detect a distance effect in resin-sorbed N within the throughfall collectors. In contrast, previous studies found clear distance patterns: for example, Bignal and others (2008) revealed a fertility effect of $NO_x$, $NH_3$ derived from roads on bryophyte species but only up to 200 m from roads. $NO_2$ levels dropped to normal levels after 125 m. A distance effect was also reported by Bernhardt-Römmermann and others (2005): N compounds in tree rings, soil nutrients, and minerals (N, C, K, Mg) were negatively correlated with distance to motorways. However, we have to consider the background N deposition at different study sites. The background N deposition at our study sites ranges between 1

**Table 2.** Summary of ANCOVA: *F* values for Resin-Sorbed Nutrients ($NH_4^+$, $NO_3^-$, DOC, DON)

| | Resin-$NH_4^+$ | Resin-$NO_3^-$ | Resin-DOC | Resin-DON |
|---|---|---|---|---|
| Road (DF = 1) | 2.84 | **384***** | **11.0**** | **7.01**** |
| Site (DF = 6) | **28.7***** | **19.8***** | 0.56 | 2.85 |
| Distance (DF = 5) | 1.91 | 3.23 | 0.32 | 3.13 |
| Road × site (DF = 6) | **12.4***** | 0.13 | **4.46*** | **13.5***** |
| Site × distance (DF = 30) | **7.38**** | 4.28* | 0.32 | **4.93*** |
| Road × distance (DF = 5) | 1.11 | 1.83 | 0.93 | 0.52 |
| Road × site × distance (DF = 30) | **7.64**** | **4.48*** | 2.02 | 2.89 |

*Effects of road-type, forest sites, distance from the roads and interactions. Values in bold represent significant results. Significant codes: * 0.05; ** 0.01; *** 0.001.*

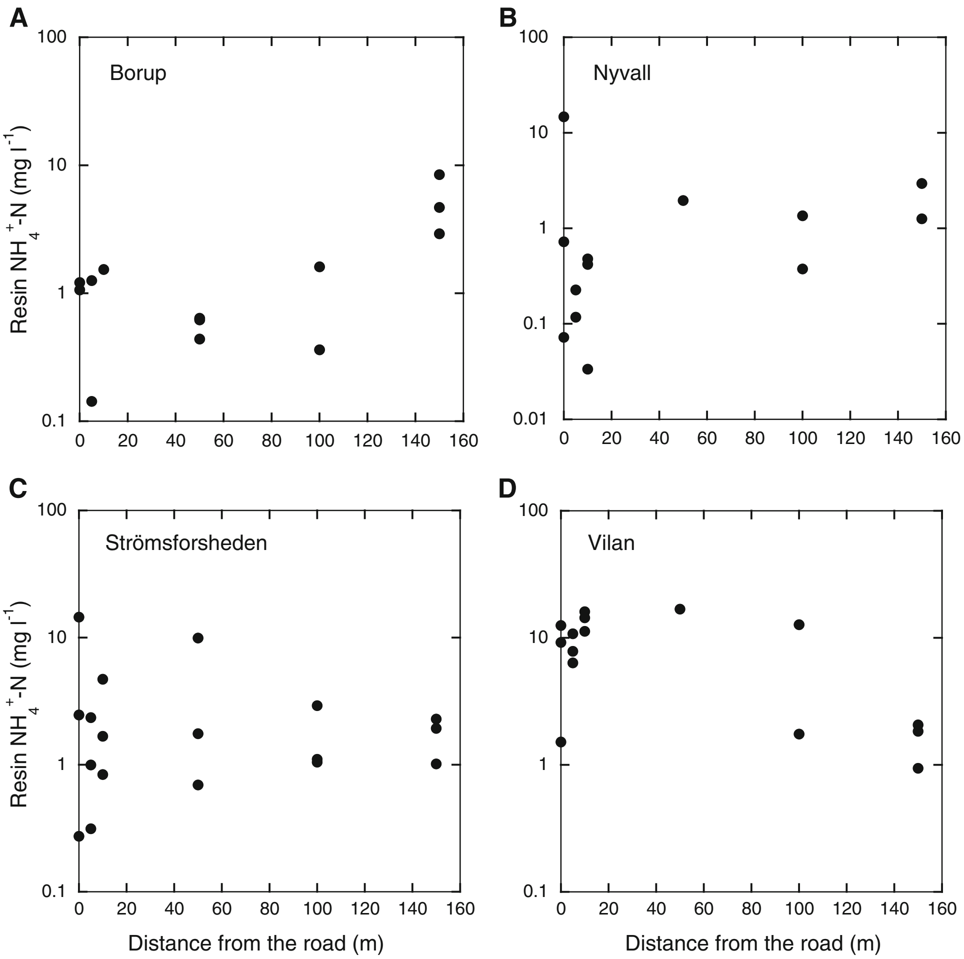

**Figure 5.** Resin-sorbed $NH_4^+$-N (mg $l^{-1}$) at six distances from the four busy roads in northern Sweden. Three replicates of resin extracts at each distance are shown.

and 2 kg N $ha^{-1}$ $y^{-1}$ (Gundale and others 2011), whereas previous studies were mostly conducted in high N deposited areas, which could lead to more distinctive effects on plants, soils, and microbes. Furthermore, the overall lack of distance effect in the resin-sorbed nutrients could be due to different traffic speeds along the busy and remote roads: fast traffic along busy roads could lead to more effective dispersion of particulates and dust via enhanced winds (Pearson and others 2000), whereas slow traffic along remote roads would be more likely to cause an accumulation of pollutants, which is balanced by the low traffic intensity along the remote roads. Also, the presence of moss could affect resin-sorbed nutrients: Jackson and others (2011) found a negative relationship between N in resins and the presence of moss (*P. schreberi*). In addition, they found that *P. schreberi* promotes substrate-induced

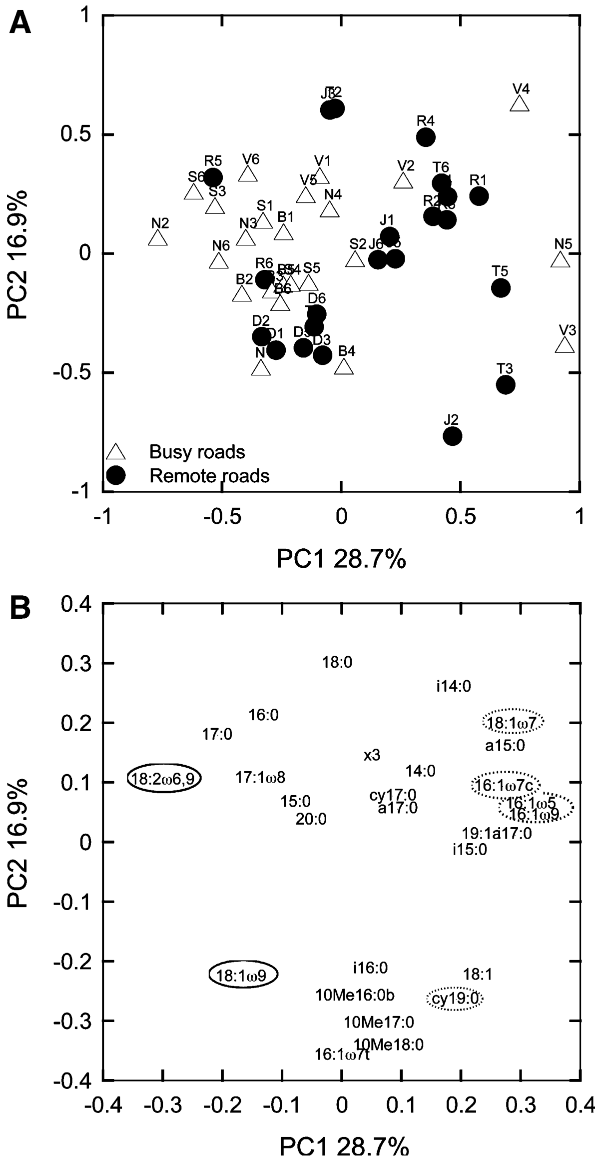

**Figure 6.** **A**, **B** PCA case scores of the PLFA data from each site and each distance. *Filled circles* represent remote roads, open triangles represent busy roads. *Letters* represent the different sites (*B* Borup, *N* Nyvall, *S* Strömsforsheden, *V* Vilan, *D* Dötternoive, *J* Jegge, *R* Reivo, *T* Tjädness), numbers (*1*–*6*) represent the distances from the roads: (*1*) 0 m, (*2*) 5 m, (*3*) 10 m, (*4*) 50 m, (*5*) 100 m, (*6*) 150 m. **B** PCA variable loadings of the PLFA data. *Solid lines* indicate the main fungal biomarker; *dotted lines* indicate the main bacterial biomarker.

respiration (SIR). Both findings suggest that the low N availability underneath the resins is a result of greater immobilization of N by microbes and mosses. However, we did not detect a decrease in ground cover of *P. schreberi* with distance from the busy roads, reflecting the lack of distance effect in throughfall N, indicating that feather moss biomass was not negatively affected by road-derived nutrient loads in this study system. Also, resin-sorbed nutrients were not correlated with $N_2$ fixation in the moss, which is in accordance with recent findings by Jackson and others (2011).

Our results revealed higher total PLFA biomass close to busy roads and because moss carpets seem to promote microbial biomass in soils (Biasi and others 2005; Jackson and others 2011), higher nutrient immobilization rates close to busy roads could have eliminated distance effects in soil nutrients, as PLFA concentrations decreased with distance to roads and thus, possibly, immobilization of nutrients. We detected a tendency for a separation of the microbial PLFA composition along PC1, suggesting different community compositions in the remote and busy roads. Rousk and others (2011) found a clear separation of PLFA biomarkers along a pH and a N fertilization gradient in a grassland experiment in which the biomarkers 17:0, 14:0, i17:0 16:0 were linked to high fertility conditions and the biomarkers 18:1, 18:2ω6, a17:0 to low fertility conditions. These PLFA biomarkers did not show a consistent pattern in our samples (Figure 5B). However, the difference in N load levels between the studies must be considered, with levels up to 150 kg N $ha^{-1}$ $y^{-1}$ in the grassland soils (Rousk and others 2011) compared to below 3 kg N $ha^{-1}$ $y^{-1}$ of these boreal systems.

The clustering of bacterial biomarkers toward remote road sites and the clustering of the fungal marker toward the busy road sites seem counterintuitive due to the common belief that fungal biomass is reduced under higher nutrient loads (for example, Alden and others 2001). However, microbes could experience higher nutrient loads from $N_2$ fixation under active moss layers, thereby promoting bacterial growth in these soils. Another explanation could be that, although the environmental conditions are similar in the two road types, the busy road sites are slightly more productive, but still low-nutrient systems, and therefore promote both bacteria and fungi. In accordance to that, we found higher PLFA concentrations for both fungal and bacterial markers in the busy road samples. The differences between our sites in pH, soil nutrients, and so on, are too subtle to distinctively separate the microbial community according to road type, in contrast to forest sites which can dramatically vary in abiotic factors between samples sites due to groundwater influence, and so on (for example, Nilsson and others 2005). Further, the distribution of fungi in our samples could also reflect the correlation between tree species and type of fungi. Ectomycorrhizal (EM) mycelia production for example, is linked to fine root biomass (Nilsson and others 2005). Spruce trees root shallower than pine trees, with that, depending on the depth of the soil samples and on the dominant trees species at our sites, we could have more EM in the busy road sites leading to higher fungal PLFA concentrations in these samples compared to the remote road sites which are older on average and host more pine trees than spruce trees.

## CONCLUSIONS

The most sensitive indicator for the negative effects of road pollution in boreal forest systems was $N_2$ fixation in feather moss–cyanobacteria associations. In contrast with this responsive bio-indicator for $N_2$ deposition, we did not detect significant patterns in soil nutrients along the roads, whereas microbial communities (PLFA) and resin data showed only subtle differences between the road types and a marginal distance effect. Although, the deposition of road pollutants in northern Sweden could be too low to be detectable in soil, our results show that the activity of cyanobacteria in feather mosses can be used as an indicator of increased N loads, even at low N dosages. Our results suggest that increased N deposition is likely to alter N flow through natural systems due to the suppression of $N_2$ fixation. Yet, total influx of N into the system might not change as long as N input is relatively low, however, the way N enters the system will fundamentally change: from moss derived, mainly organic N to atmospheric inorganic N. N deposition below 3 kg N $ha^{-1}$ $y^{-1}$ is likely to nominate $N_2$ fixation in feather mosses as the main N source in the boreal forest (Gundale and others 2011), above this threshold, $N_2$ fixation is inhibited and N enters the system by short-circuiting the moss layer. N deposition at our study sites is below this threshold to bypass the moss layer, as we found no consistent distance effect in soil nutrients. However, N deposition was high enough to inhibit $N_2$ fixation in the feather moss close to the busy roads. Thus, given the dominance of feather mosses in the boreal understory vegetation, the size of the boreal biome and its importance in global biogeochemical cycles, increased N loads are likely to fundamentally affect N flow in pristine northern ecosystems.

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