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1 **Asymbiotic nitrogen fixation on woody roots of Norway spruce and Silver birch**

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Abstract

High rates of asymbiotic nitrogen fixation have been measured in woody roots in temperate forests, but this rate has not been quantified in boreal forests. We studied the asymbiotic N₂ fixation associated with living and decomposing woody roots of Norway spruce in three sites in Finland. In addition, tree species effect was studied in one site that included Norway spruce and Silver birch monocultures and mixed stands. The rate of N₂ fixation measured as nitrogenase activity was affected by host tree species, spruce roots being the most active (in spruce monocultures 0.67 C₂H₄ d⁻¹ (g dry mass)⁻¹). The activity was not statistically different in decayed and living root samples; and moisture content did not explain the observed high variability in nitrogenase activity. In a birch-spruce mixed stand the average N₂ fixation in woody roots was 0.17 kg N ha⁻¹ yr⁻¹, whereas in Norway spruce dominated sites, the activity ranged from 0.06 to 0.15 kg N ha⁻¹ yr⁻¹. The N₂ fixation in decaying and living woody roots is an important contributor to the long-term total N balance of the forest. However, the estimated rate of N₂ fixation is low compared to atmospheric N deposition.

Keywords Acetylene reduction assay (ARA); coarse woody debris; *Picea abies*; *Betula pendula*; forest nitrogen supply

35 **Introduction**

36

37 Plants require nitrogen (N) in larger amounts than any other mineral nutrient, and in boreal forests plant
38 available N is generally the most growth limiting factor (Kukkola and Saramäki 1983; Nohrstedt 2001;
39 Saarsalmi and Mälkönen 2001; Weetman et al. 1997). In a boreal forest, where the annual N use for plant
40 growth was 50 kg N ha⁻¹ yr⁻¹, nutrient retranslocation and litter decomposition were the main N sources for the
41 plants, while N originating from atmospheric deposition contributed less than 30 % of the annual demand
42 (Korhonen et al. 2013). In mature Norway spruce stands, the amount of N returned back to soil in litterfall is 30
43 kg N ha⁻¹ yr⁻¹ (Ukonmaanaho et al. 2008). Furthermore, biological N₂ fixation may add to the N supply, if there
44 is substrate that acts as host for the N₂ fixing bacteria (Sponseller et al. 2016). The range for N fixed in above
45 ground woody residues is between 0.16 and 2.1 kg N ha⁻¹ year⁻¹ depending on the wood decay phase and the
46 mass of woody debris available for asymbiotic N₂-fixing bacteria (Brunner and Kimmins 2003 and review by
47 them; Jurgensen et al. 1987). In comparison, the activity of cyanobacteria living in association with feather
48 mosses may contribute up to 3 kg N ha⁻¹ year⁻¹ in boreal forests (Gundale et al. 2012; Leppänen et al. 2013;
49 Lindo et al. 2013). However, current estimates of the amounts of asymbiotic N₂ fixation in forests may be
50 underestimates, since all ecosystem components where N₂ fixation occurs have not been included. Early results
51 by Granhall and Lindberg (1978) showed that in boreal upland forests, where the overall rate of N₂ fixation
52 varied from 0.35 to 3.2 kg N ha⁻¹ year⁻¹, the activity of the N₂-fixing bacteria was found mainly in association
53 with feather mosses, both above- and belowground decaying wood, and in the rhizosphere. Their results also
54 showed that the measured N₂-fixation activity was positively affected by the vicinity of roots.

55

56 The rates of asymbiotic N₂ fixation associated with rhizosphere and decomposing woody roots have rarely been
57 evaluated, and the few published reports deal with temperate forests (Burgoyne and DeLuca 2009; Chen and
58 Hicks 2003). According to Chen and Hicks (2003), N₂-fixation rates in decaying roots are four times higher than
59 the rates reported for on other substrates such as dead wood and litter. The N content of decaying stumps
60 increases during the first years after harvesting and retain N at the rate of 1.4-1.8 kg N ha⁻¹ year⁻¹, which is
61 suggested to be partly explained by N₂ fixation (Palviainen et al. 2010). The biomass of coarse roots is two-fold
62 in comparison to stump biomass (e.g. Hakkila 1989) and this relatively large pool of woody residue is a

potentially important substrate for N₂ fixation (Chen and Hicks 2003). Based on the study where high rates of asymbiotic N₂ fixation (2.1-10.4 kg N ha⁻¹ yr⁻¹) were found in decomposing woody roots in Oregon U.S.A. (Chen and Hicks 2003), we hypothesize that N₂ fixation in decaying roots is an important primary source of N also in boreal forest soils.

The importance of the various factors on root associated asymbiotic N₂-fixing activity has not been tested in boreal conditions. Earlier studies on above- and belowground woody residues have resulted in conflicting results on the effect of tree species on N₂-fixation rates (reviewed by Son 2001). However, the activity is affected by the soil pH (Nohrstedt 1985) and we hypothesize that deciduous tree species, which tend to increase the pH in boreal forest soils (Augusto et al. 2015; Smolander and Kitunen 2011), have a positive influence on the N₂-fixation activity associated to decaying roots.

The aims of this study were (1) to determine the rate of asymbiotic N₂ fixation associated with living and decomposing woody roots of Norway spruce (*Picea abies* (L.) H. Karst.) and Silver birch (*Betula pendula*, Roth.) in southern boreal forests, (2) to evaluate differences in the rates of nitrogenase activity (that was used as a measure of N₂ fixation) between tree species in mixed stands and monocultures, (3) to analyse the relationship between nitrogenase activity and root moisture content and (4) to upscale the results for N₂-fixation activity to forest stand level (kg N ha⁻¹ y⁻¹).

Materials and methods

Study sites, root sampling and preparation of incubation

N₂ fixation was investigated in decaying and living spruce roots on three geographically different sites that formed a latitudinal gradient from south to north-east (Table 1). All study sites were mesic sites (*Vaccinium myrtillus* site type, which is intermediate fertility level according to applied site type classification (Hotanen et al. 2008)).

On one site (Eno) the effect of tree species growing on either monocultures or in mixed stands on N_2 -fixation rate was studied. The Eno study site ($62^{\circ}47'20.4''$ N, $30^{\circ}05'38.4''$ E) is situated in eastern Finland, and has previously been described in detail (Smolander et al. 2005). It is a birch-spruce experiment, which had been planted in 1964-1965. We chose two single-species birch and spruce plots and two mixed-species plots for our study. The birch plots were last thinned in 1985 and the spruce and mixed plots in 2007. Concurrently with the 2007 thinning, trees were also felled on the birch plots in order to create logging tracks; and the resulting stumps were used for the sampling of this study. Thus, all sampled dead roots were decayed for 7 years. The plot size was 40 m x 40 m. Both stand characteristics and soil properties were determined in 2008. The ground vegetation varied between the plots; herbs and grasses were abundant on the birch plots, while the spruce plots were covered almost solely by mosses and needle litter. The birch plots were pure single-species stands. The spruce plots contained 93.8% and 99.1% spruce respectively, and the proportion of spruce in the mixed stands was 62% and 78% (Table A1).

The two other sites were Norway spruce-dominated stands located in Heinävesi ($62^{\circ}24'32.4''$ N $28^{\circ}42'25.2''$ E), eastern Finland, and Heinola ($61^{\circ}10'08.4''$ N $26^{\circ}02'52.8''$ E), southern Finland. The stands were regenerated for Norway spruce in 1931 (Heinävesi) and in 1949 (Heinola). The plot size was 30 m x 30 m in Heinävesi and 30 m x 25 m in Heinola. The stands were thinned in 1999 (Heinävesi) and in 1990 and 2004 (Heinola). For more details of the Heinävesi site see Smolander and Mälkönen (1994) and Saarsalmi et al. (2014) (experiment 35, control plot used in the present study), and for the Heinola site see Saarsalmi et al. (2014) (experiment 155, control plot).

The study focus was on living and decaying woody root material larger than 5 mm in diameter (varied between 5 mm and 60 mm, the most common being 20 mm). Roots of living trees and those attached to decaying stumps of harvested trees were sampled with spade and scissors in August 2014. The decay age of the sampled dead roots was 7 years in the Eno site and 10 and 15 years in the Heinola and Heinävesi, respectively. The sample size is shown in Table A1. The sampled trees and stumps were selected randomly, and one root sample per sample tree was extracted by cutting it off and storing it in a plastic bag. The root samples were stored in the dark at $+4^{\circ}\text{C}$ for 1-3 weeks until the N_2 fixation incubation experiment was started.

Prior to incubation, the roots were lightly cleaned from soil, cut into pieces (length 6-7 cm) and 1-3 pieces were placed into 125 ml glass bottles. The fresh weight of the incubation samples varied from 7.9 to 49.4 g, the mode value being 31 g. The average volume of the incubated root samples was 18.5 ml. Two days before incubation 2

ml of distilled water was added to allow optimal moisture conditions for N_2 fixation (tested in a pilot experiment), and the samples were moved from storage temperature to the incubation temperature + 15 °C, which is near the average temperature in the organic layer of the studied forest sites during the growing season. The openings of the vials were loosely covered with tin foil. When the experiment started, the bottles were closed with gas-tight rubber septa.

Acetylene reduction assay (ARA)

The N_2 -fixation activity associated with living and decaying woody roots was measured with the acetylene reduction assay (ARA; Hardy et al. 1968). ARA is the most commonly used method for estimating biological N_2 fixation; and this assay takes advantage of the fact that the nitrogenase enzyme of the N_2 -fixing bacteria also catalyzes the reduction of acetylene (C_2H_2) to ethylene (C_2H_4), which is easily detected using a gas chromatograph. The activity of the nitrogenase measured with the ARA correlates with N_2 fixation (Vessey 1994). When nitrogenase acts in saturating concentrations of acetylene and H_2 production is inhibited, the conversion factor is 4.0 under ideal conditions (Capone 1993; Margaret and Deborah 2004), which value was used in our calculations. Earlier studies have calibrated the conversion factor for decaying woody roots using $^{15}N_2$ gas and they found that the conversion factors were 4.5 and 4.4 (Burgoyne and DeLuca 2009; Chen and Hicks 2003). In general, the incubation time in previous studies has been 24 hours (e.g. Brunner and Kimmins 2003; Chen and Hicks 2003; Griffiths et al. 1993; Leppänen et al. 2013), but incubation periods ranging from 12 h to 100 h have been used (e.g. Granhall and Lindberg 1978; Brunner and Kimmins 2003). We tested 24-h and 48-h incubations and found that nitrogenase activity is not saturated due to incubation conditions within 48-h (incubations of 24-h and 48-h gave estimates of N_2 -fixation rates that were both within the same range); and we selected an incubation time of 48 h in order to accumulate higher concentrations for the measurements. We incubated our samples in + 15°C in a dark room.

After the incubation vials were closed, 10% of the headspace air was removed and replaced with acetylene. When this portion of the headspace is filled with acetylene it is assumed to be above the saturation limit (Knowles and Bergersen 1980; Turner and Gibson 1980). In previous pilot experiments, no ethylene production had been detected without acetylene addition. The evolved ethylene was measured after 48 h (varied between 46.9 and 48.4 h) with a gas chromatograph (HP 6890) equipped with a flame ionization detector and a HP-Plot Q column (30.0 m × 530 µm × 40 µm), using He as carrier gas. The gas chromatograph settings have previously been described in detail (Leppänen et al. 2013). The total number of the measured samples was 311 and

measurements were repeated for 37 samples, which showed either very low or very high nitrogenase activities. A second measurement produced results that were consistent with the original measurement, and the mean values of the first and second measurements were used for the repeated samples.

Dry mass, water content and density of the root samples

The dry mass and water content of the samples were determined by drying the samples to constant weight at 70 °C for 1-2 days. The moisture content was calculated by subtracting the oven dry sample weight from the fresh sample weight. The wood density was measured by determining the root sample volume of frozen samples by the water-displacement method and measuring the dry weight of the samples after they had been dried at 102-105 °C for 1 day. The dry mass divided by the root volume gives the root density. The average wood density of the decaying spruce roots was 91.6% of the average density of the living spruce root density; the average density of the decayed birch roots was 80.4% of the average density of the living birch root.

Total root biomass and upscaling to stand-level N₂ fixation

The total root biomass of the standing and dead trees was estimated by the diameter (D1.3 m or stump diameter) of each tree by applying biomass equations (Marklund 1988) (Table A2). According to the density measurements of the sampled roots, we knew that the density of the sampled dead roots was 8.4% (spruce) and 19.6% (birch) lower than that of respective living spruce and birch trees. Thus the root biomass estimates, which were calculated with the biomass equations that apply to living trees, were reduced by this percentage to give a rough estimate for the dead root biomasses.

We used the following assumptions for the estimates of the annual N₂-fixation rates: A period of 184 days with nitrogenase activity was used based on the temperature conditions of our study sites, leaving the period November to April outside the calculations, and assuming a minimum temperature of 5 °C for significant N₂-fixation activity (Englund and Meyerson 1974). Some N₂-fixation activity may be possible during mild winters at temperatures between 0 and 5 °C, but limitations in the carbohydrate supply might reduce this activity to insignificance. The mean annual daily temperatures on our study sites were: Eno 2.9, Heinävesi 3.6, and Heinola 4.6 °C (Table A3). Brunner and Kimmins (2003) used a conservative assumption of 240 with N₂-fixation activity for their study site with an average annual daily temperature of 8 °C. A conversion factor of 4 was used

for conversion of acetylene reduction to N_2 fixation, which corresponds to the theoretical conversion factor (Bergersen et al. 1991).

Statistical analyses

Generalized linear mixed models (GLMM) were applied in the statistical analyses. Due to the highly skewed distribution of the nitrogenase activity log-normal distribution with identity link was used. The models were fitted by using the residual maximum likelihood (REML) method of estimation with type of root (decayed/living), tree species (spruce/birch) and type of forest (spruce/birch/mixed) as fixed effects. The root-size effect was tested by non-parametric Mann-Whitney U test after pooling the entire data for all study sites and dividing them into size classes under (5-25 mm, $n=121$) and over (30-60 mm, $n=38$) 30 mm, and found to be non-significant. Our research hypotheses on monoculture/mixed forest were tested separately by using one site from where we had measurements for all combinations, i.e. the difference in the nitrogenase activity associated to Norway spruce and Silver birch roots between monocultures and spruce-birch stand was analyzed with the Eno site data only. In addition, spruce monocultures were also compared between all three sites. All the plots from sites were used as blocks, and these random effects were assumed to be independent and normally distributed. All samples within block were randomly selected. The models took account that trees within block might have been somewhat correlated. The block factor explained third of total variance from analysis of type of root and tree species (Fig. 1) and tenth from spruces in different forest types from site Eno (Fig. 2), respectively. In comparison of spruce monocultures between sites, block factor did not explain variance at all.

The appropriateness of the models was studied by residual analyses. The residuals were tested for normality with boxplots and normal probability plots (Tukey 1977). The residuals were also plotted against the fitted values. These plots indicated that the assumptions of the models were adequate. For pairwise comparison of means, the Tukey-Kramer post hoc test was used. A significance level of $\alpha=0.05$ was used in all analyses. Degrees of freedom were calculated using the containment method. The analyses were performed using the MIXED and GLIMMIX procedures of the SAS Enterprise Guide 7.1 (SAS Institute Inc., Cary, NC, USA).

Results

Tree species' influence on N_2 fixation in living and dead woody roots

According to our results, the tree species had a significant effect on the rate of N_2 fixation ($p < 0.001$) and spruce roots produced clearly higher N_2 -fixation rates measured as nitrogenase activity (Fig. 1). Although spruce was overall more active, one birch plot in Eno (plot 57) showed very high nitrogenase activities compared to the other plots, but it also showed higher pH, net nitrification, net N mineralization and C mineralization values, which indicated overall high biological activity (Table A1).

The N_2 -fixation activities did not differ significantly between the decayed and living root samples of Norway spruce or Silver birch on site Eno, where both tree species were present ($p=0.920$) (Fig. 1). Overall, decayed roots had 1.5 times higher activities ($p=0.0479$).

The average N_2 -fixation rate of spruce roots in spruce monocultures was $0.68 \text{ C}_2\text{H}_4 \cdot \text{d}^{-1}(\text{g dry mass})^{-1}$ and the differences between site locations were statistically significant ($p = 0.042$) (Fig. 2). In Eno, which is located about 100 km and 330 km northeast of Heinävesi and Heinola, the mean rate of N_2 fixation was 1.9 and 1.7 times higher, respectively. In the spruce-birch mixed stands, the N_2 -fixation activity in spruce roots was $1.17 \text{ nmol C}_2\text{H}_4 \cdot \text{d}^{-1}(\text{g dry mass})^{-1}$. The difference in the N_2 -fixation rates between spruce roots sampled from monocultures and mixed stands in Eno site were not statistically significant ($p=0.757$).

Effect of root moisture content and root size on N_2 fixation activity

In this study the water content of the decayed roots had no significant impact on the nitrogenase activity ($p=0.49$). In our data, the overall variation in water content was between 40.8% and 698.2% of the dry mass, and the within-site variation was higher than the between-site variation. The previously mentioned Eno birch plot 57, which showed the highest nitrogenase activity, also had the highest root moisture content (average 319.5% dry weight compared to other plots, which varied between 173.3% and 297.3% dry weight). According to our data, smaller ($<30 \text{ mm}$) and larger ($>30 \text{ mm}$) woody roots did not differ significantly ($p=0.504$) with regard to their N_2 -fixation activity.

Overall N_2 fixation capacity at stand scale

The measured N_2 -fixation activities in the living and decomposing root systems were upscaled based on the stand-scale estimates of the root biomasses (Table A2). The biomass of living roots ranged from 21.2 Mg ha^{-1} to

42.3 Mg ha⁻¹, and the biomass of dead roots from 5.3 Mg ha⁻¹ to 16.5 Mg ha⁻¹. The total combined annual N₂ fixation per hectare for spruce was 0.06 kg N ha⁻¹ yr⁻¹ (Heinola), 0.06 kg N ha⁻¹ yr⁻¹ (Heinävesi) and 0.15 kg N ha⁻¹ yr⁻¹ (Eno) (Fig. 3). The nitrogen fixation in birch roots was studied only in Eno, and the average nitrogen fixation per hectare was 0.16 kg N ha⁻¹ yr⁻¹ in the birch stands and 0.17 kg N ha⁻¹ yr⁻¹ in the mixed birch-spruce stands (Fig. 3).

Discussion

The asymbiotic N₂ fixation associated with woody roots in our study sites varied, but nitrogenase activity was detected in all the three southern boreal forest stands, and in both tree species, and in both living and decaying roots. This external source of N is additional to the known N₂ fixation in boreal forests reported earlier for coarse woody debris (reviewed by Brunner and Kimmins 2003) and bryophytes (reviewed by Lindo et al. 2013). According to our results, the nitrogenase activity in Norway spruce roots was higher than that in birch roots, which result do not support our hypothesis that higher N₂-fixation activity is associated to deciduous trees. Earlier studies have not found differences between N₂-fixation activity of decaying roots of different tree species in temperate forests (Chen and Hicks 2003), where soil N availability is higher than in boreal forests. In our study sites, soil total N, N in the microbial biomass and the rate of net N mineralization were all lower in the soil surrounding spruce roots than birch roots (Table A1). Palviainen et al. (2010) have shown that both the initial N concentration and the rate of N accumulation as well as the rate of decomposition were lower in decaying Norway spruce stumps than in birch stumps. The N₂ fixation is an energy-consuming process, which is stimulated by lack of available N. Thus, the found higher nitrogenase activity in Norway spruce root may result from a deficiency of available N. Seven years had elapsed after the trees had been felled in the mixed stand and the decay stage as well as the microbial communities associated with birch and spruce roots might deviate, but the difference observed in the living roots between species remained the same. Communities of the N fixing bacteria associated to decaying wood are known to be dependent on host tree species (Hoppe et al. 2014). Furthermore, Augusto et al. (2015) suggested that, on a given site, both the chemical composition of roots and the root morphology of deciduous species indicate that they are more prone to decomposition than are the roots of evergreen species. The observed differences in the N₂-fixation activity between the studied tree species are most likely linked to chemical composition and morphology of roots as well as community composition of active microbes.

258

259 We found that microbes associated with decaying woody roots are able to fix N_2 as efficiently as those attached
260 to living woody roots. The stages of decay of the dead root samples varied considerably even though the trees
261 were felled at the same time. Thus, it is possible that the magnitude of the amounts of N_2 fixation may depend
262 more on the availability of the easily accessible carbon. Supporting this assumption, Hendrickson (1991)
263 demonstrated a positive correlation between carbohydrate supply and nitrogenase activity in decaying wood. It
264 has also been suggested that some phenolic compounds support nitrogenase activity, but the materials
265 remaining in later stages of decomposition, such as lignin, are poor energy sources for N-fixing bacteria (Chan
266 1986; Jurgensen et al. 1989). Our wood and root samples lost about between 8.4% (Norway spruce) and 19.6%
267 (Silver birch) of the initial density after 7-15 years of decomposition. This, together with the results that living
268 and decayed samples had no statistically significant difference in N_2 -fixing rates indicates that the remaining
269 material at this stage of decay is as favorable a habitat for N_2 -fixing micro-organisms as the living roots.

270

271 We estimated that the overall mean nitrogenase activity across our samples was $0.762 \text{ nmol C}_2\text{H}_4 \text{ d}^{-1} (\text{g dry}$
272 $\text{mass})^{-1}$, which was a single observation of nitrogenase activity in time of the active period. In our root samples,
273 the nitrogenase activity ranged between 0 and $40.3 \text{ nmol C}_2\text{H}_4 \text{ d}^{-1} (\text{g dry mass})^{-1}$. Some individual samples
274 indicated much higher activities than the highest reported values for CWD or dead roots (Brunner and Kimmins
275 2003; Chen and Hicks 2003), but the overall mean rate of activity was notably lower due to the high variability
276 between the samples. In comparison, the nitrogenase activities measured for forest floor bryophytes ranged from
277 2.6 to $240 \text{ nmol C}_2\text{H}_4 \text{ d}^{-1} (\text{g dry mass})^{-1}$ (Leppänen et al. 2013). The N_2 fixing rates resulting from Frankia-Alnus
278 symbiosis, which is the form of N_2 fixing symbiosis that is common in early successional boreal forests, are
279 much higher than those mentioned above, but these require the presence of alder in the forest (Benson and
280 Silvester 1993).

281

282 In this study, nitrogenase activity was measured with 48-h incubation using ARA method, which has been tested
283 and found to be best suited for screening a large number of samples for their ability to fix N_2 (Leppänen et al.
284 2013). Our reported rates of nitrogenase activity might nevertheless underestimate the real rates. A conversion
285 factor of 4 from acetylene reduction to N_2 fixation was used; whereas a factor of 3 is applied in many studies
286 (reviews by Brunner and Kimmins 2003; Son 2001). Furthermore, some bacteria are able to use ethylene
287 produced during ARA as their growth substrate (Capone 1993), which may cause underestimations in the ARA

results. On the other hand, plant material may produce ethylene, but in our root samples, endogenous ethylene production was not detected. When combined, these assumptions yield a conservative estimate of N_2 -fixation rate. Since acetylene reduction activity, an indicator of N_2 fixation, is affected by temperature and moisture (Chen et al. 2000), also other factors than conversion from ethylene production to the actual amount of fixed N_2 , have importance in scaling to areal and annual N_2 fixation values. Assuming a minimum temperature of 5 °C for significant activity (Englund and Meyerson 1974) we used a period of 184 days with nitrogenase activity in the calculations. This was based on temperature conditions on our study sites, leaving the months from November to April outside the calculations.

Nitrogenase activity in decaying roots under natural conditions depends on several environmental factors, of which study site location, root size and moisture were investigated in this study. The N_2 -fixation activity of mosses has been reported to be higher when the moss samples were collected from sites in northern Finland compared to more southern sites, which was explained by the atmospheric deposition of N, which is lower in the north (Leppänen et al. 2013). In our experiments, the same trend was seen for decaying root samples. In contrast to many other studies (e.g. Brunner & Kimmins 2003; Hicks 2000; Wei & Kimmins 1998), we found no positive correlation between nitrogenase activity and water content. The same amount of water results in different water contents on a dry mass basis, depending on the density of the material. Consequently, the water content on a dry mass basis does not provide complete information about differences in the degree of water saturation between samples, and comparisons should thus be made with caution. In our study, the soil properties vary at the stand scale, but also between the study sites. We know that the most southern site (Heinola) was more fertile, having a productivity than the Heinävesi site (Saarsalmi et al 2014). However, we found no relationship between site differences and nitrogenase activity.

On the study sites, the estimates of N_2 fixation in woody roots ranged from 0.06 kg N ha⁻¹ yr⁻¹ in a Norway spruce stand to 0.17 kg N ha⁻¹ yr⁻¹ in a mixed spruce-birch stand depending on the nitrogenase activity and on the biomasses of living and dead roots. The amounts found here are substantial, given that an overall nitrogen fixation rate of 0.5 kg N ha⁻¹ yr⁻¹ has been reported by Rosén & Lindberg (1980) for coniferous boreal forests, 3.2 kg N ha⁻¹ yr⁻¹ for a mixed Scots pine and Norway spruce stand (Granhall & Lindberg 1978), while bryophyte-associated nitrogen fixation in boreal forests ranges from 0.01 to 3.5 kg N ha⁻¹ yr⁻¹ (reviewed by

Lindo et al. 2013). However, the estimated N₂-fixation capacity (0.06-0.16 kg N ha⁻¹ yr⁻¹) is far less than the current supply by N deposition (2.5-2.8 kg N ha⁻¹ yr⁻¹)(Lindroos et al. 2013)).

Conclusions

In ecosystems where symbiotic N₂ fixation and atmospheric inputs are low, asymbiotic N₂ fixation in decaying and living woody roots makes an important long-term contribution to the total N balance. The estimated N₂-fixation activity in decaying woody roots is, however, relatively low in comparison to current atmospheric N deposition, which compensates for minor N losses in a forest ecosystem.

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Tables

Table 1. Description of the study sites. Time since thinning gives decay age of analyzed root samples.

	Latitude	Longitude	Stand age,	Time since	Soil type	Soil texture	Humus type
	°N	°E	years	thinning, years			
Eno	62.789	30.094	50	7	Podzol	Loamy sand	Mor
Heinävesi	62.409	28.707	65	15	Podzol	Sandy loam	Mor
Heinola	61.169	26.048	83	10	Podzol	Sandy loam	Mor

493 **Appendices**

494 **Table A1** Stand and soil characteristics as well as total sample numbers (n) of collected decayed and living spruce and birch roots. In the Eno site, soil analysis was
495 conducted in 2008 by methods that have been described previously (Smolander et al. 2005). Soil characteristics for Heinävesi and Heinola sites were obtained from Saarsalmi
496 et al (2014) data. The results are expressed on organic matter basis (per kg organic matter). The area of the plots used was 1600 m² each in Eno, 900 m² in Heinävesi and 750
497 m² in Heinola.

498

Site/plot	Tree species	Birch/ spruce %	n spruce (dead)	n spruce (living)	n birch (dead)	n birch (living)	pH	Org.ma tter % of. d.m.	C/N	N g/kg	Microbial biomass C g/kg	Microbial biomass N g/kg	Net nitrification NO ₃ -N mg/kg/d	Net N mineralization (NH ₄ +NO ₃)-N mg/kg/d	C mineralization, CO ₂ -C mg/kg/d
Eno 29	Spruce	6/94	24	14			3,9	80.5	25,1	21,0	8,9	1,5	0,0	4,5	374
Eno 56	Spruce	1/99	24	7			4.0	78.9	25,7	19,9	10,9	1,6	0,0	4,3	397
Eno 30	Birch	100/0			22	14	4,4	58.3	18,3	31,6	12,0	2,1	0,0	7,6	406
Eno 57	Birch	100/0			12		5.0	51.4	18,3	26,5	15,7	2,2	1,3	11,2	621
Eno 32	Mixed	37/63	25	8	26	10	4,1	69.0	20,0	27,5	10,7	1,8	0,0	8,5	394
Eno 58	Mixed	23/77	23	10	12	7	4,1	67.4	22,9	23,7	12,1	1,7	0,0	5,8	436
Heinävesi	Spruce	0/100	18	19			4.0	82.7	26.3	20.9					
Heinola	Spruce	0/100	18	18			4.3	72.0	23.7	23.8					

499

Table A2 Total living and decayed root biomasses (kg ha^{-1}) estimated with biomass equations (Marklund 1988).

	Eno 29 spruce	Eno 56 spruce	Eno 30 birch	Eno 57 birch	Eno 32 mixed	Eno 58 mixed	Heinävesi spruce	Heinola spruce
	kg ha^{-1}	kg ha^{-1}	kg ha^{-1}	kg ha^{-1}	kg ha^{-1}	kg ha^{-1}	kg ha^{-1}	kg ha^{-1}
Spruce living roots	22800	20500	50		16200	25300	36000	42900
Birch living roots	1400	700	22100	22800	8000	5000		
Living roots total	24200	21200	22150	22800	24200	30300	36000	42900
Spruce dead roots	11900	12900			8000	12600	5300	10600
Birch dead roots	850		12300	7600	6700	3900		
Dead roots total	12750	12900	12300	7600	14700	16500	5300	10600
All total	36950	34100	34450	30400	38900	46800	41300	53500

Table A3 Average monthly temperature (°C) and precipitation (mm) on the study sites (calculated as 30-year averages over the period of 1985-2014). Source: Finnish Meteorological Institute.

Eno	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec
Average temp.°C	-9.5	-9.1	-4.9	1.3	8.2	14.3	16.8	14.5	9.1	3.5	-2.7	-7.5
Precipitation mm	40.7	32.2	32.3	33.5	39.2	61.8	73.8	82.1	61.2	62.4	55	47.2
Heinävesi	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec
Average temp.°C	-9.5	-9.1	-4.9	1.3	8.2	14.3	16.8	14.5	9.1	3.5	-2.7	-7.5
Precipitation mm	40.7	32.2	32.3	33.5	39.2	61.8	73.8	82.1	61.2	62.4	55	47.2
Heinola	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec
Average temp. °C	-6	-6.4	-3.3	2.2	8.6	14.1	16.9	15.5	10.7	5.4	0.4	-3.4
Precipitation mm	47	36.6	34.7	37	41.9	47.5	61.7	74.9	64.4	69.7	66.1	54.6

510 **Figure captions**

511

512 **Fig. 1** Estimated mean ($\pm$ SE) nitrogenase activities (C_2H_4 nmol $\text{g}^{-1}\text{d}^{-1}$) of decayed ($n=132$) and living ($n=76$)
513 spruce (*Picea abies*) roots and decayed ($n=72$) and living ($n=30$) birch (*Betula pendula*) roots.

514

515

516 **Fig. 2** Estimated mean ($\pm$ SE) nitrogenase activities (C_2H_4 nmol $\text{g}^{-1}\text{d}^{-1}$) of spruce (*Picea abies*) roots in Eno ($n=$
517 135), Heinävesi ($n=37$) and Heinola ($n=36$).

518

519

520 **Fig. 3** Total potential N_2 -fixing activities ($\text{kg N ha}^{-1}\text{year}^{-1}$) associated to woody roots on the study sites.

Fig. 1 Estimated mean ($\pm$ SE) nitrogenase activities (C₂H₄ nmol g⁻¹ d⁻¹) of decayed (n= 132) and living (n= 76) spruce (*Picea abies*) roots and decayed (n= 72) and living (n= 30) birch (*Betula pendula*) roots.

Fig. 2 Estimated mean ($\pm$ SE) nitrogenase activities (C₂H₄ nmol g⁻¹ d⁻¹) of spruce (*Picea abies*) roots in Eno (n= 135), Heinävesi (n= 37) and Heinola (n= 36).

Fig. 3 Total potential N₂-fixing activities (kg N ha⁻¹ year⁻¹) associated to woody roots on the study sites.