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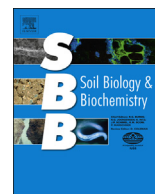
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Shifts in soil microbial community structure, nitrogen cycling and the concomitant declining N availability in ageing primary boreal forest ecosystems



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ABSTRACT

Plant growth in boreal forests is commonly limited by a low supply of nitrogen, a condition that may be aggravated by high tree below-ground allocation of carbon to ectomycorrhizal (ECM) fungi and associated microorganisms. These in turn immobilise N and reduce its availability to plants as boreal ecosystems develop. Here, we studied a boreal forest ecosystem chronosequence created by new land rising out of the sea due to iso-static rebound along the coast of northern Sweden. We used height over the ocean to estimate ecosystem age and examined its relationship to soil microbial community structure and the gross turnover of N. The youngest soils develop with meadows by the coast, followed by a zone of N₂-fixing alder trees, and primary boreal conifer forest on ground up to 560 years old. The young soils in meadows contained little organic matter and microbial biomass per unit area. Nitrogen was turned over at low rates when expressed per area (m⁻²), but specific rates (per gram soil carbon (C)) were the highest found along the transect. In the zone with alder, the amounts of soil C and microbial biomass were much higher (bacterial biomass had doubled and fungal biomass quadrupled). Rates of gross N mineralisation (expressed on an area basis) were highest, but the retention of added labelled NH₄⁺ was lowest in this soil as compared to other ages. The alder zone also had the largest extractable pools of inorganic N in soil and highest N % in plant foliage. In the older conifer forest ecosystems the amounts of soil C and N, as well as biomass of both bacteria and fungi increased. Data on organic matter ¹⁴C suggested that the largest input of recently fixed plant C occurred in the younger coniferous forest ecosystems. With increasing ecosystem age, the ratio of microbial C to total soil C was constant, whereas the ratio of microbial N to total soil N increased and gross N mineralization declined. Simultaneously, plant foliar N % decreased and the natural abundance of ¹⁵N in the soil increased. More specifically, the difference in δ¹⁵N between plant foliage and soil increased, which is related to relatively greater retention of ¹⁵N relative to ¹⁴N by ECM fungi as N is taken up from the soil and some N is transferred to the plant host. In the conifer forest, where these changes were greatest, we found increased fungal biomass in the F- and H-horizons of the mor-layer, in which ECM fungi are known to dominate (the uppermost horizon with litter and moss is dominated by saprotrophic fungi). Hence, we propose that the decreasing availability of N to the plants and the subsequent decline in plant production in ageing boreal forests is linked to high tree belowground C allocation to ECM fungi, a strong microbial sink for available soil N.

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1. Introduction

Despite the abundance of nitrogen (N) in the atmosphere and soil, plant productivity in many forest ecosystems is constrained by the lack of accessible N (Vitousek and Howarth, 1991; LeBauer and Treseder, 2008). This is typically the case for boreal forests (Tamm,

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1991), where higher N availability occurs only locally (Lahti and Väisänen, 1987), especially in groundwater discharge areas (Giesler et al., 1998).

Plant available N is produced as microorganisms decompose organic matter releasing peptides and amino acids, and the further processing of some of this N to ammonium (NH_4^+) and nitrate (NO_3^-). Until a few decades ago, mineralisation of organic N to NH_4^+ and nitrification to NO_3^- were the main foci in this context, but the common observation of lack of net release of inorganic N during the first weeks of soil incubations (eg., Hart et al., 1994a; Priha and Smolander, 1999) called for a shift in thinking. Subsequently, it became more widely known that the plants also take up organic N (Näsholm et al., 1998, 2009) and that various forms of organic N may dominate N uptake in ecosystems with a low soil N supply (Schimel and Bennett, 2004). The occurrence of inorganic N could be viewed as a supply of organic N in excess of the current biological demands of ecosystems (Schimel and Bennett, 2004), which is also dependent on the supply of carbon to the organisms (Hart et al., 1994a). Like peptides and amino acids, inorganic forms of N are rapidly taken up when the supply is low, and may, therefore not be detected when their pool sizes are measured. Thus, low abundances are not in themselves proof that they are not produced or important (e.g., Davidson et al., 1991, 1992; Hart et al., 1994a,b).

Why are pools of available N so low in boreal forests despite inputs of N from N_2 -fixation (DeLuca et al., 2002)? Several mechanisms are possible, for example, losses of N from the soil in recurrent fires typical of boreal forests (Zackrisson, 1977). Another possible mechanism addressed in numerous studies, is that there is a potential for chemical reactions between soil organic matter and inorganic N, e.g. NH_3 (Nömmik and Vahtras, 1982), NO_2^- (Azhar et al., 1986) or organic N forms (Knicker, 2004). Yet another possibility is related to the microorganism's supply of C and N as indicated by the frequent observations of relatively slow release of N from litter or organic matter with a high C/N ratio (Booth et al., 2005, and references therein). This stimulated research on the role of biotic factors like plant-microbial competition for available N (Kaye and Hart, 1997), plant characteristics, and plant and microbial community composition, (e.g., Merilä et al., 2002a,b; Leckie et al., 2004; Jerabkova et al., 2006; Boyle-Yarwood et al., 2008).

A study by Lindahl et al. (2007) highlighted the fact that the upper part of the organic layer in a typical boreal forest soil, which consists of a layer of mosses or lichens mixed with above-ground plant litter, is mainly dominated by saprotrophic fungi, but has very few ectomycorrhizal (ECM) fungi, the root symbionts of boreal forest trees. These observations are important, because it means that studies of release of available N forms from litter in litter-bags focus on a stage where saprotrophic fungi dominate and where there are no plant roots. Plants access N through ECM fungi, which dominate in F- and H-horizons deeper down in the organic horizon (Lindahl et al., 2007; Clemmensen et al., 2013) and further down (Rosling et al., 2003). It is in these lower F + H horizons that the availability of N to plants is of greatest interest. Near the litter layer surface the saprotrophs have a higher availability of C and are able to retain more N. Deeper in the profile C supply to saprotrophs is exhausted but ECM fungi are supplied directly with recent photosynthate C from their tree hosts (Yarwood et al., 2009). Therefore, ECM fungi become superior competitors for available N. This is analogous to the combat between saprophytic and ECM fungi for phosphorus demonstrated by Lindahl et al. (2001).

A number of our previous studies support ideas that the links between soil microbial community structure, the supply of C and their combined effects on the microbial N sink capacity and N cycling are of pivotal interest. For example, the microbial cytoplasm rather than abiotic mechanisms was found to be the major immediate sink for ^{15}N (as $^{15}\text{NH}_4^+$ or glycine) injected into the F + H

horizons (Näsholm et al., 1998). Likewise, the immediate retention of $^{15}\text{NH}_4^+$ label was high when cation exchange capacity (CEC) was low and fungal/bacterial ratio was high and the reverse was true when CEC was high and fungal/bacterial ratios were low (Högberg et al., 2003, 2006, 2007a). Because fungi in these (F + H) layers should predominantly be ECM (Högberg and Högberg, 2002; Lindahl et al., 2007; Clemmensen et al., 2013), high fungal/bacterial ratios and N retention, but low N cycling rates (Högberg et al., 2007b) may indicate a role of ECM fungi in N immobilisation.

The idea of ECM mycelium as an efficient N immobiliser was recently supported by Näsholm et al. (2013), based on the fates of labelled C supplied as $^{13}\text{CO}_2$ to the tree canopies and of N injected as $^{15}\text{NO}_3^-$ into the soil. In addition, survey data from N deposition gradients and data from long-term N addition experiments support that the contribution by fungi and bacteria to the soil microbial community may be a useful predictor of the N retention capacity (Demoling et al., 2008; Högberg et al., 2011, 2013, 2014a; Zechmeister-Boltenstern et al., 2011; Blaško et al., 2013). Moreover, the common increase in the natural abundance of the stable isotope ^{15}N , denoted $\delta^{15}\text{N}$, with increasing soil depth in this type of soil is indicative of immobilisation of N in the soil mycelium of ECM (e.g. Högberg et al., 1996; Lindahl et al., 2007; Hobbie and Ouimette, 2009). Thus, the microbial community structure and the microorganisms physiology appear to be pivotal in this context. A key aspect of the F + H horizons of the mor-layer is that the supply of fresh plant photosynthate C to roots, and thus also ECM fungi and associated microorganisms, is low under conditions of high soil N supply, but higher under conditions of low N availability (Hermans et al., 2006; Mäkelä et al., 2008; Högberg et al., 2010).

To test the role of soil microorganisms, especially ECM fungi, as key immobilisers of available N in the soil, we followed changes in C and N cycling with soil age in a replicated chronosequence of boreal forest ecosystems created by iso-static rebound after the last glaciation. The rebound, causes land to rise at a constant rate along the Gulf of Bothnia, creating new land that is colonized by plants and on which developing boreal ecosystems can be studied. More specifically, we address the following questions: (i) How long time does it take for newly developing boreal ecosystems to progress into typical N limitation? (ii) Do shifts in microbial biomass and fungal/bacterial ratios coincide with shifts in N cycling rates and N availability? (iii) Can the decline in N availability be linked to a key role of ECM fungi? Some previous studies have used coastal chronosequences in the same larger region for similar studies. For example, Wallander et al. (2009) found an enrichment in ^{15}N in the soil along with soil depth and increasing age of the ecosystems in a study encompassing systems of up to 7800 years old; they concluded that this enrichment was likely caused by ECM fungi. Merilä et al. (2002a,b, 2010) used younger systems to examine the links between available N and plant-microbial interactions. We focus more than these previous studies on the early and potentially rapid changes of relations among ecosystem age, microbial community composition, and the decline in available N using three replicated transects across ecosystem ages from 25 to 560 years.

2. Methods

2.1. Study sites

The study sites were located within a nature reserve on the island Bjuren (N 63°44', E 20°35') in the Gulf of Bothnia, 15 km SE of Umeå, Sweden. The mean annual (2003–2012) temperature is 5 °C and precipitation is 550 mm. The mean annual N deposition during the same period is 2 kg ha⁻¹. On average, the study area is covered by snow from late October until early May. The rate of the isostatic rebound of the coastline is 8.16 mm per year (Ekman, 1996; Vestøl,

2006). Five sites, representing ecosystems that differ in ground age and vegetation cover, were selected and replicated along three parallel transects separated by 150–200 m and running perpendicular to the shoreline (Fig. 1). The ground age of these five sites was estimated from the elevation above sea level (Trimble R6 GPS Rover, Trimble Navigation Limited, California, USA, Sweref 99 coordinate system) and the land-uplift rate (Ekman, 1996). The youngest ecosystem is 25 ± 12 years old (mean ± 1 SE) and has meadow vegetation (e.g., *Agrostis stolonifera* var. *Bottnica*, *Juncus balticus*). The 115-year-old ecosystem (112 ± 13 years) is dominated by grey alder (*Alnus incana* (L.) Moench), which forms root nodule symbioses with N_2 -fixing actinobacteria of the genus *Frankia* (Huss-Danell, 1997; Pölmé et al., 2014). In the third, 150-year-old ecosystem (151 ± 15 years) there is a Norway spruce (*Picea abies* (L.) Karst forest (Fig. 1). The age of the next ecosystem, a Norway spruce forest, was 215 ± 24 years. The oldest ecosystem, 560-year-old (564 ± 31 years), is dominated by Norway spruce and Scots pine (*Pinus sylvestris* (L.)). The soils throughout were sandy and silty glacial deposits (FAO) with many boulders. The soils with continuous organic mor-layer with F- and H-horizons (150, 215, and 560-year-old ground) are classified as Haplic Podzol (FAO classification).

2.2. Experimental design and sampling

We sampled soil at five sites of different ecosystem age along three parallel transects ($N = 3$) between 16 and 23 August 2010. One separate transect was sampled at each of three sampling days, and processed the same day in the laboratory. At each of the five sites (Fig. 1), we collected five ($n = 5$) composite samples within a $10 \text{ m} \times 15 \text{ m}$ area (five composite samples from each of five sites across three transects, $5 \times 5 \times 3 = 75$ samples). At the two youngest sites, which lacked a continuous organic layer, five cm of the uppermost soil layer was sampled using a spade and a plastic frame

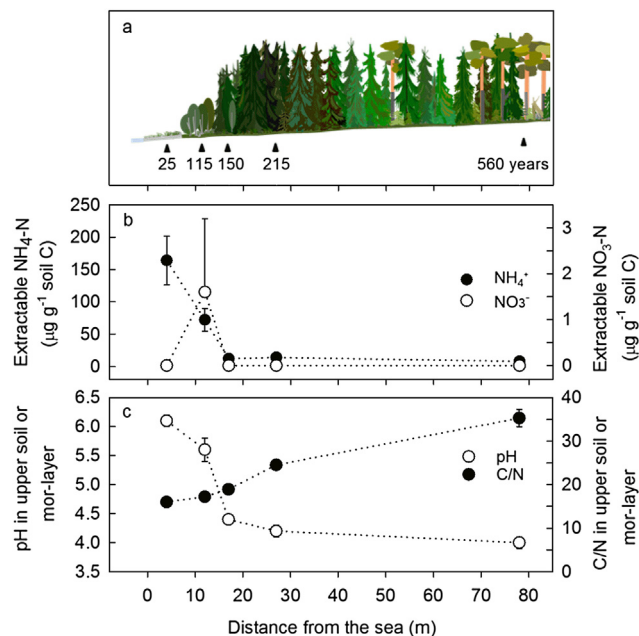


Fig. 1. Ecosystem age (here ground age), and vegetation communities at Bjuren, Sweden. Study sites are indicated by black arrows. (a) The vegetation ranged from meadow (25 years), alder (115 years), spruce–alder (150 years), spruce (215 years), and spruce–pine (560 years) vegetation. (b) Soil ammonium concentration (filled), nitrate (unfilled). (c) Soil pH (unfilled) and C/N (filled) across the three transects. Soil data were from the upper 5 cm surface soil at ages 25 and 115 years and from the F + H horizon at the higher ages. Means (± 1 SE), $N = 3$ per ecosystem age.

(30 cm \times 30 cm). The composite samples contained soil from the area of one to two frames. In the coniferous sites we used a 10 cm diameter corer to sample the entire organic (F + H) horizon (c. 10 cm thick and corresponding approximately to Oa and Oe horizons). Each of the five composite samples consisted of material from more than three soil cores. The soil was kept in cooling boxes during transportation and kept at 4 °C in the laboratory. The soil was sieved (5 mm mesh) and the remaining roots > 1 mm diameter were discarded. Microbial biomass C and N, pH_{H_2O} , ammonium (NH_4^+), nitrate (NO_3^-), N retention, and gross N mineralisation were measured on fresh root-free F + H soil on the day of sampling. On 10 and 16 July 2012 a separate sampling for determination of total CEC and radiocarbon age of the organic matter was performed for F + H soil and mineral horizons (at 0–10 cm, and 10–20 cm depths below the organic horizon). For this, soils were sampled from one pit at each site along the three transects using a spade (one pit per site, three depths, five sites, three transects, $1 \times 3 \times 5 \times 3 = 45$ samples). As in the main sampling the uppermost c. 5 cm of the soil profile were collected at the two sites lacking organic layers.

Foliage from the top whorls of the dominant trees was sampled on 20 and 21 July 2011. Three alder, three spruce, and one to three pine trees were sampled in each of three transects, depending on tree density.

2.3. Soil C and N, ¹⁵N analysis of soil and foliage, and cation exchange capacity

Subsamples of root-free surface soil (youngest ecosystems) or F + H horizon (older forest ecosystems) were freeze-dried, and the foliage was oven-dried (70 °C, 48 h) prior to grinding in a ball mill. Organic (surface 5 cm or F + H horizon) and mineral soils sampled from pits were sieved (5 and 1.6 mm mesh, respectively) and oven-dried at 105 °C for 48 h, and at 28 °C for 2.5 weeks, respectively. Mineral soil samples were ground using a ball mill. Foliage and soil subsamples were analysed for C %, N %, and ¹⁵N abundance on an Elemental Analyzer (Carlo Erba CHN 1115, Italy) coupled online to an Isotope Ratio Mass Spectrometer (EA-IRMS, Delta V, Thermo Fischer Scientific, USA). Variations in the isotopic ratio of ¹⁵N/¹⁴N are expressed in per mil (‰), δ , denoting parts per thousand deviation from the standard atmospheric N_2 .

$$\delta^{15}N_{\text{sample}} = \left(\left(\frac{R_{\text{sample}}}{R_{\text{standard}}} \right) - 1 \right) * 1000, \quad (1)$$

where R is the ratio ¹⁵N/¹⁴N. The standard deviation of the $\delta^{15}N$ isotopic measurements for the reference material did not exceed 0.2‰. For measurements of CEC, soil was extracted with 1.0 M ammonium acetate ($NH_4C_2H_3O_2$) solution at pH 7.0 and the extract analysed by Inductively Coupled Plasma Atomic Emission Spectroscopy (ICP-AES) for Ca^{2+} , Mg^{2+} , Na^+ , K^+ , and Mn^{2+} . For analysis of Al^{3+} subsamples of soil were extracted with 1 M KCl and analysed on ICP-AES. Extracts were titrated with NaOH to pH 7 to estimate the total acidity ($TA_{7.0}$). The molar charges of cations per kg of dry soil and $TA_{7.0}$ were summed representing total CEC at pH 7.0. CEC and TA were analysed at the Soil-Water laboratory, SLU, Uppsala, Sweden.

2.4. Radiocarbon analysis and modelling

The ¹⁴C content of organic matter at three depths across the soil profiles ($N = 3$) were analysed at the Max Planck Institute in Jena, Germany (Steinhof et al., 2004). To remove inorganic C, samples were treated with 2 M HCl and neutralized with 2 M NaOH prior to analysis by accelerator mass spectrometry (AMS). ¹⁴C

concentrations are given as percent modern carbon (pMC) (Mook and van der Plicht, 1999), which is 100 times the ratio of $^{14}\text{C}/^{12}\text{C}$ compared to the $^{14}\text{C}/^{12}\text{C}$ of CO_2 in the atmosphere in 1950. Radiocarbon data reported as pMC are normalized to a common $\delta^{13}\text{C} = -25\text{‰}$ using measured $\delta^{13}\text{C}$ values to correct for any mass dependent fractionation of ^{13}C (Stuiver and Polach, 1977). Values greater than 100% indicate the presence of carbon produced by atmospheric weapons testing in the early 1960s. For soils of known age that are accumulating C over time, the total amount of C and its radiocarbon content can be used to provide a unique solution to the equations that express C and ^{14}C mass balances (see Trumbore and Harden, 1997):

$$dC(t)/dt = I - kC(t) \quad (2)$$

$$d(F_C(t) * C(t))/dt = I * F_{\text{atm}}(t - t_{\text{lag}}) - (k + \lambda)F_C(t) * C(t) \quad (3)$$

Here, $C(t)$ is the amount of C accumulated at time (t) since the initiation of soil development (assumed to be $t = 0$), $F_C(t)$ is the percent modern radiocarbon signature of the O horizon or mineral C, $F_{\text{atm}}(t)$ is the changing atmospheric content of radiocarbon (in percent Modern C), t_{lag} is a time lag to account for addition of pre-aged C as inputs, as in the case of woody roots (Gaudinski et al., 2010), I is the annual rate of C input (assumed constant over time) and k is the rate constant for first order decomposition (also assumed constant). For simplicity, we assumed each of the sites on the chronosequence had only a single, average I and k , as the age of accumulated C was mostly very young. We calculated I and k values separately for organic horizons and total mineral soil C (0–20 cm), assuming a C-weighted average per cent modern value for the radiocarbon. After the appearance of tree roots in the chronosequence (>100 years) we assumed a lag time of 15 years for inputs to the organic and mineral soils, which includes lags in long-lived needles and roots (Gaudinski et al., 2010). In some cases, two combinations of I and k fit the amount of accumulated C and its radiocarbon content; in these cases we report both values. An example of how the model works is to consider a certain amount of C that has accumulated over the past 100 years. If decomposition rates are very slow, that C will be approximately half pre-bomb carbon (<100 per cent modern) and half post-bomb carbon (>100 per cent modern). Annual C input rates must also be small (to balance the fact that little of the accumulated C is lost). However, if decomposition rates are fast, most of the C will have been fixed in the last few decades (pMC > 0; old bomb-derived C); input rates must be higher than in the slow-decomposition case to accumulate the same amount of C in a century.

2.5. Soil microbiology

Microbial biomass C and N were measured using the chloroform fumigation extraction technique (Brookes et al., 1985; Vance et al., 1987; Högberg et al., 2003). Subsamples of root-free fresh soil (12–30 g) were fumigated in a desiccator with 45 ml of ethanol-free chloroform for 20 h at room temperature. Non-fumigated soil samples were extracted with 50 ml of 0.5 M K_2SO_4 , shaken on a rotary shaker (30 min at 120 rpm) and filtrated by gravity (Munktell 00H, Munktell Filter AB, Grycksbo, Sweden). Fumigated soil samples were extracted in the same way as described above after the chloroform was evacuated from the samples. Extracts were kept frozen at -20 °C before they were analysed for total organic C (TOC-VCPH/CPN, with TNM-1 module, Shimadzu corp., Kyoto, Japan). The sum of organic N and $\text{NH}_4\text{-N}$ in the extracts was determined after Kjeldahl digestion and $\text{NH}_4\text{-N}$ analysis by flow injection analysis (FIAStar™ 5012, FOSS, Höganäs, Sweden). For conversion of C and N in microbial

cytoplasm to microbial biomass C and N (C_{mic} , N_{mic}), we used conversion factors $k_{\text{EC}} = 0.4$ and $k_{\text{EN}} = 0.4$ (Martikainen and Palojarvi, 1990).

To determine the microbial community composition microbial neutral lipid fatty acids, phospholipid fatty acids (PLFAs), and fungal DNA for subsequent Q-PCR were extracted from subsamples of freeze-dried and cryo-milled soil. The signature phospholipid fatty acids were used as biomarkers indicating microbial groups and microbial community composition (Frostegård and Bååth, 1996; Frostegård et al., 2011), following the same procedures and instrumentation as were described in Högberg et al. (2014a). In short, neutral lipid fatty acids and PLFAs were extracted from soil equivalent to 0.15 g organic matter using the Bligh and Dyer (1959) extraction method as modified by Frostegård et al. (1991, 1993). Silica gel columns (Varian, HF Bond Elut LRC-Si, 100 mg, particle size 120 μm , Palo Alta, USA) were used to separate the extracts into fractions of glyco-, neutral-, and phospholipids (only the two latter fractions were analysed). The mild alkaline methanolysis of the lipid fraction (White et al., 1979) was followed by derivatisation of the PLFAs to their respective fatty acid methyl esters (FAMES). Methylnonadecanoic acid (Me19:0) was used as internal standard. FAMES were injected (250 °C) into a gas chromatograph (GC-FID equipped with a HP-5MS column of 60 m length, 0.25 mm i.d., film thickness 0.32 μm , Agilent Technologies 19091S-416H, JandW Scientific, Santa Clara, CA, USA) and brought to a final temperature of 295 °C . Standard nomenclature was used to describe PLFA fatty acids: that is, by the total number of C atoms: number of double bonds, and ω followed by the position of the double bond from the methyl end of the molecule. The prefixes a, i, and cy, refer to anteiso, iso, cyclopropyl branching. 10Me indicates a methyl group on the 10 th carbon from the carboxyl end of the molecule.

We used the ratio between the abundance of neutral lipid fatty acids and PLFA 16:1 ω 5 to distinguish between arbuscular (AM) mycorrhizal fungi and bacteria. The ratio spans from 1 to 200 in AM fungi, and we used the data above 1.0 as indicative of AM fungi (Olsson, 1999). We used the PLFA 18:2 ω 6, 9 as a biomarker for fungi across the ecosystem ages and for ectomycorrhizal fungi in the coniferous ecosystems in which F + H horizons were studied (Högberg et al., 2011, 2014a). The PLFA 18:2 ω 6,9 is the crown biomarker for fungi (Ratledge and Wilkinson, 1988; Frostegård et al., 2011). Here, we use this PLFA as biomarker for ECM fungi, in particular. This was supported by reports from similar coniferous forests in this area (i) of a strong relationship ($R^2_{\text{adj}} = 0.9$) between this biomarker and DNA-sequences of ECM fungi (Högberg et al., 2011), (ii) of a significant relationship ($R^2_{\text{adj}} = 0.9$) between the ^{13}C labelling of the biomarker in soil and ECM fungal sporocarps in a large-scale ^{13}C tracer pulse-chase experiment in a similar forest (Näsholm et al., 2013), (iii) of high sensitivity of this biomarker and ECM sequences to terminated tree below ground C allocation (Yarwood et al., 2009), and of (iv) a dominance of DNA sequences of ECM fungi in F- and H-horizons of the mor layer (Lindahl et al., 2007; Clemmensen et al., 2013), i.e. the soil horizon sampled and studied here. The other putative fungal biomarker 18:1 ω 9, which does not always correlate with the fungal biomarker ergosterol (Högberg, 2006) and does not respond to tree-girdling (Högberg et al., 2007a), is also indicative of bacteria (Frostegård et al., 2011) and is therefore not used as a fungal biomarker. The three most abundant terminally branched PLFAs (i.e., i15:0, i16:0, a17:0) were used as signature lipid biomarkers for gram-positive bacteria, while the three most abundant PLFAs characteristic of gram-negative bacteria were: 16:1 ω 7, 18:1 ω 7, and cy19:0. PLFAs 10Me17:0 and 10Me18:0 were used as biomarkers of actinobacteria. To validate the use of the fungal phospholipid

biomarker quantitative polymerase chain reaction (qPCR) was performed by using a StepOne Plus Real Time Thermocycler (Life Technologies, Carlsbad, CA) using KiCqStart[®] Sybr Green Ready Mix with ROX (Sigma–Aldrich, St. Louis, MO). Each reaction was performed in triplicate using 2 µl of DNA per reaction. Gene copy numbers of the fungal internal transcribed spacer region (ITS) was quantified using primers 5.8S/ITS1F. Reaction conditions have been described previously (Fierer et al., 2005; Yarwood et al., 2010). All gene copy numbers were determined by comparison to dilution series of DNA of known concentration. All standard curves had an $R^2 > 0.95$ and the amplification efficiency was 90–105%.

2.6. Inorganic N pools, gross N mineralisation, and N retention

Gross N mineralisation rates were estimated using the ^{15}N pool dilution technique (Kirkham and Bartholomew, 1954; Hart et al., 1994b). We followed a procedure described in detail previously (Blaško et al., 2013; Högberg et al., 2014a). We added approximately the same amount of ^{15}N per gram soil C in the samples (Högberg et al., 2006). Surface layer soil was labelled with $0.01 \mu\text{g}$ of $^{15}\text{N g}^{-1}$ dry matter. The organic horizon soil was labelled with $0.84 \mu\text{g}$ of $^{15}\text{N g}^{-1}$ dry matter. $^{15}\text{N-NH}_4\text{Cl}$ (99 atom % $^{15}\text{N-NH}_4\text{Cl}$, Cambridge Isotope laboratory, Inc., Andover MA, USA) was dispersed into each soil sample in three separate injections using a syringe. Sub-samples of soil were extracted immediately (samples denoted t_0) with 1 M KCl solution (soil/solution ratio, 1/5, Merck Emsure, Merck KGaA, Darmstadt, Germany) or incubated (17°C , 24 h) and then extracted (t_{24}). After extraction at 120 rpm for 1 h and filtration (Munktell 00H) by gravity NH_4^+ and NO_3^- concentrations in the t_0 and t_{24} extracts were determined by flow injection analysis (FIAsstar 5012, FOSS, Höganäs, Sweden) and stored at -20°C until further analysis.

We used the micro-diffusion technique to prepare solid samples for ^{15}N measurements (EA-IRMS) and the calculated-blank correction (Stark and Hart, 1996) to calculate gross N mineralization rates according to Hart et al. (1994b):

$$m = ([\text{NH}_4^+]_{t_0} - [\text{NH}_4^+]_{t_1})/t * \log(\text{APE}_{t_0}/\text{APE}_{t_1})/\log([\text{NH}_4^+]_{t_0}/[\text{NH}_4^+]_{t_1}) \quad (4)$$

where m = gross N mineralisation rate, t = incubation time (one day), $[\text{NH}_4^+]_{t_0} = \text{NH}_4^+$ pool size at time 0, and $[\text{NH}_4^+]_{t_1} = \text{NH}_4^+$ pool size after 1 day of incubation, $\text{APE} = \text{atom percentage excess} = \text{atom \% } ^{15}\text{N}$ enrichment of the NH_4^+ pool $- 0.3663 \text{ atom \%}$, $\text{APE}_{t_0} = \text{atom \% } ^{15}\text{N}$ excess at time 0, $\text{APE}_{t_1} = \text{atom \% } ^{15}\text{N}$ excess after 1 day of incubation.

Nitrogen retention was calculated as the immobilised ^{15}N at time zero, t_0 (here within 30 s from tracer addition). First, the salt extractable ^{15}N recovered from the 1 M KCl extractable NH_4^+ pool at time zero (Hart et al., 1994b) was calculated as follows:

$$\text{Recovery (\%)} = \left(\text{APE}_{t_0} * [\text{NH}_4^+]_{t_0} / ^{15}\text{N}_{\text{injected}} \right) * 100\% \quad (5)$$

Then, the fraction of ^{15}N retained in soil was calculated by subtracting the fraction of salt extractable ^{15}N from that of added (Högberg et al., 2014a):

$$\text{Retention (\%)} = 100 (\%) - \text{Recovery (\%)} \quad (6)$$

2.7. Statistical analyses

Statistical analyses were performed in SigmaStat (v4.0, Systat Software Inc., CA, USA). The strength of the relationships among the measured variables was tested by Pearson product moment correlation ($n = 15$). We used two-way ANOVA with ecosystem age and transect as factors to test for significant differences among ecosystem ages, transects, and for the interaction between these two factors. This was followed by Holm-Sidak *post hoc* test and multiple pair-wise comparisons. Twenty-five lipid fatty acids biomarker signatures reflecting the microbial community composition were examined in PC-ORD Multivariate Analysis of Ecological Data (version 6.0, MjM software, Gleneden Beach, OR, USA). We normalised amounts of lipid fatty acids and other measures to the C content in the soil. We used (i) proportion data (mol % out of total abundance of PLFAs) for describing microbial community composition, (ii) moles PLFA normalized to amount organic matter (per gram C) as a relative quantification of biomarkers, and (iii) moles PLFA per unit area (m^2) as a quantitative estimate at an ecosystem level to describe the microbial communities. In all subsequent analyses, we used fatty acids that were identifiable and present at 0.5 mol % or higher. Mole percent values sum to a constant value (100%), and thus are not normally distributed. The proportion data were arcsine square root transformed before analysis as recommended (Zar, 1984) to improve multivariate normality (Balsler and Firestone, 2005). Non-metric Multidimensional Scaling (NMS) ordination ended up in linear relationships between the ordination scores (individual variables) and axes, and Pearson product moment correlations were used to examine these. The analysis was followed by multi-response permutation procedures (MRPP) using non-transformed data. The same distance was used (Bray–Curtis) in NMS and MRPP ensuring agreement between these two analyses. The resulting test statistics, T and A, describes the separation between the groups and within-group homogeneity, respectively. The more negative T is, the stronger the separation. An A-statistic equal to 1, indicates that all items in the group are identical; when heterogeneity within groups equals expectation by chance $A = 0$.

An A-value of 0.3 is considered high for environmental data (McCune and Grace, 2002). P -values < 0.05 were considered significant. Means are reported as means ± 1 SE unless otherwise stated.

3. Results

3.1. Soil pH, soil C and N

The pH decreased by more than two units from 6.1 in the uppermost soil from the recently exposed to 4.0 in the F + H horizon of the oldest sites (Fig. 1). The most profound pH decline and the largest accumulation of organic C and N occurred at about 115–150 years ecosystem age (Table 1). The C/N ratio doubled from 16 in the recently exposed 25-year-old to 35 in the oldest 560-year-old site and the latter differed from all the other sites ($P < 0.05$) (Table 1). Soil pH was negatively related to ecosystem age ($R = -0.74$, $P < 0.01$), soil N % ($R = -0.88$, $P < 0.001$), C/N ratio ($R = -0.76$, $P < 0.01$), and to contents of organic matter, C, and N (R between -0.78 and -0.56 , $P < 0.05$). The C/N ratio correlated

Table 1

Site characteristics on the Bjuren island. Values are means (1 SE), N = 3. Row by row, means followed by the same letter are not significantly different ($P > 0.05$). n.a. = not applicable. Ellipses (...) indicate not detectable.

Properties	Ecosystem age (years)				
	25	115	150	215	560
<i>Trees</i>					
Basal area ($\text{m}^2 \text{ha}^{-1}$)	n.a.	13.0 (0.3) ^a	16.3 (2.5) ^a	32.3 (5.0) ^a	27.6 (6.3) ^a
<i>Soil</i> [†]					
N (%)	0.1 (0.0) ^a	0.3 (0.1) ^a	1.9 (0.0) ^b	1.9 (0.2) ^b	1.3 (0.1) ^c
$\text{NH}_4^+ - \text{N}$ (mg m^{-2})	18.9 (2.2) ^a	34.1 (9.9) ^a	26.1 (10.7) ^a	18.5 (5.5) ^a	15.1 (3.6) ^a
$\text{NO}_3^- - \text{N}$ (mg m^{-2})	...	1.1 (1.1)
H_2O ($\text{g g}^{-1}\text{C}$)	28.2 (4.6) ^a	3.5 (0.8) ^b	3.0 (0.4) ^b	4.7 (1.1) ^b	4.6 (1.2) ^b
CEC (cmol kg^{-1})	5.3 (1.1) ^a	13.8 (4.2) ^a	66.3 (14.6) ^b	96.4 (2.9) ^b	100.4 (13.0) ^b
Organic matter (kg m^{-2})	0.2 (0.0) ^a	0.7 (0.1) ^a	3.1 (0.7) ^a	2.4 (0.5) ^a	3.1 (0.9) ^a
Total C (kg m^{-2})	0.1 (0.0) ^a	0.5 (0.1) ^{ab}	2.2 (0.5) ^b	1.4 (0.3) ^{ab}	2.0 (0.3) ^b
Total N (g m^{-2})	8.2 (1.7) ^a	28.9 (4.5) ^{bc}	118.6 (27.2) ^c	57.5 ^{bc} (15.0) ^{bc}	58.5 (13.2) ^{ab}
<i>Microorganisms</i>					
Microbial C (mg g^{-1} soil C)	30.5 (1.2) ^a	12.1 (0.5) ^b	8.5 (1.0) ^b	11.0 (2.2) ^b	12.7 (1.3) ^b
Microbial N (mg g^{-1} soil C)	4.0 (0.5) ^a	2.1 (0.1) ^a	1.2 (0.1) ^a	1.4 (0.2) ^a	1.6 (0.2) ^a
Microbial C/N	7.8 (0.7) ^a	5.9 (0.4) ^a	7.2 (0.3) ^a	7.9 (0.7) ^a	7.7 (0.5) ^a

[†]The F + H horizon, except at ages 25 and 115 years, where the upper 5 cm surface soil was sampled.

strongly with ecosystem age ($R = 0.98$, $P < 0.001$) and negatively to gross N mineralisation ($R = -0.54$, $P < 0.05$).

3.2. Nitrogen content and natural abundance of ^{15}N in soil and foliage

Soil and tree foliar N concentrations (%) (Table 1) and the enrichment factor ($\epsilon_{\text{ffs}} = \delta^{15}\text{N}$ of foliage – $\delta^{15}\text{N}$ of surface or organic soil) declined. A smaller difference in $\delta^{15}\text{N}$ between soil and needles, i.e. ϵ_{ffs} values closer to zero, means higher efficiency of N transport between the soil and trees. The $\delta^{15}\text{N}$ and N% of tree foliage and soil are shown in Fig. 2a and b. The ϵ_{ffs} was -1.0 (0.2), -3.5 (0.9), -3.9 (0.5), and -6.5 (0.4) ‰ in the 115-, 150-, 215- and 560-year-old ecosystems, respectively. The enrichment factor in the 115- and 560-year-old ecosystems differed significantly from the other ages. The decrease with ecosystem age was even more pronounced when the mineral soil was used for the calculation of ϵ_{ffs} .

3.3. Radiocarbon signatures and modelled estimates of C input and turnover rates

Together with the amount of C accumulated over time, radiocarbon signatures of litter and soil organic matter are indicative of

the balance of inputs and loss by decomposition (Trumbore and Harden, 1997). Radiocarbon signatures in the organic layer increased from values close to those expected for recent photosynthetic products in 2012 (about 100.2 percent modern C) in the youngest soil to values of ~120 percent modern C in the 150 and 215-year-old ecosystems, declining again in the 500-year-old ecosystem (Table 2). Radiocarbon values generally declined with soil depth except in the 115-year-old site with alder. The mineral soil radiocarbon signatures were similar in the 150-, 215-, and 560-year-old ecosystems ($P > 0.05$), but the 150-year-old ecosystem differed from the two youngest ones ($P = 0.05$). The concentration of modern C in the organic layer correlated negatively with NH_4^+ pools ($R = -0.84$, $P < 0.001$) and gross N mineralisation when expressed per gram C ($R = -0.79$, $P < 0.001$), but positively with the % of soil N ($R = 0.86$, $P < 0.001$).

A simple model of inputs and decomposition (equations above) can reproduce the C and ^{14}C content of organic layer and 0–20 cm mineral horizons (Table 2). Reproducing the highest ^{14}C values observed (>110 percent modern C) required the introduction of a lag time of at least 15 years for the inputs to both the mor-layer and the mineral soil, starting at the 115 year old ecosystem i.e., when trees started to become the dominant plants. For organic layers, the estimated time-averaged inputs ranged from ~0.01 to

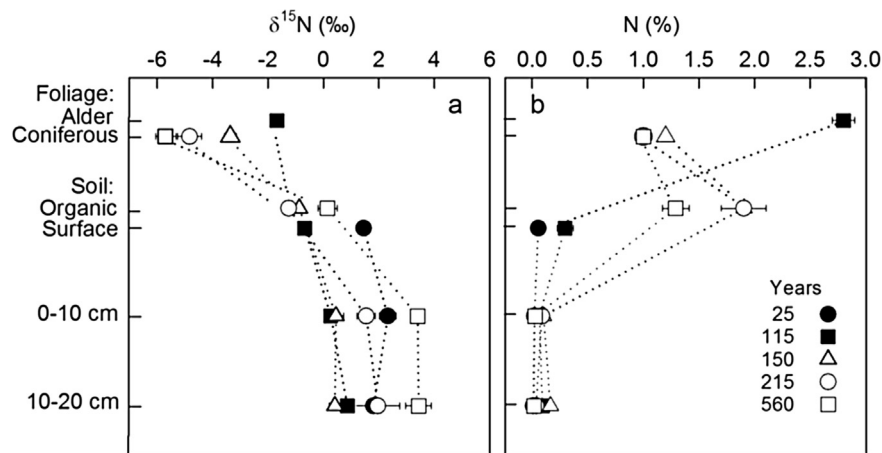


Fig. 2. Natural abundance of ^{15}N , $\delta^{15}\text{N}$ (a), and N% of foliage of dominant trees and across soil profiles (b) in 25-, 115-, 150-, 215-, and 560-year-old ecosystems on Bjuren island in northern Sweden. Soil data were from the upper 5 cm (surface soil) at ages 25 and 115 years and from F + H horizon (organic soil) at the higher ages. Mineral soil was sampled at 0–10 and 10–20 cm depth at all ages. The values are means (± 1 SE), N = 3 per ecosystem age.

Table 2
Summary of outcomes of modelling of soil C turnover. Accumulated C at time (t) since the initiation of soil development, modern C, C input rate, time lag, and turnover time are denoted C(t), FC(t), I, Lag and TT in Equations (2) and (3). I, TT, and k (is the rate constant for first order decomposition) are assumed constant. Lag is a time lag to account for addition of pre-aged C as inputs, as in the case of roots (Gaudinski et al., 2010). n.a. = not applicable.

Organic soil [†]					
Age (t)	Accumulated C at time t (kg C m ⁻²)	Modern C (%)	C Input rate (kg C m ⁻² year ⁻¹)	Time lag (year)	Turnover time = 1/k (year)
25	0.1	101.32	0.055	0	1
115	0.9	108.85	0.064	0	14.5
150	1.9	120.36	0.01	15	19
215	2.2	121.05	0.115	15	19
560	1.7	112.25	0.350 or 0.025	15	4.5 or 65
560	1.7	112.25	0.075 or 0.034	0	23 or 50
0–20 cm Depth mineral					
25	56	95.1	n.a.	n.a.	n.a.
115	352	112.6	5.4	0	85
150	609	113.4	10	15	66
215	344	106.7	2.5	15	200
560	128	99.8	0.29	15	1000
560	128	99.8	0.3	0	>400

[†]The F + H horizon, except at ages 25 and 115 years, where the upper 5 cm surface soil was sampled.

0.12 kg C m⁻² year⁻¹ for organic horizons, with highest inputs in the 215-year-old ecosystem. The youngest mineral soil, which contains inherited C from sediments, could not be modelled (Table 2).

3.4. Soil microbiology

3.4.1. Microbial biomass

We observed a decline in microbial biomass C and N in response to ecosystem age when expressed per g⁻¹ of soil C with a non-significant minimum in the 150-year-old ecosystem (Table 1). When expressed per unit area microbial biomass C and N were positively related to ecosystem age ($R = 0.72$, $P < 0.01$ and $R = 0.69$, $P < 0.01$, respectively) and increased from 4.0 to 0.5 g m⁻², respectively, in the youngest to 26.7 and 3.7 g m⁻², respectively, in the oldest ecosystem (Fig. 3a). Thus, microbial C and N increased eight-fold with age of the ecosystem when expressed per m², a pattern that was in agreement with the increase in total abundance of PLFA biomarkers per unit area. The summed abundance of PLFAs and microbial biomass C correlated ($R = 0.85$, $P < 0.001$), indicating a strong agreement between these measures of microbial biomass C. The mean C/N ratio of the microbial biomass varied between 7.2 and 7.9 at four of the sites, but there was a non-significant minimum at 5.9 in the youngest forest. The microbial C/N ratio correlated negatively to gross N mineralisation ($R = -0.57$, $P < 0.05$), but positively to ¹⁵N retention ($R = 0.68$, $P < 0.01$).

3.4.2. Microbial community composition

The summed abundance of PLFA biomarkers declined per gram C with increasing ecosystem age (Table 3), but the opposite pattern was observed in total abundance of PLFAs when expressed per unit area. Fungal and bacterial PLFA biomarkers followed the same general patterns (Fig. 3b). An increase was also seen in the fungi/bacteria ratio, which doubled from c. 0.3 to 0.6 with increasing ecosystem age. According to the quantitative DNA analyses (Fig. 3b), the PLFA 18:2ω6,9 was of fungal origin at all ecosystem ages as indicated by the high correlation between the two fungal biomarkers ($R = 0.77$, $P < 0.001$). The biomarkers for AM fungi were present only in the two youngest ecosystems; the recently exposed and in the forested 115-year-old ecosystem (Table 3). Total, bacterial, and fungal PLFAs per unit area, and fungi/bacteria ratio were positively related to the ecosystem age ($R = 0.55$ – 0.76 , $P < 0.05$). The molar contribution by PLFAs indicative of gram-negative and

gram-positive bacteria showed opposite trends, and the ratio gram negative/gram positive bacteria decreased by almost 50% ($P < 0.05$) towards the older ecosystems. The abundance of actinobacteria was on average 0.8 mol % and rather stable through ecosystems of all ages.

NMS ordination of PLFAs resulted in a 3-dimensional solution with a final instability 0.00000 and stress 3.2 after 48 iterations, with no prospect of misinterpretation (McCune and Grace, 2002). Two axes described in total 92% of the variation (75 and 17%, respectively), and the third described a few percentages. A Monte-Carlo test (250 runs on randomized data) confirmed the significance of axes ($P = 0.004$). After MRPP (general model, $T = -5.3$, $A = 0.39$, $P = 0.0004$), and pairwise comparison of community composition among ecosystem ages T varied between -1.1 and -3.0, A between 0.1 and 0.5 which is unusually high for ecological data (McCune and Grace, 2002), and P varied between 0.02 and 0.13. The microbial communities in the 25- and 115-year-old ecosystems differed significantly from those in the 150-, 215- and 560-year-old ecosystems. We thus observed major shifts in the composition of microbial communities during the first 150 years of the ecosystem development (Fig. 4).

3.5. Inorganic N, N retention, net and gross N mineralisation

The NH₄⁺ pool size was similar among the ecosystem ages (Table 1). Gross N mineralisation rate per unit C was significantly higher in the recently exposed ecosystem and in the youngest forest (alder) ecosystem (Table 4). The areal gross N mineralisation rates, however, showed no significant trends although the highest mean value was found in the alder forest ecosystem, where at the same time the retention of ¹⁵N tracer was the lowest (~19%). This ecosystem was also the only one where NO₃⁻ was detected (Table 1) and where net N mineralisation (1.7 ± 7.1 mg N m⁻² day⁻¹) occurred. Nitrogen retention declined sharply between the recently exposed and the alder forest ecosystem from 94% to 19% and at the same time the gross N mineralisation rate more than doubled from 33 to 81 mg N m⁻² day⁻¹. Thereafter, with increasing ecosystem age up to 560 years, gross N mineralisation rates decreased from 81 to 23 mg N m⁻² day⁻¹, while at the same time ¹⁵N retention increased from 17 to 88% (Table 4). In the recently exposed soil neither ¹⁵N retention, nor gross N mineralisation per unit area differed from that found in the >150 year-old ecosystems. The inverse

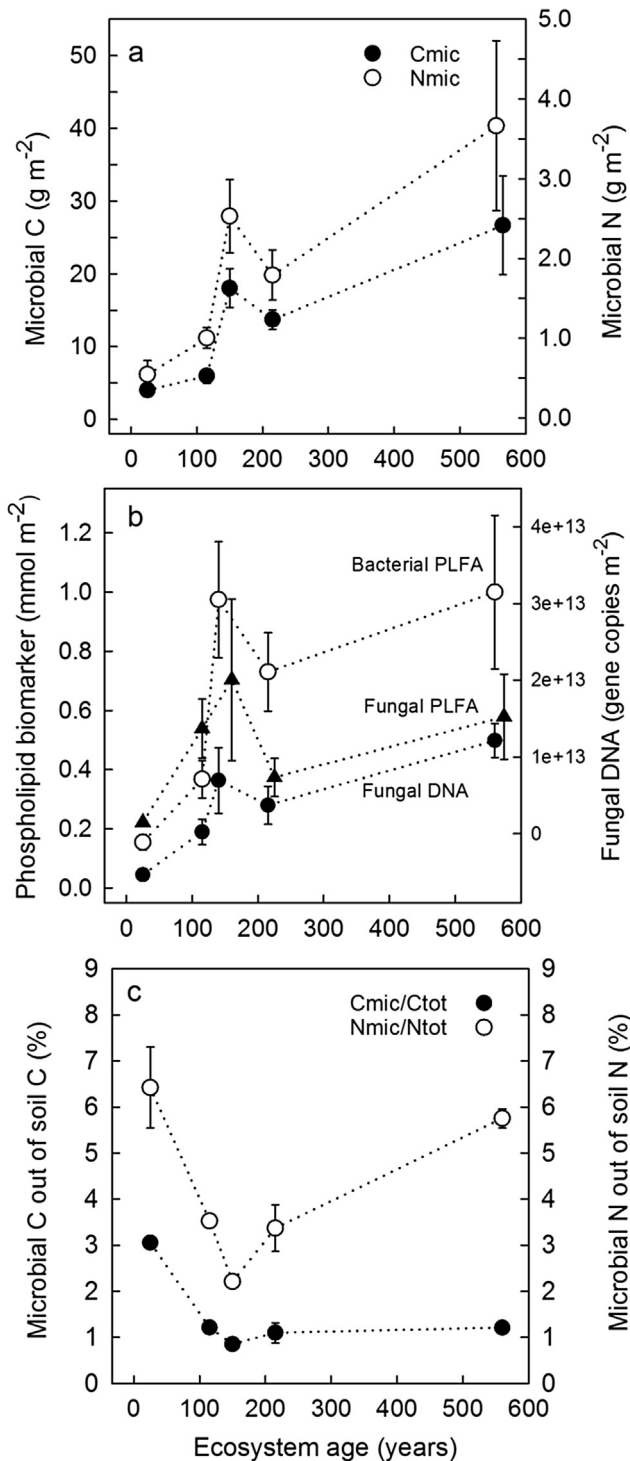


Fig. 3. (a), Microbial biomass C and N per unit area (ecosystem ages are adjusted for clarity), (b), fungal and bacterial abundance per unit area, (c), fractions of microbial C out of total soil C and microbial N out of total soil N versus ecosystem age on the Bjuren island. Soil data were from the upper 5 cm surface soil at ages 25 and 115 years and from F + H horizon at the higher ages. The values are means (± 1 SE), $N = 3$ transects per ecosystem age.

relationship between gross N mineralisation and ^{15}N retention was illustrated by a strong negative correlation ($R = -0.80$, $P < 0.001$). Retention of ^{15}N did not co-vary with CEC of the organic layer ($P > 0.05$). Gross N mineralisation correlated positively to extractable pools of inorganic N ($R = 0.81$, $P < 0.001$), but negatively to soil

C/N ratio ($R = -0.54$, $P < 0.05$). In addition, there were correlations between microbial biomass C and N and soil pH ($R = 0.64$ and 0.58 , $P < 0.05$, respectively), and between fungi/bacteria ratio and soil pH ($R = 0.55$, $P < 0.05$).

4. Discussion

4.1. At which ecosystem age do boreal forests start to progress into N limitation?

The radiocarbon modelling implies decreasing decomposition rates (increasing turnover times), which also implies decreasing N release rates, for material in the organic horizon in sites of higher age. Inputs required to accumulate the large amounts of C in the younger mineral soils tended to be much higher ($2.5\text{--}10 \text{ kg m}^{-2} \text{ year}^{-1}$), then declined to long term averages of $\sim 0.3 \text{ kg C m}^{-2} \text{ year}^{-1}$ in the oldest soil thus approaching rates previously found in boreal forests (Trumbore and Harden, 1997). Decomposition rates in the mineral soils were also lower than in organic layers and similarly decreased with time (turnover time > 66 years) (Table 2). Since the oldest site has lost C in both the mineral and organic layers, compared to the next younger site, the results of a long-term accumulation model are likely not applicable. Instead of very long turnover times at this site, the C and ^{14}C values likely reflect reduced C inputs that may be combined with destabilisation of previously accumulated (high ^{14}C) carbon. A model reproducing these changes would however, require more constraints, such as independent knowledge of annual C inputs.

However, there was evidently a large accumulation build-up of C and total and plant available N during the age dominated by N_2 -fixing alder, in particular. In the meadow, there was likely a contribution by N_2 -fixing free-living cyanobacteria, and in the alder zone also by *Myrica gale*, which like the alder forms N_2 -fixing root nodule symbiosis with actinobacteria (Sellstedt and Richau, 2013), and by the legume *Vicia cracca* forming root nodule symbiosis with rhizobia (Roscher et al., 2011). In systems older than these, N_2 -fixation is likely dominated by cyanobacteria living on the surfaces of mosses and occurs at lower rates (DeLuca et al., 2002). In the phase dominated by alder, the inputs of N can be estimated to be large. It is here the mor-layer starts to form, and during the time between 115 years (alder zone) and 150 years (the youngest conifer system) the build-up of N in the mor-layer amounts to 900 kg N ha^{-1} (Table 1) at a rate of $22.5 \text{ kg N ha}^{-1} \text{ year}^{-1}$. This build-up cannot be attributed to redistribution from the mineral soil, because in the mineral soil horizons there was no evidence of a decrease in % N (Fig. 2b). Rates of N_2 -fixation of $20 \text{ kg N ha}^{-1} \text{ year}^{-1}$ are not exceptional for alder forest, which can fix even more (Johnsrud, 1978; Huss-Danell et al., 1991; Binkley et al., 1992). Using the ^{15}N natural abundance method to estimate the contribution of N_2 -fixation to plant N (see equation in Myrold and Huss-Danell, 2003) and using *Sorbus aucuparia* as the reference plant (data not shown), the relative amount of N acquired by *Alder incana* through N_2 -fixation was 54 and 64% at ecosystem ages 115- and 150-years, respectively. However, the $\delta^{15}\text{N}$ of $-1.6 \pm 0.2\text{‰}$ (SD) for *A. incana* (Fig. 4) are similar to the $-1.8 \pm 0.2\text{‰}$ (SD) reported when all foliage N originated from N-fixation (Domenach et al., 1988) and indicates that our estimate may be conservative.

It is also clear that the availability of N was high in the alder zone, which concurs with the higher decomposition rates suggested by the radiocarbon modelling. The N % in the foliage of the alder was as high as 2.8% and the pool of extractable NH_4^+ in the soil was high, although not significantly higher than elsewhere due to a very high variability between samples. The fact that extractable NO_3^- could only be detected in the alder site indicates that this is the system with highest N supply in relation to the biological sinks.

Table 3
Selected soil microbial community characteristics, based on signature lipid biomarkers. Values are means (1 SE), N = 3. Row by row, means followed by the same letter are not significantly different ($P > 0.05$). Ellipses (...) indicate not detectable.

Properties [†]	Ecosystem age (years)				
	25	115	150	215	560
PLFA (nmol g ⁻¹ soil C)	3244 (271) ^a	2240 (266) ^{ab}	1193 (103) ^b	1481 (247) ^b	1405 (143) ^b
Bacteria (nmol g ⁻¹ soil C)	1195 (95) ^a	752 (61) ^b	446 (27) ^b	362 (82) ^b	476 (58) ^b
Fungi (nmol g ⁻¹ soil C)	326 (3) ^a	402 (100) ^a	164 (22) ^a	218 (47) ^a	254 (49) ^a
Arbuscular fungi (mol%)	3.6 (1.0) ^a	0.8 (0.4) ^b
Fungi/bacteria	0.28 (0.02) ^a	0.52 (0.11) ^a	0.36 (0.04) ^a	0.38 (0.04) ^a	0.55 (0.10) ^a
G-neg bacteria (mol%)	22.0 (1.3) ^a	18.2 (1.3) ^a	18.5 (0.4) ^a	18.5 (0.0) ^a	17.5 (1.8) ^a
G-pos bacteria (mol%)	7.1 (0.6) ^a	8.9 (0.9) ^a	11.6 (0.8) ^a	12.1 (0.8) ^a	10.2 (0.5) ^a
G-neg/G-pos bacteria	3.2 (0.5) ^a	2.1 (0.1) ^{ab}	1.6 (0.1) ^b	1.6 (0.1) ^b	1.7 (0.1) ^b
Actinobacteria (mol%)	0.8 (0.2) ^a	0.8 (0.3) ^a	0.8 (0.0) ^a	0.7 (0.1) ^a	0.9 (0.1) ^a

[†]The F + H horizon, except at ages 25 and 115 years, where the upper 5 cm surface soil was sampled.

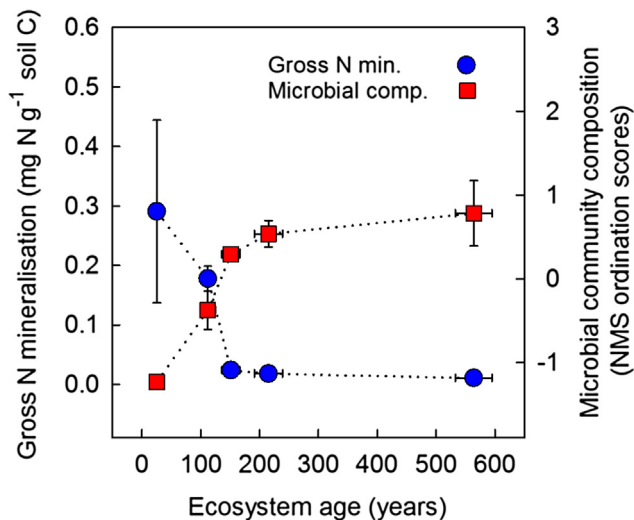


Fig. 4. The relationship between gross N mineralisation rate and soil microbial community composition as summarized by non-metric multivariate scaling (NMS) of microbial biomarkers at different ecosystem ages at Bjuren. The NMS axis describes 75% of the variation. Soil data were from the upper 5 cm surface soil at ages 25 and 115 years and from F + H horizon at the higher ages. The values are means (± 1 SE), N = 3 transects per ecosystem age.

The latter was confirmed by the data on retention of added $^{15}\text{NH}_4^+$, which was below 20% as compared to around 90% elsewhere (Table 4), a difference that was not related to the CEC (Table 1), but should be biologically related. In studies of older boreal forest systems of the region, we commonly found retention of tracer N to be around 80–90% (Högberg et al., 2006, 2014a). Low retention, as found here in the alder zone, has only been recorded in an exceptionally N-rich groundwater discharge area (Högberg et al., 2006) and in plots subject to high levels of N additions (Högberg et al., 2014a).

Table 4
Gross N mineralisation (gross N min), mean residence time (MRT), and retention of ^{15}N label across the ecosystems of different age at Bjuren in 2010. Mean residence time is the average number of days an N atom resides in the $\text{NH}_4^+ - \text{N}$ pool. Values are means (1 SE), N = 3. Row by row, means followed by the same letter are not statistically different ($P > 0.05$).

Properties [†]	Ecosystem age (years)				
	25	115	150	215	560
Gross N min ($\mu\text{g g}^{-1} \text{C day}^{-1}$)	290.6 (153.5) ^a	177.8 (21.0) ^a	24.0 (6.1) ^b	18.2 (5.2) ^b	10.7 (2.0) ^b
Gross N min ($\text{mg m}^{-2} \text{day}^{-1}$)	33.0 (14.7) ^a	80.8 (10.5) ^a	50.2 (14.2) ^a	22.4 (6.5) ^a	22.6 (7.0) ^a
MRT (days) [‡]	0.8 (0.3) ^a	0.4 (0.1) ^a	0.6 (0.1) ^a	1.9 (1.3) ^a	1.0 (0.2) ^a
Retention of ^{15}N (%)	93.7 (1.6) ^a	18.6 (10.3) ^b	87.8 (5.9) ^a	92.5 (2.9) ^a	93.5 (5.1) ^a

[†]The F + H horizon, except at ages 25 and 115 years, where the upper 5 cm surface soil was sampled. [‡] $\text{NH}_4^+ - \text{N}$ pool size divided by gross N mineralization rate.

Several indicators related to N availability declined as sites aged, although not significantly so. However, the fact that N % in foliage, C/N ratio of soil, soil extractable inorganic N and gross N mineralisation (Tables 1 and 2) all declined for sites older than the alder site, along with the significant increase in tracer N retention, suggest that N availability is declining in these systems. A similar declining trend in N availability can be inferred from the increased soil C/N and declining net N mineralisation rates with age of the ecosystems created by iso-static rebound in Finland (Merilä et al., 2002a,b). We would like to stress that after a rapid build-up of the N capital, and rates of N cycling, the availability of N as tested by the retention of the tracer N, rapidly decreased between the alder and the youngest conifer system, i.e. between 115 and 150 years, and remained low thereafter. The nitrogen concentration of 1.0% found in current needles in the old systems is low and typical of strongly N-limited boreal forests (e.g. Sikström, 1997; Reich and Oleksyn, 2004). We conclude that the strong N limitation typical of boreal forest can develop in less than 150 years, a conclusion which is consistent with the rapid decline in N availability found when long-term experimental N additions have been terminated (Högberg et al., 2011, 2014a,b; Blaško et al., 2013).

4.2. Do shifts in microbial biomass and fungal and bacterial contributions coincide with shifts in N cycling rates and N availability?

With the exception of the large early inputs of N, which clearly are made by N_2 -fixing bacteria, especially those symbiotic with alder trees, it is difficult to unequivocally attribute the changes in the N capital and cycling described above to changes in microbial biomass and structure. One important issue is the expression of measures in terms of g soil C versus area (per m²). Areal measures are the most relevant from an ecological perspective. For example, the amount of microbial PLFA biomarkers decline clearly with ecosystem age when expressed per g soil C, but the reverse is true when data are based on microbial PLFA per m² (Fig. 3b). The

proportion of microbial C and N out of total soil C and N pools from the youngest to the oldest ecosystem (Fig. 3c) approached the levels commonly observed in boreal coniferous forests (Bauhus and Khanna, 1999; Högberg and Högberg, 2002; Högberg et al., 2003). Importantly, we observed a doubling in microbial N immobilisation from 2.3 to 5.5% out of total soil N, whereas at the same time the proportion of microbial C out of total C declined from 3 to 1.3% which indicate that the proportion of biomass C out of total C declines whereas the biomass N out of total N increases. This means that at higher ecosystem age of boreal forests a larger share of the soil N but a smaller share of soil C is immobilised in microbial biomass.

It is commonly assumed that bacteria cycle N at higher rates than fungi under C limited conditions, and that this is associated with lower C demand relative to that of N (low C/N ratios) and the lower carbon use efficiency of bacteria (Keiblinger et al., 2010; Franklin et al., 2011). Here, gross N mineralisation per gram soil C (but not per unit area) was significantly higher in the youngest system and in the alder zone compared with the coniferous sites. This parameter, the soil tracer N retention and foliar N % all indicate the highest N supply in relation to biological demand in the alder zone, where microbial cytoplasm C/N showed a non-significant minimum of 5.9 (Table 1), and where microbes thus could be more likely to release N in excess of their own demand. Again, these differences are not all significant, but the N retention capacity was significantly lower (Table 4), which supports the idea that N is cycled at faster rates in the alder zone.

Across the whole range of sites studied, the fungal/bacterial ratio increased significantly with ecosystem age. Simultaneously the rate of gross N mineralisation decreased per gram soil C. The relation between the fungal/bacterial ratio and the rate of soil N cycling was less clear than in previous studies of local natural gradients in N availability (Högberg et al., 2006) or comparisons between N-loaded plots and N-limited control forests (Blaško et al., 2013; Högberg et al., 2014a). Merilä et al. (2002a,b) studied the ecosystem ages from ~100 to 1000 years and found no significant changes in gross N mineralisation rates or in the fungal/bacterial ratio. Our results from multivariate analysis showed that the largest changes in microbial community structure occurred in the youngest systems and that once the conifers became dominant (at 150 years) there was little further change (Fig. 4). The low rates of gross N mineralization in the conifer systems were thus associated with a microbial community that differed significantly in composition from that in younger soils and coincide with the appearance of ECM as the dominant mycorrhizal association (Fig. 2a).

4.3. Can the decline in N availability be linked to a key role of ECM fungi?

The high natural abundance of ^{15}N in ECM fungi (Gebauer and Dietrich, 1993; Taylor et al., 1997; Hobbie and Högberg, 2012) is caused by isotope fractionation and the relatively stronger retention of this isotope by ECM fungi as N is passed through them from the soil to their host trees (Högberg et al., 1999). As a consequence, the plant foliage becomes depleted in ^{15}N relative to the fungi to the extent that plant N is depleted relative to soil total N (Hobbie and Ouimette, 2009). This leads to the development of a soil profile with a ^{15}N -depleted surface soil and a gradual enrichment with increasing soil depth, as observed here (Fig. 2a). It was recently demonstrated that there is no change of ^{15}N in the soil as long as the saprotrophic fungi dominate the uppermost horizons, but enrichment in ^{15}N occurs when ECM fungi appear (Lindahl et al., 2007).

We can safely assume that sedges (Muthukumar et al., 2004), grasses, and herbs in the meadow are non-mycorrhizal or have AM

(as indicated by the presence of AM biomarker there). Alder roots may form both AM and ECM, while spruce and pine only form ECM (Smith and Read, 2008). Hence, we expect a profound increase in the potential influence of ECM fungi in the next older, the coniferous ecosystem. The difference in natural abundance of ^{15}N between soil and foliage, the enrichment factor, ϵ_{fs} , is related to soil N availability (Garten and van Miegroet, 1994). Clearly, the increasing difference in $\delta^{15}\text{N}$ between foliage and soil, especially for conifers, suggests an important influence of ECM fungi over the N cycle (Fig. 2a). This influence changes the difference between $\delta^{15}\text{N}$ of foliage and the 10–20 cm mineral soil in the alder zone –1‰ to less than –9‰ in the oldest conifer forest (Fig. 2a). The latter difference and slightly larger ones are found in soil profiles in many and much older ECM forest ecosystems around the world (Hobbie and Ouimette, 2009). Wallander et al. (2009) also found the same type of soil $\delta^{15}\text{N}$ profile development, but did not concentrate as much on the younger forests as we have done here.

Näsholm et al. (2013) recently showed that the large investment of C made by the trees in their ECM fungi under conditions of strong N limitation actually leads to a large N retention by the fungi, rather than a more active promotion of their contribution to tree N uptake. Here, we find isotopic evidence of a role of ECM in soil N retention (Fig. 2a), along with high immediate retention of tracer N (Table 4). We cannot unequivocally ascribe the increasing N limitation to the action of ECM fungi. The strong circumstantial evidence provided here, however, points in that direction and calls for further detailed and more conclusive studies.

5. Conclusions

We found evidence for declining N availability in an ageing primary boreal forest ecosystem. The declines in soil and foliage N %, extractable pools of NH_4^+ , and gross N mineralisation rates coincided with increasing microbial biomass C and N, abundance of microbial PLFA biomarkers, microbial immobilisation of N, tracer N retention, and shifts in the microbial community structure towards microbial communities dominated by ECM fungi. Carbon modelling indicated that these shifts are associated with a slowing of average decomposition rates for organic matter, and declines in C inputs to soil. We propose that higher percentage modern C in the F + H horizon of the coniferous ecosystems relative to percentage modern C in the upper 5 cm soil from 25-year-old meadow and 115-year-old *Alnus* ecosystems (Table 2) reflects higher inputs of recently fixed C because of higher C-allocation to ECM fungi in the conifer ecosystems under declining N-supply. This is associated with an increasing difference between $\delta^{15}\text{N}$ signatures of tree foliage and deeper soil horizons (Fig. 2a), suggesting an increasing role of ECM fungal mycelium and associated microorganisms as sink for N and the creation of the strong N limitation typical of boreal forests.

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