University of South Bohemia in České Budějovice
Faculty of Science

The Effect of Temperature on Nitrogen Mineralization in Spruce Forest Soils

Bachelor thesis

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Annotation:
The effect of temperature on gross nitrogen mineralization was studied in soils of research site at the Gårdsjön Lake using the ^{15}N pool dilution technique at four temperatures (5, 10, 15, 25°C). Nitrogen mineralization rates in organic and mineral horizons of nitrogen loaded soils were compared to soils with natural nitrogen deposition and an eventual effect of chronic increased nitrogen deposition was examined.

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Introduction

Access to nitrogen (N) sources is one of the key factors limiting growth and development of plants. Their nutrition depends on the availability of N in soil for which plants compete with microorganisms (Jingguo & Bakken 1997). Although many forests have developed on soils with a growth-limiting deficiency of N (Vitousek & Howarth 2007), those conditions have changed by human activities (Aber et al. 2003). Originally N-limited ecosystems, using biological fixation as the primary source of available N, experience increased atmospheric deposition of fossil-fuel combustion and agriculture by-products. Chronic N deposition may disrupt ecosystem's structures and functions, system becomes N-saturated and N is lost in leachate (Dise & Wright 1995). Together with increasing temperature (Rogelj et al. 2012) and the overall global change, soils become even more fragile.

Soil represents the main source of nourishment for plants and changes in environmental conditions, caused by human activities (e.g. N input and temperature), may affect the whole ecosystem. Microorganisms play the pivotal role for plants nutrition, as only they can provide them with bioavailable ammonium ion (NH_4^+) via mineralization of N-containing organic compounds. Thus, when the microbial mineralization is altered, the overall soil N cycle may be affected. To study the process, the rate by which microorganisms utilize N-compounds into NH_4^+ can be measured using the ^{15}N dilution pool technique (Murphy et al. 2003). Experimental measurements of N mineralization rate in soils from regions with increased N deposition together with simulations of global warming in laboratory conditions can therefore serve as background researches for prediction of soil responses to human activities. Determination of N mineralization with respect to the environmental factors thus helps to understand soil responses to global changes and consequently conditions to which plants have to adapt.

Nitrogen Cycle in Soil

Nitrogen (N) is essential to all living organisms. Its most abundant form is non-reactive atmospheric nitrogen (N_2) and its bioavailability is therefore limited. N primarily enters the soil by means of simple deposition of N species from the atmosphere, but also via biological fixation of N_2, which only prokaryotic organisms are capable of (Rascio & La Rocca 2008) (*Fig 1.*). Microorganisms in the soil then participate on the decomposition of the Soil Organic Matter (SOM), comprising of dead organisms, underground plant structures, exudates and litter. During the decomposition of SOM, N is released in soluble organic forms (Dissolved Organic Nitrogen (DON)) which might be converted into ammonium ions (NH_4^+) in the process of N mineralization. Plants and microorganisms can immobilize this reactive N and assimilate it into their bodies, or it can be oxidized into nitrite (NO_2^-) and further into nitrate (NO_3^-) by nitrifying microorganisms (Sinha & Annachhatre 2007). The nitrates may be (i) immobilized by plants and microorganisms (assimilative nitrate reduction), (ii) converted back into ammonium ion (Dissimilative Nitrate Reduction (DNRA)), or (iii) released back into to the atmosphere as N oxides (NO, N_2O) and N_2 via denitrification. The remaining nitrates can be easily leached from the soil due to their high mobility. During the transformations of N in the soil, one form of N can be converted into another without accumulation in living structures (Kariminiaae-Hamedaani et al. 2004). Molecules might enter the cycle at any point as well as they can be absorbed, and the N pathway is therefore one of the most complex among nutrient cycles.

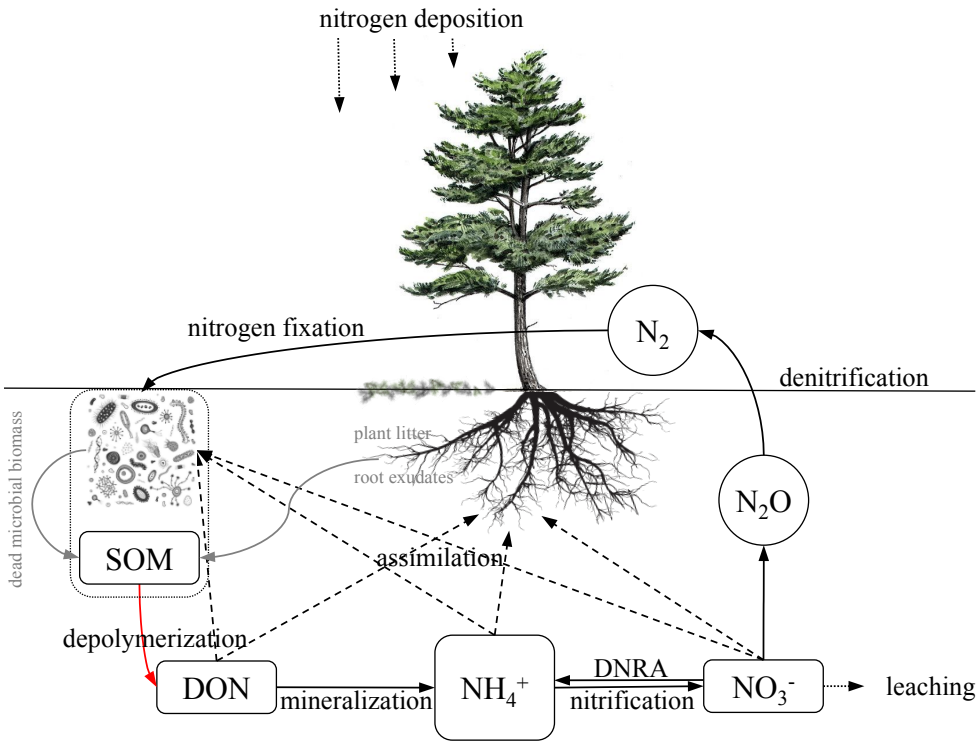

Fig. 1: Basic N transformations in soil. N enters the soil via wet and dry deposition of atmospheric N species (nitrogen oxides, nitric acid vapour, gaseous ammonia, nitrate, ammonium, partly organic nitrogen in DON) and by nitrogen fixation. N as a component of SOM undergoes depolymerization into DON, following by mineralization into ammonium cation and nitrification into nitrate anion. N_2O is the intermediate and N_2 is the final product of the denitrification cascade. Either DON, NH_4^+ and NO_3^- can be immobilized and assimilated by plants or microorganisms.

Nitrogen Fixation

The initial step in the incorporation of N into the soil is N fixation carried out exclusively by prokaryotes (diazotrophic microorganisms). The atmospheric nitrogen (N_2) is captured, cleaved, converted into ammonia (NH_3) and incorporated into living structures. The N_2 molecule is rather inert, comprising two N atoms held together by a triple covalent bond. The cleavage is therefore coupled to the hydrolysis of 16 equivalents of ATP and is thus energetically highly demanding[1]. The reaction also requires an electron and hydrogen ion supply in anaerobic conditions. The necessary energy, which is obtained from sugars in the form of ATP, can be produced by the prokaryotes themselves (e.g. photoautotrophic cyanobacteria), alternatively these microorganisms may live in intimate symbiotic associations with plants or with other organisms (e.g. protozoa). Beside the energy, symbionts provide them with an oxygen-free environment. As soon as N is integrated into NH_3, it is released from the nitrogenase complex and can be directly assimilated by prokaryotic organisms. The excess NH_3 is easily protonated in the soil medium resulting in NH_4^+ which may be immobilized by a plant or another soil microorganism. Subsequent incorporation into proteins and other organic N structures proceeds during the assimilation processes (Rascio & La Rocca 2008).

Nitrogen Mineralization and Assimilation

N mineralization used to be recognized as the principal process of the N cycle, however, this paradigm has changed over the last decade (Schimel & Bennett 2004). The importance of N mineralization was supported by the two former core assumptions that (i) plants use only inorganic N forms and (ii) they poorly compete for N with soil microorganisms. However, the utilisation of organic N by plants and their successful competition for N with microorganisms was proved in the late 1990s (Jingguo & Bakken 1997; Nasholm et al. 1998). As mineralization is no longer recognized as the crucial step for plant nutrition, the regulation of N cycle has shifted towards the depolymerisation as the initial process during which N-containing compounds are decomposed.

[1] The N fixation can be summarized into equation: $N_2 + 8H^+ + 8e^- + 16\ ATP = 2NH_3 + H_2 + 16ADP + 16\ Pi$ (Hoffman et al. 2014). The reaction is catalysed by the nitrogenase enzyme complex with iron-molybdenum cofactor (Fe-Mo-cofactor). Electrons released upon the oxidation of ferredoxin are passed via series of oxidation and reduction steps of the Fe-Mo-cofactor and used for the reduction of N_2 into HN=NH, further into H_2N-NH_2 and finally two molecules of NH_3 are produced. The reduced ferredoxin, required as the electron donor, is generated by photosynthesis, respiration or fermentation, depending on the type of microorganism.

Since N in soil is frequently bound in large organic polymers (e.g. proteins, nucleic acids, peptidoglycan, chitin) forming the SOM, its bioavailability and subsequent transformation depends upon the depolymerisation of such complexes into DON. During this process, N-containing polymers, originated from either animal or plant remains, are cleaved into smaller fractions[2] by extracellular enzymes of heterotrophic microorganisms. The resulting monomers may be absorbed either by microorganisms or plants. It induces their competition for the available N, and therefore the depolymerisation step regulates the entire N cycle (Schimel & Bennett 2004).

N mineralization itself is the direct microbial transformation of N-monomers into NH_4^+. The reaction occurs under aerobic conditions and involves oxidation of DON into mineral N. In return, it provides ammonifying bacteria with carbon (C) for building their biomass. The turnover of DON into NH_4^+ itself is often called Gross Nitrogen Mineralization. The resulting NH_4^+ can be either assimilated during the process, excreted and immobilized by other microorganisms and plants or it can serve as the substrate for number of further reactions. The assimilation of the NH_4^+ proceeds via two pathways[3] depending on the concentration of the ion. Excess NH_4^+ can be utilized during nitrification. The remaining available amount of mineralized N, which has not been consumed (assimilated or further transformed) by microorganisms or absorbed by plants, is referred to as Net Mineralization (Bruun et al. 2006).

The mineralization of N depends on the C/N ratio of the organic material (substrate) (Bengtsson et al. 2003). It determines the decomposability of material and the equilibrium between microbial mineralization and immobilization. Substrates with high C/N ratios (thus low N) induce the immobilisation of mineral N available in soil as microorganisms require additional N to decompose organic material with the high content of C. Low C/N ratio substrates (with high content of N) provide sufficient N supply during the decomposition and excess N (NH_4^+) is released into the soil via mineralization. Every soil system has a different

[2] Proteins are broken down by variety of proteinases, proteases and peptidases into individual amino acids. Cell wall polymers are split into single amino sugar monomers. Nucleic acids are degraded by RNases and DNases into nucleotides. Urea is hydrolysed into carbon dioxide and ammonium.

[3] Either ammonium ion is joined with 2-oxoglutarate (α-Ketoglutarate) to form glutamate in the glutamate dehydrogenase complex catalysed by the oxidation of $NADPH+H^+$ into $NADP^+$, or during the ATP-dependent assimilation of ammonium ion into the glutamate amide group producing glutamine. The glutamine is then transferred to the 2-oxoglutarate, and the following cleavage yields two molecules of glutamate by the glutamine synthetase–glutamine synthase concerted reaction. The resulting glutamate is used for building up amino acids and further the microbial mass.

threshold C/N ratio value between N mineralization and immobilization (Gundersen et al. 1998). This threshold is estimated to be 20–40 globally, and lower than 32 for European boreal forest ecosystems (Šantrůčková et al. 2006), below which mineralization prevails. The equilibrium between immobilization and mineralization further depends on the composition of the microbial community i.e. fungi-to-bacteria ratio. Fungi have a C/N ratio around 15–20 and start to release NH_3 via mineralization when the C/N ratio of the substrate drops below 20, whereas bacteria with C/N ratio around 5–8 would still immobilize N. However, there processes are also influenced by soil properties, substrate availability and composition (other nutrients, e.g. phosphorus) as well as the temperature and moisture of the soil.

Nitrification

The next step in the N in cycle is the biological oxidation of NH_4^+ via nitrite (NO_2^-) to nitrate (NO_3^-) known as nitrification, which is enabled by the microbial activity of chemolithoautotrophs. The process is presumed to be a two-step reaction[4] conducted by ammonia-oxidizing bacteria (AOB) or archaea (AOA) and nitrite oxidizing bacteria (NOB), coupled to a high-energy gain. During the reactions[5], two molecules of hydrogen ion (H^+) are released for each NH_4^+, contributing to an acidification of the soil. Moreover, in contrast to positively charged NH_4^+ ions which are usually adsorbed to negatively charged clay particles and SOM, the resulting NO_3^- is highly mobile and can be easily washed out of the soil (Van Miegroet & Cole 1984). Both the substrate (NH_4^+) and the product (NO_3^-) can be utilized by plants and microbes (immobilization and assimilative nitrate reduction). Therefore, net and gross nitrification should be distinguished. Gross Nitrification is recognized as the conversion of NH_4^+ to NO_3^- regardless of consumption, whereas Net Nitrification describes the change of NO_3^- pool available in the soil over a period of time (Verchot et al. 2001). There are various physical, environmental and chemical factors that affect soil nitrification (Sahrawat 2008). Of these factors, soil pH, temperature, level of oxygen, moisture, substrate (NH_4^+) concentration and its availability are the most important as well at the population of nitrifying organisms. Nitrate which has not been immobilized

[4] However, based on the presumption that one complete oxidation of ammonium would yield more energy than two separate steps, the completely nitrifying bacterium from the genus *Nitrospira* has been discovered (Daims et al. 2015)

[5] $2NH_4^+ + 3O_2 \rightarrow 2NO_2^- + 2H_2O + 4H^+$
$2NO_2^- + 1O_2 \rightarrow 2NO_3^-$

may be converted back to NH_3 (Dissimilative Nitrate Reduction (DNRA[6])) or further transformed via denitrification.

Denitrification

Denitrification is the microbial process reducing nitrate (NO_3^-) to gaseous N. Denitrifying bacteria[7] are anaerobes that use nitrate as a terminal electron acceptor instead of O_2 during the electron transport chain, since it has, like oxygen, high reduction potential. During this sequential reduction[8], catalysed by corresponding reductases, protons are transported across the membrane. The arising electrochemical gradient is crucial for oxidative photorespiration and other related energy transduction processes. Therefore, denitrification along with DNRA is the highest-energy-yielding system in anoxic environments (Strohm et al. 2007). Besides the energetic benefits, denitrification is also the only N transformation that actively removes N from the soil ecosystem. Not only is the final product (N_2) rapidly released into the atmosphere, but also several intermediates are developed and can escape. Among the most environmentally important of those are nitric (NO) and nitrous oxides (N_2O). The increase in NO concentration in the atmosphere contributes to an acid rain[9] (Pilegaard 2013) and ozone depletion. N_2O is an important greenhouse gas (among CO_2 and CH_4) (Wrage et al. 2001) which can also be converted to NO and damage the ozone layer (Ravishankara et al. 2009). Therefore, the intermediates of denitrification may have a strong environmental impact.

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[6] Direct reduction of nitrate to ammonium: $NO_3^- \rightarrow NO_2^- \rightarrow NH_4^+$. The dissimilatory pathway uses nitrate as the electron acceptor and generates energy. DNRA does not contribute to N-removal, but rather recycles fixed N in the environment.

[7] Recently has been discovered also denitrification activity of fungi (Shoun et al. 1992)

[8] $NO_3^- \rightarrow NO_2^- \rightarrow NO \rightarrow N_2O \rightarrow N_2$

[9] $\cdot NO + HO_2\cdot \rightarrow \cdot NO_2 + \cdot OH$

$\cdot NO_2 + \cdot OH \rightarrow HNO_3$

Human Impact on The Nitrogen Mineralization in Spruce Forest Soil

Since the beginning of the industrial revolution in the mid-18th century, human activity has significantly altered biogeochemical cycling at regional and global scales. Progressive industrial and agricultural activities have been causing an increase in concentration of atmospheric N-species and consequently promote the global warming (Vitousek et al. 1997). Major questions are how terrestrial ecosystems respond to the increase of N inputs and changes in temperature and if those shifts can be predictable in the future.

The Effect of Elevated Nitrogen Deposition

Moderate temperature of the temperate and boreal climate is apart from the parental material, relief, organisms and time one of the principal soil formation factors (Bockheim et al. 2014). It induces a rather slow decomposition rate and forest soils developed in such conditions have in general larger SOM pool containing N that cannot be easily utilized by microorganisms and plants. Consequently, as the N-bioavailability is directly linked to the decomposition and depolymerization processes, temperate and boreal forest soils are traditionally N-limited (Vitousek & Howarth 2007; Lupi et al. 2013). The internal N cycle is therefore considered as largely closed system, where an eventual microbial mineralization is tightly coupled to the immobilization. Under the limiting conditions, N is lost mainly as DON and leaching of nitrates (NO_3^-) is negligible. N is removed from the soil ecosystem as harmless inert dinitrogen (N_2) rather than nitrous oxide (N_2O), a greenhouse gas (Rennenberg & Dannenmann 2015).

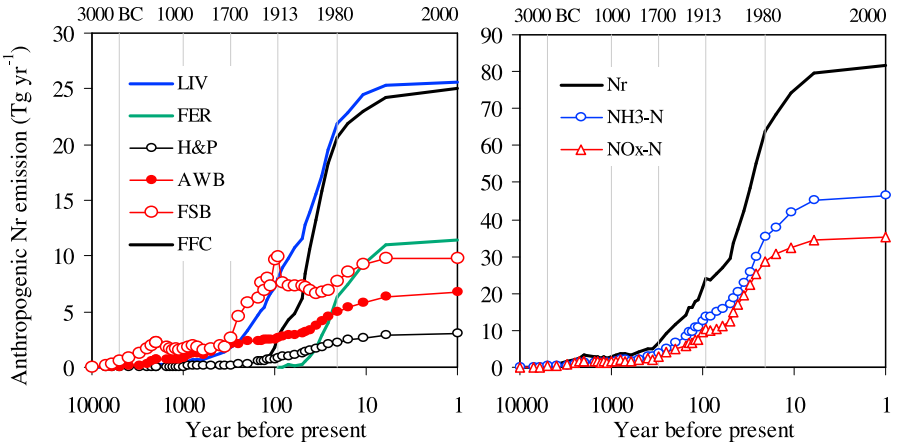

Fig. 2a and b: Global N emissions. From livestock production (LIV), application of synthetic N fertilizers (FER), human excreta and domestic pets (H&P), burning of agricultural waste and biofuel (AWB), forest and savannah burning (FSB), fossil fuel combustion (FFC), and total emission of NH_3–N, NOx–N, and total reactive N (Nr). Time is expressed in logarithmic scale (1 = year 2000; numbers on top are calendar years). Adapted from Kopáček & Posch 2011.

Although human activities have influenced the overall N transformations since the advent of agriculture (8000 B.C) (Kopáček & Posch 2011), the N emissions have been steeply increasing since the beginning of industrial revolution (*Fig. 2a* and *b*). Emissions of reactive N (Nr) species, including N oxides (NO_x) from combustion processes and NH_3 from agricultural activities, have changed the input of N into the soil (Galloway et al. 2008). N is thus no longer the limiting nutrient (Aber et al. 2003). Forests can accumulate available N, released by microbial decomposition, by an enhanced plant growth or by mycorrhizal assimilation[10]. However, with subsequent decomposition of plant tissues, concentration of N in SOM further increases. When the mineralized N can no longer be assimilated (by plants or microorganisms), the N deposition exceeds the total retention capacity and the system is N-saturated (Aber et al. 1989; Tamm et al. 1995). Nitrifying bacteria utilize the large NH_4^+ pool and leaching of highly mobile NO_3^- indicates the N-saturated state (Dise & Wright 1995; Peterjohn et al. 1996; Rogora 2007). Besides, it also contributes to gradual acidification and degradation of the soil (Adams et al. 2007).

The N retention requires an effective conversion of a mineral N to an organic form that will reside within the soil system for an extended period. Critical factors are the pool sizes and transformations of NH_4^+ and NO_3^-, for which plants, heterotrophic microbes and nitrifiers compete. The balance between the gross production (mineralization and nitrification) and gross consumption (assimilation and further transformation) is the net production. In the N-limited system, the consumption of NH_4^+ and NO_3^- is more effective, resulting in much lower rates of net mineralization and net nitrification (10–20 times), than in N-saturated conditions (Aber 1990; Verchot et al. 2001). Such low rates can indicate that assimilation is effective and N is maintained within the closed system. The opposite is true for N-saturated systems, where the retention capacity has been exceeded, the rates of net productions are higher and N can no longer be retained.

However, based on the few existing studies, there is no clear trend of gross mineralization in N-saturated soils. With an increasing availability of N, the mineralization rates may increase as microorganisms utilize the available organic N. This is confirmed by studies revealing higher gross N mineralization in N-saturated soils (Tietema 1998; Chen & Högberg 2006)

[10] which uses its photosynthetic host as the carbon source and can therefore utilize the N input without consumption of carbon from the soil. N can be assimilated and retained in the mycorrhizal system for years (Aber et al. 1998).

than in soils N-limited. Contrary to such results, other found N mineralization negatively correlated with high N additions (e.g. Venterea et al. 2004). Apart from functional differences between soils, duration and level of N deposition are of great importance. Under a chronic high N input, the microbial activity may be suppressed (Bowden et al. 2004; DeForest et al. 2004; Freedman et al. 2013) and the gross N mineralization rate may be therefore reduced. However, when the N input was reduced on sites previously determined as N-saturated with low mineralization rates, the rates increased again, suggesting a recovery of microbial activity. Such results indicate a reversibility of N-saturation (Corre & Lamersdorf 2004).

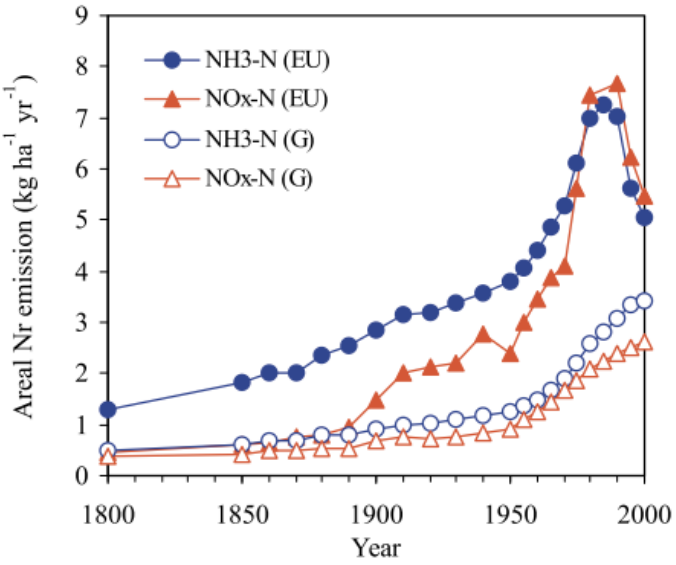

Fig. 3: Areal European (EU; 1.05 Gha) and global (G; 13.6 Gha, excluding Antarctica) Nr emissions rates calculated on a land area basis. Adapted from Kopáček & Posch 2011.

Though N emissions and consequently depositions have decreased during the last 50 years especially in the central Europe (*Fig. 3*), once an ecosystem enters the N-saturated state, it is not easy to fully recover even after a decade. (Chen & Högberg 2006). Moreover, the emissions of Nr are continuous threats for majority of ecosystems globally as they are still increasing especially in the eastern Asia (e.g. Liu et al. 2013). Therefore, it is important to understand the N transformation processes in N-saturated soil, which may also help to predict consequences of further increase in atmospheric N species concentration following by deposition.

The Effect of Elevated Temperature

The concentration of greenhouse gases in the atmosphere has been increasing since the mid 18th century (Houghton et al. 1990). These gases can trap the heat in the atmosphere and cause a considerable heating of the Earth (Arrhenius 1897). New findings repeatedly confirm the rise in global average surface temperatures since 1900. Over the period 1880–2012 temperature data show the linear trend of global surface warming 0.85 [0.65–1.06] °C (Hartmann et al. 2013). The even further increasing trend is likely to continue as the atmosphere temperature is about to increase 2–4.5°C over the 21st century (Rogelj et al. 2012) (*Fig. 4*). Soil temperatures can change accordingly to atmospheric temperatures, but are also influenced by the presence or absence of snow layer on the surface. The increase in air temperature may lead to snow and ice melting during the winter season. As the soil surface is then less insulated and the surface more exposed, its temperature could be temporarily reduced (Wisser et al. 2011). However, the climate change includes not only the increasing atmospheric and surface temperature, but also changes in humidity and nutrient cycles globally.

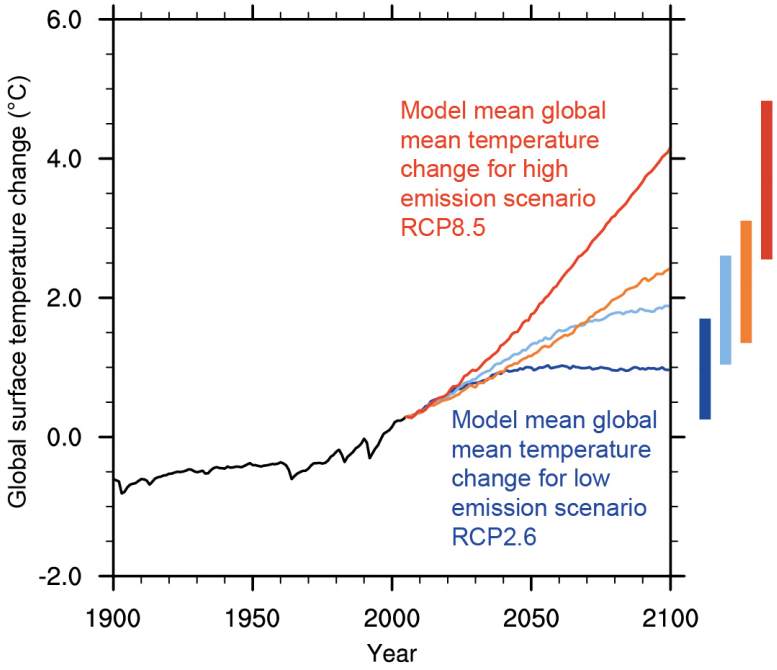

Fig. 4: Global mean temperature change averaged across all Coupled Model Intercomparison Project Phase 5 (CMIP5) models (relative to 1986–2005). For the four Representative Concentration Pathway (RCP) scenarios: PCP2.6 (dark blue), RCP6.0 (orange) and RCP8.5 (red). Likely ranges for global temperature change by the end of 21st century are indicated by vertical bars (these ranges apply to the difference between two 20-year means, 2081–2100 relative to 1986–2005). Adapted from IPCC: Collins et al. 2013.

The principal effect of the increasing soil temperature is the enhancement of microbial activity, as with the increasing temperature the rate of chemical reactions generally becomes higher (Aquilanti et al. 2010). The rates of reactions which involve microorganisms and are catalysed by enzymes increase likewise, until the point beyond which their activity is progressively inactivated (Zantua & Bremner 1977). The activity soil of microbial enzymes, responsible for the decomposition and depolymerization of organic matter, correlates positively to temperature (Fraser et al. 2013; Koch et al. 2007; Schindlbacher et al. 2011). With elevated temperature, the decomposition of organic matter is faster. With the faster decomposition, the quantity of DON (bioavailable N pool) and the quality of dissolved substrate (C/N ratio) are higher (Cookson et al. 2007). Temperature is therefore one of the key factor controlling the N soil transformations.

DON can be mineralized by microorganism and resulting NH_4^+ immobilized or further transformed. Net rates refer to balance between consumption and mineralization both affected by temperature. While some studies confirm significant increase in the rate of net mineralization under the effect of increasing temperature (Sierra 1997; Rustad et al. 2001; Bai et al. 2013; Schütt et al. 2014), others refer no effect (e.g. Auyeung et al. 2013). However, the net transformation rates factor in the immobilization and other consumptive processes and they might not provide the clear information about the temperature dependency of the mineralization itself (Schmidt et al. 1999). When consumption exceeds the mineralization capacity the net mineralization appears negative (Stottlemyer et al. 2001).

Gross rates describe the actual rate at which N is being mineralized from DON (Schütt et al. 2014). When compared to the net mineralization rate, gross rates are an order of magnitude higher than the net one (Hart et al. 1994; Zaman & Chang 2004). Higher gross N mineralization rates with increasing temperature have been also measured, but they are not correlated with the net rates (Hart et al. 1994). Such imbalance suggests that microorganisms do not mineralize N at the same rate as they immobilize or utilize resulting NH_4^+ in other consumption processes. It indicates, that factors controlling those processes do not affect them equally.

The response of soil microorganisms to the temperature increase is tightly connected to their temperature sensitivity[11], substrate quality and availability and other environmental factors such as soil moisture and aeration. Based on the Arrhenius enzyme kinetic theory, the larger the activation energy (E_a), the stronger the system responds and the bigger is temperature sensitivity (Q_{10}). Processing a low-quality (i.e. complex) substrate requires larger E_a and the system responds stronger to the temperature change, whereas simple substrate is easier to decompose and the response is mild (Fierer et al. 2005). The Q_{10} of N mineralization is therefore inversely related to the quality of SOM (Koch et al. 2007; Schütt et al. 2014). Similarly, when the substrate is stabilized and its availability is low or microorganisms are limited by pH, water or oxygen supply, the temperature responses are mild as well (Gershenson et al. 2009). However, the Q_{10} is not constant over the range of temperature changes, as activity of microbial enzymes, responsible for the soil N transformations, can adopt to increased temperatures (Fraser et al. 2013). Microorganisms of soils from colder climatic zones (with mean annual temperatures < 2°C) respond to rising temperature by a steep increase in their activity, compared to those originated in warmer regions (Dessureault-Rompré et al. 2010). Consequently, Q_{10} decreases with the temperature increase (Dalias et al. 2002; Auyeung et al. 2013).

Faster decomposition together with increase of gross N mineralization rates promoted by elevated temperature may provide new evidence that the rate of soil N mineralization in colder regions are likely to increase under global warming scenarios. One of the reasons may be higher Q_{10} values of soil microbial activity in such regions. However, as N mineralization is affected not only by the temperature, such assumptions cannot be applied in general. The arising question is, in which conditions the increasing temperature stimulates the overall N soil transformation. Moreover, response of soil microorganisms and their enzymes is of great concern because of potentially increasing global temperatures and their positive feedback on the emission of oxides from soil that contributes to the climate change (Saad & Conrad 1993; Kirschbaum 1995).

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[11] temperature sensitivity (Q_{10}) is the response of system to change in temperature by 10 K;
$Q_{10} = (k_2/k_1)^{\{10/(T_2-T_1)\}}$, k_2 and k_1 are rate constants for a process of interest observed at two temperatures T_2 and T_1 with $\Delta T = 10$
The temperature sensitivity is also related to the Arrhenius equation: $k = Ae^{-E_a/(RT)}$ which serves as the theoretical justification of the system behaviour; k is the rate constant, absolute temperature T, A is the pre-exponential constant (frequency factor), E_a is the activation energy for the reaction and R is the universal gas constant

Aims of the Study

The aims of presented study were to: (i) determine the effect of temperature on gross N mineralization in soils of Sweden Gårdsjön Lake catchment using the ^{15}N pool dilution technique and (ii) compare N mineralization rates in organic and mineral soil horizons and to understand whether the chronic increased N deposition has any influence on the N mineralization rate.

Hypotheses

Based on literature revised, two principal hypotheses have emerged: (i) With increasing temperature, the rate of gross N mineralization will be increasing as well as the net N mineralization, but rates do not mutually correlate. (ii) Under a chronic high N deposition, gross and net N mineralization will be higher than in not impacted soils.

Experimental Set-up and Procedure

Study Site Description

The research site at the Gårdsjön Lake is located 14 km from the west coast of Sweden (58° 04' N, 12° 01' E). The catchment covers 0.52 ha at the elevation 135–145 m above sea level and it is covered by mature forest (mainly Norway spruce; *Picea abies* (L.) Karst). Soils are predominantly silty and sandy podzols with mean soil depth 38 cm (Kjønaas et al. 1998). The site naturally receives moderate deposition (throughfall) of oxidised (NO_x) and reduced (NH_3) N species of 13 kg $N \cdot ha^{-1} \cdot y^{-1}$. N deposition has been increased at the catchment during the NITREX project[12] started in 1991 by weekly or fortnightly sprinkling of ammonium nitrate (NH_4NO_3) with average annual additions 39.8 kg $N \cdot ha^{-1} \cdot y^{-1}$. The sum loading of about 53 kg $N \cdot ha^{-1} \cdot y^{-1}$ continues to this day. The untreated site served as a control with similar conditions as the experimental NITREX site.

Soil Sampling

Soil samples from the Gårdsjön Lake catchment were collected in September 2015 from organic (O) horizon (partially decomposed Oe (fermentation F layer) + well-decomposed Oa (humification H layer); summary noted as organic horizon) and mineral topsoil A horizon (5–10 cm; noted as mineral horizon), in total 32 samples: 16 from the experimental site (treatment N) and 16 from the control site (treatment C), from each 8 samples were from wet and 8 samples from dry areas. Immediately after the sampling, soil samples were further processed.

Experimental Design and ^{15}N Labelling

Fresh soil samples were sieved (0.4 cm) and 25 g portions were weighed into glass flasks (250 ml). Soil was then moistened to 50–60 % of maximal water holding capacity, covered with perforated parafilm and kept incubated for three weeks at four discrete temperatures: 5, 10, 15 and 25°C. Soil moisture was controlled during the whole period.

After the incubation period, 5 g from each soil sample were weighted into 50 ml centrifuged tubes and labelled with ^{15}N in the form of ^{15}N-NH_4 to achieved N addition of 2 µg N (99 % ^{15}N) per 1 g of soil dry weight. Soil samples were then manually stirred and kept incubated at the initial temperatures for another 4 (t_0) or 24 (t_1) hours.

[12] Nitrogen Saturation Experiments designed to study the effect of chronic N deposition to or removal from European coniferous forest catchments (Wright & van Breemen 1995)

Samples were then extracted by shaking (45 min, 150 opm) with K_2SO_4 solution (0.5 M; soil/extractant, 1:4, w/v) on horizontal shaker. The soil slurry was centrifuged for 15 min at 4000 rpm and the supernatant was filtrated through glass fibre filter (Machery–Nagel, GmbH & Co., Germany, ø 240 mm). Filtrates were kept frozen at –20°C prior to analysis itself. The concentrations of NH_3 were measured using the flow injection analyser (FIA; QuickChem 8500, Lachat Instruments, USA)[13].

Isotope Ratio Mass spectrometry (IRMS) Analyses

Filtrates were prepared for ^{15}N isotopic analysis using the diffusion procedure derived from Brooks et al. (1989) and Stark & Hart (1996) according to (Goerges & Dittert 1998). Extracts were put into glass flasks (250 ml), NaOH (10 M) was added and flasks were immediately enclosed by double lid (air tight) containing filter paper acid traps (⌀ = 0,5 cm, ashless, Whatman filter papers, UK) treated with $KHSO_4$ solution (2.5 M; 5 µl) and embedded in two PTFE (Teflon) tape strips (*Fig. 5*). Diffusion of NH_4^+ proceeded on horizontal shaker (6 days, 140 opm, 24°C). After the period, filters were dried overnight in desiccator with H_2SO_4), packed in tin capsules and measured for $^{15}N/^{14}N$ ratio and total N on IRMS (IR-MS Delta X Plus, Finnigan, Germany) connected to NC analyser (Elementar analyser FLASH 2000, Thermofisher Sci., Germany)

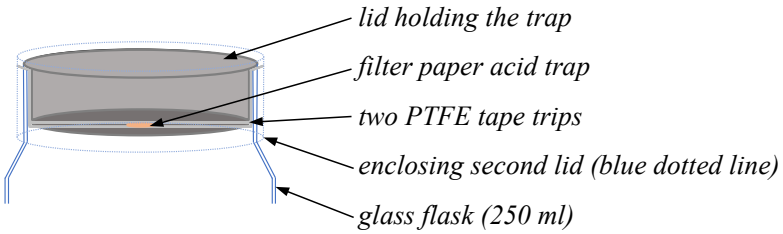
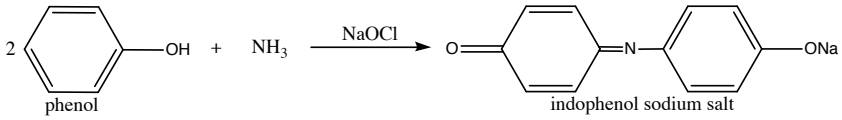


Fig. 5 Set-up for diffusion procedure – air-tight double lid

[13] i) The method for determination of total ammonia (NH_3) concentration is based on the Berthelot reaction:



Absorbance of the resulting indophenol (blue) was measured at 630 nm.

Calculations

Data were expressed on soil dry weight basis. Dry weight of soil samples was determined by weighing the naturally wet soil before and after being dried in oven (105°C for 5 hours) according to the equation:

$$dry\ weight = \frac{total\ weight\ [g]\ dry}{total\ weight\ [g]\ wet}$$

The net mineralization rates were calculated as the total N mass difference (Δ) during the first (t_0) and the second (t_1) time per unit of soil dry weight. The rate was calculated per one day.

$$net\ N\ mineralization = \ (NH_4^+)_{t_1} - (NH_4^+)_{t_0} [\mu g\ N\ g^{-1} soil\ dry\ weight\ day^{-1}]$$

The data of IRMS analyses (δ ^{15}N [‰] vs. AT-air) of IRMS analyses were converted into atom percent (*atm %*) according to the equation:

$$atm = \frac{100 \cdot AR \cdot \frac{\delta}{1000+1}}{1 + AR \cdot \frac{\delta}{1000+1}}\ [\%]$$

where $AR = 0.0036764$ (molar ratio $^{15}N/^{14}N$ of atmospheric N_2). The correction of actual *atm %* ^{15}N enrichments (E_s) using a calculated blank was based on Stark & Hart (1996). The mass of N in the blank (M_b) was calculated by comparing diffused and non-diffused labelled standards as:

$$M_b = \frac{M_{std}(E_d - E_n)}{(E_b - E_d)}$$

where M_{std} is the mass of N in the standard, E_d is the ^{15}N enrichment in diffused standards, E_n is the ^{15}N enrichment in non-diffused standards, E_b is the *atm %* ^{15}N enrichment of the blank (assumed to be 0.366 %). The calculated mass of N in the blank (M_b) was then used to calculate the blank-corrected *atm %* ^{15}N enrichment of samples (E_s) using the equation:

$$E_s = E_m + \frac{M_b(E_m - E_b)}{M_s}$$

where M_b is the calculated mass of N in the blank, E_m is the *atm %* of measured sample, E_b is the assumed *atm %* of blank and the M_s is the mass of N in the sample.

The calculations of gross mineralization rates (m) were derived from the theoretical equation of Kirkham & Bartholomew (1954) by Hart et al (1994):

$$m = \frac{[NH_4^+]_0 - [NH_4^+]_t}{t} - \frac{log(APE_0/APE_t)}{log\left([NH_4^+]_0/[NH_4^+]_t\right)} [\mu g\ N\ g^{-1} soil\ dry\ weight\ day^{-1}]$$

where $[NH_4^+]_0$ is the total NH_4^+ amount (µg of N g^{-1} soil dry weight) at time-0, $[NH_4^+]_t$ is the total NH_4^+ amount (µg of N g^{-1} soil dry weight) at time-t, t is the length of the incubation

(days), APE_0 is the atom percent ^{15}N excess of NH_4^+ pool at time-0, APE_t is the atom percent ^{15}N excess of NH_4^+ pool at time-t. *APE = Es – Eb, (Eb assumed 0.366).* The rate was calculated per one day.

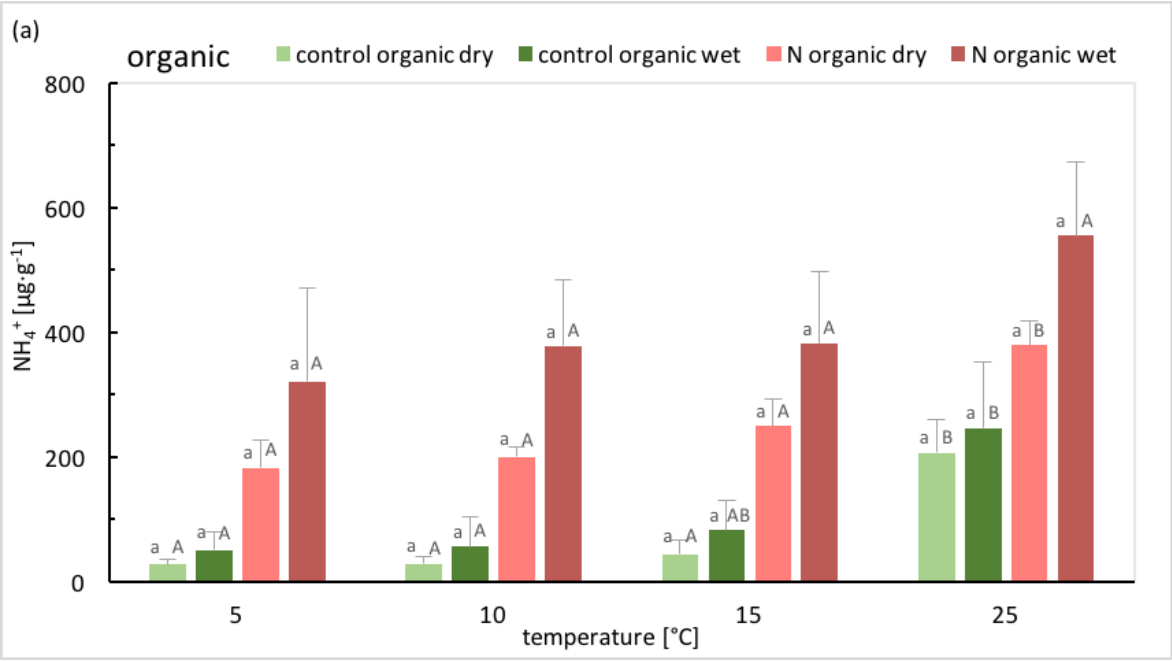
Statistical Analysis

The normality of the data was evaluated using the Kolmogorov-Smirnov test and logarithmic (ln) transformation was used to stabilize the distribution to approximate normality, except for net mineralization data which were used non-transformed. General linear models (GLM) were used to determine the dependency of variables (NH_4^+, gross mineralization, net mineralization) on factors (temperature, moisture, treatment, horizon) and Tukey HSD post-hoc test was used to determine significant differences among means. The correlation of gross mineralization and NH_4^+ pool was tested. Significance for all statistical analysis was accepted at $\alpha = 0.05$. For the statistical analysis of the data, Statistica software (Dell Inc. (2016), version 13) was used.

Results

Initial NH_4^+ Availability

Across all groups, the amount of NH_4^+ (t_0) was dependent on the temperature ($F = 15.018$, $p < 0.001$) and it was on average higher in soils from N treated sites than from control ones (on average 171.715 ± 44.695 $\mu g \cdot g^{-1}$ and 50.907 ± 22.510 $\mu g \cdot g^{-1}$, N and control respectively, $F = 76.684$, $p < 0.001$). When groups were compared regardless of horizon, the positive effect of temperature on NH_4^+ availability was revealed at 25°C. When compared separately, in organic horizon NH_4^+ pool increased already at 15°C (*Fig. 6a*). However, the differences between temperatures were mostly not significant, namely in the mineral horizon (*Fig 6b*), due to big data variability of four real soil replications. Availability of NH_4^+ was significantly different in each horizon, as it was higher is the upper organic horizon (*Fig. 6a*) than in the lower mineral horizon (*Fig. 6b*) (on average 211.276 ± 59.989 $\mu g \cdot g^{-1}$ and 11.346 ± 7.307 $\mu g \cdot g^{-1}$, organic and mineral respectively, $F = 567.101$, $p < 0.001$). Moreover, moisture also significantly affected soil NH_4^+ availability, so that wet soils contained higher amount of NH_4^+ than dry soils (on average 136.389 ± 50.821 $\mu g \cdot g^{-1}$ and 86.233 ± 16.384 $\mu g \cdot g^{-1}$, wet and dry respectively, $F = 17.126$, $p = 0.001$).



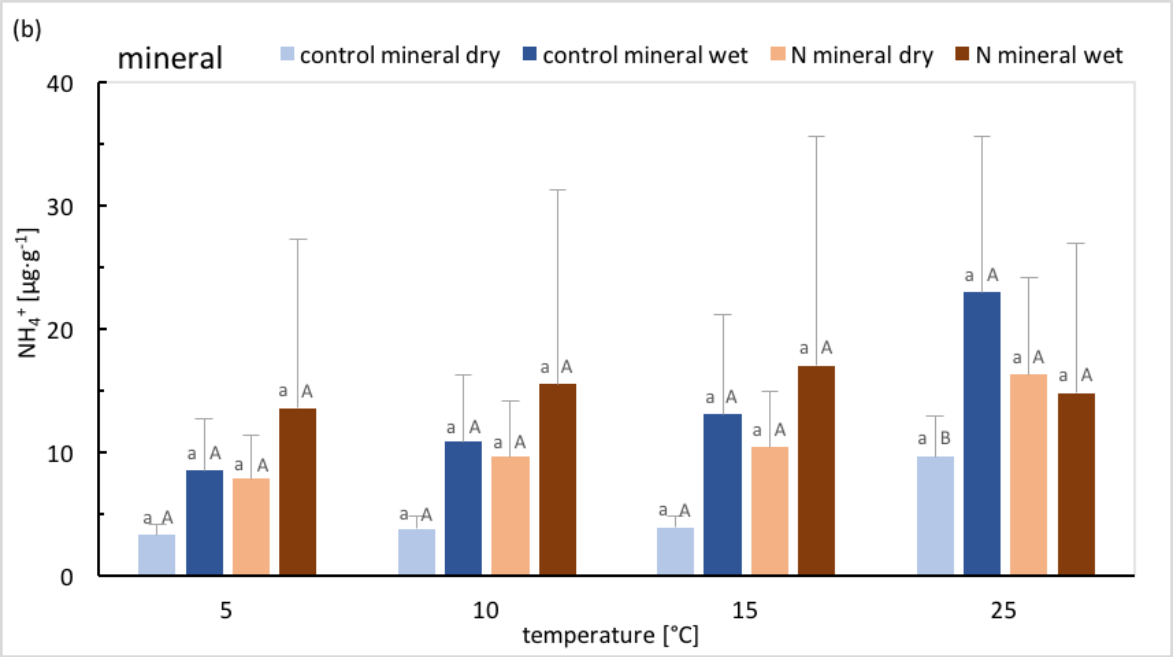


Fig. 6a and b: Dependence of NH_4^+ pool on temperature with respect to moisture (wet and dry) and treatment (C and N) in organic (upper graph) and mineral (lower graph) horizon. Small letters note statistical differences among the four groups (columns) at given temperature. Capitals note statistical differences within one of four soil groups (C dry, C wet, N dry, N wet) in one of the two horizons with changing temperatures. See the differences between y-axis scales of Fig. 6a and b. Values represent means of the non-transformed data set, error bars show standard deviations.

Gross Nitrogen Mineralization

In total, gross N mineralization rate changed significantly with temperature ($F = 13.074$, $p < 0.001$). The lowest rate was at 10°C, but when soils were kept at 5°C the gross NH_4^+ production was comparable to the one at 15°C. The fastest rates were measured at 25°C (except for N organic dry soils). The gross N mineralization differed between both horizons greatly (on average 41.066 ± 25.854 $\mu g \cdot g^{-1} \cdot day^{-1}$and 2.897 ± 2.139 $\mu g \cdot g^{-1} \cdot day^{-1}$, organic and mineral respectively, $F = 145.716$, $p < 0.001$), being on average more than 15 times higher in organic than in mineral horizon (*Fig. 7a* and *b*). Mainly in organic horizon, N mineralization in wet soils tended to be higher, but the effect was not significant ($F = 3.516$, $p = 0.064$). Across all groups, N addition did not affect gross N mineralization rates. However, when the interaction between treatment and horizons was tested, an increase of the rate in soils from organic horizon with N treatment was revealed ($F = 12.285$, $p = 0.0001$), while the opposite was observed in mineral horizon.

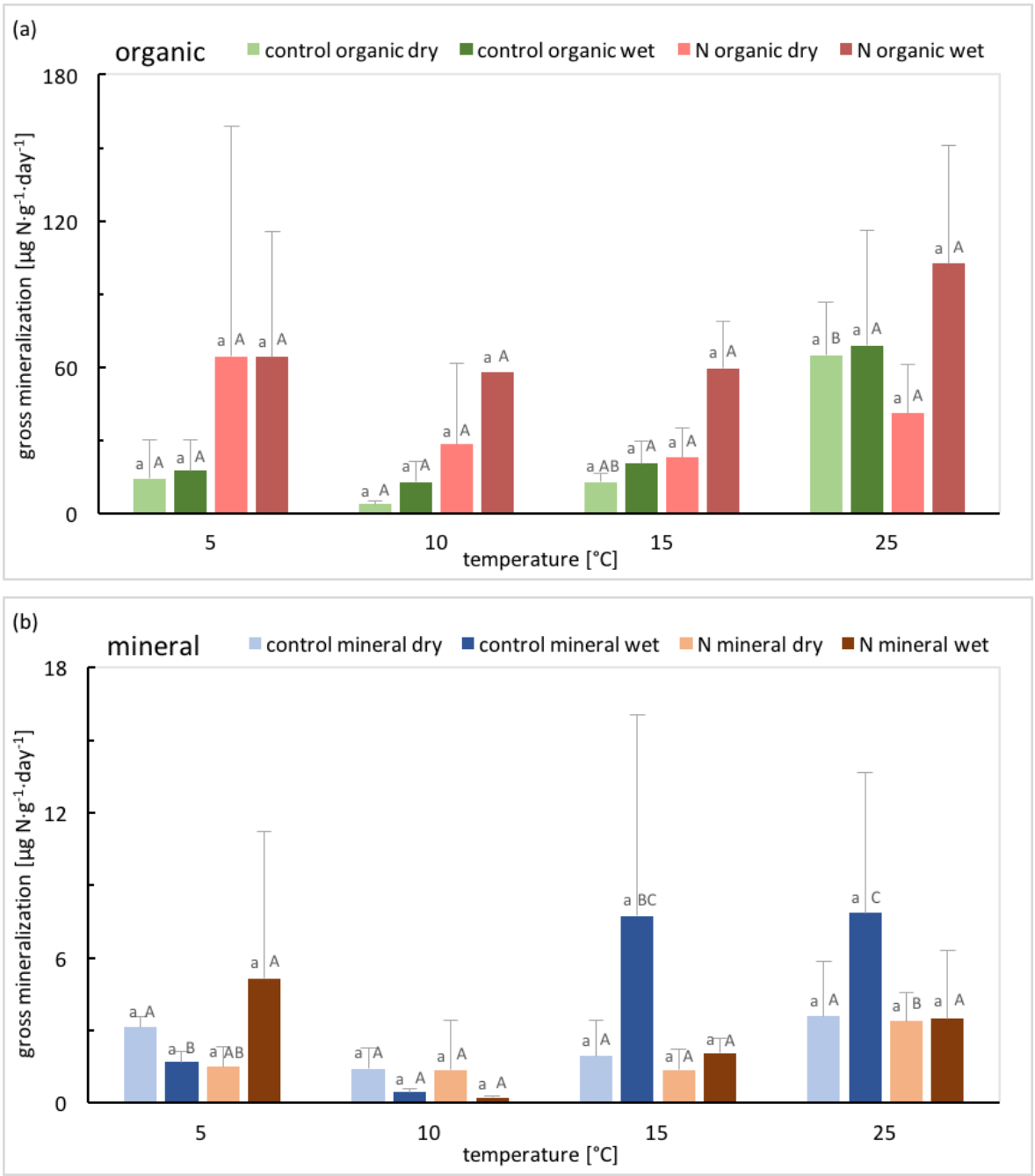


Fig. 7a and b: Dependence of gross N mineralization rates on temperature with respect to moisture (wet and dry) and treatment (C and N) in organic (upper graph) and mineral (lower graph). Small letters note statistical differences among the four groups (columns) at given temperature. Capitals note statistical differences within one of four soil groups (C dry, C wet, N dry, N wet) in one of the two horizons with changing temperature. See the differences between y-axis scales of Fig. 7a and b. Values represent means of the non-transformed data set, error bars show standard deviations.

Gross N mineralization was linearly correlated with initial NH_4^+ pool (t_0) (*Fig. 8*) with Pearson correlation coefficient $r = 0.772$. Correlation was positive with parameter 0.686 and linear equation $y = 2.266 + 0.686x$.

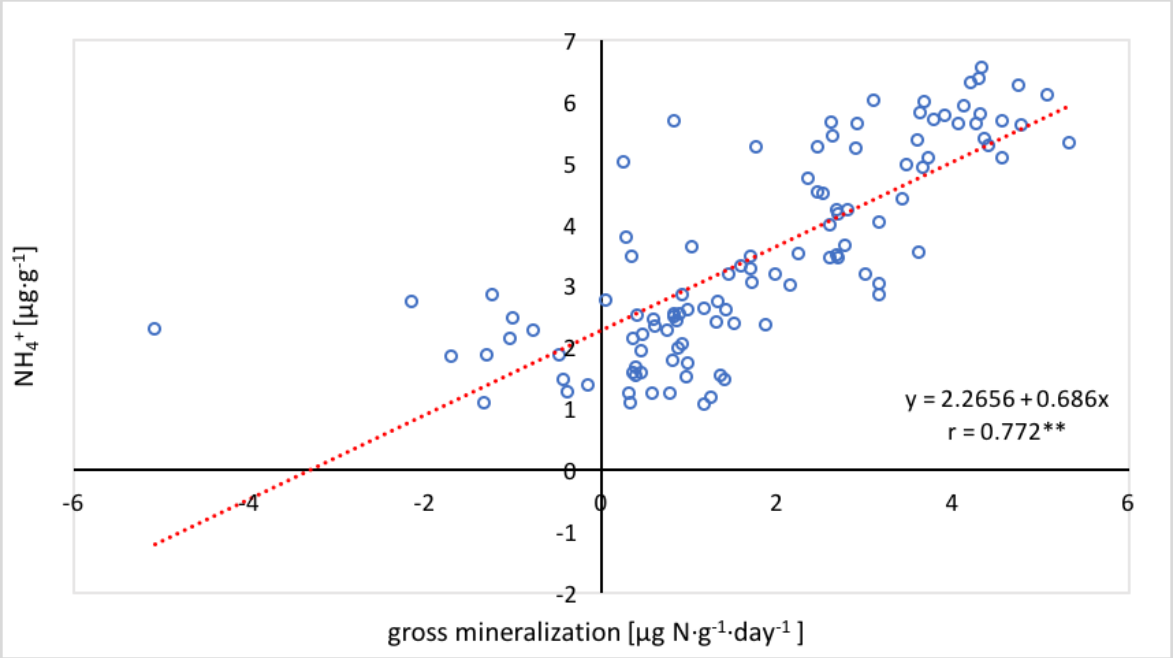


Fig. 8: Correlation of gross mineralization (x) and NH_4^+. Values represent the ln-transformed data set.

Net Nitrogen Mineralization

Net N mineralization rate, measured as the difference between the total amount of NH_4^+ at t_0 and t_1, decreased on average with the increasing temperature ($F = 2.754$, $p = 0.045$). However, systematic changes occurred only in the organic horizon (*Fig. 9a*), whereas in mineral horizon there was no general trend (*Fig. 9b*). This result was confirmed by significant interaction of temperature and horizon factors ($F = 3.579$, $p = 0.017$). The net N mineralization rate was independent of treatment (C and N), moisture and horizon. Nevertheless, in organic horizon the rate was almost always negative while in mineral horizon positive values prevailed.

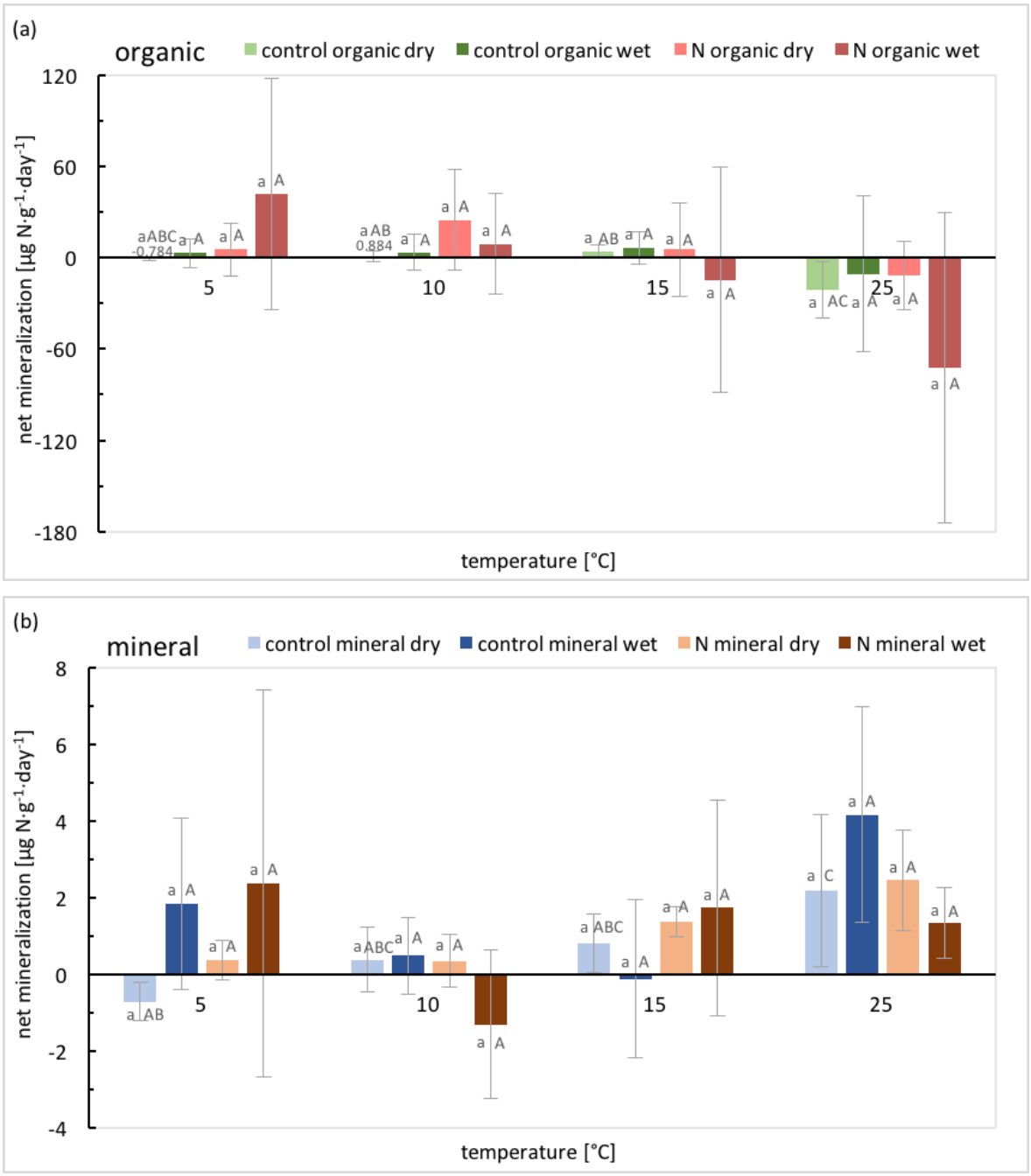


Fig. 9a and b: Dependence of net mineralization on temperature with respect to moisture (wet and dry) and treatment (C and N) in organic (upper graph) and mineral (lower graph). Small letters note statistical differences among the four groups at given temperature. Capitals note statistical differences within one of four soil groups (C dry, C wet, N dry, N wet) in one of the two horizons. See the differences between y-axis scales of Fig. 9a and b. Values represent means of the non-transformed data set, error bars show standard deviations.

Discussion

The Effect of Temperature on Gross Nitrogen Mineralization and NH_4^+ Availability

Rates of gross N mineralization (*Fig. 7a* and *b*) (ranging from 3.8 to 102.5 $\mu g \cdot g^{-1} \cdot day^{-1}$ in organic horizon and from 0.2 to 7.8 $\mu g \cdot g^{-1} \cdot day^{-1}$ in mineral horizon) were in agreement with rates found in similar conditions (organic horizon: Bengtsson et al. 2003 in range 0–142 $\mu g \cdot g^{-1} \cdot day^{-1}$; Chen & Högberg 2006 in range 15–60 $\mu g \cdot g^{-1} \cdot day^{-1}$; Schütt 2014 in range 6–40 $\mu g \cdot g^{-1} \cdot day^{-1}$, organic+mineral horizon: Lang et al. 2010 in range 0.5–6 $\mu g \cdot g^{-1} \cdot day^{-1}$; Cheng et al. 2013 0.1–7.8 $\mu g \cdot g^{-1} \cdot day^{-1}$, mineral horizon: Schütt 2014 in range 0–6 $\mu g \cdot g^{-1} \cdot day^{-1}$). Temperature affected gross N mineralization rate, however, the temperature response did not follow the Arrhenius equation[11]. The rate was not gradually increasing with the temperature (5–25°C), contrary to studies, which determined linear relationship between temperature and gross N mineralization (Hoyle et al. 2006 with temperature range 5–40°C in Red-Brown Earth organic horizon of arable land; Cheng et al. 2015 with temperature range 5–35°C in organic+mineral horizon of subtropical soil). On average, surprisingly lower mineralization was measured at 10°C, the rate increased again at 15°C to the level of initial 5°C and was further higher at 25°C. Moreover, each of the total of eight groups of samples (wet and dry, N and control in organic and mineral horizon separately) responded to temperature differently. These inconsistent trends suggest a linkage of temperature with other factors, e.g. substrate availability and moisture.

Clearly higher rates of gross N mineralization observed at 15 and 25 than at 10°C in most of the groups could have been related to the greater microbial activity and the faster substrate (organic matter) decomposition. As microorganisms require not only N but also stoichiometrically equivalent amount of readily available C to sustain their metabolism and growth, the mineralization and assimilation of N proceeds along with mineralization and assimilation of C. Higher temperature may therefore induce rapid decomposition and mineralization of SOM and consequently higher rates of N mineralization (Murphy et al. 2003). Organic matter decomposition, as well as the mineralization, is dependent on the microbial activity and the activity of enzymes responsible for those processes (Schindlbacher et al. 2011). For the enzymatic activity, the increasing trend of its temperature response was found and the optimum temperature for forest microbial community was determined around 25°C by Pietikäinen et al. 2005, which would correlate with the measured significant increase of N mineralization at 25°C. The increasing trend of enzymatic activity could have

been related also to the NH_4^+ availability. The size of NH_4^+ pool, accumulated during three weeks of incubation, was gradually increasing over the measured temperature range (5–25°C) and was accordingly highest at 25°C (*Fig 6a* and *b*). Connection of both mineralization and NH_4^+ availability was shown by the mutual correlation (*Fig. 8*).

The increasing trend of temperature response of microbial activity is apparently inconsistent with the decrease of gross N mineralization (mainly in mineral horizon) at 10°C. This contradiction can be explained for example by increased direct usage of organic N at 10°C. Utilization of DON, without a release of mineralized N, would have bypassed the labelled inorganic NH_4^+ pool, so the isotopic ratio would remain unchanged and measured gross mineralization would drop (Verburg et al. 1999). When the temperature further increased, the DON pool amplified and microbes released more N via mineralization. This hypothesis was supported by measured microbial N (and C), which was the highest mostly at 10°C (in some cases at 15°C) and thus showed maximal N assimilation (Tahovska, personal communication). However, the decrease in gross N mineralization was not consistent for all groups and for the denotation of it as a trend, further investigations are needed.

Surprisingly higher gross N mineralization rates measured at 5°C than at 10°C can be explained by an adaptation of soil microorganisms from boreal region (with the mean annual temperature at Gårdsjön 6.4°C ranging from –2.4°C in winter to 15.6°C in summer; (Kjønaas et al. 1998)) for such low temperatures. Microorganisms are able to mineralize N at temperatures even below zero (Schütt 2014) and 5°C is comparable to temperatures they are accustomed to live in. Activity of their enzymes was therefore probably not supressed by such low temperature and rates of N mineralization were comparable to those ones at 15°C.

Apart from temperature, the enzyme production is also regulated by substrate availability and moisture (Sinsabaugh 1994). Organic matter content usually decreases down with the soil profile in spruce forests. Microbial activity and consequently organic matter decomposition should be higher in upper horizons (Andersson et al. 2004), which was proved by the more than order of magnitude faster gross N mineralization in organic than in mineral horizon on average (*Fig. 7a* and *b*). The effect of higher organic matter mineralization was further related to the NH_4^+ availability (suggested by Verburg et al. 1999), as it was more than 10x larger in organic than in mineral horizon (in accordance with Vervaet et al. 2004).

Increasing moisture conditions can stimulate N transformations in general (Tietema et al. 1992) until the point at which lack of aeration can become a problem for processes dependent on oxygen (e.g. mineralization). The positive effect of moisture on N mineralization have been proved (Binkley et al. 1994; Stottlemyer et al. 2001; Zaman & Chang 2004; Booth et al. 2005), but could have been supressed in this study, as each of the soil samples were incubated at the same moisture content and differences between soils originated from either wet or dry sites could have been hindered. Nevertheless, an increase in gross N mineralization rate was observed in soils from wet sites, compared to those from dry sites, in most cases (see *Fig. 7a* and *b*). It suggested more suitable water availability for the metabolism of microbial community and for its movement to access substrate in soil, yet still good soil aeration (Zaman & Chang 2004).

The Effect of Temperature on Net Mineralization

Net N mineralization rates were lower than gross rates on average (Tietema 1998; Cheng et al. 2013) and rates were not correlated to each other (contrary to other studies, e.g. Hoyle et al. 2006). Net rates soils of organic horizon were decreasing with rising temperature, suggesting greater rates of consumptive processes at higher temperatures. Measured net rates (*Fig. 9a* and *b*) were different in mineral and organic horizon as they were probably related to the amount of organic matter as well as were the gross rates. In upper horizon (*Fig. 9a*) gross NH_4^+ consumption most probably prevailed over gross N mineralization at the highest temperature (25°C) resulting in negative net N mineralization. NH_4^+ might have been assimilated as well as further transformed (via nitrification) at higher rates than it was produced at the given time. However, for the exact quantification, gross consumption rates (assimilation, nitrification and microbial biomass remineralization) and pool sizes will be required. When compared to rates in lower horizon (*Fig. 9b*), where the availability of organic matter was likely limited, microorganisms rather mineralized available N but the rates were very low.

The Effect of Chronic Nitrogen Deposition

Contrary to studies, which suggest higher (Tietema 1998; Chen & Högberg 2006) or lower (Fisk & Fahey 2001) gross and net N mineralization under a chronic high N deposition, mineralization was on average not affected by increased long term N input. Nevertheless, N deposition promoted mineralization in organic horizon (*Fig. 7a*) as the substrate was probably rich in organic N. Likely for the same reason, NH_4^+ pool in impacted soils of

organic horizon (*Fig. 6a*) was higher than in soils with natural N deposition. Both higher mineralization and NH_4^+ availability might have been directly connected with the NH_4NO_3 experimental loading to the soil which was not fully assimilated or further transformed and remained adsorbed to soil sorption complex. Interestingly, in mineral horizon (*Fig. 7b*) there was some evidence that gross N mineralization was supressed by increased N input, while the NH_4^+ availability was enhanced (*Fig. 6b*). The NH_4^+ pool was probably originated from sorption soil complex, where experimentally loaded NH_4NO_3 remained adsorbed and the NH_4^+ availability was consequently higher.

Conclusions

In accordance with the hypothesis, rather positive effect of rising temperature on gross N mineralization was shown. Microorganisms mineralized available organic N into NH_4^+ faster at higher temperatures (except 10°C) and an increase in their enzymatic activity can be suggested for an explanation. Microbial enzymes also shown adaptation to the lowest temperature (5°C). The rate of NH_4^+ production was correlated with the cumulative availability of NH_4^+ in soil after three weeks of incubation, which was increasing with rising temperature. The available NH_4^+ was utilized by microorganisms which was shown by rates of net mineralization being lower than the gross ones. At the highest temperature (25°C), microbial consumption exceeded mineralization in organic horizon, resulting in negative net N mineralization. Both processes, N mineralization and consumption do not need to respond to temperature equally, which was suggested as gross and net N mineralization was not correlated. With respect to N deposition, chronically N loaded soils did not show an increase of gross neither net N mineralization on average, and so the hypothesis was not supported. However, different responses of soils from the two horizons to N loading were observed, so that the rate of gross N mineralization rather increased in organic horizon, while in mineral it was reduced. This result indicates the importance of soil organic matter availability and quality in predicting gross N mineralization changes after loading of mineral N.

Results can be related to worldwide conditions, where the global warming can contribute to faster decomposition of soil organic matter and subsequent faster N mineralization, but also consumption. However, to assess the general effect of increasing temperature and N input, further determination of microbial activity and its temperature sensitivity is required as well as investigations of overall N soil transformations (e.g. N consumption, nitrification, microbial biomass turnover). This research can serve as a background study for further analyses and coupled to the other data will serve as a basis for field experiments and for models predicting temperature responses of highly complex N transformations in N-loaded soils.

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