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Impact of nitrogen deposition on forest biogeochemical processes investigated using a tool kit of stable isotope methods

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Zusammenfassung

Über 50 Prozent der gegenwärtigen atmosphärischen Stickstoffdepositionen werden durch anthropogene Aktivitäten verursacht und durch den atmosphärischen Stickstofftransport teils kilometerweit transportiert. Diese anthropogenen Veränderungen der biogeochemischen Kreisläufe von Stickstoff und folge dessen auch von Kohlenstoff verstärken die aktuelle Klimakrise in vielen Bereichen. Terrestrische Ökosysteme spielen eine essentielle Rolle in der Regulierung des Klimawandels da terrestrische Böden die signifikantesten Kohlenstoffsenken an Land sind. Mikrobielle Gemeinschaften in Waldböden sind ein essentieller Teil des terrestrischen Stickstoffkreislaufs da sie Stickstofftransformationen vermitteln und den Kohlenstoffhaushalt beeinflussen.

In dieser Studie wurden die Effekte einer Langzeitbelastung durch erhöhten Stickstoffeintrag in temperate Waldböden in 4 Ländern durch stabile Isotopenmethoden gemessen. ^{15}N und ^{13}C Konzentrationen wurden in Bodenproben und in mikrobiellen Bodengemeinschaften mittels verschiedener Tracer-Experimente gemessen. Ungeachtet der unterschiedlichen klimatischen und bodenchemischen Parameter verhielten sich alle Böden der untersuchten Waldgebiete hinsichtlich der Reaktionen in Stickstoff- und Kohlenstoffparametern gleich. Mittels statistischer Analysen konnten wir signifikante Unterschiede zwischen Kontrollböden und mit Stickstoff gedüngten Böden feststellen. Auch bei PLFA Untersuchungen verhielten sich die untersuchten Böden der verschiedenen Standorte gleich, mit signifikanten Unterschieden zwischen den Behandlungen, wobei wir hier eine Reduktion des gram-negative zu gram-positive Verhältnisses beobachteten. Bodenrespirationsmessungen und Messungen des in der mikrobiellen Biomasse enthaltenen Stickstoffes ergaben widersprüchliche Ergebnisse. Unsere Hypothese besagte, dass sich die Zersetzung des biologischen Bodenmaterials durch eine höhere Stickstoffzufuhr erhöhen wird, was wir in unseren Versuchsstandorten in Deutschland und Schweden bestätigen konnten, jedoch nicht in Österreich und Großbritannien. Diese Ergebnisse könnten bedeuten, dass in diesen Waldböden andere Parameter wichtiger für die mikrobielle Aktivität sind.

General introduction

All organisms require nitrogen as an essential macronutrient. It is inherently necessary for organisms to build crucial compounds of life like proteins, DNA and ATP. Nitrogen is also a major limiting factor in most ecosystems on Earth.

Nitrogen cycle

The biogeochemical cycle of nitrogen is almost entirely dependent on reduction-oxidation reactions primarily mediated by microorganisms (Canfield et al., 2010). Nitrogen occurs in different forms and at several redox stages, from ammonia and organic compounds at an oxidation state of -3 to nitrate at an oxidation state of +5. In soil, nitrogen takes nine different chemical forms which correspond to those oxidation stages. In the atmosphere, dinitrogen gas is the most common form of nitrogen and makes up 79%. Therefore, dinitrogen gas is the most abundant form of nitrogen on Earth.

As a short description of the nitrogen cycle, dinitrogen gas is transformed by biological N_2 fixers and is converted into biologically available forms (ammonium) which can be used by most organisms. Biological nitrogen fixation is the dominant natural process by which nitrogen enters biological pools. In terrestrial or marine ecosystems, the resulting ammonium can be assimilated into living organisms (organic N forms) or be further oxidized to nitrite and subsequently to nitrate by nitrifiers. Organic nitrogen which is assimilated into living biomass can also be mineralized into inorganic forms by saprotrophic microorganisms. The conversion of ammonium to nitrate is called nitrification and occurs under aerobic conditions. Nitrate can be turned into nitrous oxide and dinitrogen gas by denitrification under anoxic conditions. Both forms can re-enter the atmosphere. Other forms of nitrogen are involved in these conversions primarily as intermediates and can escape during these conversations into the atmosphere as well (Robertson and Groffman, 2015). Currently, a big concern is the high anthropological impact on the natural nitrogen cycle. Anthropogenic sources are double the natural rate of terrestrial nitrogen fixation and contribute around 45% of the total fixed nitrogen annually produced on Earth (Canfield et al., 2010). This extensive human impact started with the development of processes for reducing dinitrogen gas to ammonium which is used as fertilizer in agriculture. Before the 20th century, nitrogen was a limiting factor in agriculture. Employing nitrogenous fertilizer produced by an industrial process called the Haber-Bosch process led to an enormous increase in food production worldwide. These anthropogenic activities have altered the nitrogen cycle in many ways, leading to eutrophication of freshwaters and coastal

zones and global acidification (Gruber & Galloway, 2008). Agricultural nitrogen sources include industrial fertilizer production and biological fixation of nitrogen in agriculture, which combined account for 80% of anthropogenic nitrogen fixation (Fowler et al., 2013; Stevens et al., 2018). There is also an increased nitrogen input into the atmosphere due to anthropogenic activities, like nitrous oxide, which is a potent greenhouse gas. Nitrogen oxides are emitted during fossil fuel combustion, and ammonia volatilizes as a result of extensive agricultural practices (Canfield et al., 2010). More atmospheric dinitrogen gas is converted to biologically reactive forms through anthropogenic activities than all other natural conversations combined (Nadelhoffer et al., 1999). It is the higher anthropogenic nitrogen fixation rates that are causing a global imbalance between nitrogen fixation and denitrification, which have been historically balanced within the natural nitrogen cycle. This shift is causing nitrogen to become a significant pollutant. There is compelling evidence that anthropogenic alterations of the nitrogen cycle are negatively affecting the environment (Galloway et al., 2008).

Atmospheric nitrogen deposition

Atmospheric nitrogen deposition results from emissions of reactive nitrogen species and their atmospheric transport. They are emitted by natural sources like soils, by lightning, as well as anthropogenic sources (Neff et al., 2002; Dentener et al., 2006; Galloway et al., 2008; Kanakidou et al., 2016). Before anthropogenic activities like industrial agriculture based on inorganic fertilizer usage and fossil fuel combustion, atmospheric nitrogen deposition was a relatively unimportant nitrogen source in terrestrial and marine ecosystems. Since the industrial and agricultural revolution, nitrogen deposition gradually became an important and sometimes even the most dominant nitrogen source in terrestrial and marine ecosystems. Contemporary sources of atmospheric nitrogen are approximately over 50% associated with human activities (Fowler et al., 2013; Kanakidou et al., 2016; Stevens et al., 2018). The rapid increase of atmospheric nitrogen deposition is closely related to human activities (Canfield et al., 2010; Galloway et al., 2008; Peñuelas et al., 2012; Huang et al., 2015).

Atmospheric nitrogen deposition occurs as organic and inorganic nitrogen forms and returns to the Earth's surface as wet or dry deposition, a factor determined by the solubility of the compounds. Atmospheric inorganic nitrogen forms include ammonium (NH_4^+) and nitrate (NO_3^-) as aerosol forms and ammonia (NH_3), nitrogen dioxide (NO_2), nitric acid (HNO_3) and nitrous oxide (N_2O) as gaseous forms. Even though organic nitrogen deposition is a significant fraction of atmospheric nitrogen deposition, it is still poorly characterized. It is believed that

peptides and dissolved free amino acids make up between 20% and 50% of all dissolved organic nitrogen (Kieber et al., 2005; Nakamura, Matsumoto and Uematsu, 2005; Mace et al., 2003; Cornell, 2011), while amines make up less than 10% (Calderon et al., 2007; Gibb et al., 1999; Gorzelska et al., 1992; Mopper and Zika, 1987; Cornell, 2011). Urea could account for under 10% to almost all of the organic nitrogen deposition (Timperley et al., 1985; Seitzinger and Sanders, 1999; Mace et al., 2003; Cornell, 2011). Bulk deposition of inorganic nitrogen has been measured more extensively than organic nitrogen because until recently, organic nitrogen was considered to be a natural background of locally recycled material (Cornell, 2011). Reactive nitrogen is removed from the atmosphere and deposited in the biosphere through precipitation (rain, snow, fog or as clouds) which is called wet deposition and by a direct interception by surfaces of aerosols and gaseous nitrogen forms, which is called dry deposition (Bertolini et al., 2016). Compositions of wet or dry depositions vary depending on location, anthropogenic activities and season. Ammonium and nitrate make up the bulk nitrogen depositions on Earth (Liu et al., 2013). Wet nitrogen deposition is relatively easy to measure using rain collectors, while the measurement of dry deposition is more challenging (Lamb and Bowersox, 2000; Sickman et al., 2019). Therefore, most available data are on wet deposition due to those challenges, yet dry deposition accounts for 30% to 60% of the total nitrogen deposition (Song et al., 2017; Xu et al., 2018; Benedict et al., 2018; Deng et al., 2019). Current global nitrogen deposition is estimated between 125 and 132 tera-gram nitrogen (TgN) per year, out of which organic nitrogen is about 20% to 30% or 27 to 36 TgN per year (Kanakidou et al., 2016). Inorganic nitrogen deposition is estimated at approximately 93.6 TgN per year (Ackerman et al., 2019). There has been a threefold increase of nitrogen deposition over land and a twofold increase over the ocean since the industrial and agricultural revolutions (Kanakidou et al., 2016). Reactive nitrogen emissions in Europe have decreased or stabilized since the early 1990s due to the implementation of stricter legislation. In Southeast Asia, especially China, reactive nitrogen emissions are still on an upward trend (Billen et al., 2013; Song et al., 2017). Based on changes in emissions, inorganic nitrogen deposition is declining or staying at similar levels in areas such as Europe and increasing in others, such as East Asia and Southern Brazil (Ackerman et al., 2019). In contrast to the USA and Europe, ammonium has often been the primary source of atmospheric nitrogen deposition in China, which is mostly connected to extensive agriculture, the excessive use of fertilizers and traditional farming practices which involve extensive use of manures (Xiao et al., 2010; Huang et al., 2015).

It has to be noted that it is hard to make exact estimations even just for one region because it is challenging to accurately quantify all the different nitrogen inputs of wet, particulate and

gaseous forms due to different measurement requirements (Fenn et al., 2009; Sickman et al., 2019). Due to atmospheric transport of reactive nitrogen, nitrogen is often introduced into nitrogen-limited ecosystems which do not have other external sources of anthropogenic nitrogen (Phoenix et al., 2006; Duce et al., 2008). Atmospheric reactive nitrogen transport has a wide transport range expanding to tens to thousands of kilometres (Phoenix et al., 2006; Duce et al., 2008; Bobbink et al., 2010). Different nitrogen forms have different ranges of transport. Organic nitrogen compounds are essential in the long-range transport of nitrogen because their removal processes are less effective than those of ammonium and nitrate, which are generally deposited closer to their source (Neff et al. 2002; Nakamura, Matsumoto and Uematsu, 2005; Cornell, 2011). Ammonia cannot be transported such long distances as well due to its conversion to ammonium. Its maximum transport distance reaches about 50 km downwind of the source (Van Herk et al., 2003; Huang et al., 2015). Atmospheric nitrogen deposition appears to be seasonal in tropical regions with a dry and a wet season, characterized by a bimodal pattern with gaseous nitrogen compounds being more evident during the dry season and aerosol and bulk nitrogen deposition rates being higher during the wet season (Bertolini et al., 2016).

Nitrogen deposition occurs at different rates and composition across the world due to various anthropogenic and natural nitrogen sources and due to nitrogen transport and transformation through meteorology and atmospheric chemistry. While the main source of reactive nitrogen in developing countries is extensive agriculture, the main sources in industrialized countries are industrial processes and traffic, as well as extensive agriculture. These various sources are shaping nitrogen depositions in their immediate and more distant environments (Dentener et al., 2006; Bertolini et al., 2016).

Urbanization is a crucial player in atmospheric nitrogen deposition as well. The highest levels of nitrogen deposition are detected in highly populated regions like North America, Europe and parts of Asia (Stevens et al., 2018). Urban areas are impacting the pattern of wet inorganic nitrogen deposition and seem to be more affected by nitrogen deposition then rural and remote locations. Cities in Europe and the USA always received higher rates of nitrogen deposition than rural areas (Lovett et al., 2000; Power and Collins, 2010; Huang et al., 2015). There is a significant negative correlation with wet inorganic nitrogen deposition and atmospheric nitrate deposition with distance from urban areas due to increased NO_x emissions within cities (Huang et al., 2015). It is believed that land-use types could affect nitrogen deposition as well, although this topic is still relatively less studied (Deng et al., 2019).

Effects of atmospheric nitrogen deposition

Due to the large variety of atmospheric nitrogen deposition, the effects of additional input of reactive nitrogen are manifold, far-reaching and highly complex upon biological systems (Bobbink and Hettelingh, 2011). Effects of additional nitrogen input into ecosystems depend on the amount and forms of nitrogen deposition as well as on the receiving ecosystem and its microbial community (Robertson and Groffman, 2015). Excessive nitrogen deposition can have negative impacts on ecosystems (Townsend and Howarth, 2010; Song et al., 2017), such as increased gaseous and mineral nitrogen losses from the ecosystem, changes in the plant community functions, changes in biodiversity, soil acidification and reduction of phosphorus availability (Zaehle et al., 2011; Clark et al., 2013; Throop and Lerdau, 2004; Matson et al., 1999; Bertolini et al., 2016). Additional nitrogen can be taken up by microbial communities which results in emissions of different nitrogen forms like dinitrogen gas, nitrous oxide, nitric oxide or leached nitrogen into watersheds. The two main effects of atmospheric nitrogen deposition are eutrophication and soil acidification. Nitrogen deposition indirectly mediates changes in soil chemistry and other biotic and abiotic interactions which can impact ecosystems at significant distances (Janssens et al., 2010; Bobbink et al., 2010; Stevens et al., 2018). Direct toxicity of nitrogen gases and aerosols, on the other hand, can solely occur in at the vicinity of locations with high atmospheric concentrations of ammonia close to a substantial nitrogen source (Bobbink et al., 2010; Stevens et al., 2018). Effects of atmospheric nitrogen deposition are recognized in all oligotrophic natural systems in boreal, temperate and Mediterranean climates including forests, aquatic and marine habitats, coastal habitats, oligotrophic wetland, grassland and heathland (Achermann and Bobbink, 2003; Bobbink and Hetelingh, 2011; Gilliam, 2006; Pardo et al., 2011; Van Dobben and de Vries, 2016).

Additional nitrogen input into terrestrial ecosystems also impacts soil microbial communities (Bobbink and Willems, 1987; Clark and Tilman, 2008; Horswill et al., 2008; Van den Berg et al., 2011; Stevens et al., 2018). Soil microbial communities are crucial parts of the nitrogen cycle as microbes are mediating all nitrogen transformations like nitrogen mineralization and immobilization, as well as biological nitrogen fixation, nitrification and denitrification. Microbes are able to take up nitrate, ammonium and organic nitrogen as monomers. It was long believed that microbes prefer nitrate and ammonium over organic nitrogen. Now it is well established that organic nitrogen contributes significantly to nitrogen nutrition of soil microorganisms as well (Luxhøi et al., 2006; Geisseler et al., 2010).

Microbes which are able to adapt to nutrient availability have a competitive advantage, but those organisms which are adapted to low nitrogen soils regulate the synthesis and activity of

different enzyme systems more tightly (Koch, 1985). Numerous studies reported that the microbial community shifts when additional nitrogen is introduced (Janssens et al., 2010). Additional nitrogen deposition could also cause a shift in the saprotrophic community and therefore cause changes in the decomposition of soil organic matter (SOM). Reasons for a change could be that the saprotrophic community could switch food sources or change its carbon use efficiency. In boreal and temperate forests, soil microbial biomass declined by 20% on average when additional nitrogen was introduced to the ecosystem (Janssens et al., 2010). Microbial transformations of reactive nitrogen also influence soil fertility, water quality and atmospheric chemistry on both small and large scales (Robertson and Groffman, 2015).

Soil acidification due to additional nitrogen input can change soil chemistry, influence species compositions and subsequently lower biodiversity of an ecosystem. In nitrogen-low ecosystems, microorganisms usually use most acquired nitrogen to meet their own needs. When additional nitrogen is introduced to the system, microbes consume more carbon to grow and subsequently produce nitrate. This upregulated nitrification process triggered by an additional ammonium supply is a significant source of soil acidification in many terrestrial ecosystems (Robertson and Groffman 2015; Van Dobben and De Vries, 2016). Reduced soil pH also impacts the chemical availability of metals and other nutrients, as well as the toxicity of aluminium (Tyler and Olsson, 2001; Schuster and Diekmann, 2003; Dise and Wright, 1995; Stevens et al., 2018). Soil pH is vital for enzyme functioning, thus influencing microbial activity and the decomposition of soil organic matter and plant litter (Janssens et al., 2010). Impacts of acidification have been observed primarily in acidic soils which are poorly buffered (Maskell et al., 2010, Stevens et al., 2018). A large fraction of soil organic matter is physically or chemically protected from microbial decay and is consequently buffering potential effects of additional nitrogen input in terrestrial soils. If all fractions of SOM were available for the microbial community to use, it would affect the soil respiration and decomposition rates of an ecosystem (Janssens et al., 2010).

Plants take up nitrogen either directly through root cell membranes, through nitrogen fixation via symbiosis with soil rhizobial bacteria or scavenge nitrogen through associations with mycorrhiza. (Geisseler et al., 2010). An increase in biologically available nitrogen results in lower biodiversity due to changes in species composition caused by increased competitive interactions between oligotrophic species and fast-growing species, making conditions unfavourable for oligotrophs(Ceulemans et al., 2017; Hautier, Niklaus and Hector, 2009; Stevens et al., 2018, Bobbink et al., 2010). Many plant species of nitrogen-limited ecosystems are adapted to nutrient-poor conditions and can only compete successfully in these conditions

(UNECE, 2016). Additional nitrogen uptake by plants can also lead to changes in plant biochemistry and nitrogen tissue content. Higher growth rates associated with elevated nitrogen depositions have been linked to increased sensitivity to extreme weather like frost, wind and drought (Caporn, Ashenden, and Lee, 2000; Friedrich et al., 2012; Stevens et al., 2018). Additional nitrogen stored in foliage could enhance herbivory and would consequently have substantial impacts on primary and secondary consumers as well. Effects on primary and secondary consumers could include changes in the quality and quantity of food due to changes in the plant communities and changes due to variations in vegetation structure, such as different nesting places (Berg and Meentemeyer, 2002).

Effects of atmospheric nitrogen deposition on soil carbon cycling and carbon storage is an important current field of interest as well, as additional reactive nitrogen in terrestrial ecosystems might play a relevant role in carbon cycle processes and interactions. Nitrogen-limited terrestrial ecosystems might enhance carbon sequestration, the process of capturing and storing atmospheric carbon dioxide, due to enhanced nitrogen fertilization. Nitrogen deposition could be therefore linked to the carbon cycle, in particular to carbon dioxide removal from the atmosphere due to additional primary production (Bertolini et al., 2016; Zaehle et al., 2011; Duce et al., 2008; Galloway et al., 2008; Kanakidou et al., 2016). The dominant carbon input into terrestrial ecosystems is through photosynthesis (Luo et al., 2015). Carbon is then partitioned among three pools within plants, their leaves, stems and roots. Plant litter can be divided in a metabolic litter pool which describes a labile or fast decomposing fraction, and a structural litter pool which describes the slowly decomposing fraction and represents mostly cell wall compounds with bound proteins and lignified structures. Carbon stored in leaves and roots can either become metabolic litter or structural litter, while carbon stored in stems is likely to become structural litter (Corbeels, 2001). Litter carbon can then be used by the microbial community, which then fuels the slow SOM pool and the passive SOM pool. SOM pools consist of structural plant litter, plant and animal residues at various stages of decomposition, cells and tissues of soil organisms, dead microorganisms and their metabolites. Carbon transfers are donor-pool dominated with the donors generally being trees in forested systems, with input rates to litter and soil pools being dependent on the output rates of their tree-donor pools. In the absence of litter, subsequent carbon pools decay and the remaining carbon in the ecosystem, which would have been stored otherwise, is released into the atmosphere through respiration (Luo et al., 2015). Additional nitrogen input could have various effects on carbon cycling in soils. Short-term studies tend to detect positive effects like enhanced ecosystem productivity, while long-term studies stated significant decreases in

decomposition rates (Janssens et al., 2010). A meta-data analysis of Janssens et al. (2010) stated that additional nitrogen input might hinder organic SOM decomposition and therefore stimulate carbon sequestration in nitrogen-limited ecosystems. Another meta-analysis by Chen et al. (2015) investigating 88 studies states that nitrogen addition increased the above-ground plant carbon pool and slightly increased plant litter due to enhanced net primary production. No effects of additional nitrogen on either OH or mineral soil carbon pools were reported, however other meta-analyses state that nitrogen deposition increased soil carbon storage, while other reports suggest an increased carbon pool in OH but not in mineral horizons (Hyvonen et al., 2008; Janssens et al., 2010; Nave et al., 2009). The effects of nitrogen deposition on soil organic carbon storage therefore seem to be highly context-dependent. Additional nitrogen input may also lead to increased litterfall and decreased litter decomposition and soil respiration, both of which foster carbon sequestration. Soil carbon pools do not seem to change with additional nitrogen input, which could be partly explained by the decrease of below-ground plant carbon allocation (Chen et al., 2015), while others reported increased plant root biomass by nitrogen deposition (Xia and Wan, 2008; Lu et al., 2011). Several studies pointed out that the impact of additional nitrogen on ecosystem carbon dynamics depends on ecosystem type and rates of nitrogen input (Liu and Greaver, 2010; Chen et al., 2015), which could be explained by the nitrogen saturation hypothesis. This hypothesis states that once an ecosystem reaches nitrogen saturation, additional input of nitrogen will not increase its net primary production but reduce plant performance and soil functions (Aber et al., 1998; Chen et al., 2015). Although differences in results, all meta-studies suggest that atmospheric nitrogen deposition into terrestrial ecosystems is likely to change the global carbon cycle because nitrogen and carbon cycles are interdependent, forming the basis of biochemical cycles and energy flows (Chapin et al., 2009; Chen et al., 2015).

Anthropogenic changes to the biogeochemical cycles of nitrogen and subsequently of carbon add to the current climate crisis. Terrestrial ecosystems play a crucial role in regulating global climate, with terrestrial soils being the most significant carbon sink on land (Luo et al., 2015). All forests exposed to long-term nitrogen deposition will eventually run into nitrogen saturation, a state in which the forest nitrogen cycle is no longer closed (Janssens et al., 2010). Effects are dependent on the duration, total amount and forms of additional nitrogen input, the intrinsic sensitivities of plant communities and the abiotic conditions of individual ecosystems (Bobbink et al., 2010). Potential changes to carbon sequestration in terrestrial soil would influence its carbon sink activity and therefore its carbon dioxide output into the atmosphere (Nadelhoffer et al., 1999). Additional nitrogen within ecosystems also enhances nitrous oxide

emissions into the atmosphere through increased nitrification and denitrification activities. Nitrous oxide is a greenhouse gas, whose warming potential is 300 times higher than that of carbon dioxide, and which additionally destroys the ozone layer in the stratosphere (Canfield et al., 2010; Robertson and Groffman, 2015).

Accurate measurements are required to understand and mitigate the effects of atmospheric nitrogen deposition, which is especially important when establishing critical loads of nitrogen deposition for different ecosystems (Sickman et al., 2019). The critical loads concept was developed under the *Convention on Long-Range Transboundary Air Pollution* (UNECE LRTAP Convention) and is defined as "a quantitative estimate of an exposure to one or more air pollutants below which significant harmful effects on specified sensitive elements of the environment do not occur according to present knowledge". Atmospheric nitrogen deposition should not exceed critical load thresholds in order to enable long-term protection of ecosystems. Most of the ecosystems in Europe exceeded critical loads of nitrogen between 2 and up to 30 kgN ha^{-1} a^{-1} and higher, most of Scandinavia, parts of Portugal and Spain, Romania and the Northern UK as exceptions (UNECE, 2016). The variety of nitrogen forms which have to be measured separately and in different ways makes it hard to gather reliable estimations. Organic nitrogen is often disregarded due to its various constituents and lack of previous research, which is also potentially problematic when adapting the critical loads approach to regions where yet insufficient data have been gathered. Relative contributions of organic and inorganic nitrogen forms to total nitrogen deposition may vary greatly between regions where the critical loads approach has been developed (Cornell, 2011). Another approach defining and assessing anthropogenic influences on ecosystems is a framework of planetary boundaries proposed by Johann Rockström and Will Steffen in 2009. This framework was created to define a safe operating space for humanity based on the fundamental biophysical processes that regulate the stability of Earth as a functioning system. Within this framework, the biogeochemical flow of nitrogen is a control variable which crossed this defined boundary of a safe operating space and is at a high risk to globally change the Earth system (Steffen et al., 2015).

Due to the projected increase of the human population, it is not likely that atmospheric nitrogen deposition will drop significantly in the near future. Therefore, changes due to additional nitrogen input are likely to intensify (Canfield et al., 2010; Gruber and Galloway, 2008; Kanakidou et al., 2016). The function of forest soils as a carbon sink is potentially under threat, with major implications for global climate change.

This study reports a trans-European manipulation experiment which investigated the functional response of temperate forest soils to elevated atmospheric nitrogen deposition, focusing on potential changes in soil microbial community composition, litter decomposition and soil organic carbon turnover, as well as subsequent implications on climate change using a toolbox of stable isotope methods. We investigated (1) if elevated nitrogen inputs affect soil microbial activity in forest soils and (2) if soil microbial community composition responds to elevated nitrogen input. We hypothesized that increased soil N availability will lead to increased rates of organic matter breakdown in these nutrient –poor forest ecosystems by promoting the activity of free-living saprophytic microorganisms. Furthermore, we hypothesized that additional nitrogen input will induce a shift in microbial community composition away from populations adapted to nitrogen poor conditions towards copiotrophic communities.

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Abstract

Contemporary sources of atmospheric reactive nitrogen are to approximately 50% associated with human activities. Due to atmospheric transport of reactive nitrogen, nitrogen is often introduced into nitrogen-limited ecosystems like temperate forest ecosystems. Those anthropogenic changes to the biogeochemical cycle of nitrogen and subsequently of carbon add to the current climate crisis in many ways. Terrestrial ecosystems play a crucial role in regulating climate and affect climate change, with terrestrial soils being the most significant carbon sink on land. Soil microbial communities are a crucial part of the nitrogen cycle as microbes mediate most soil nitrogen transformations. In this report, we investigated the effects of medium-term exposure to elevated nitrogen input in temperate forest soils (80 to 100 kg ha^{-1} yr^{-1} for 3-7 years) by tracing ^{15}N- and ^{13}C-labelled litter material in bulk soils as well as in soil microbial communities at multiple sites across Europe (Austria, Germany, UK, and Sweden). Irrespective of differences in climatic parameters and soil chemistry, all study sites showed a faster decomposition in bulk soil nitrogen measurements. A significantly increased soil ^{13}C and ^{15}N retention was found across all four study sites. Phospholipid acid (PLFA) analysis showed the same pattern at both investigated study sites, with decreasing the gram-negative to gram-positive bacteria ratios under elevated nitrogen inputs. Soil respiration and soil microbial biomass nitrogen measurements revealed contradictory results. We hypothesised an increased rate of organic matter breakdown through added nitrogen inputs, which could be confirmed in Germany and Sweden, but not in Austria and the UK. This indicates that other parameters than nitrogen availability might be more important to control soil microbial activity in those forests.

Introduction

Atmospheric nitrogen deposition results from emissions of reactive nitrogen species and their atmospheric transport. They are emitted by natural sources like soils, lightning, plants as well as anthropogenic sources (Neff et al., 2002; Dentener et al., 2006; Galloway et al., 2008; Kanakidou et al., 2016). Before anthropogenic activities like industrial agriculture based on inorganic fertilizer usage and fossil fuel combustion, atmospheric nitrogen deposition was a relatively unimportant nitrogen source in terrestrial and marine ecosystems. Since the industrial and agricultural revolutions, reactive nitrogen deposition gradually became an important and sometimes even the most dominant nitrogen source in terrestrial and marine ecosystems. Contemporary sources of atmospheric nitrogen are approximately 50% associated with human activities (Fowler et al., 2013; Kanakidou et al., 2016; Stevens et al., 2018). There has been a threefold increase of nitrogen deposition over land and a twofold increase over the ocean since the industrial and agricultural revolutions (Kanakidou et al., 2016). Reactive nitrogen emissions in Europe have decreased or stabilized since the early 1990s due to implementations of stricter legislations. In Southeast Asia, especially China, atmospheric nitrogen deposition is still on the rise (Billen et al., 2013; Song et al., 2017). It has to be noted that it is hard to make exact estimations even just for one region because it is challenging to accurately quantify all the different nitrogen inputs by wet, particulate and gaseous forms due to different measurement requirements (Fenn et al., 2009; Sickman et al., 2019). However, due to atmospheric nitrogen transport, nitrogen is often introduced into nitrogen-limited ecosystems (Phoenix et al., 2006; Duce et al., 2008). Atmospheric nitrogen transport can have a wide transport range expanding to tens to thousands of kilometres depending on the composition of atmospheric reactive nitrogen (Phoenix et al., 2006; Duce et al., 2008; Bobbink et al., 2010).

Due to the large range in the quantity and quality of atmospheric nitrogen deposition, the effects of additional input of reactive nitrogen are manifold, far-reaching and highly complex upon biological systems (Bobbink and Hettelingh, 2011). Effects of additional nitrogen input into ecosystems depend on the amount and forms of nitrogen deposition as well as on the receiving ecosystem and its plant and microbial community (Robertson and Groffman, 2015). Excessive nitrogen deposition can have a negative impact on terrestrial ecosystems (Townsend and Howarth, 2010; Song et al., 2017), resulting in increased gaseous and mineral losses from the ecosystem, changes in the plant community functions and in biodiversity, soil acidification and reduction of phosphorus availability (Zaehle et al., 2011; Clark et al., 2013; Throop and Lerdau, 2004; Matson et al., 1999; Bertolini et al., 2016). Nitrogen is a limiting factor in most terrestrial and marine ecosystems and therefore controls primary productivity in those ecosystems (Mills

et al., 2004; Kanakidou et al., 2016; Stevens et al., 2018). Additional nitrogen input into terrestrial ecosystems also impacts soil microbial communities (Bobbink and Willems, 1987; Clark and Tilman, 2008; Horswill et al., 2008; Van den Berg et al., 2011; Stevens et al., 2018). Soil microbial communities are crucial players of the nitrogen cycle as microbes are mediating most soil nitrogen transformations like nitrogen mineralization and immobilization, as well as biological nitrogen fixation, nitrification and denitrification (Luxhøi et al., 2006; Geisseler et al., 2010). Microbes which are able to adapt to nutrient availability have a competitive advantage, but those organisms which are adapted to low nitrogen soils regulate the synthesis and activity of different enzyme systems more tightly (Koch, 1985). Numerous studies reported that the microbial community shifts when additional nitrogen is introduced. Additional nitrogen deposition could also cause a shift in the saprotrophic community and therefore cause changes in the decomposition of soil organic matter (SOM). In boreal and temperate forests, soil microbial biomass declined by 20% on average when additional nitrogen was introduced to the ecosystem (Janssens et al., 2010). Microbial transformations of reactive nitrogen also influence soil fertility, water quality and atmospheric chemistry on both small and large scales (Robertson and Groffman, 2015).

Effects of atmospheric nitrogen deposition on the soil carbon cycle and carbon storage are an important current research topic. Additional reactive nitrogen deposition in terrestrial ecosystems might play a relevant role in carbon cycle processes and interactions. Nitrogen-limited terrestrial ecosystems might enhance carbon sequestration, the process of capturing and storing atmospheric carbon dioxide, due to enhanced nitrogen fertilization. Nitrogen deposition could be therefore linked to the carbon cycle, in particular to carbon dioxide removal from the atmosphere due to increased primary production (Bertolini et al., 2016; Zaehle et al., 2011; Duce et al., 2008; Galloway et al., 2008; Kanakidou et al., 2016). Although different in their main outcome, all current meta-analyses suggest that atmospheric nitrogen deposition into terrestrial ecosystems is likely to change the global carbon cycle because nitrogen and carbon cycles are interdependent forming the basis of biochemical cycles and energy flows (Chapin et al., 2009; Chen et al., 2015). Several studies pointed out that the impact of additional nitrogen on ecosystem carbon dynamics depends on ecosystem type and rate of nitrogen input (Liu and Greaver, 2010; Chen et al., 2015), which could be explained by the nitrogen saturation hypothesis. This hypothesis states that once an ecosystem reaches nitrogen saturation, additional input of nitrogen will not increase its net primary production but reduce plant performance and soil functions (Aber et al., 1998; Chen et al., 2015). Additional nitrogen input may also lead to increased litterfall and decrease litter decomposition as well as decrease soil

respiration, both of which can foster carbon sequestration. Soil carbon pools do not seem to change with additional nitrogen input, which could be partly explained by the decrease of below-ground plant carbon allocation (Chen et al., 2015), however contrasting results state that plant root biomass was increased by nitrogen deposition (Xia and Wan, 2008; Lu et al., 2011). Anthropogenic changes to the biogeochemical cycles of nitrogen and subsequently of carbon add to the current climate crisis. Terrestrial ecosystems play a crucial role in regulating climate change, with terrestrial soils being the most significant carbon sink on land (Luo et al., 2015). All forests exposed to long-term nitrogen deposition will eventually run into nitrogen saturation, a state in which the forest nitrogen cycle is no longer closed (Janssens et al., 2010). Effects are dependent on the duration, cumulative amount and forms of additional nitrogen input, the intrinsic sensitivities of plant communities and the abiotic conditions of individual ecosystems (Bobbink et al., 2010). Potential changes to carbon sequestration in terrestrial soil would influence its carbon sink activity and therefore its carbon dioxide output into the atmosphere (Nadelhoffer et al., 1999). Additional nitrogen within ecosystems also enhances nitrous oxide emissions into the atmosphere through additional nitrification and denitrification activities. (Canfield et al., 2010; Robertson and Groffman, 2015). Due to the projected increase of the human population, it is not likely that atmospheric nitrogen depositions will drop significantly worldwide in the near future. Therefore, changes due to additional nitrogen input are likely to intensify (Canfield et al., 2010; Gruber and Galloway, 2008; Kanakidou et al., 2016). The function of forest soils as a carbon sink is potentially under threat, with major implications for global climate change.

This study reports a trans-European manipulation experiment which investigated the functional response of temperate forest soils to elevated atmospheric nitrogen deposition, focusing on potential changes in soil microbial community composition, litter decomposition and soil organic carbon turnover, as well as subsequent implications on climate change using a toolbox of stable isotope methods. We investigated (1) if elevated nitrogen is affecting soil microbial activity in forest soils and (2) whether soil microbial community composition responds to elevated nitrogen input. We hypothesized that increased nitrogen availability will promote organic matter breakdown in these nutrient –poor forest ecosystems through stimulation of free-living saprophytic microorganisms. Furthermore, we hypothesized that additional nitrogen input will induce a shift in microbial community composition from oligotrophic populations adapted to nitrogen poor conditions towards copiotrophic communities.

Materials and methods

Study sites

This study included four study sites distributed across Europe, representing nitrogen-limited temperate forests in Austria, Germany, Sweden and the UK. Austrian, German and Swedish sites were part of the trans-European ALTER-net-MSII network. Study sites were located in Zöbelboden, Austria, in Bayreuth, Germany, in Kindla, Sweden and in Moose House, UK. The Austrian study site was located in a mixed coniferous-broadleaf forest on Leptosol soil. The study site in Germany was located within a broadleaf forest dominated by *Betula pendula* on Cambisol soil. The study sites in Sweden and the UK were both located in broadleaf forests, stocking on Podzol in Sweden and Histosol in the UK. In the UK, nitrogen fertilization treatments started in 2014, in all other countries nitrogen treatments started in June 2010. Study site locations ranged from 23 meters above sea level (m. a.s.l.) in the UK to 632 m. a.s.l. in Austria. Mean annual temperatures ranged between 4 °C in Sweden to 9 °C in the UK. Mean annual precipitation (MAT) was 1800 mm at the Austrian study site, and ranged between 640 and 850 mm at the other sites. Forest soil C:N ratios varied between 15.3 in Germany and 33.9 in Sweden (Table 1).

Table 1: Characteristics of the study sites in Austria, Germany, Sweden and the UK: Location (longitude and latitude in decimal degrees [DD]), ecosystem type, soil type, meters above sea level [m a.s.l.], mean annual precipitation MAP [mm/yr], mean annual temperature MAT [°C/yr], soil C:N ratio, soil total nitrogen [µg/g] and decomposition rate.

Study site	Location (DD)	Ecosystem	Soil type	m a.s.l.	MAP	MAT	C:N ratio	Total N	Decomposition rate
Austria Zöbelboden	47.83333 14.43333	Forest - Coniferious and broadleaf	Leptosol	632	1800	7.20	21.4	10550	84
Germany Beyreuth	49.96666 11.50000	Forest - Birch	Cambisol	342	638	8.30	15.3	3459	83
Sweden Kindla	59.75000 14.90000	Forest - Broadleaf	Podsol	365	850	4.00	33.9	8358	-
UK Moore House	54.08330 -2.80000	Forest - Broadleaf	Histosol	23	676	9.00	21.8	17483	92

Experimental design

This experiment was carried out as a randomized block design with five plots per study site. Each plot was subdivided into a nitrogen treatment subplot and a control treatment subplot. Each plot measured 5 m^2, and subplots measured 1 m^2. The plots were randomly distributed within an area of about 20 x 20 m with at least 2 m between plots. Nitrogen treatment subplots were watered once a month with tap water containing ammonium nitrate fertilizer. According

to literature, we simulated an additional nitrogen input of 80 to 100 kg N/ha/yr, which is equivalent to 8 to 10 g N/m²/yr. Control treatment subplots were watered once a month with tap water only. Each subplot received one litter of solution every 3rd week. Fertilization was not conducted during heavy rains. For the labelling experiment, all study sites received mesh bags containing isotope labelled, pre-leached birch litter mixed with topsoil. Soils of one treatment of all plots within a study site were mixed to counteract variabilities between plots. 2.65 g of dry litter was mixed with 240 g of their respective soils. Mixed soils were then filled into mesh bags of 50 ± 10 microns. This mesh size is big enough to allow access to bacteria, fungal hyphae, most nematodes and protozoa while restricting access to mesofauna and macrofauna. These mesh bags were each filled with 5 g of soil and re-buried at the top of the A horizon. Eight mesh bags were re-buried on each plot, four mesh bags per treatment. At subsequent sampling times, mesh bags were simultaneously collected from all sites and dispatched by post for analysis. Additionally, samples were collected at the start of the experiment with and without added labelled material on the 1st of June 2017 (T0 unlabelled, T0 labelled). After samples were re-buried, they were collected on four sampling times, on the 14th of June 2017 (T1), on the 28th of June 2017 (T2), on the 26th of July 2017 (T3) and on the 30th of September 2017 (T4). 336 samples were collected in total.

Production of stable isotope labelled litter material

In order to produce isotopically labelled material, 2-year-old *Betula pendula* were collected. They were then repeatedly isotopically labelled with ^{15}N by soil amendment of ^{15}N fertilizer, and with $^{13}CCO_2$, using a Perspex isotopic labelling chamber. $^{13}CCO_2$ labelling was achieved by pulse labelling using sodium bicarbonate ($NaH^{13}CO_3$, Sigma Aldrich, 98 atom% ^{13}C). $NaHCO_3$ was used according to Hood et al., (2004). We used 10 mL of 0.5 M $NaH^{13}CO_3$ solution per injection. The ^{15}N labelling solution was prepared from 10 atom% ^{15}N at a solution concentration of 50 mM ammonium nitrate (NH_4NO_3). Labelling was carried out weekly over 120 days. Trees were harvested on the 15th of September 2015. Beech saplings were separated into leaf, stem and root fractions, which were dried, weighed and coarsely ground. Leaf samples were subsequently leached twice with boiling water and re-dried at 40 °C to reduce the soluble N and C fractions, in order to assure even labelling and reduce the risk of leaching losses compounding the results. Subsamples of leached materials were dried and finely ground for subsequent isotope analysis. The produced leached leaf litter material that was isotopically enriched and had $\delta^{13}C$ values of 4.38 ‰ and $\delta^{15}N$ values of 266 ‰.

Bulk soil analysis

Soil samples were immediately dried at 60 °C for three days and ball-milled afterwards. Bulk soil analysis was conducted using 20 mg of dried soil samples. The isotopic composition of C is reported in the delta (δ) notation relative to the Vienna Pee Dee Belemnite (V-PDB) standard. The isotopic composition of N is reported in the delta (δ) notation relative to the atmospheric N_2 standard. The precision (standard deviation) of the $\delta_{13}C_{tot}$ and $\delta_{15}N_{tot}$ measurements was ≤ 0.2‰. Every soil sample was analyzed using the Dumas combustion method with an elemental analyser linked to an isotope ratio mass spectrometer (IRMS). Total nitrogen and organic carbon contents as well as $\delta_{15}N_{tot}$ and $\delta_{13}C_{tot}$ of bulk soils were measured using an EA IsoLink™ IRMS System by Thermo Scientific™, detecting the mass-to-charge ratios (m/z) 44, 45 and 46 (CO_2) and 28, 29 and 30 (N_2).

Soil microbial biomass analysis

Total soil microbial biomass was measured using the chloroform-fumigation-extraction (CEF) method (Hood et al., 2010; Makarov et al., 2015).

Aliquots of one gram fresh soil were weighed into 12 mL vacutainers. Fumigated samples were prepared by 24-hour fumigation in ethanol-free chloroform (MTBE stabilized) in an evacuated darkened desiccator, while un-fumigated controls were left at room temperature without chloroform exposure. After 24 h, 8 mL 0.05 M K_2SO_4 was added to all samples, shaken for 1 hour and centrifuged at 3000 rpm for 10 minutes (Brookes et al., 1985; Amato and Ladd, 1988). The supernatant was extracted using a syringe filter set-up. Aliquots (1 mL) of each sample was dried in round-bottomed Eppendorf tubes at 60 °C until dry. Samples of 60-80 mg salt residue were prepared for carbon and nitrogen content and isotope analysis using an EA IsoLink™ IRMS System by Thermo Scientific™. Calculation of the isotopic signature of microbial biomass was done using isotope and mass balance equations:

$$\delta_{15}N_{MB} = (\delta_{15}N_{FUM} * N_{FUM} - \delta_{15}N_{UNFUM} * N_{UNFUM}) / N_{MB}$$

where FUM is the fumigated and UNFUM is the unfumigated fraction of $CHCl_3$-labile microbial biomass (Widmer et al., 1989). Samples were analysed for isotope composition of total microbial biomass ($\delta_{15}N_{MB}$), as well as percentage nitrogen and percentage carbon.

Soil respiration analysis

Soil respiration (rate of CO_2 production) was measured as described by Kitzler et al. (2006). Respiration measurements were made on one-gram fresh soil samples accurately weighed into 12 mL gas-tight Labco Vacutainers, which were left to stand in the lab at 20°C for one day before analysis. Sample vials were capped, repeatedly evacuated and manually refilled with high purity helium. Empty jars without soils were used as controls. Samples were automatically injected and measured at 120- and 620-minutes following evacuation, giving a sampling interval of approximately 406 minutes. This was necessary to account for very low backgrounds and to obtain flux measurements. Sampling was done using a CTC analytics CombiPal autosampler injecting 500 µL gas withdrawn from the 12 mL vial held at 20 °C into a Thermo-Fisher Trace Gas Chromatograph Ultra. Chromatographic separation was achieved with a helium carrier pressure of 60 kPa, via an Agilent PoraPlot Q (25 m x 0.32 mm). The starting temperature was 35 °C which was held for 3.5 min and raised to 105 °C by 40 °C steps per minute. All gases were passed through a high-temperature conversion reactor (HTC, maintained at 200°C to prevent sample re-condensation or physical conversion) installed within a ThermoFisher GC-Isolink. $\delta_{13}C_{CO2}$ was measured by a ThermoFisher DeltaV isotope ratio mass spectrometer detecting the mass-to-charge ratios (m/z) 44, 45 and 46.

Phospholipid fatty acid analysis

To investigate metabolically active soil microbial communities, phospholipid fatty acid (PLFA) analysis was coupled with stable isotope probing (Boschker and Middelburg, 2002; Johnsen et al., 2002; Wawra et al., 2018), with a HP5890 Series II (Agilent, Santa Clara, USA) connected to a Delta S via a Combustion II Interface (Finnigan, Bremen, Germany). PLFAs were analysed according to Watzinger (2015). Briefly, one gram of fresh soil was extracted in a chloroform-methanol solution, the lipid fraction retained, and the phospholipid fraction chromatographically separated from the bulk lipids. Methylation was achieved via an alkaline methylation procedure and samples re-solubilized in iso-octane. 13:0 and 19:0 standards as well as two blanks were run within all batches. Isotopic ratios and concentrations of PLFAs of the resulting fatty acid methyl esters (FAMES) were calculated according to Watzinger (2015) and 13:0 standards were used for corrections.

A total of 22 different PLFA were detected and identified by GC-combustion-IRMS: Straight-chain saturated PLFAs (14:0, 15:0, 16:0, 18:0), monounsaturated PLFAs (16:1ω5, 16:1ω7+6, 17:1ω8, 18:1ω5c, 18:1ω7c/9t, 18:1ω9c), polyunsaturated PLFAs (18:2ω6,9), terminally

branched saturated PLFAs (i14:0, i15:0, i16:0, i17:0, a15:0, a17:0), cyclic PLFAs (cy17:0, cy19:0+cy19:1) and saturated PLFAs with mid-chain branching (10Me17:0, 10Me18:0+12Me18:0, i17ω1:8+10Me16:0). Due to overlapping peaks in the chromatogram, separation of 16:1ω7 and 16:1ω6, i17:1ω8 and 10Me16:0, 10Me18:0 and 12Me18:0, as well as cy19:0 and 19:1 was not possible, and the peaks were integrated and analysed combined. Some PLFAs were not included in further investigations and statistical analysis due to low and therefore uncertain concentrations in samples, e.g. 14:0, 15:0, 10Me17:0, 17:1ω8, a17:0, i14:0 and i17:0.

$\delta^{13}C_{PLFA}$ of total PLFA and ^{13}C excess of total PLFA were investigated for the Austrian and German study sites. Additionally, fatty acids were summed into biomarker groups to investigate changes in soil microbial communities over time. Excluding weaker biomarkers, a15:0, i15:0, i16:0 and 10Me18:0+12Me18:0 were used as biomarkers for gram-positive bacteria and 16:1ω7+6, 18:1ω5c, 18:1ω7c/9t, cy17:0 and cy19:0+19:1 as biomarkers for gram-negative bacteria. 18:2w6,9 was used as a biomarker for fungal organisms (Wawra et al., 2018; Watzinger et al., 2014; McKinley et al., 2005; Frostegård and Bååth, 1996; Zak et al., 1996).

Data analysis

In order to investigate potential effects of elevated nitrogen input in forest soils, incorporation of isotopically labelled litter material was investigated, which was followed as ^{13}C excess and ^{15}N excess. Atom percent (at%) ^{13}C and ^{15}N were calculated from the isotopic signatures of $\delta^{15}N_{tot}$, $\delta^{15}N_{MB}$, $\delta^{13}C_{tot}$, $\delta^{13}C_{CO2}$ and $\delta^{13}C_{PLFA}$.

$$at\% = 100 * ((\delta + 1000) / (\delta + 1000 + (1000 / R_{Standard})$$

where $R_{Standard}$ is the ratio R (^{13}C:^{12}C) of PeeDee Belemnite (PDB) for ^{13}C excess, or the ratio R (^{15}N:^{14}N) of atmospheric air N_2 (AIR) for ^{15}N excess. Atom percent excess (APE) is then calculated by the following approximation:

$$APE = at_s - at_n$$

Where at_n is the atom percent of soil at natural stable isotope abundance and at_s is the atom percent of soils treated by labelled material amendment. To estimate incorporation of ^{13}C (and ^{15}N) into bulk soil nitrogen and carbon, microbial biomass nitrogen, soil respired CO_2 and PLFA, APE was used:

^{13}C excess (^{15}N excess) = m_s * (APE / 100)

where m_s is the mass of the measured compound and ^{13}C excess (^{15}N excess) is the mass of the labelled fraction in the measured compound.

Statistical analysis was performed with IBM SPSS statistical software version 25.0 for Mac OS (SPSS Inc.). Graphical representation of data was performed with SigmaPlot 14.0 (Systat Software Inc.). In order to investigate treatment effects on bulk soil nitrogen, bulk soil carbon, soil respired CO_2 and soil microbial nitrogen, as well as their isotope excesses two-way mixed analysis of variance (ANOVA) was used testing for the main effects of treatment and time, as well as their interaction. Due to fewer sampling times, one-way analysis of covariance (ANCOVA) was used to investigate treatment effects on PLFA results.

Results

Added ^{13}C and ^{15}N labelled material was utilized over time by soil microbial communities

A successful incorporation of added ^{15}N and ^{13}C labelled material could be observed through significant changes of isotope enrichments between unlabelled and labelled soil (Fig. 2 and Fig. 4). Overall, values of microbial ^{15}N excess and ^{13}C excess revealed a decrease over a four-month sampling period between first and last measurements in all experiments and from all four study sites. Statistical analysis further emphasised these results by showing significant differences over time for microbial ^{15}N excess as well as for ^{13}C excess in all experiments and all study sites as well. These results indicate the decomposition and microbial incorporation of added labelled material in these forest soils. Additionally, isotopic excess values did not drop to initial values of unlabelled soils in the four-month sampling time, indicating significant retention of labelled matter.

Response of isotope excess in bulk soil N, bulk soil C, soil microbial biomass N and soil respired CO_2 to elevated nitrogen input

Two-way ANOVA of ^{15}N excess of bulk soil nitrogen showed significant differences between treatments in all four study sites. Higher ^{15}N excess values in nitrogen treatment subplots of bulk soils were observed at all sampling times in all four study sites, with the last two measuring points in Germany as exceptions. Especially strong differences of bulk soil ^{15}N excess values between nitrogen treatment and control treatment subplots could be observed in Sweden.

Similarly, high values in nitrogen treatment subplots could be detected for ^{13}C excess values of bulk soil carbon, with exceptions again observed in Germany as well as especially high differences found in Sweden. Statistical analysis of ^{13}C excess of bulk soil carbon showed significant differences between treatments for all countries. Contrary to bulk soil nitrogen results, ^{13}C excess of bulk soil carbon showed significant interaction terms in all four countries, highlighting that ^{13}C excess did not change monotonously for both treatments over time.

The isotope excess of soil microbial biomass nitrogen and respiratory CO_2, except for microbial biomass nitrogen measurements in Austria, showed patterns similar to bulk soil trends. Contrary to our expectation, significant differences of ^{15}N excess of soil microbial biomass nitrogen between treatments were observed in Germany and Sweden but not in Austria and the UK. In response to elevated nitrogen treatment, soil respired CO_2 showed significant differences to control treatments in Germany and Sweden. However, statistical analysis did not confirm any significant differences for Austria and the UK^{13}C excess of soil respired CO_2 showed significant interaction terms in all study sites except Austria, highlighting that ^{13}C excess did not change monotonously for elevated nitrogen input subplots compared to control subplots over time in those study sites.

Significant interaction terms of treatment effect and time for bulk soil nitrogen as well as soil microbial biomass nitrogen were found only in Germany, indicating that in German forest soils ^{15}N excess responded differently over time between unamended soils and nitrogen fertilized soils.

Response of soil microbial community composition and ^{13}C-PLFA incorporation to elevated nitrogen input

Phospholipid fatty acid analysis revealed high ^{13}C uptake in gram-negative bacteria relative to fungi and gram-positive bacteria. Results of statistical analysis using one-way ANCOVA revealed significant differences between treatments for ^{13}C excess of total PLFA for both investigated study sites. Closer inspections of ^{13}C incorporation into PLFA of different organism groups revealed that ^{13}C excess in PLFA of gram-negative bacteria showed significant differences, with values of ^{13}C excess decreasing more rapidly in nitrogen treatment subplots of both Austrian and German study sites. Additionally, statistical analysis did not confirm any significant differences between treatments in ^{13}C uptake for fungi or gram-positive bacteria in forest soils.

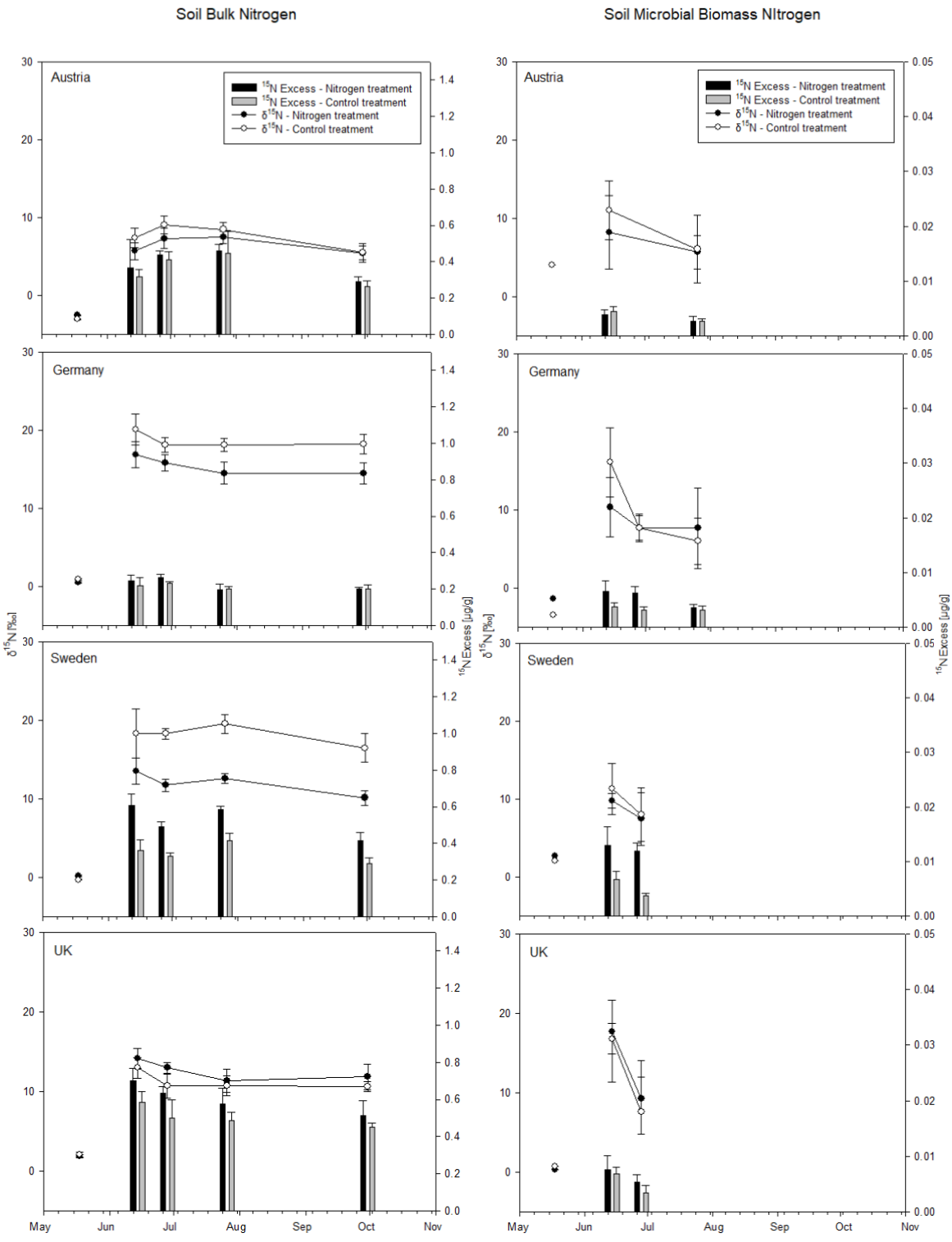

Fig.2: Delta 15N values (per mil) and 15N excess (µg/g) of bulk soil nitrogen (left column) and soil microbial biomass nitrogen (right column). Delta 15N values of unlabelled soils are shown in the beginning (mid May).

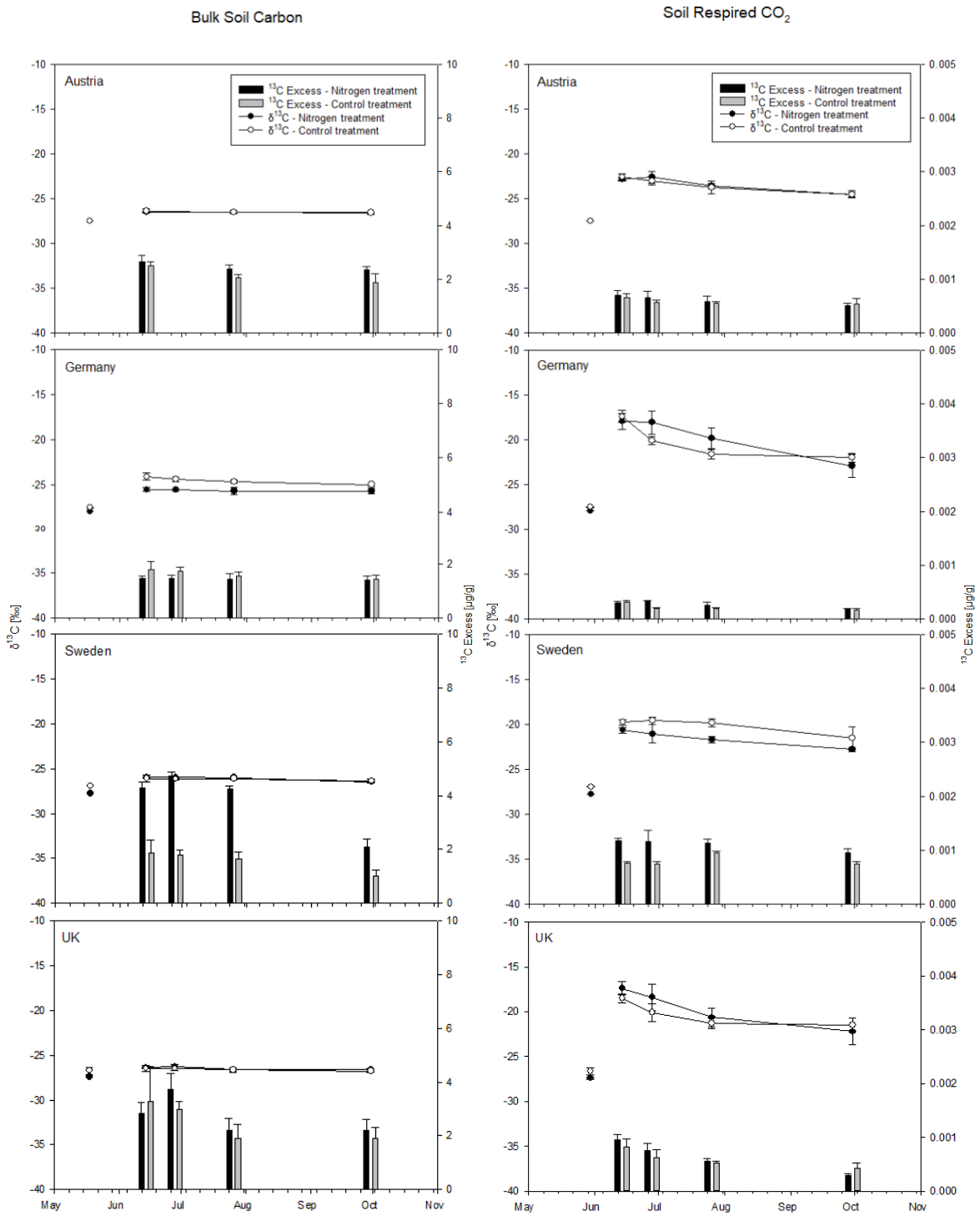

Fig. 3: Delta 13C values (per mil) and 13C excess (µg/g) of bulk soil carbon (left column) and soil respired CO_2 (right column). Delta 13C values of unlabelled soils are shown in the beginning (mid May).

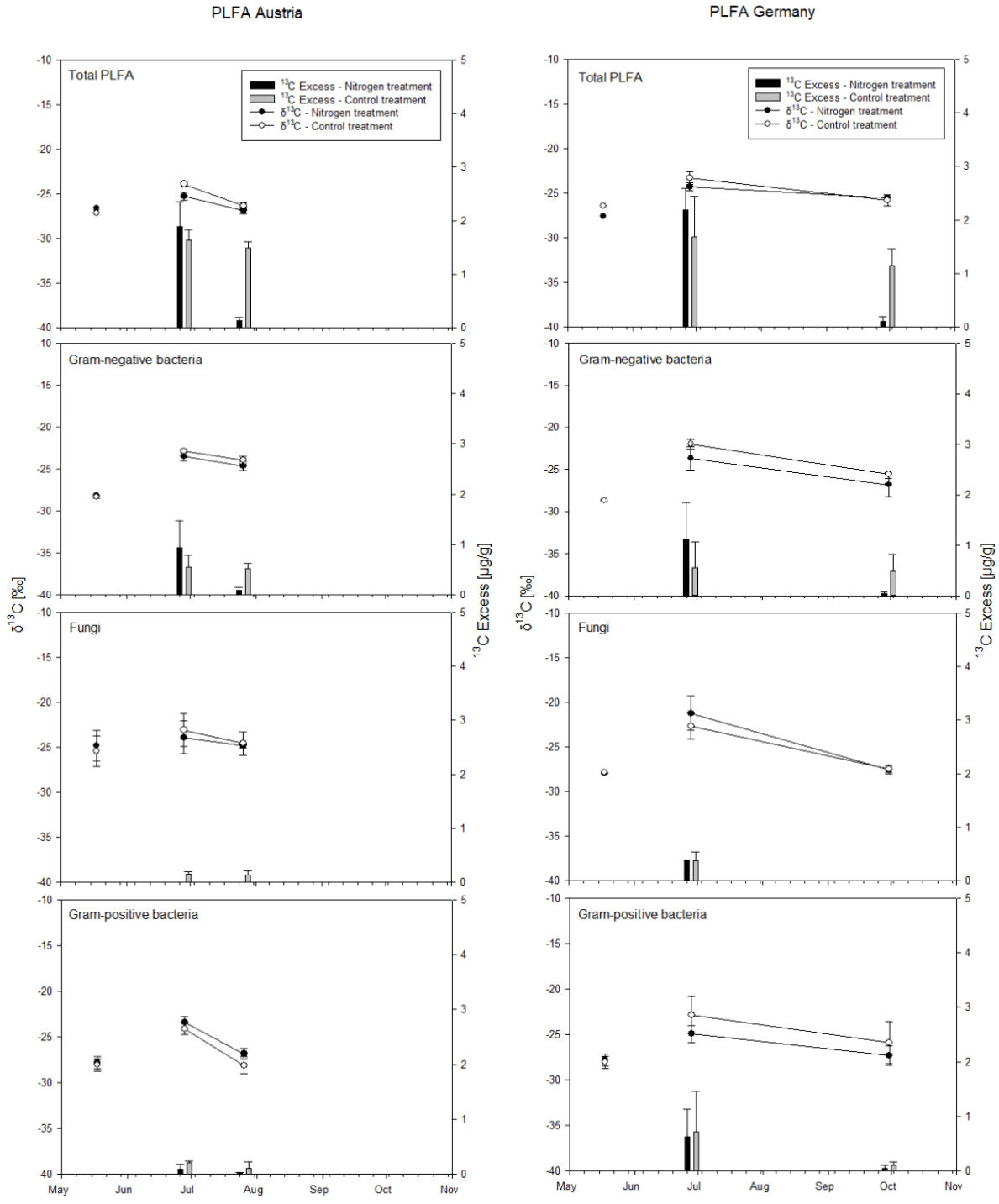

Fig. 4: Delta 13C values (per mil) and 13C excess (nmol/g) of total PLFA, gram-negative bacteria, fungi and gram-positive bacteria of the Austrian and German study sites. Delta 13C values of unlabelled soils are shown in the beginning (mid May).

Discussion

Added isotopically labelled material was utilized by soil microbial communities

We could confirm that labelled carbon of the added litter material was released by decomposition and subsequently utilized by the soil microbial communities. Soil CO_2 efflux showed the highest ^{13}C enrichment shortly after labelled litter addition and thereafter steadily declined, suggesting the rapid utilization of the added labelled litter. A continued decrease in microbial ^{13}C-incorporation could also be observed in all organism groups which were investigated via PLFA analysis.

The results revealed that ^{15}N-labelled litter was utilized as well, with microbial biomass ^{15}N enrichment decreasing over time. However, ^{15}N enrichment of the soil microbial biomass showed relatively high fluctuation across all study sites. It is therefore plausible that the choice of method, chloroform fumigation extraction (CFE) caused the higher variation in microbial biomass ^{15}N results. Despite the limitations of the CFE method, and consequently high variations in microbial biomass ^{15}N, the findings do nevertheless suggest that added labelled nitrogen was utilized by soil microbial communities.

Responses of bulk soil nitrogen and carbon isotopic enrichment to elevated nitrogen input are significant across all investigated study sites

The effects of elevated nitrogen input on biogeochemical processes in forest soils were investigated using replicated experiments in four forest ecosystems across Europe. The study sites differed in mean annual temperature, mean annual precipitation and site elevation, forest composition, as well as in soil physicochemical characteristics like soil C:N ratios, and soil nitrogen and soil carbon concentration. Despite these differences, bulk soil isotope measurements of all four study sites showed similar responses to elevated nitrogen inputs. All four study sites showed significant treatment effects to elevated nitrogen input in bulk soil nitrogen and carbon isotopes.

Elevated nitrogen input shows different effects on soil respiration and soil microbial biomass nitrogen depending on study site

Contrary to bulk soil measurements, soil respiration and microbial biomass nitrogen isotopes showed contradictory results across study sites. Both parameters showed significant treatment effects in Germany and Sweden, where a faster decomposition rates in nitrogen treatment subplots could be detected, while no effects were observed in Austria and the UK. A meta-

analysis of effects of elevated nitrogen exposure by Chen et al. (2015) stated that soil respiration in temperate forest soils decreases when exposed to elevated nitrogen input, which we could confirm for our German and Swedish study sites. The reason for our rather contradictory results in Austria and the UK suggests that other parameters-controlled forest soil microbial activity in those forest soils.

Elevated nitrogen input causes a shift in soil microbial community compositions

We found significantly faster decomposition of ^{13}C labelled litter into total PLFA and gram-negative bacteria when exposed to elevated nitrogen input. These results were consistent across both investigated study sites. Differences in ^{13}C excess of total PLFA were most likely attributed to parallel changes in ^{13}C excess of gram-negative bacteria. Numerous studies reported that the microbial community shifts when additional nitrogen is introduced into forest ecosystems (Janssens et al., 2010), which we could also confirm. Our findings are in agreement with a meta-study of previous nitrogen-addition experiments published by Zhou et al. (2017), which showed that nitrogen addition consistently decreased gram-negative to gram-positive bacteria ratios.

Conclusion

This study explored the effects of elevated nitrogen deposition on temperate forest soil microbial communities and their functions by tracing added ^{15}N- and ^{13}C-labelled litter into bulk soil, microbial biomass nitrogen, soil respiration and PLFA. The experiments were conducted in four forest ecosystems across Europe. Irrespective of differences in climate parameters and soil chemistry, all study sites showed similar results in bulk soil carbon and nitrogen dynamics, where a fast decomposition could be observed with elevated nitrogen input in all study sites except Austria. Same trend could be observed for gram-negative bacteria. Elevated nitrogen also affected the composition of soil microbial communities by decreasing the gram-negative to gram-positive bacteria ratio.

Soil respiration and soil microbial biomass nitrogen measurements revealed contradictory results. We expected faster decomposition of soil organic matter through additional nitrogen inputs for both experiments, which could be confirmed in Germany and Sweden, but not in Austria and the UK. This highlights that other environmental factors exerted greater control than nitrogen availability on soil microbial activity in those forests.

Appendix

Results of bulk soil measurements as well as soil microbial biomass nitrogen, respiration and PLFA analysis

Table 2: Arithmetic means and standard deviations of $_{15}N$ excess [µg/g] and $\delta_{15}N$ [per mil] of bulk soil measurements. Samples were taken in Austria, Germany, Sweden and the UK over a four-month sampling time.

Bulk Soil Measurments		15N Excess [µg/g]		δ15N [‰]	
		Nitrogen treatment	Control	Nitrogen treatment	Control
Austria	unlabelled soil	0	0	-2.52 ± 0.04	-3.04 ± 0.18
	17.06.14	0.37 ± 0.15	0.32 ± 0.03	5.69 ± 1.07	7.81 ± 0.89
	17.06.28	0.44 ± 0.02	0.41 ± 0.04	7.30 ± 1.28	9.04 ± 1.15
	17.07.26	0.46 ± 0.03	0.44 ± 0.11	7.48 ± 0.78	8.47 ± 0.87
	17.09.30	0.29 ± 0.02	0.26 ± 0.03	5.45 ± 0.92	5.49 ± 1.20
Germany	unlabelled soil	0	0	0.54 ± 0.13	0.94 ± 0.15
	17.06.14	0.25 ± 0.03	0.22 ± 0.04	16.90 ± 1.68	20.11 ± 1.96
	17.06.28	0.26 ± 0.01	0.23 ± 0.01	15.85 ± 1.02	18.49 ± 1.53
	17.07.26	0.20 ± 0.03	0.19 ± 0.01	14.51 ± 1.38	18.11 ± 0.81
	17.09.30	0.19 ± 0.01	0.19 ± 0.02	14.50 ± 1.34	18.24 ± 1.25
Sweden	unlabelled soil	0	0	0.18 ± 0.09	-0.28 ± 0.07
	17.06.14	0.60 ± 0.07	0.36 ± 0.05	13.57 ± 1.65	18.32 ± 3.13
	17.06.28	0.48 ± 0.02	0.33 ± 0.01	11.74 ± 0.77	18.30 ± 0.70
	17.07.26	0.58 ± 0.01	0.41 ± 0.03	12.61 ± 0.65	19.55 ± 1.18
	17.09.30	0.41 ± 0.04	0.29 ± 0.03	10.15 ± 0.94	16.49 ± 1.84
UK	unlabelled soil	0	0	1.82 ± 0.18	1.81 ± 0.12
	17.06.14	0.70 ± 0.06	0.58 ± 0.05	14.19 ± 1.26	13.01 ± 1.31
	17.06.28	0.63 ± 0.03	0.50 ± 0.09	13.02 ± 0.67	10.69 ± 1.54
	17.07.26	0.57 ± 0.08	0.48 ± 0.04	11.37 ± 1.42	10.76 ± 1.23
	17.09.30	0.51 ± 0.07	0.45 ± 0.02	11.87 ± 1.61	10.61 ± 0.59

Table 3: Arithmetic means and standard deviations of $_{15}N$ excess [µg/g] and $\delta_{15}N$ [per mil] of soil microbial biomass nitrogen measurements. Samples were taken in Austria, Germany, Sweden and the UK over a four-month sampling time.

Microbial Biomass Measurements		15N Excess [µg/g]		d15N [‰]	
		Nitrogen treatment	Control	Nitrogen treatment	Control
Austria	unlabelled soil	0	0	NA	4.01
	17.06.14	0.0038 ± 0.0008	0.0045 ± 0.0008	8.24 ± 4.67	11.05 ± 3.72
	17.06.28	NA	NA	NA	NA
	17.07.26	0.0027 ± 0.0008	0.0027 ± 0.0003	5.68 ± 2.15	6.08 ± 4.31
Germany	unlabelled soil	0	0	-1.30	-3.40
	17.06.14	0.0066 ± 0.0018	0.0036 ± 0.0008	10.37 ± 3.82	16.11 ± 4.42
	17.06.28	0.0063 ± 0.0011	0.0032 ± 0.0005	7.71 ± 1.58	7.71 ± 1.80
	17.07.26	0.0035 ± 0.0006	0.0031 ± 0.0007	7.66 ± 5.16	5.99 ± 3.00
Sweden	unlabelled soil	0	0	2.67	2.11
	17.06.14	0.0130 ± 0.0034	0.0067 ± 0.0013	9.82 ± 0.93	11.35 ± 3.28
	17.06.28	0.0118 ± 0.0015	0.0036 ± 0.0005	7.45 ± 3.35	8.00 ± 3.42
	17.07.26	NA	NA	NA	NA
UK	unlabelled soil	0	0	0.28	0.71
	17.06.14	0.0076 ± 0.0024	0.0068 ± 0.0012	16.51 ± 5.12	16.81 ± 1.95
	17.06.28	0.0053 ± 0.0013	0.0034 ± 0.0013	9.46 ± 4.62	8.37 ± 3.60
	17.07.26	NA	NA	NA	NA

Table 4: Arithmetic means and standard deviations of $_{13}C$ excess [µg/g] and $\delta_{13}C$ [per mil] of bulk soil measurements. Samples were taken in Austria, Germany, Sweden and the UK over a four-month sampling time. Samples of Austria T2 (17.06.28) were removed due to unrealistic results.

Bulk Soil Measurments		13C Excess [µg/g]		δ13C [‰]	
		Nitrogen treatment	Control	Nitrogen treatment	Control
Austria	unlabelled soil	0	0	-27.48 ± 0.01	-27.48 ± 0.02
	17.06.14	2.51 ± 0.25	2.49 ± 0.15	-26.46 ± 0.14	-26.38 ± 0.22
	17.06.28	NA	NA	NA	NA
	17.07.26	2.38 ± 0.14	2.04 ± 0.12	-26.50 ± 0.14	-26.50 ± 0.09
	17.09.30	2.34 ± 0.12	1.87 ± 0.31	-26.54 ± 0.14	-26.57 ± 0.18
Germany	unlabelled soil	0	0	-27.79 ± 0.32	-27.55 ± 0.07
	17.06.14	1.44 ± 0.10	1.77 ± 0.30	-25.50 ± 0.21	-24.10 ± 0.41
	17.06.28	1.46 ± 0.11	1.71 ± 0.16	-25.57 ± 0.13	-24.36 ± 0.24
	17.07.26	1.42 ± 0.21	1.55 ± 0.13	-25.67 ± 0.41	-24.64 ± 0.17
	17.09.30	1.38 ± 0.16	1.43 ± 0.14	-25.67 ± 0.34	-24.96 ± 0.18
Sweden	unlabelled soil	0	0	-27.77 ± 0.03	-26.93 ± 0.03
	17.06.14	4.29 ± 0.20	1.88 ± 0.46	-25.95 ± 0.04	-26.10 ± 0.38
	17.06.28	4.71 ± 0.18	1.80 ± 0.18	-25.94 ± 0.04	-26.11 ± 0.13
	17.07.26	4.24 ± 0.10	1.65 ± 0.26	-25.97 ± 0.03	-26.11 ± 0.18
	17.09.30	2.07 ± 0.30	1.01 ± 0.20	-26.49 ± 0.07	-26.36 ± 0.19
UK	unlabelled soil	0	0	-27.42 ± 0.11	-27.26 ± 0.37
	17.06.14	2.82 ± 0.43	3.29 ± 1.30	-26.36 ± 0.07	-26.46 ± 0.31
	17.06.28	3.74 ± 0.59	2.97 ± 0.31	-26.24 ± 0.21	-26.47 ± 0.19
	17.07.26	2.20 ± 0.43	1.89 ± 0.52	-26.58 ± 0.21	-26.61 ± 0.29
	17.09.30	2.18 ± 0.41	1.82 ± 0.34	-26.60 ± 0.18	-26.76 ± 0.15

Table 5: Arithmetic means and standard deviations of $_{13}C$ excess [µg/g] and $\delta_{13}C$ [per mil] of respiration measurements. Samples were taken in Austria, Germany, Sweden and the UK over a four-month sampling time.

Respiration Measurements		13C CO2 Excess [µg/g]		δ13C CO2 [‰]	
		Nitrogen treatment	Control	Nitrogen treatment	Control
Austria	17.06.14	0.00071 ± 0.00008	0.00065 ± 0.00007	-22.85 ± 0.21	-22.58 ± 0.34
	17.06.28	0.00061 ± 0.00008	0.00057 ± 0.00004	-22.62 ± 0.65	-23.02 ± 0.48
	17.07.26	0.00057 ± 0.00010	0.00055 ± 0.00003	-23.58 ± 0.31	-23.74 ± 0.75
	17.09.30	0.00051 ± 0.00001	0.00053 ± 0.00001	-24.54 ± 0.40	-24.54 ± 0.26
Germany	17.06.14	0.00030 ± 0.00002	0.00031 ± 0.00002	-17.96 ± 0.89	-17.40 ± 0.66
	17.06.28	0.00032 ± 0.00002	0.00019 ± 0.00001	-18.06 ± 1.28	-20.06 ± 0.44
	17.07.26	0.00025 ± 0.00005	0.00019 ± 0.00001	-19.82 ± 1.13	-21.58 ± 0.53
	17.09.30	0.00017 ± 0.00003	0.00017 ± 0.00002	-22.90 ± 1.26	-22.00 ± 0.46
Sweden	17.06.14	0.00117 ± 0.00004	0.00076 ± 0.00003	-20.64 ± 0.33	-19.75 ± 0.29
	17.06.28	0.00116 ± 0.00020	0.00075 ± 0.00004	-21.04 ± 1.03	-19.57 ± 0.42
	17.07.26	0.00113 ± 0.00007	0.00094 ± 0.00003	-21.72 ± 0.37	-19.82 ± 0.44
	17.09.30	0.00094 ± 0.00008	0.00073 ± 0.00005	-22.80 ± 0.29	-21.54 ± 1.29
UK	17.06.14	0.00096 ± 0.00009	0.00082 ± 0.00014	-17.36 ± 0.73	-18.50 ± 0.52
	17.06.28	0.00076 ± 0.00001	0.00062 ± 0.00014	-18.36 ± 1.49	-20.10 ± 1.03
	17.07.26	0.00055 ± 0.00005	0.00052 ± 0.00001	-20.62 ± 1.07	-21.30 ± 0.57
	17.09.30	0.00029 ± 0.00003	0.00042 ± 0.00001	-22.22 ± 1.49	-21.48 ± 0.82

Table 6: Arithmetic means and standard deviations of $_{13}C$ excess [nmol/g] and $\delta_{13}C$ [per mil] of PLFA analysis in our Austria study site.

Phospolipid Fatty Acid Analysis Austria		13C Excess [nmol/g]		d13C PLFA (‰)	
		Nitrogen treatment	Control	Nitrogen treatment	Control
Total PLFA	unlabelled soil	0.00	0.00	-27.63	-27.17
	17.06.14	NA	NA	NA	NA
	17.06.28	1.895 ± 0.45	1.62 ± 0.19	-25.26 ± 0.41	-23.87 ± 0.27
	17.07.26	0.129 ± 0.06	1.480 ± 0.12	-26.90 ± 0.37	-26.31 ± 0.36
	17.09.30	NA	NA	NA	NA
Gram-negative bacteria	unlabelled soil	0.00	0.00	-28.10	-29.86
	17.06.14	NA	NA	NA	NA
	17.06.28	0.944 ± 0.52	0.555 ± 0.22	-23.51 ± 0.48	-22.84 ± 0.14
	17.07.26	0.087 ± 0.05	0.513 ± 0.11	-24.62 ± 0.54	-23.92 ± 0.44
	17.09.30	NA	NA	NA	NA
Fungi	unlabelled soil	0.00	0.00	-26.61	-26.68
	17.06.14	NA	NA	NA	NA
	17.06.28	0.001 ± 0.00	0.150 ± 0.04	-23.89 ± 1.84	-23.07 ± 1.81
	17.07.26	0.00 ± 0.00	0.130 ± 0.07	-24.84 ± 0.20	-24.57 ± 1.27
	17.09.30	NA	NA	NA	NA
Gram-positive bacteria	unlabelled soil	0.00	0.00	-27.11	-28.41
	17.06.14	NA	NA	NA	NA
	17.06.28	0.093 ± 0.08	0.209 ± 0.02	-23.41 ± 0.66	-24.13 ± 0.63
	17.07.26	0.018 ± 0.00	0.145 ± 0.04	-26.83 ± 0.62	-28.09 ± 0.95
	17.09.30	NA	NA	NA	NA

Table 7: Arithmetic means and standard deviations of $_{13}C$ excess [nmol/g] and $\delta_{13}C$ [per mil] of PLFA analysis in our German study site.

Phospolipid Fatty Acid Analysis Germany		13C Excess [nmol/g]		d13C PLFA (‰)	
		Nitrogen treatment	Control	Nitrogen treatment	Control
Total PLFA	unlabelled soil	0.00	0.00	-27.58	-26.47
	17.06.14	NA	NA	NA	NA
	17.06.28	2.191 ± 0.40	1.679 ± 0.75	-24.27 ± 0.47	-23.27 ± 0.72
	17.07.26	NA	NA	NA	NA
	17.09.30	0.110 ± 0.08	1.148 ± 0.30	-25.55 ± 0.12	-25.79 ± 0.65
Gram-negative bacteria	unlabelled soil	0.00	0.00	-28.65	-28.50
	17.06.14	NA	NA	NA	NA
	17.06.28	1.111 ± 0.72	0.548 ± 0.52	-23.93 ± 1.54	-21.98 ± 0.58
	17.07.26	NA	NA	NA	NA
	17.09.30	0.039 ± 0.03	0.494 ± 0.31	-26.80 ± 1.41	-25.72 ± 0.57
Fungi	unlabelled soil	0.00	0.00	-28.00	-27.87
	17.06.14	NA	NA	NA	NA
	17.06.28	0.368 ± 0.01	0.364 ± 0.17	-21.22 ± 1.92	-22.70 ± 1.42
	17.07.26	NA	NA	NA	NA
	17.09.30	0.00 ± 0.00	0.00 ± 0.00	-27.55 ± 0.50	-27.53 ± 0.29
Gram-positive bacteria	unlabelled soil	0.00	0.00	-27.34	-28.54
	17.06.14	NA	NA	NA	NA
	17.06.28	0.623 ± 0.50	0.708 ± 0.75	-24.91 ± 0.94	-22.85 ± 2.04
	17.07.26	NA	NA	NA	NA
	17.09.30	0.047 ± 0.05	0.103 ± 0.06	-27.33 ± 1.05	-25.85 ± 2.33

Statistical analysis of $_{15}N$ excess / $_{13}C$ excess and $\delta_{15}N$ / $\delta_{13}C$ values

Table 8: Results of statistical analysis (Two-way ANOVA) of $_{15}N_{tot}$ excess and $_{15}N_{MB}$ excess values of our study sites in Austria, Germany, Sweden and the UK. Significant results are shown in bulk.

		F statistics	p	partial η^2
Austria				
$^{15}N_{tot}$ Excess	- Treatment*Time	F(3,54) = 0.11	p = 0.952	0.069
	- Time	F(3,54) = 28.10	p < 0.001	1.000
	- Treatment	F(1,18) = 4.47	p = 0.049	0.517
$^{15}N_{MB}$ Excess	- Treatment*Time	F(1,10) = 0.90	p = 0.363	0.139
	- Time	F(1,10) = 17.50	p = 0.002	0.964
	- Treatment	F(1,10) = 1.65	p = 0.228	0.214
Germany				
$^{15}N_{tot}$ Excess	- Treatment*Time	F(3,54) = 2.66	p = 0.057	0.129
	- Time	F(3,54) = 17.41	p < 0.001	0.492
	- Treatment	F(1,18) = 5.18	p = 0.035	0.224
$^{15}N_{MB}$ Excess	- Treatment*Time	F(2,20) = 13.62	p < 0.001	0.994
	- Time	F(2,20) = 21.19	p < 0.001	1.000
	- Treatment	F(1,10) = 18.41	p = 0.002	0.971
Sweden				
$^{15}N_{tot}$ Excess	- Treatment*Time	F(3,54) = 6.77	p = 0.001	0.967
	- Time	F(3,54) = 52.75	p < 0.001	1.000
	- Treatment	F(1,18) = 468.29	p < 0.001	1.000
$^{15}N_{MB}$ Excess	- Treatment*Time	F(1,10) = 1.06	p = 0.326	0.150
	- Time	F(1,10) = 5.15	p = 0.046	0.340
	- Treatment	F(1,10) = 103.72	p < 0.001	0.912
UK				
$^{15}N_{tot}$ Excess	- Treatment*Time	F(3,54) = 1.05	p = 0.376	0.270
	- Time	F(3,54) = 21.39	p < 0.001	1.000
	- Treatment	F(1,18) = 40.31	p < 0.001	1.000
$^{15}N_{MB}$ Excess	- Treatment*Time	F(1,10) = 1.19	p = 0.299	0.107
	- Time	F(1,10) = 29.80	p < 0.001	0.749
	- Treatment	F(1,10) = 2.85	p = 0.122	0.222

Table 9: Results of statistical analysis (Two-way ANOVA) of $\delta_{15}N_{tot}$ and $\delta_{15}N_{MB}$ values of our study sites in Austria, Germany, Sweden and the UK. Significant results are shown in bulk.

		F statistics	p	partial η^2
Austria				
$d^{15}N_{tot}$	- Treatment*Time	F(3,54) = 2.60	p = 0.061	0.608
	- Time	F(3,54) = 32.06	p < 0.001	1.000
	- Treatment	F(1,18) = 21.92	p < 0.001	0.993
$d^{15}N_{MB}$	- Treatment*Time	F(1,10) = 1.10	p = 0.318	0.159
	- Time	F(1,10) = 10.78	p = 0.008	0.841
	- Treatment	F(1,10) = 0.71	p = 0.418	0.120
Germany				
$d^{15}N_{tot}$	- Treatment*Time	F(3,54) = 1.23	p = 0.305	0.064
	- Time	F(3,54) = 11.75	p < 0.001	0.395
	- Treatment	F(1,18) = 101.69	p < 0.001	0.850
$d^{15}N_{MB}$	- Treatment*Time	F(2,20) = 4.982	p = 0.018	0.748
	- Time	F(2,20) = 15.90	p < 0.001	0.998
	- Treatment	F(1,10) = 16.60	p = 0.379	0.133
Sweden				
$d^{15}N_{tot}$	- Treatment*Time	F(3,54) = 2.08	p = 0.113	0.504
	- Time	F(3,54) = 14.42	p < 0.001	1.000
	- Treatment	F(1,18) = 239.83	p < 0.001	1.000
$d^{15}N_{MB}$	- Treatment*Time	F(1,10) = 0.26	p = 0.616	0.076
	- Time	F(1,10) = 8.96	p = 0.013	0.770
	- Treatment	F(1,10) = 0.54	p = 0.478	0.103
UK				
$d^{15}N_{tot}$	- Treatment*Time	F(3,54) = 1.61	p = 0.198	0.400
	- Time	F(3,54) = 16.68	p < 0.001	1.000
	- Treatment	F(1,18) = 23.34	p < 0.001	1.000
$d^{15}N_{MB}$	- Treatment*Time	F(1,10) = 0.11	p = 0.012	0.061
	- Time	F(1,10) = 14.75	p = 0.003	0.932
	- Treatment	F(1,10) = 0.11	p = 0.739	0.061

Table 10: Results of statistical analysis (Two-way ANOVA) of $^{13}C_{tot}$ excess and $^{13}C_{CO2}$ excess values of our study sites in Austria, Germany, Sweden and the UK. Significant results are shown in bulk.

		F statistics	p	partial η^2
Austria				
$^{13}C_{tot}$ Excess	- Treatment*Time	F(2,36) = 3.76	p = 0.033	0.173
	- Time	F(2,36) = 32.32	p < 0.001	0.642
	- Treatment	F(1,18) = 30.39	p < 0.001	0.628
$^{13}C_{CO2}$ Excess	- Treatment*Time	F(3,24) = 1.11	p = 0.364	0.262
	- Time	F(3,24) = 8.18	p = 0.001	0.979
	- Treatment	F(1,8) = 1.28	p = 0.290	0.170
Germany				
$^{13}C_{tot}$ Excess	- Treatment*Time	F(3,54) = 2.60	p = 0.061	0.607
	- Time	F(3,54) = 5.43	p = 0.002	0.920
	- Treatment	F(1,18) = 22.28	p < 0.001	0.994
$^{13}C_{CO2}$ Excess	- Treatment*Time	F(3,24) = 17.35	p < 0.001	1.000
	- Time	F(3,24) = 56.86	p < 0.001	1.000
	- Treatment	F(1,8) = 20.20	p = 0.002	0.974
Sweden				
$^{13}C_{tot}$ Excess	- Treatment*Time	F(3,54) = 51.79	p < 0.001	1.000
	- Time	F(3,54) = 192.44	p < 0.001	1.000
	- Treatment	F(1,18) = 1290	p < 0.001	1.000
$^{13}C_{CO2}$ Excess	- Treatment*Time	F(1.310,24) = 9.17	p = 0.009	0.843
	- Time	F(1.310,24) = 5.77	p = 0.029	0.650
	- Treatment	F(1,8) = 97.87	p < 0.001	1.000
UK				
$^{13}C_{tot}$ Excess	- Treatment*Time	F(3,54) = 2.841	p = 0.046	0.650
	- Time	F(3,54) = 20.51	p < 0.001	1.000
	- Treatment	F(1,18) = 4.92	p = 0.040	0.555
$^{13}C_{CO2}$ Excess	- Treatment*Time	F(3,24) = 4.99	p = 0.008	0.224
	- Time	F(3,24) = 66.08	p < 0.001	1.000
	- Treatment	F(1,8) = 0.29	p = 0.293	0.169

Table 11: Results of statistical analysis (Two-way ANOVA) of $\delta_{13}C_{tot}$ and $\delta_{13}C_{CO2}$ values of our study sites in Austria, Germany, Sweden and the UK. Significant results are shown in bulk.

		F statistics	p	partial η^2
Austria				
$d^{13}C_{tot}$	- Treatment*Time	F(2,36) = 0.73	p = 0.488	0.039
	- Time	F(2,36) = 3.867	p = 0.030	0.177
	- Treatment	F(1,18) = 0.36	p = 0.555	0.020
$d^{13}C_{CO2}$	- Treatment*Time	F(3,24) = 0.94	p = 0.364	0.226
	- Time	F(3,24) = 34.53	p < 0.001	1.000
	- Treatment	F(1,8) = 0.23	p = 0.641	0.071
Germany				
$d^{13}C_{tot}$	- Treatment*Time	F(3,54) = 6.08	p = 0.001	0.948
	- Time	F(3,54) = 14.00	p < 0.001	1.000
	- Treatment	F(1,18) = 251.60	p < 0.001	1.000
$d^{13}C_{CO2}$	- Treatment*Time	F(3,24) = 11.99	p < 0.001	0.998
	- Time	F(3,24) = 88.72	p < 0.001	1.000
	- Treatment	F(1,8) = 1.85	p = 0.210	0.226
Sweden				
$d^{13}C_{tot}$	- Treatment*Time	F(3,54) = 3.16	p = 0.032	0.702
	- Time	F(3,54) = 23.48	p < 0.001	1.000
	- Treatment	F(1,18) = 6.06	p = 0.024	0.644
$d^{13}C_{CO2}$	- Treatment*Time	F(3,24) = 1.01	p = 0.402	0.242
	- Time	F(3,24) = 19.10	p < 0.001	1.000
	- Treatment	F(1,8) = 41.43	p < 0.001	1.000
UK				
$d^{13}C_{tot}$	- Treatment*Time	F(3,54) = 0.71	p = 0.548	0.192
	- Time	F(3,54) = 7.92	p < 0.001	0.985
	- Treatment	F(1,18) = 13.65	p = 0.002	0.937
$d^{13}C_{CO2}$	- Treatment*Time	F(3,24) = 2.71	p = 0.067	0.585
	- Time	F(3,24) = 29.94	p < 0.001	1.000
	- Treatment	F(1,8) = 4.32	p = 0.071	0.449

Table 12: Results of statistical analysis (One-way ANCOVA) of $_{13}C$ excess and $\delta_{13}C$ values of total PLFA as well as PLFA of gram-negative bacteria, fungi and gram-positive bacteria of Austria. Significant results are shown in bulk.

Austria	**F statistics**	**p**	**partial η^2**
Total PLFA			
$^{13}C_{PLFA}$ Excess	F(1,7) = 400.94	**p < 0.001**	0.983
$d^{13}C_{PLFA}$	F(1,7) = 0.03	p = 0.862	0.005
Gram-negative bacteria			
$^{13}C_{PLFA}$ Excess	F(1,7) = 37.45	**p < 0.001**	0.843
$d^{13}C_{PLFA}$	F(1,7) = 0.22	p = 0.648	0.031
Fungi			
$^{13}C_{PLFA}$ Excess	F(1,7) = 0.26	p = 0.622	0.037
$d^{13}C_{PLFA}$	F(1,7) = 0.04	p = 0.831	0.007
Gram-positive bacteria			
$^{13}C_{PLFA}$ Excess	F(1,7) = 3.67	p = 0.097	0.344
$d^{13}C_{PLFA}$	F(1,7) = 3.78	p = 0.093	0.351

Table 13: Results of statistical analysis (One-way ANCOVA) of $_{13}C$ excess and $\delta_{13}C$ values of total PLFA as well as PLFA of gram-negative bacteria, fungi and gram-positive bacteria of Germany. Significant results are shown in bulk.

Germany	**F statistics**	**p**	**partial η^2**
Total PLFA			
$^{13}C_{PLFA}$ Excess	F(1,7) = 37.45	**p < 0.001**	0.843
$d^{13}C_{PLFA}$	F(1,7) = 0.09	p = 0.765	0.014
Gram-negative bacteria			
$^{13}C_{PLFA}$ Excess	F(1,7) = 5.99	**p = 0.044**	0.461
$d^{13}C_{PLFA}$	F(1,7) = 0.57	p = 0.473	0.076
Fungi			
$^{13}C_{PLFA}$ Excess			
$d^{13}C_{PLFA}$	F(1,7) = 0.00	p = 0.935	0.001
Gram-positive bacteria			
$^{13}C_{PLFA}$ Excess	F(1,7) = 0.03	p = 0.855	0.005
$d^{13}C_{PLFA}$	F(1,7) = 1.05	p = 0.338	0.131

Concentrations of bulk soil, soil microbial biomass, respiration and PLFA measurements

Table 14: Concentration nitrogen [µg/g], concentration carbon [µg/g] and C:N ratio of bulk soil measurements.

Bulk Soil Measurments		Concentration Nitrogen (µg/g)		Concentration Carbon (µg/g)		C:N ratio	
		Nitrogen treatment	Control	Nitrogen treatment	Control	Nitrogen treatment	Control
Austria	unlabelled soil	10685 ± 55	10373 ± 31	218838 ± 587	221650 ± 340	20.48 ± 0.12	21.37 ± 0.07
	17.06.01	11474 ± 31	9330 ± 59	256119 ± 247	199539 ± 1191	22.32 ± 0.04	21.39 ± 0.12
	17.06.14	11905 ± 3625	8495 ± 402	236103 ± 13468	185762 ± 5143	21.98 ± 0.40	22.22 ± 0.49
	17.06.28	11636 ± 334	9318 ± 254	231675 ± 6511	185031 ± 6108	19.92 ± 0.44	19.86 ± 0.58
	17.07.26	12811 ± 786	12467 ± 4860	235693 ± 19556	189635 ± 16977	18.29 ± 0.99	16.66 ± 4.38
	17.09.30	10454 ± 666	8142 ± 546	240397 ± 7032	179021 ± 9258	22.41 ± 0.43	22.01 ± 0.51
Germany	unlabelled soil	3671 ± 143	3227 ± 137	56963 ± 3067	49407 ± 2299	15.52 ± 0.38	15.31 ± 0.31
	17.06.01	4555 ± 142	3197 ± 75	78430 ± 1905	52296 ± 1071	17.22 ± 0.14	16.36 ± 0.15
	17.06.14	3981 ± 576	3136 ± 334	63834 ± 7673	48446 ± 4025	16.16 ± 1.66	15.49 ± 0.69
	17.06.28	4700 ± 313	3688 ± 171	70919 ± 6418	50974 ± 3913	15.09 ± 0.91	13.81 ± 0.71
	17.07.26	3949 ± 441	3264 ± 348	62118 ± 5115	48896 ± 4114	15.80 ± 0.90	15.05 ± 1.13
	17.09.30	3918 ± 194	3200 ± 281	65031 ± 3027	50529 ± 4116	16.61 ± 0.63	15.82 ± 0.91
Sweden	unlabelled soil	12888 ± 23	4751 ± 66	177098 ± 1398	436390 ± 1077	37.28 ± 0.38	33.86 ± 0.07
	17.06.01	12658 ± 103	5865 ± 39	212360 ± 3426	425128 ± 308	36.21 ± 0.10	33.59 ± 0.10
	17.06.14	12178 ± 418	5075 ± 514	423085 ± 8974	188839 ± 17660	34.38 ± 0.71	37.32 ± 2.49
	17.06.28	11755 ± 297	4912 ± 185	437587 ± 7503	194750 ± 9161	37.40 ± 0.80	39.53 ± 0.71
	17.07.26	13285 ± 164	6001 ± 577	434745 ± 9522	201327 ± 17894	32.72 ± 0.53	33.58 ± 0.88
	17.09.30	11461 ± 356	4871 ± 293	434993 ± 8125	190308 ± 12909	37.97 ± 0.78	39.07 ± 1.43
UK	unlabelled soil	17430 ± 984	17548 ± 1958	405555 ± 26645	382569 ± 21127	23.27 ± 1.49	21.80 ± 1.25
	17.06.01	16934 ± 777	17777 ± 1096	387999 ± 32420	369519 ± 19351	22.91 ± 0.68	20.79 ± 0.61
	17.06.14	14964 ± 1783	15118 ± 913	351216 ± 17200	376160 ± 9787	22.90 ± 1.28	24.95 ± 1.33
	17.06.28	15451 ± 333	15844 ± 513	361595 ± 22445	382005 ± 11790	23.10 ± 1.00	24.13 ± 0.94
	17.07.26	16456 ± 544	16524 ± 235	363610 ± 17220	376820 ± 17278	22.10 ± 0.75	23.20 ± 0.60
	17.09.30	14420 ± 1299	14842 ± 598	351452 ± 39972	375236 ± 11306	24.33 ± 1.01	25.30 ± 0.72

Table 15: Concentration nitrogen [µg/g], concentration carbon [µg/g] and C:N ratio of soil microbial biomass nitrogen measurements.

Microbial biomass measurements		Concentration N (µg/g)		Concentration C (µg/g)		C:N ratio	
		Nitrogen treatment	Control	Nitrogen treatment	Control	Nitrogen treatment	Control
Austria	17.06.01	112	284	205	683	2.78	2.78 ± 1.72
	17.06.14	207 ± 103	109 ± 19	2272 ± 313	1687 ± 111	6.72 ± 0.80	6.81 ± 0.95
	17.06.28	NA	NA	NA	NA	NA	NA
	17.07.26	303 ± 133	249 ± 101	994 ± 221	855 ± 154	2.98 ± 0.66	3.46 ± 0.67
	17.09.30	325 ± 60	187 ± 84	1872 ± 575	1484 ± 252	4.99 ± 0.91	5.11 ± 1.34
Germany	17.06.01	NA	NA	NA	NA	3.70	8.17
	17.06.14	100 ± 6	36 ± 30	465 ± 100	373 ± 5	5.23 ± 1.09	6.02 ± 1.78
	17.06.28	126 ± 21	60 ± 15	841 ± 110	525 ± 31	6.31 ± 0.98	6.50 ± 0.68
	17.07.26	92 ± 29	99 ± 33	365 ± 277	672 ± 382	3.84 ± 0.88	5.03 ± 0.78
	17.09.30	91 ± 24	58 ± 21	329 ± 92	249 ± 62	4.19 ± 0.37	4.58 ± 0.75
Sweden	17.06.01	NA	NA	NA	NA	5.27	4.11
	17.06.14	246 ± 64	120 ± 31	1459 ± 325	502 ± 171	7.38 ± 0.90	7.31 ± 0.58
	17.06.28	338 ± 61	105 ± 36	1843 ± 333	9334 ± 394	6.98 ± 1.32	9.31 ± 1.72
	17.07.26	481 ± 88	125 ± 32	1935 ± 315	747 ± 383	5.58 ± 0.41	8.39 ± 0.94
	17.09.30	NA	NA	NA	NA	NA	NA
UK	17.06.01	NA	NA	NA	NA	5.76	6.25
	17.06.14	102 ± 41	95 ± 24	792 ± 246	1014 ± 313	9.19 ± 0.62	10.33 ± 1.28
	17.06.28	260 ± 73	168 ± 66	1027 ± 536	1056 ± 295	7.43 ± 2.15	7.69 ± 1.47
	17.07.26	NA	NA	NA	NA	NA	NA
	17.09.30	NA	NA	NA	NA	NA	NA

Table 16: Concentration of respired CO_2 [µg/g] of soil respiration measurements

Respiration Measurements		Concentration C-CO2 (µg/g)	
		Nitrogen treatment	Control
Austria	17.06.14	12.98 ± 2.59	12.50 ± 1.45
	17.06.28	12.37 ± 2.52	10.64 ± 1.39
	17.07.26	16.08 ± 3.46	16.51 ± 2.10
	17.09.30	16.01 ± 1.84	16.78 ± 3.72
Germany	17.06.14	2.90 ± 0.27	2.64 ± 0.54
	17.06.28	3.07 ± 0.31	2.43 ± 0.05
	17.07.26	3.26 ± 0.56	3.14 ± 0.27
	17.09.30	3.27 ± 0.70	3.03 ± 0.51
Sweden	17.06.14	16.49 ± 1.53	8.15 ± 0.75
	17.06.28	20.29 ± 4.59	9.05 ± 0.73
	17.07.26	22.23 ± 1.88	11.90 ± 0.84
	17.09.30	21.96 ± 1.39	11.57 ± 1.54
UK	17.06.14	8.69 ± 0.38	8.58 ± 1.24
	17.06.28	7.85 ± 0.48	8.12 ± 1.65
	17.07.26	8.15 ± 1.36	8.018 ± 0.58
	17.09.30	6.22 ± 0.58	6.50 ± 1.04

Table 17: Concentration carbon [nmol PLFA/g] of phospholipid acid analysis

Concentration PLFA analysis [nmol PLFA/g]		Austria		Germany	
		Nitrogen treatment	Control	Nitrogen treatment	Control
Total PLFA	unlabelled soil	0.00	0.00	0.00	0.00
	17.06.14	NA	NA	NA	NA
	17.06.28	6401 ± 2832	3504 ± 759	10532 ± 1962	3233 ± 2956
	17.07.26	616 ± 244	6402 ± 1590	NA	NA
	17.09.30	NA	NA	608 ± 289	3663 ± 1550
Gram-negative bacteria	unlabelled soil	0.00	0.00	0.00	0.00
	17.06.14	NA	NA	NA	NA
	17.06.28	3487 ± 1837	1211 ± 340	1611 ± 1100	1363 ± 1103
	17.07.26	138 ± 74	1808 ± 1429	NA	NA
	17.09.30	NA	NA	308 ± 171	1246 ± 965
Fungi	unlabelled soil	0.00	0.00	0.00	0.00
	17.06.14	NA	NA	NA	NA
	17.06.28	538 ± 668	216 ± 61	1224 ± 160	245 ± 141
	17.07.26	31 ± 29	1554 ± 631	NA	NA
	17.09.30	NA	NA	6 ± 1	58 ± 41
Gram-positive bacteria	unlabelled soil	0.00	0.00	0.00	0.00
	17.06.14	NA	NA	NA	NA
	17.06.28	459 ± 265	1365 ± 334	4511 ± 1203	2856 ± 3372
	17.07.26	79 ± 31	2735 ± 1340	NA	NA
	17.09.30	NA	NA	168 ± 50	163 ± 104

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