Gross N_2O fluxes across soil-atmosphere interface and stem N_2O emissions from temperate forests

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Summary

Although nitrous oxide (N_2O) is a minor constituent of the atmosphere, it is still of great concern. This is because N_2O can significantly affect the physics and chemistry of the atmosphere and thus influence the climate on Earth. Soil is a major source of N_2O, and microbial nitrification and denitrification are the dominant N_2O producing processes. Soil N_2O fluxes usually exhibit significantly spatio-temporal variability since the microbial processes of N_2O production and consumption are both affected by the substrate availability, redox potential and temperature. Moreover, plants can influence soil N_2O fluxes through altering soil properties and microbial communities and through serving as additional conduits for transport of soil-generated N_2O. However, we are still struggling to fully understand the complexity of N_2O production, consumption and transportation processes in soil, and the links to abiotic (e.g. soil climate, physics and chemistry) and biotic (e.g. microbial–plant–soil interactions) factors. The difficulty of measuring gross N_2O production and consumption in soil impedes our ability to predict N_2O dynamics across the soil-atmosphere interface.

The aim of the first study was to disentangle gross N_2O production and consumption in soil by comparing $^{15}N_2O$ pool dilution (PD) and gas-flow soil core (GFSC) measurements. Intact soil cores were taken from grassland, cropland, beech and pine forests, covering different vegetation, soil types and climatic conditions. Across sites, gross N_2O production and consumption measured by $^{15}N_2OPD$ were only 10% and 6%, respectively, of those measured by GFSC. Hence, we proposed to use different terminologies for the two methods. 'Gross N_2O emission and uptake' are appropriate for $^{15}N_2OPD$, which encompasses gas exchange within the $^{15}N_2O$-labelled, soil air-filled pores; while 'gross N_2O production and consumption' can be used for GFSC, which includes N_2O directly reduced to N_2 in anaerobic microsites. Although the $^{15}N_2OPD$ could measure only part of gross N_2O production in soil, it is the only method that can be used under field conditions to quantify atmospheric N_2O uptake, an important process commonly unquantified in many ecosystems.

The aim of the second study was to quantify temporal variability and environmental controls of gross N_2O fluxes. We measured gross N_2O emission and gross N_2O uptake using the $^{15}N_2OPD$ technique that we validated in the first study. Asymbiotic N_2 fixation was also measured to infer the gaseous N balance. This experiment was conducted in adjacent spruce and beech forests in central Germany. Our results showed that the beech stand had higher soil gross and net N_2O emissions and asymbiotic N_2 fixation than the spruce stand. Seasonal variation of gross N_2O emission was mainly controlled by soil NO_3^- concentration; gross N_2O

uptake was largely influenced by soil extractable organic C; and asymbiotic N_2 fixation was correlated with soil extractable organic C and temperature. Asymbiotic N_2 fixation was an order of magnitude lower than gross N_2O uptake in these highly acidic, N-enriched forest soils.

The aim of the third study was to determine tree-mediated N_2O fluxes under field conditions as well as their contributions to total forest N_2O fluxes. Here, we quantified *in situ* stem N_2O fluxes from mature alder trees on poorly-drained soil and mature beech and spruce trees on well-drained soils in central Germany. Alder, beech and spruce consistently emitted N_2O via stems and all displayed higher emission rates in summer than in spring and autumn. Stem N_2O fluxes from alder were higher than beech and spruce due to the presence of aerenchyma and lenticels as well as higher soil water content and soil C and N availability in the alder stand. Stem N_2O fluxes represented 8-11% of the total (soil + stem) N_2O fluxes in the spruce and beech stands, whereas in the alder stand with its large soil N_2O emission stem emission contributed only 1% of the total flux.

Overall, this research provides new insights into gross N_2O fluxes and their environmental factors, and also provides an estimate of tree-mediate N_2O fluxes which can improve N budgets of forest ecosystems. Our findings show that the $^{15}N_2O$ PD technique was a valuable tool to separate the net N_2O flux into gross N_2O emission and gross N_2O uptake in the gas phase of the soils, but probably did not allow measuring gross N_2O production and consumption in anaerobic microsites. Gross N_2O emission played an important role in controlling the direction and magnitude of net N_2O flux. And the regression relationships between gross N_2O emission and net N_2O fluxes also open the possibility of making estimates of soil gross N_2O emissions based on measured soil net N_2O emissions. Tree species had a large influence on gross N_2O emission, net N_2O flux and asymbiotic N_2 fixation, and thus large-scale field quantification under similar soil types and climatic conditions can be based on tree-species stratification as a promising basis to scale up these rates. Lastly, both wetland trees and upland trees act as important conduits for soil-generated N_2O and the relative contribution of tree-mediated N_2O fluxes to the total N_2O fluxes is more important in upland trees than in wetland trees.

Zusammenfassung

Obwohl Distickstoffmonoxid (N_2O) zu den Nebenbestandteilen der Atmosphäre zählt, ist es doch von großer Bedeutung. Es hat signifikanten Einfluss auf die Physik und Chemie der Atmosphäre und beeinflusst damit das Klima auf der Erde. Der Boden ist eine wichtige N_2O-Quelle, mit mikrobieller Nitrifikation und Denitrifikation als bestimmende Prozesse der N_2O-Produktion. Boden-N_2O-Flüsse zeigen gewöhnlich hohe räumlich-zeitliche Variabilität, denn mikrobielle Prozesse von N_2O-Produktion und -Verbrauch sind jeweils von der Substratverfügbarkeit, dem Redoxpotential und der Temperatur abhängig. Auch können Pflanzen die N_2O-Flüsse beeinflussen, indem sie Bodeneigenschaften und mikrobielle Gesellschaften verändern und als zusätzlicher Transportkanal für bodenbürtiges N_2O dienen. Dennoch fällt es schwer, die volle Komplexität von Prozesse der N_2O-Produktion, des -Verbrauchs und des -Transports zu verstehen sowie ebenfalls die Verbindungen zu den abiotischen Faktoren (z.B. Bodenklima, -physik und -chemie) und biotischen Faktoren (z.B. Interaktion mikrobielle Biomasse/Pflanze/Boden). Die Schwierigkeit, N_2O-Produktion und -Verbrauch im Boden zu messen wirkt sich auf die Möglichkeit der Vorhersage von N_2O-Dynamiken im System Boden/Atmosphäre aus.

Das Ziel der ersten Studie war es, N_2O-Produktion und-Verbrauch voneinander zu trennen, indem $^{15}N_2O$ *pool dilution* (PD)- und *gas-flow soil core* (GFSC)-Messungen verglichen wurden. Intakte Bodenzylinder wurden in Grasland, Ackerland, Buchen- und Kiefernwäldern genommen um verschiedene Vegetation, Bodentypen und Klimabedingungen abzudecken. Über die Versuchsflächen war die N_2O-Produktion und -Verbrauch, gemessen mit $^{15}N_2O$PD nur 10% bzw. 6% derer, die mit GFSC gemessen wurden. Daher schlagen wir eine unterschiedliche Terminologie für die N_2O-Flüsse nach den jeweiligen Methoden vor: 'Brutto-N_2O-Emmission und -Aufnahme' sind für $^{15}N_2O$PD geeignet, welche den Gasaustausch zwischen den $^{15}N_2O$-markierten luftgefüllten Bodenporen umfasst; 'Brutto-N_2O-Produktion und -Verbrauch' kann für GFSC genutzt werden, welches das N_2O beinhaltet, welches in anaeroben Mikroarealen zu N_2 reduziert wird. Obwohl die $^{15}N_2O$PD nur einen Teil der Brutto-N_2O-Produktion im Boden messen konnte ist sie die einzige Methode, die unter Feldbedingungen zur Quantifizierung der atmosphärischen N_2O-Aufnahme genutzt werden kann, welches ein wichtiger, oft nicht quantifizierter Prozess vieler Ökosysteme ist.

Das Ziel der zweiten Studie war es, zeitliche Variabilität und Umwelteinflüsse auf N_2O-Flüsse zu quantifizieren. Es wurden die Brutto-N_2O-Emission und Brutto-N_2O-Aufnahme mit Hilfe der $^{15}N_2O$PD-Technik gemessen, welche in der ersten Studie validiert wurde. Die

asymbiotische N_2-Fixierung wurde ebenfalls gemessen, um Rückschlüsse auf das gasförmige N-Gleichgewicht zu ziehen. Dieses Experiment wurde in angrenzenden Fichten- und Buchenwäldern in der Mitte Deutschlands durchgeführt. Unsere Ergebnisse zeigen, dass die Buchenbestände höhere Boden-Brutto- und -Netto-N_2O-Emissionen und asymbiotische Stickstoff(N_2)-Fixierung aufwiesen als die Fichtenbestände. Die saisonale Variabilität der Brutto-N_2O-Emission wurde hauptsächlich durch die Boden-Nitrat(NO_3)-Konzentration bestimmt; die Brutto- N_2O-Aufnahme war stark durch den aus dem Boden extrahierbaren organischen Kohlenstoff(C) beeinflusst; und die asymbiotische N_2-Fixierung korrelierte mit dem aus dem Boden extrahierbaren C und der Temperatur. Die asymbiotische N_2-Fixierung war eine Magnitude niedriger als die Brutto-N_2O-Aufnahme in diesen stark sauren, N-angereicherten Waldböden.

Das Ziel der dritten Studie war es, die baumbürtigen N_2O-Flüsse unter Feldbedingungen zu ermitteln und ihren Anteil an den N_2O-Gesamtflüssen im Wald zu bestimmen. Hierbei wurden die *in situ*-Stamm-N_2O-Flüsse von großen Erlen auf schlecht abfließenden Böden und großen Buchen und Fichten auf gut abfließenden Böden quantifiziert. Erle, Buche und Fichte emittierten konsistent N_2O über den Stamm und zeigten alle höhere Emissionsraten im Sommer als im Frühjahr und Herbst. Stamm-N_2O-Flüsse von Erle waren höher als von Buche und Fichte, auf Grund der Anwesenheit von Parenchym und Lentizellen sowie höherem Bodenwasseranteil und der Boden-C- und -N-Verfügbarkeit im Erlenbestand. Die Stamm-N_2O-Flüsse bildeten 8-11% der Gesamt(Boden + Stamm)-N_2O-Flüsse im Fichten- und Buchenbestand, wobei sie im Erlenbestand mit seinen hohen N_2O-Emissionen nur 1% der Gesamtflüsse ausmachten.

Insgesamt bietet die Studie neue Einblicke in die Brutto-N_2O-Flüsse und asymbiotische N_2-Fixierung welche bisher nicht in anderen Ökosystemen untersucht wurden, und bringt eine Abschätzung von baumbürtigen N_2O-Flüssen, die das N-Budget von Waldökosystemen verbessern kann. Unsere Befunde zeigen, dass die $^{15}N_2O$PD-Technik ein wertvolles Werkzeug darstellt, um die Netto-N_2O-Flüsse von Brutto-N_2O-Emission und -Aufnahme in der Gasphase von Böden zu separieren. Sie erlaubte es jedoch wahrscheinlich nicht, Brutto-N_2O-Produktion und -Verbrauch in anaeroben Mikroarealen zu messen. Brutto-N_2O-Emission spielte eine bedeutende Rolle in der Änderung der Richtung und der Magnitude der N_2O-Flüsse und ihre regressiven Beziehungen eröffnen auch die Möglichkeit, Schätzungen der Boden-Brutto-N_2O-Emissionen basierend auf den gemessenen Boden-Netto-N_2O-Emissionen. Die Baumart hatte großen Einfluss auf die N_2O-Emission, den Netto N_2O-Fluss und die asymbiotische N_2-Fixierung. Somit ist eine großskalige Quantifizierung im Feld bei

vergleichbaren Bodentypen und klimatischen Bedingungen auf der Basis von Baumartenstratifizierung erfolgversprechend. Bäume feuchter Gebiete und solche höhergelegener Gebiete funktionieren als Kanal von bodengeneriertem N_2O und der relative Beitrag von baumbürtigen N_2O-Flüsse ist wichtiger für Bäume hochgelegener Gebiete als für Bäume feuchter Gebiete.

Chapter 1

General Introduction

1.1. Nitrous oxide production and consumption in soil

The nitrous oxide (N_2O) is the one of the main greenhouse gases, contributing approx. 6% to the anticipated global warming (IPCC, 2001). It also plays a significant role in atmospheric photochemical reactions that contribute to stratospheric ozone depletion (Ravishankara et al., 2009). In the past few decades, the atmospheric concentration of N_2O has increased nearly linearly by a rate of 0.2-0.3% yr^{-1} (IPCC, 2007). Although atmospheric N_2O has been intensively studied using fluxes measurement and global models, there are still many uncertainties concerning the global budget of N_2O and the mechanisms involved in its formation and loss in the atmosphere. This is mainly because the concentration of N_2O is relatively low (325.1 ppb) and the residence time in the atmosphere is rather long (ca. 120 years), and also because there is a variety of natural and anthropogenic sources of N_2O (WMO, 2013).

At present, soils are thought as the main N_2O source in the terrestrial ecosystem: emission from natural soils are estimated to be 4.3-5.8 Tg N_2O-N yr^{-1}, while emissions from agricultural soils are estimated to be 6-7 Tg N_2O-N yr^{-1} (Syakila and Kroeze, 2011). Although there is a wealth of biotic and abiotic processes that can form N_2O in soil, nitrification and denitrification are recognized as the dominant processes and contribute ca. 70% of global N_2O emissions (Syakila and Kroeze, 2011). N_2O produced in soil can subsequently be consumed by the last step of denitrification, i.e. reduction of N_2O to N_2. Substantial fractions of N_2O produced in subsoil have been found to be consumed by the last step of denitrification either in the same denitrifier cell (Knowles, 1982) or along the diffusion pathway towards soil surface (Conen and Neftel, 2007; Koehler et al., 2012). Reduction of N_2O to N_2 is of ecological significance since it is the prevailing natural process that converts reactive nitrogen back to inert form of nitrogen, N_2 (Dannenmann et al., 2008).

The conceptual ‘hole-in-the-pipe’ model considered two levels of controls regulating N_2O production: (1) factors influencing the rates of nitrification and denitrification (i.e. ‘the flow through the pipe’); and (2) factors regulating the proportions between the gaseous end products (i.e. ‘the size of the holes’; Firestone and Davidson, 1989). Proximal environmental

factors, which influence ‘the flow through the pipe’ and ‘the size of the holes’, are NO_3^- concentration, C availability, temperature and O_2 concentration (Saggar et al., 2013). Those factors can directly affect microbial communities and thus lead to instantaneous changes in denitrification rates and the $N_2O:N_2$ ratio. Specifically, NO_3^- and C availabilities control the denitrification rate, because they are important substrate and energy source of denitrification and also because they act as electron acceptor and donor for this process. Furthermore, high NO_3^- concentration usually results in a high $N_2O:N_2$ ratio, since NO_3^- is preferred as an electron acceptor over N_2O (Chapuis-Lardy et al., 2007). High soil moisture and soil respiration trigger denitrification as they consequently lower the oxygen content in the soil and thus result in the formation of N_2 rather than N_2O (Butterbach-Bahl et al., 2013). Temperature is also an important controlling factor not only because nitrification and denitrification are enzymatic processes but also because it can change soil respiration rates and thus soil oxygen concentrations (Butterbach-Bahl et al., 2013). Lastly, soil pH influences N_2O production since the activity of nitrous oxide reductase increases with increasing pH values (Dannenmann et al., 2008).

Owing to the dependency of microbial-mediated nitrification and denitrification on similar environmental factors and availability of substrates, these two processes often occur in close area and interact on each other. Hence, N_2O fluxes at the soil surface usually exhibit significantly spatial and temporal variability due to the complicated production and consumption processes in soil. Disentangling gross N_2O production and consumption in soil will help us understand the underlying mechanisms controlling N_2O fluxes. However, it is difficult to quantify these processes in soil since the large heterogeneity of denitrification products and the large background of atmospheric N_2 (Davidson and Seitzinger, 2006). Although acetylene inhibition and ^{15}N tracing are two methods that are usually applied to separate N_2O and N_2 production, both methods have obvious disadvantages since they either modify the denitrification process or add ^{15}N-labelled substrate. A better method is needed to quantify N_2O production, consumption and controlling factors across the range of ecosystems.

1.2. Nitrous oxide emission and uptake at the soil surface

The flux of N_2O measured at the soil-atmosphere interface is a composite of source and sink terms within the soil profile. Although soils are identified to be significant sources of atmospheric N_2O, net N_2O uptakes by soils have also been frequently observed in various natural and managed ecosystems (Chapuis-Lardy et al., 2007; Schlesinger, 2013). Schlesinger (2013) compiled 118 values of N_2O uptake potential in soils of different ecosystems and

demonstrated that net N_2O uptake ranged from <1 to 207 μg N m^{-2} h^{-1}, with a median of 4 μg N m^{-2} h^{-1}. In temperate forest soils, net N_2O uptake ranged from 0.55 to 66.6 μg N m^{-2} h^{-1} (Butterbach-Bahl et al., 2002, 1998; Dong et al., 1998; Goossens et al., 2001). Furthermore, IPCC (2013) report mentions for the first time a global surface N_2O sink of 0-1 Tg N_2O-N yr^{-1}.

The sink strength depends on the ease of N_2O diffusion from the atmosphere to soil and the potential for N_2O reduction to N_2. Hence, soil texture and particle size distribution may significantly affect N_2O production and consumption (Wlodarczyk et al., 2005). Soil water status is also one of the most important driving factors for the N_2O sink strength. Net N_2O uptake is thought to occur in wet soils since denitrification prefers anoxic condition. This is also because high soil water content decrease gas diffusion and convection, as well as increase N_2O entrapment, which extends the time for potential reduction of N_2O to N_2 (Clough et al., 2005). However, recent studies have also shown that soils can take up N_2O from the atmosphere and reduce it to N_2 under dry and oxic conditions (Goldberg and Gebauer, 2009; Wu et al., 2013). Goldberg and Gebauer (2009) for instance showed that long drought periods can lead to drastic decreases of N_2O fluxes from soils to the atmosphere or even turn forest soils temporarily to N_2O sink. In those cases, the N_2O uptake from the atmosphere is usually linked to low NO_3^- concentrations in soils, highlighting again that NO_3^- availability is a major regulator for source and sink of N_2O. Since atmospheric N_2O is the only electron acceptor left for denitrification when NO_3^- concentration is limited, the consumption of atmospheric N_2O by denitrification via N_2O reductase can explain the observed uptake of atmospheric N_2O under the low NO_3^- concentration (Butterbach-Bahl et al., 1998).

A 'compensation concentration' concept was proposed to explain the direction of net N_2O fluxes depending on concentrations in soils and in the atmosphere (Conrad, 1994). However, if only the N_2O concentrations are considered, the observed net N_2O fluxes would probably be interpreted as low rates of N_2O production but not as a combination of gaseous input and output at the soil-atmosphere interface. N_2O fluxes at the soil surface, measured by chamber method, should be composed of gross N_2O emission and gross N_2O uptake (Conen and Neftel, 2007). The terminologies 'gross N_2O emission and gross N_2O uptake' are used to avoid confusion with 'gross N_2O production and gross N_2O consumption' in soils. Gross N_2O emission indicates that N_2O produced in soil finally escapes to the atmosphere, while gross N_2O uptake indicates that atmospheric N_2O diffuses in soil and reduces to N_2. These two processes occur at the soil atmosphere interface simultaneously, and their relative flux rates decide the magnitude and direction of net N_2O fluxes. Hereby, net N_2O uptake can only be observed when gross N_2O uptake rates are higher than gross N_2O emission rates (Conen and

Neftel, 2007). Splitting net N_2O fluxes at the soil-atmosphere into gross N_2O emission and gross N_2O uptake activity would help to better estimate global N_2O turnover, and open new perspectives on the mitigation of N_2O emissions from soil. However, gross N_2O emission and uptake at the soil surface have never been evaluated due to the absence of a proper measurement technique.

1.3. Effects of plants on nitrous oxide emission

The influence of plants on soil properties can determine N_2O production and consumption in soil (Rückauf et al., 2004). Differences in the structure of leave litter on soil surface may alter air diffusivity, and thus soil moisture and soil oxygen conditions. Earlier studies have reported that deciduous forests typically act as stronger sources of N_2O than coniferous forests since litter from broad leaved trees restrict oxygen diffusion into the soil more than litter from spruce of pine needles, particularly under wet conditions (Ambus et al., 2006). Furthermore, differences in C/N ratios of litter quality and root exudation can influence turnover rates of organic material (Butterbach-Bahl et al., 1997). Lower C/N ratios probably promote faster N cycling and consequently higher N_2O production, which have been reported for 11 different sites across Europe (Ambus et al., 2006). Moreover, plants affect nitrification and denitrification processes by influencing the structure of soil microbial community and/or through competition with microbes for mineral N (Cavieres and Badano, 2009).

Plants also contribute to N_2O emission from terrestrial ecosystem as plants can act as conduits of soil-produced N_2O (Smart and Bloom, 2001). N_2O in soil may diffuse into plant roots directly or indirectly by water uptake. Afterwards, N_2O is transported though plants via aerenchyma system or transpiration stream (Díaz-Pinés et al., 2016; Machacova et al., 2016). Hereby, plant-mediated N_2O are reported to be influenced by soil water status, temperature and N availability. High soil moisture can stimulate denitrifying enzymes and thus promote N_2O production in soil and therein N_2O emission at the soil surface. Machacova et al. (2013) observed that flooding caused a dramatic transient increase of stem N_2O emission by factors of 740 for Alder and 14,230 for beech. Increasing stem N_2O emission in response to temperature have also been observed in previous studies (e.g. Machacova et al., 2013), since temperature is the major factor responsible for the belowground N_2O production. Moreover, stem N_2O emission appears to be regulated strongly by soil N availability, as shown by the rapid increase following fertilization (e.g. Pihlatie et al., 2005; Díaz-Pinés et al., 2016).

Currently, the estimates of N_2O emissions from terrestrial ecosystems are mainly restricted to emission from soils excluding the contribution of plant to the trace gas exchange

with the atmosphere (Gauci et al., 2010). An exception are studies on exchange of N_2O between agricultural ecosystem and the atmosphere, where N_2O are mainly quantified as total emission from soil plus crop plants (e.g. canola, barley, rice, wheat, soybean and maize; Chang et al., 1998; Chen et al., 2002; Yan et al., 2000; Zou et al., 2005). Contribution of plant-mediated N_2O to total plant-soil emission is on average 25% for wheat plants (Zou et al., 2005), 6-11% for soybean plants and 8.5-16% for maize plants (Chen et al., 2002). While in rice, the contribution ranged from 17.5% to 87.3% depending on soil water status (Yan et al., 2000; Yu et al., 1997). These results demonstrate that N_2O emissions from soil-crop systems are greatly affected by plants.

Although forests cover 31% of the terrestrial area worldwide (FAO, 2010), tree-mediated N_2O flux is one of the least studied N_2O emission pathways. Previous studies are mostly restricted to seedlings and saplings under laboratory conditions, and information of mature trees under field conditions is lacking. Therefore, current estimates of N_2O emission from forest ecosystem are only based on chamber-based measurement of soil N_2O fluxes and do not include tree-mediated N_2O flux. As far as we know, only two studies were conducted in the field to estimate the contribution of tree-mediated N_2O to the total N_2O fluxes. One study found that tree-mediated N_2O accounted for 1-3% of the total forest N_2O fluxes, and concluded that N_2O emission from tree stem is not important (Díaz-Pinés et al., 2016). Another paper, however, reported that stem N_2O emission contributed up to 18% of the total pine forest, and accordingly demonstrated that stem emissions play a significant role in N_2O emission (Machacova et al., 2016). Overall, these conflicting results may arise from different tree species, environmental conditions and measurement time and hence highlights the need for more detailed measurements of tree-mediated N_2O fluxes in various forest ecosystems. If the large contribution of tree-mediated N_2O fluxes can be proved in other forest ecosystems, the N_2O source strength of forest ecosystems may have been underestimated. Moreover, this may be a reason to explain the discrepancy between bottom-up emissions-based estimates and top-down inverse or satellite-based N_2O emission sources.

1.4. Temperate forests in central Germany

Ecosystems in central Europe, especially in Germany, have received high element inputs by atmospheric deposition since the beginning of industrialization. As a result, geochemical cycles and biological processes in many German forest soils have significantly changed and soil characteristic like pH, base saturation and C:N ratios have also altered (Brummer et al., 2009a, b; Meesenburg et al., 2009). N deficiency, which has been common feature of forest

stands in the temperate region (Tamm, 1991), does not occur any more due to high N deposition rates. On the contrary, N saturation occurs in many forest ecosystems. Previous studies conducted on German N saturated forest soils found decreased gross N mineralization, increased gross nitrification (Corre and Lamersdorf, 2004; Corre et al., 2003) and increased NO_3^- leaching (as indicated by high leaching:throughfall ratio: 4.2; Corre et al., 2007) compared to low N deposition forests. Morover, N_2O fluxes from German forests were even as high as that from tropical forest (Butterbach-Bahl et al., 1997; Schulte-bisping and Brumme, 2003).

This research was primarily conducted in the Solling forest, which is located in the central Germany. Our study sites have received elevated N deposition rates for the past few decades and have evidence of high N leaching (Meesenburg et al., 1995; Corre et al., 2003; Corre and Lamersdorf, 2004; Corre et al., 2007). Since N-saturated soils increase susceptibility for N_2O losses to the atmosphere, exploring the mechanisms and processes responsible for variabilities of N_2O emissions at this site is important. We will conduct three studies to investigate gross N_2O production and consumption, gross N_2O emission and uptake, and tree-mediated N_2O fluxes. These studies provide the much needed information on the mechanisms underlying soil N_2O fluxes and associated controlling factors, as well as providing new insights into the effect of trees on atmospheric N_2O concentration and their contributions to the total ecosystem fluxes.

1.5. Aims and hypotheses

The aim of the first study was to test whether $^{15}N_2O$ pool dilution (PD) technique is a suitable method for disentangling gross N_2O production and consumption. Since this technique was reported as a robust method that can disentangle gross N_2O production and consumption in the field without inhibiting any step of processes or adding additional substrate, we would like to validate this method in order to apply this method in subsequent stages of my research on N_2O dynamics. Hence, we compared this method with an established gas-flow-soil core (GFSC) method by measuring soil intact cores from different ecosystems. We hypothesized that the $^{15}N_2OPD$ and GFSC methods would yield comparable estimates of gross N_2O production and consumption in soil.

The aims of the second study were to quantify gross N_2O emission and uptake and asymbiotic N_2 fixation in soils under beech and spruce forests, and to determine the controlling factors of these processes. After we tested the $^{15}N_2OPD$ technique and defined the processes that can be measured using this approach (gross N_2O emission and uptake), we

applied the $^{15}N_2O$ PD technique to disentangle gross fluxes of N_2O at the soil-atmosphere interface. We also measured asymbiotic N_2 fixation, in order to test the hypothesis that N_2 fixation could compensate N_2O emissions. In this study, we hypothesized: (1) the beech forest will have higher gross N_2O emission and uptake in the soil than the spruce forest; (2) gross N_2O emission and uptake in soil will mainly be regulated by soil N availability and moisture content, whereas soil temperature and available C will additionally influence asymbiotic N_2 fixation; (3) at both forests, with acidic soil and high N deposition, asymbiotic N_2 fixation will be lower than gross N_2O uptake (or N_2 flux from the soil).

The aims of the third study were to quantify *in situ* tree-mediated N_2O emissions and their seasonal patterns, and to assess their controlling factors in order to infer the mechanisms responsible for tree-mediated N_2O emissions. In this study, we test following hypotheses: (1) tree-mediated N_2O fluxes will be higher in alder than in beech and spruce stands as the former is a wetland and is an N_2-fixing tree species, of which anaerobic and high soil N conditions may promote high soil N_2O production, whereas the latter are upland and non N_2-fixing tree species; (2) N_2O transport in alder stem will be dominated by N_2O diffusion from the soil to the aerenchyma tissue and lenticels, and thus alder stem N_2O emission will be influenced by the amount of N_2O produced in the soil; (3) N_2O transport in beech and spruce stems will mainly be through dissolved form via xylem sap flow and thus will be limited by the sap flow rate which, in turn, will be influenced by air temperature, vapor pressure deficit and soil water content.

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Chapter 2

Disentangling gross N_2O production and consumption in soil

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Abstract

The difficulty of measuring gross N_2O production and consumption in soil impedes our ability to predict N_2O dynamics across the soil-atmosphere interface. Our study aimed to disentangle these processes by comparing measurements from gas-flow soil core (GFSC) and $^{15}N_2O$ pool dilution ($^{15}N_2OPD$) methods. GFSC directly measures soil N_2O and N_2 fluxes, with their sum as the gross N_2O production, whereas $^{15}N_2OPD$ involves addition of $^{15}N_2O$ into a chamber headspace and measuring its isotopic dilution over time. Measurements were conducted on intact soil cores from grassland, cropland, beech and pine forests. Across sites, gross N_2O production and consumption measured by $^{15}N_2OPD$ were only 10% and 6%, respectively, of those measured by GFSC. However, $^{15}N_2OPD$ remains the only method that can be used under field conditions to measure atmospheric N_2O uptake in soil. We propose to use different terminologies for the gross N_2O fluxes that these two methods quantified. For $^{15}N_2OPD$, we suggest using 'gross N_2O emission and uptake', which encompass gas exchange within the $^{15}N_2O$-labelled, soil air-filled pores. For GFSC, 'gross N_2O production and consumption' can be used, which includes both N_2O emitted into the soil air-filled pores and N_2O directly consumed, forming N_2, in soil anaerobic microsites.

2.1. Introduction

N_2O is one of the most important long-lived greenhouse gases and is expected to be the single most important ozone-depleting substance throughout the 21st century[1]. Soils account, globally, for about 60% of the total N_2O flux to the atmosphere, with 6.6 Tg N yr^{-1} from natural ecosystems and 4.1 Tg N yr^{-1} from agricultural systems[2]. Although it is generally known that microbial nitrification and denitrification in soils are the major sources of atmospheric N_2O, it remains a struggle to disentangle and quantify gross rates of microbial N_2O production and consumption in soil which, in turn, determine the net N_2O flux across the soil-atmosphere interface.

Under anaerobic conditions, incomplete denitrification produces N_2O whereas the terminal step of denitrification (i.e. the reduction of N_2O to N_2) consumes N_2O. Hence, microbial N_2O production and consumption can occur simultaneously in soil via the activities of different microorganisms or even by a single denitrifying cell[3]. In addition, within the soil profile and in the soil air-filled pores, N_2O can be further reduced to N_2 during its transport to the soil surface[4–6]. Soil physical (e.g. water or oxygen content, temperature, porosity) and biochemical factors (e.g. pH, concentrations of electron donors and acceptors) influence the balance between soil N_2O production and consumption[7], and consequently the net N_2O flux to the atmosphere. Soil net N_2O uptake has been complied in a review[8], which specifically refers to the net flux of N_2O from the atmosphere to the soil and can be detected only if soil N_2O consumption exceeded production. Soil N_2O consumption, however, is often ignored because it is prone to be masked by the much larger N_2O production[4] and is difficult to measure directly (e.g. as soil N_2 flux) against a very high (78%) atmospheric background[9].

The static chamber method, commonly used to measure net N_2O flux on the soil surface, cannot quantify the simultaneously occurring gross N_2O production and consumption within the soil. One possibility to measure gross N_2O production and consumption in soil is the $^{15}N_2O$ pool dilution ($^{15}N_2OPD$) technique, which entails adding $^{15}N_2O$ to the chamber headspace and subsequently measuring the changes in $^{14}N_2O$ and $^{15}N_2O$ over time[10]. So far, this $^{15}N_2OPD$ technique has been used in managed grassland and cropland soils and in salt marsh landscape, all located in northern California, by the same authors who first evaluated this method under field conditions[10–12].

In 2013, when the first $^{15}N_2OPD$ measurements were reported[10], a debate emerged as to what extent this technique is able to quantify gross N_2O production and consumption in soil. Well & Butterbach-Bahl[13] questioned the key assumptions of the $^{15}N_2OPD$ technique: the exchange and mixing of soil-derived N_2O and $^{15}N_2O$ label between aerobic and anaerobic soil

microsites. They argued that gross N_2O production and consumption in soil would be underestimated if produced N_2O is immediately reduced to N_2 without first mixing with the $^{15}N_2O$-labelled air in interconnected soil pore spaces. This may occur within denitrifier cells and between different microorganisms[3] in anaerobic microsites, which here we infer to include not only microsites saturated with water but also isolated pores filled with or enclosed by water and water-entrapped N_2O[14]. Yang *et al.*[15] replied that such constraints could only occur when the soil has a high proportion of anaerobic microsites, and argued that the $^{15}N_2O$ label and soil-derived N_2O are likely distributed homogeneously in the chamber headspace from which the calculation of gross N_2O fluxes is derived. In summary, the efficacy of the $^{15}N_2OPD$ technique to estimate gross N_2O production and consumption is still not settled, and so far this technique has only been compared with results from acetylene inhibition and ^{15}N tracing methods. These latter methods, however, have their own limitations for determining gross N_2O production and consumption in soil since they either modify the entire denitrification process as well as its single steps (acetylene inhibition method) or require the addition of ^{15}N-labelled substrate (^{15}N tracing method) with the need to label the soil homogeneously including its anaerobic microsites[9,16].

To date, the enigmatic lack of measurements of gross N_2O production and consumption in soil impedes our ability to predict N_2O dynamics across the soil-atmosphere interface. Our study aimed to disentangle gross N_2O production and gross N_2O consumption in soil by comparing measurements from $^{15}N_2OPD$ technique and gas-flow soil core (GFSC) method. The latter is an established method that directly measures gross N_2O production and consumption in soil by simultaneously quantifying N_2O and N_2 fluxes[17] without the use of an inhibitor or ^{15}N labelling of substrate[9,16]. We hypothesized that if the assumption of the $^{15}N_2OPD$ method (i.e. exchange and mixing of soil-derived N_2O and $^{15}N_2O$ label between aerobic and anaerobic soil microsites) is attained, then the $^{15}N_2OPD$ and GFSC methods should yield comparable estimates of gross N_2O production and consumption in soil. We tested this hypothesis using different soils from four ecosystems: grassland, cropland, beech and pine forests (Table 1), covering a range of soil biochemical characteristics as well as soil aeration status (e.g. water content and soil texture) and N availability.

2.2. Results

From the $^{15}N_2OPD$ measurements, gross N_2O production and consumption rates and net N_2O flux (Fig. 1a-c) were higher ($p = 0.01 - 0.03$) in the silty loam Cambisol soil in manured

grassland than in the sandy Regosol soil in unmanaged pine forests, and neither differed from the sandy loam Cambisol soil in cropland or the silty loam Cambisol soil in unmanaged beech forest. For the grassland, cropland and beech forest, net N_2O emissions accounted for 66 – 79% of gross N_2O production (Fig. 1d). For the pine forest, net N_2O uptake (Fig. 1c) was paralleled by larger gross N_2O consumption (Fig. 1b) than gross N_2O production (Fig. 1a); these fluxes were very small but still above our detection limit.

From the GFSC measurements, gross N_2O production (Fig. 1a) was higher ($p = 0.02$) in the beech forest than in the cropland and pine forest and intermediate in the grassland. Gross N_2O consumption ($p = 0.37$; Fig. 1b) and net N_2O fluxes ($p = 0.06$; Fig. 1c) did not differ among sites. Net N_2O fluxes accounted, on average, for only 24% of gross N_2O production (Fig. 1d), and hence most (76%) of the produced N_2O was further reduced to N_2.

Although significant differences in gross N_2O production and consumption between the $^{15}N_2O$PD technique and GFSC method were only found in the grassland site ($p = 0.02$ for both; Fig. 1a,b), the fluxes measured by the GFSC method were up to two orders of magnitude larger than those measured by the $^{15}N_2O$PD technique (Fig. 1a,b). The large spatial variation within each site (indicated by the large standard errors) resulted in non-statistically detectable differences between these two methods. However, for gross N_2O production, rates measured by the $^{15}N_2O$PD technique were on average 10% of those measured by the GFSC method (Fig. 1a). For gross N_2O consumption, rates measured by the $^{15}N_2O$PD technique were on average 6% of those measured by the GFSC method (Fig. 1b). Net N_2O fluxes from the soil cores used for the $^{15}N_2O$PD measurement were on average 94% of those measured by the GFSC method, which did not differ in any of the sites ($p = 0.11 – 0.61$; Fig. 1c). In three sites, except the pine forest that had very low fluxes, the ratios of net N_2O flux to gross N_2O production measured by the $^{15}N_2O$PD technique were higher ($p < 0.01 – 0.05$) than those measured by the GFSC method (Fig. 1d).

Soil water-filled pore space (WFPS), microbial C and N, and denitrification enzyme activity (DEA) were generally higher ($p \leq 0.02$) in the grassland than in the pine forest (Table 2). Soil NH_4^+ concentrations were higher ($p < 0.01$) in the grassland and beech forest compared to the cropland, whereas soil NO_3^- concentrations were higher ($p = 0.02$) in the cropland than in the grassland and pine forest (Table 2). Gross N_2O production and consumption, measured by either the $^{15}N_2O$PD technique or the GFSC method, showed positive correlations with WFPS, NH_4^+, microbial C and N, and DEA ($R = 0.56 – 0.93$, $p < 0.05$; Supplementary Table S1). Net N_2O fluxes from the soil cores used for the $^{15}N_2O$PD measurements correlated positively with the same soil properties ($R = 0.64 – 0.92$, $p < 0.01$;

Supplementary Table S1), whereas no correlation was found with net N_2O flux measured by the GFSC method.

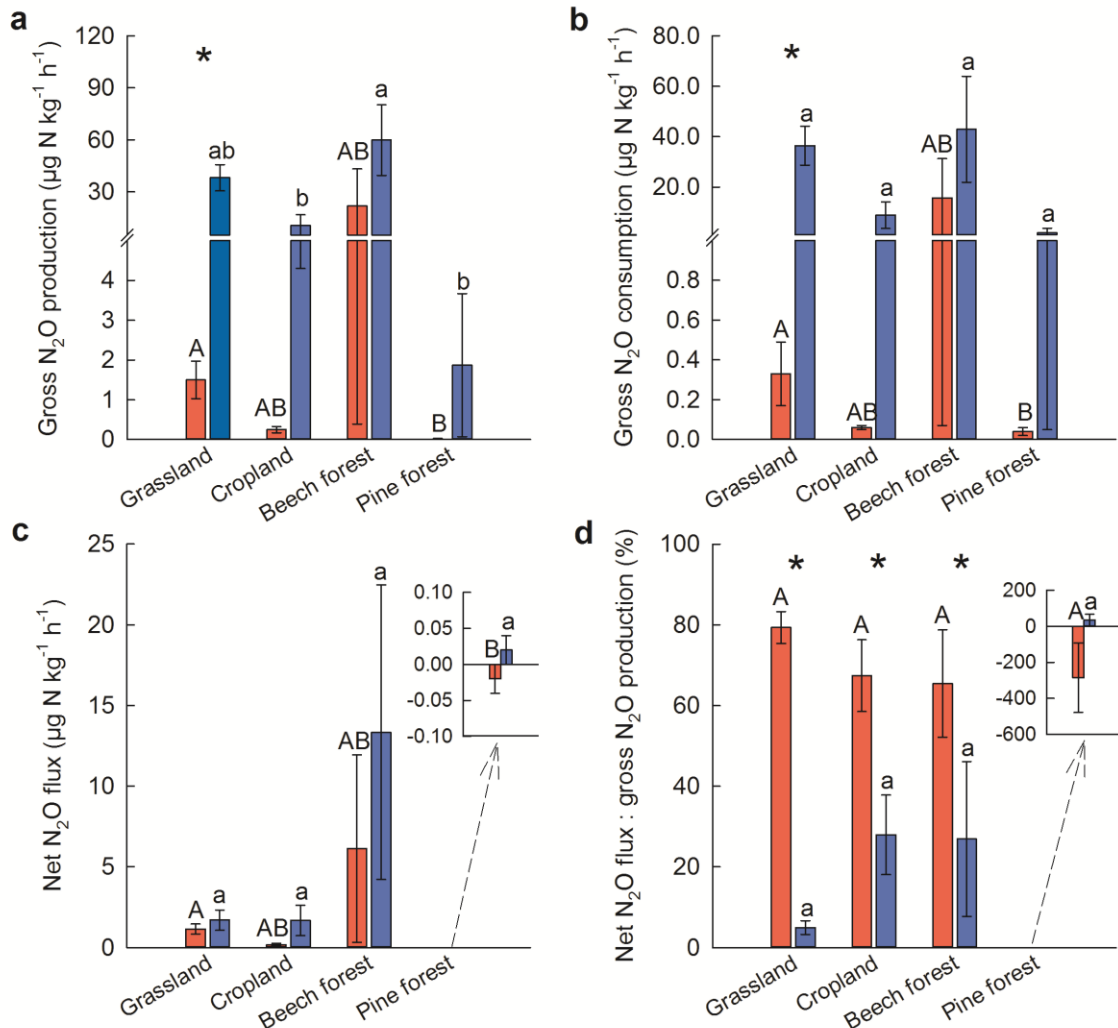

Figure 1. Soil gross and net N_2O fluxes. Gross N_2O production (**a**), gross N_2O consumption (**b**), net N_2O flux (**c**), and the ratio of net N_2O flux to gross N_2O production (**d**), measured by $^{15}N_2O$ pool dilution ($^{15}N_2OPD$; red bars) and gas-flow soil core (GFSC; blue bars). For each method, means (± s.e., n = 4 replicate sampling points) with different capital (for $^{15}N_2OPD$) and small letters (for GFSC) indicate significant differences among sites (one-way ANOVA with Fisher's LSD test at $p \leq 0.05$ or Kruskal-Wallis ANOVA with multiple comparisons of mean ranks at $p \leq 0.05$). For each site, asterisks above the bars indicate significant differences between the two methods (paired t test at $p \leq 0.05$).

Table 1. Site characteristics.

Site characteristics	Grassland	Cropland	Beech forest	Pine forest
Location	47.57°N, 11.03°E	48.19°N, 11.96°E	51.76°N, 9.58°E	43.72°N, 10.28°E
Mean annual temperature (°C)	6.7	8.5	7.3	14.1
Mean annual precipitation (mm)	1373	1029	1100	918
Elevation (m above sea level)	870	510	510	10
Vegetation/Crop	*Poaceae*; *Taraxacum*	*Zea mays*	*Fagus sylvatica*	*Pinus pinaster*
Soil type	Haplic Cambisol	Calcaric Cambisol	Dystric Cambisol	Calcareous Regosol
Soil texture (% sand/silt/clay)	10 / 68 / 23	30 / 52 / 18	12 / 54 / 34	93 / 3 / 4
Soil bulk density (g cm^{-3})	0.59	1.17	0.64	1.30
Soil pH	7.1	6.7	3.8	5.7
Soil total organic carbon (g C kg^{-1})	135	20	127	10
Soil total nitrogen (g N kg^{-1})	8.0	1.7	6.6	0.7
Soil C:N ratio	16.9	11.8	18.9	13.5

Soil characteristics in the grassland, cropland and pine forest sites were measured in the top 10 cm of mineral soil[19,21]; in the beech forest site, these were measured in the top 5 cm of mineral soil.

Table 2. Soil physical and biochemical characteristics in the top 5 cm, determined from the soil cores immediately after the measurement of gross N_2O fluxes.

Soil characteristics	Grassland	Cropland	Beech forest	Pine forest
Water-filled pore space (%)	79 ± 1 a	57 ± 2 ab	70 ± 14 ab	25 ± 1 b
NH_4^+ (mg N kg^{-1})	4.34 ± 0.97 a	0.66 ± 0.12 b	2.35 ± 0.37 a	1.30 ± 0.18 ab
NO_3^- (mg N kg^{-1})	1.00 ± 0.14 b	5.42 ± 0.60 a	4.17 ± 2.14 ab	0.71 ± 0.38 b
Microbial C (g C kg^{-1})	3.26 ± 0.13 a	0.76 ± 0.03 c	2.68 ± 0.24 ab	1.72 ± 0.10 bc
Microbial N (mg N kg^{-1})	211.02 ± 4.84 a	69.22 ± 0.90 c	160.90 ± 11.35 ab	98.70 ± 5.37 bc
Denitrification enzyme activity (g N kg^{-1} h^{-1})	5.16 ± 0.64 a	0.21 ± 0.07 bc	0.83 ± 0.17 ab	0.00 ± 0.00 c

Means ± s.e. (n = 4) within each row followed by different letter indicate significant differences among sites (one-way ANOVA with Fisher's LSD test at $p \leq 0.05$ or Kruskal-Wallis ANOVA with multiple comparisons of mean ranks at $p \leq 0.05$).

2.3. Discussion

Both the $^{15}N_2O$PD and GFSC methods have been proposed to be able to measure gross N_2O production and consumption in soils[9.10]. The comparable net N_2O fluxes determined by these methods (Fig. 1c) suggest that both methods can yield similar results in terms of the net effect of concurrently occurring production and consumption of N_2O. However, the measured gross N_2O production and consumption rates (Fig. 1a,b), and thus the ratios of net N_2O flux to gross N_2O production (Fig. 1d), differed between the two methods. Hence, we reject our hypothesis that $^{15}N_2O$PD technique and GFSC method yield comparable estimates of gross N_2O fluxes.

When using the $^{15}N_2O$PD technique, gross N_2O production is determined from the dilution of $^{15}N_2O$ label by $^{14}N_2O$ produced in the soil[15]. An implicit assumption of this approach is that the headspace-labelled $^{15}N_2O$ that diffuses into the soil results in a homogeneous mixture of $^{15}N_2O$ with soil-derived N_2O in the soil air-filled pores, which also imply that these pores must be interconnected to the soil surface for homogenous mixing to occur. Our conservative calculations of diffusive transport of $^{15}N_2O$ into interconnected soil air-filled pores suggest that $^{15}N_2O$ must have diffused into these pores and back to the headspace within 0.5 h. However, there may be two situations when gross N_2O production and consumption will be underestimated by this method: 1) produced N_2O is immediately consumed within denitrifier cells[3], and 2) produced N_2O diffuses out of denitrifier cells and is consumed by other microorganisms, which may have N_2O reductase but cannot act on the preceding substrates of the denitrification pathway[18], without being mixed first with the $^{15}N_2O$ label during the 3-hour measurement period. Both situations can occur in anaerobic microsites, which here we infer to microsites saturated with water, isolated pores filled with or enclosed by water forming a diffusion barrier, and water-entrapped N_2O as expounded by Clough *et al.*[14]. If these situations happen, disparity between $^{15}N_2O$PD and GFSC measurements would be large in a fine-textured soil with high water content whereas they would be comparable in a coarse-textured soil with low water content. The fact that our results showed the large differences between the $^{15}N_2O$PD and GFSC measurements in the silty loam soil of grassland with high WFPS and they were particularly comparable in the sandy soil of pine forest with low WFPS (Fig. 1a,b; Table 2) suggest that the $^{15}N_2O$PD technique was not able to quantify gross N_2O production in these above-mentioned two situations. With the GFSC method, gross N_2O production is measured as the sum of emitted N_2O and N_2, and thus those immediately consumed N_2O to N_2 within denitrifier cells and between different microorganims in anaerobic microsites are included in this measurement.

We summarize our results into a conceptual model in order to illustrate two decoupled pathways of N_2O production and consumption in soil (Fig. 2). In the first pathway, N_2O is produced in anaerobic microsites and reduced immediately to N_2 without first mixing with the $^{15}N_2O$ label. Based on our results, only the GFSC method but not the $^{15}N_2OPD$ technique was able to quantify this pathway. The second pathway covers the soil-derived N_2O that diffuses into the interconnected soil air-filled pores and mixes with the $^{15}N_2O$ label, which was captured by the $^{15}N_2OPD$ technique. Even if the N_2O that has moved into the soil air-filled pores is being consumed during its diffusion towards the soil-atmosphere interface[4], as long as the produced N_2O mixes with the $^{15}N_2O$ label this can be included in the $^{15}N_2OPD$ calculations of gross N_2O production. It is clear that both $^{15}N_2OPD$ and GFSC methods yield complementary important information, and thus a differentiation in the use of terminologies is needed. Since the $^{15}N_2OPD$ technique reflects the N_2O dynamics in the gas phase of the soils and its exchange with the atmosphere, we propose to use the terms 'gross N_2O emission' and 'gross N_2O uptake' to denote the gross N_2O fluxes in interconnected soil air-filled pores measured by this method. Since the GFSC method measures gross N_2O fluxes not only in interconnected soil air-filled pores but also in anaerobic microsites, we propose that the terms 'gross N_2O production' and 'gross N_2O consumption' be used (Fig. 2). Below we will use these proposed terminologies to distinguish between the processes measured by these two methods.

It is important to point out that the $^{15}N_2OPD$ technique is able to yield information on gross N_2O uptake from the atmosphere to the soil. For years there has been a discussion on the importance of N_2O uptake in the soil from the atmosphere and substantial progress has been hampered because until now only the net N_2O fluxes on the soil surface can be routinely measured with inexpensive static chamber method. With the $^{15}N_2OPD$ technique, we now have an operational approach that can be used for field measurements and can separate the net N_2O fluxes across the soil-atmosphere interface into gross N_2O emission and gross N_2O uptake. It is a significant advancement since this technique will allow us to investigate the factors that control N_2O uptake by soils under actual field conditions, which is a commonly unquantified sink of ecosystem N budgets.

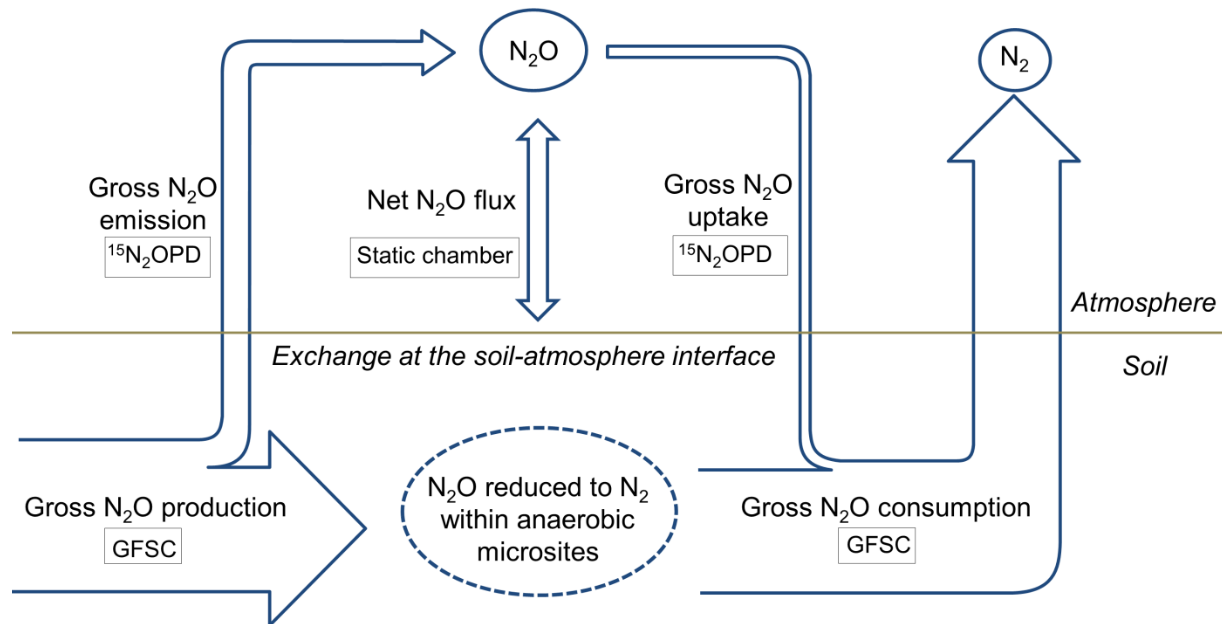

Figure 2. Conceptual diagram of gross N_2O fluxes. Gross N_2O emission and gross N_2O uptake, measured by $^{15}N_2O$ pool dilution ($^{15}N_2OPD$), which largely includes gas exchange in interconnected air-filled pores in the soil; gross N_2O uptake = gross N_2O emission – net N_2O flux. Gross N_2O production and gross N_2O consumption, measured by gas-flow soil core (GFSC), which encompasses the soil air-filled pores as well as anaerobic microsites (e.g. soil micro spots saturated with water, isolated pores filled with or enclosed by water, and water-entrapped N_2O); gross N_2O consumption = N_2 emission, and gross N_2O production = gross N_2O consumption + net N_2O flux.

Moreover, our results contrast to the notion that substantial N_2O uptake only happens in soils with net negative N_2O flux. This was shown by the larger gross N_2O uptake (measured by $^{15}N_2OPD$ technique) in the grassland that had larger net N_2O emissions than in the pine forest that had a net negative N_2O flux (Fig. 1b,c). The positive correlations of gross N_2O uptake with soil biochemical characteristics (Supplementary Table S1) suggest that high gross N_2O uptake occurs in soils with high microbial activity and high substrate availability (Table 2). The ratios of net to gross N_2O emissions (66 – 79% in grassland, cropland and beech forest; Fig.1d) were similar to the values reported by Yang *et al.*[10] and Yang and Silver[12] from managed grassland and cropland in California (net to gross N_2O emission ratio of 68 – 70%). These generally comparable ratios may open the possibility of making estimates of gross N_2O emissions and uptake based on measured net N_2O emissions.

The large fraction of gross N_2O production that was consumed to N_2 (measured by GFSC method) suggests that gross N_2O production and consumption were closely coupled,

which is in line with our aforementioned deduction (i.e. most N_2O was immediately reduced to N_2 in anaerobic microsites). Hence, the similar correlations found for gross N_2O production and consumption with soil biochemical characteristics (Supplementary Table S1) as those found for gross N_2O emission and uptake (measured by $^{15}N_2OPD$ technique) suggests that these gross N_2O fluxes were regulated by the same process, denitrification[4].

Our findings show that whereas the $^{15}N_2OPD$ technique is a valuable tool to separate net N_2O flux across the soil-atmosphere interface into gross N_2O emission and uptake, it did not allow measuring a large part of gross N_2O production and consumption in anaerobic microsites. In order to avoid misinterpretations of terminologies, we propose that the terms 'gross N_2O emission and uptake' should be used for gross N_2O fluxes measured with the $^{15}N_2OPD$ technique and 'gross N_2O production and consumption' should be used for gross N_2O fluxes measured with the GFSC method.

2.4. Methods

Study sites and soil sampling. Soil samples were collected from four ecosystems: grassland, cropland, beech and pine forests, covering different vegetation, soil types and climatic conditions (Table 1). The montane grassland is manured 2-3 times a year and cut for hay three times a year[19]. The cropland is a conventional corn-winter wheat rotation. The unmanaged beech forest (*Fagus sylvatica)* is 163 years old[20], and the unmanaged Mediterranean pine forest (*Pinus pinaster*) is 52 years old[21].

At each site, we selected four sampling points as replicates with a minimum distance of 25 m from each other. At each replicate, eight intact soil cores (250 cm^3 each) were taken using stainless-steel cores (8 cm diameter, 5 cm height): four of which were used for the $^{15}N_2OPD$ measurement and the other four for the GFSC measurement. The $^{15}N_2OPD$ measurement was conducted concurrently with the GFSC measurement, such that the soil cores for these two methods were handled similarly in all aspects. Neither soil moisture nor substrate level was adjusted.

$^{15}N_2O$ pool dilution. Four intact soil cores were placed in an incubation glass (6.6 L volume), equipped with Luer-lock stopcock for gas sampling. Upon closure of the incubation vessel, we injected into the chamber headspace 7 mL of $^{15}N_2O$ label gas, containing 100 ppmv of 98% single labelled ^{15}N-N_2O, 275 ppbv sulfurhexafluoride (SF_6, as a tracer for physical loss of N_2O) and the rest as synthetic air. This injected amount increased the N_2O concentration in

the headspace by approx. 106 ppbv N_2O with 12.5 atom% ^{15}N enrichment and SF_6 concentration of 292 pptv. At 0.5, 1, 2, and 3 h following label gas injection, 100 mL and 12 mL gas samples were taken out and stored in pre-evacuated 100 mL glass bottles and 12 mL glass tubes (Exetainer; Labco Limited, Lampeter, UK), respectively, with rubber septa. The sampled air volume was then replaced with 112 mL of a gas mixture (80% helium and 20% oxygen) to maintain the headspace at atmospheric pressure and oxygen concentration, without altering the isotopic composition of the headspace N_2O. The dilution that this replacement caused was accounted for in the calculations. The 100 mL gas samples were used to analyze isotopic composition using an isotope ratio mass spectrometer (IRMS) (Finnigan Delta^{plus} XP, Thermo Electron Corporation, Bremen, Germany). The 12 mL gas samples were used to measure N_2O and SF_6 concentrations using a gas chromatograph equipped with an electron capture detector (GC 6000 Vega Series 2, Carlo Erba Instruments, Milan, Italy). The detection limit of the entire measurement set-up and instrument precision was < 0.9 ppbv N_2O h^{-1}.

We modeled the vertical diffusive transport of $^{15}N_2O$ label through the 5 cm long soil cores, using the diffusion equation $\frac{\partial C}{\partial t} = \frac{\partial^2 C}{\partial x^2}$ in which C, t and x denote concentration, time and path length, respectively[22]. The free-air N_2O diffusion coefficient at 15 °C, 0.1582 cm s^{-1}, was used and adjusted for soil tortuosity based on the air-filled porosity[23], which was calculated using the measured bulk density and gravimetric moisture contents. Our most conservative calculations, using the lowest air-filled porosity and assuming an impervious boundary condition at bottom of the soil cores, showed that the $^{15}N_2O$ label had diffused into the 5 cm long soil cores and back to the headspace within 0.5 h. Thus, our sampling interval during the 3-hour measurement period was sufficient to allow mixing of the label gas with the soil-derived N_2O in interconnected air-filled pores and to quantify the changes in N_2O concentrations and $^{15}N_2O$ enrichments in the headspace.

Gross N_2O emission rate was calculated using the following equations modified from Yang *et al.*[10]:

$$\left[{}^{14}N_2O\right]_t = \frac{F_{14} \times P}{(k_{14}+k_l)} - \left\{\frac{F_{14} \times P}{(k_{14}+k_l)} - \left[{}^{14}N_2O\right]_0\right\} \times \exp\left\{-(k_{14}+k_l)\times(t-t_0)\right\} \quad (1)$$

$$\left[{}^{15}N_2O\right]_t = \frac{F_{15} \times P}{(k_{15}+k_l)} - \left\{\frac{F_{15} \times P}{(k_{15}+k_l)} - \left[{}^{15}N_2O\right]_0\right\} \times \exp\left\{-(k_{15}+k_l)\times(t-t_0)\right\} \quad (2)$$

where $[^{14}N_2O]_t$ is the concentration of $^{14}N_2O$ at time t, calculated as the product of N_2O concentration and ^{14}N-N_2O atom%; $[^{15}N_2O]_t$ is the concentration of $^{15}N_2O$, calculated as the product of N_2O concentration and ^{15}N-N_2O atom% excess, assuming that the ^{15}N isotopic composition of background N_2O is 0.3688 atom%[10]; t represents the time of gas sampling

from the headspace; F_{14} represents the $^{14}N_2O$ mole fraction (0.997) and F_{15} represents the $^{15}N_2O$ mole fraction (0.003) of emitted N_2O; k_{14} and k_{15} represent the first-order rate constants of $^{14}N_2O$ and $^{15}N_2O$ reduction to N_2, respectively, calculated based on the fractionation factor ($\alpha = k_{15}/k_{14}$) that has an average value of 0.9924 ± 0.0036 in literature[10]; k_l represents the first-order rate constant for loss of inert transport tracer, SF_6; P is gross N_2O emission rate. The k_{14} and k_{15} represent the biological loss, and k_l represents the physical loss. Since the changes of their concentrations in the headspace are simultaneously affected by biological consumption and physical loss, we used the sum of these constants ($k_{14}+k_l$ or $k_{15}+k_l$) in the above equations.

We estimated the parameters for P and k_{15} by simultaneously fitting the measured $[^{14}N_2O]_t$ and $[^{15}N_2O]_t$ with equation (1) and (2). The best fit of $[^{14}N_2O]_t$ and $[^{15}N_2O]_t$ was found using the least square approach and minimizing the following error function:

$$E = \sum_{t=1}^{n} \frac{(Y_{predicted}(t) - Y_{observed}(t))^2}{SD} + \sum_{t=1}^{n} \frac{(Z_{predicted}(t) - Z_{observed}(t))^2}{SD} \qquad (3)$$

where E is minimal weighted error (E); Y, Z and n indicate $^{14}N_2O$, $^{15}N_2O$ concentrations, and the number of measurements, respectively; SD refers to the standard deviation of the observed concentrations over the course of measurements[24,25]. Equation (3) was minimized using the 'fminsearchbnd' function in MATLAB (MathWorks, Version R2011b, USA). Gross N_2O uptake was calculated as the difference between gross N_2O emission and net N_2O flux[10].

Gas-flow soil core. The GFSC method is a fully automated, direct and sensitive quantification of the change of N_2O and N_2 concentrations in the headspace above the soil cores. The soil air of the four soil cores and the headspace of the incubation vessel were completely replaced by a gas mixture consisting of 20% O_2 (purity grade of 5.5), 80% He (purity grade of 5.0), N_2O (400 ppbv) and N_2 (25 ppmv). This complete exchange was done by automated repeated cycles of evacuation and gas purging, achieved through a built-in purging system in an extremely air-tight chamber that is connected directly to a gas chromatograph (Shimadzu GC-17A, Shimadzu, Munich, Germany)[17,26–28]. Eighteen hours of evacuation-purging cycles ensure a complete removal of the background atmospheric air[27], after which the headspace and tubing connections to the gas chromatograph were further purged for three hours. Subsequently, the system switched to a static chamber mode, and the headspace air of the incubation vessel was analyzed hourly over four hours through a directly connected gas chromatograph with an electron capture detector for N_2O analysis and a pulse

discharge He ionization detector (Vici AG, Schenkon, Switzerland) for N_2 analysis[26]. To sample the headspace, a slight overpressure was created by injecting 40 mL of the He-based gas mixture to the headspace, directing headspace air to the sampling loops[26]. The dilution of this non-intrusive overpressure sampling technique was accounted for in the calculation of N_2O and N_2 concentrations[26]. In order to achieve the best possible tightness of the incubation system against intrusion of atmospheric N_2, all tubing connections, valves as well as the entire incubation vessel were placed under water. Before starting the N_2O and N_2 measurements, the air-tightness of the system was always checked with an empty incubation vessel, which was connected in parallel with the measuring vessel. Based on the sensitivity and repeatability of the gas chromatograph measurements, the detection limits were < 0.03 ppmv h^{-1} for N_2 and < 0.45 ppbv h^{-1} for N_2O. The measured N_2 flux from the soil equals to gross N_2O consumption whereas the sum of N_2 and N_2O fluxes equals to gross N_2O production[17,26–28].

Soil controlling factors. Soil water content (one-day oven-drying at 105 °C and expressed as WFPS using 2.65 g cm^{-3} as particle density and the measured bulk density; Table 1), NH_4^+ and NO_3^- concentrations (0.5 M K_2SO_4 extraction), and microbial biomass C and N ($CHCl_3$ fumigation-extraction) were determined from the soil cores immediately after the gas measurements. NH_4^+ and NO_3^- concentrations in the soil extract were determined using continuous flow autoanalyzer (Skalar Scan plus system, Skalar Analytical B.V., Breda, Netherlands). Microbial biomass C and N were determined as the difference in 0.5 M K_2SO_4-extractable organic C and N (analyzed using persulfate oxidation with an infrared detector; Multi N/C 3100 TOC/TNb-Analysator, Analytik Jena, Jena, Germany) between the fumigated and unfumigated soils divided by $k_{EC} = 0.45$ and $k_{EN} = 0.68$[29]. DEA was determined from the N_2O produced during an anaerobic incubation with glucose and NO_3^- added in excess and acetylene inhibited N_2O reduction of to N_2[30].

Statistical analysis. The above soil properties, determined separately from the soil cores used for $^{15}N_2$OPD and GFSC measurements, did not differ between these two measurements ($p > 0.05$; paired t test); thus, the values from the two measurements were averaged to represent a replicate sampling point. Data sets were first tested for normal distribution (Shapiro-Wilk's test) and equality of variance (Levene's test). We used log-transformation for variables with non-normal distributions or unequal variances and assessed the differences in gross N_2O fluxes and soil properties among sites using one-way analysis of variance (ANOVA) with Fisher's least significant difference test. When none of the data

transformations were able to attain normal distribution and equality of variance, differences among sites were tested using the Kruskal-Wallis ANOVA with multiple comparisons test. The differences in gross and net N_2O fluxes between the $^{15}N_2O$PD and GFSC methods for each site were assessed using the paired t test. Relationships of gross N_2O fluxes with soil properties were assessed using spearman rank correlation test. Statistical significance was set at $p \leq 0.05$. Statistical analyses were conducted using SPSS (SPSS, Chicago, Illinois, USA).

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Author contributions

M.D.C., E.V., M.D., B.W. and K.B-B. designed the study; Y.W. and Z.C. carried out the measurements and analyzed data; B.W. modeled the diffusive transport of $^{15}N_2O$ label in soil; A.C. and Y.W. solved the $^{15}N_2OPD$ equations in MATLAB and experimentally tested them; G.W., Z.C., B.W., R.K., M.D. and K.B-B. established the GFSC method; M.D. and Z.C. conceptualized Fig. 2; M.D.C., Y.W. and E.V. wrote most parts of the manuscript; all authors reviewed and rewrote parts of the manuscript.

Competing financial interests

The authors declare no competing financial interests.

Supplementary Table S1. Relationships between soil physical and biochemical characteristics and gross N_2O production and consumption, measured by $^{15}N_2O$ pool dilution technique and gas-flow soil core method.

Explanatory soil variables	$^{15}N_2O$ pool dilution			Gas-flow soil core		
	Gross N_2O production	Gross N_2O consumption	Net N_2O flux	Gross N_2O production	Gross N_2O consumption	Net N_2O flux
pH	-0.09	-0.08	-0.07	-0.19	-0.31	-0.17
Water-filled pore space	0.92**	0.85**	0.92**	0.58*	0.56*	0.32
NH_4^+-N	0.69**	0.68**	0.64**	0.70**	0.65*	0.34
NO_3^--N	0.20	0.10	0.22	-0.17	-0.17	0.17
Microbial C	0.77**	0.72**	0.77**	0.60*	0.60*	0.16
Microbial N	0.74**	0.73**	0.71**	0.66**	0.69**	0.27
Denitrification enzyme activity	0.93**	0.80**	0.91**	0.83**	0.74**	0.42

Correlations were assessed using Spearman rank correlation test; n = 16; * indicates $p \leq 0.05$, and ** indicates $p \leq 0.01$.

Chapter 3

Gross N_2O emission, gross N_2O uptake and asymbiotic N_2 fixation in soils under temperate spruce and beech forests

Yuan Wen, Marife D. Corre, Wiebke Schrell, Edzo Veldkamp

Abstract

Soils are not only a major source but also a potential sink of atmospheric nitrous oxide (N_2O), a potent greenhouse gas and the most important substance causing stratospheric ozone depletion. Net N_2O flux at the soil surface is a result of two concurrent processes: gross N_2O emission and gross N_2O uptake. Little is known about these processes and their controlling factors because only in the last five years that the $^{15}N_2O$ pool dilution method was developed to measure these processes in the field. Here, we used this method to quantify gross N_2O emission and gross N_2O uptake in adjacent spruce and beech forests on a Cambisol soil in central Germany. Asymbiotic N_2 fixation was also measured to infer the balance between the natural input of N_2 into the soil and its output from the soil through gross N_2O uptake. Our results showed that the beech stand had higher soil gross and net N_2O emissions and asymbiotic N_2 fixation ($P < 0.01$-0.04) than the spruce stand. Seasonal variation of gross N_2O emission was mainly controlled by soil NO_3^- concentration; gross N_2O uptake was largely influenced by soil extractable organic C; and asymbiotic N_2 fixation was correlated with soil extractable organic C and temperature. The larger gross and net N_2O emissions in beech than spruce stands, together with the strong correlation between gross and net N_2O fluxes, suggest that gross N_2O emission rather than gross N_2O uptake drove the net N_2O flux from the soil. Asymbiotic N_2 fixation was an order of magnitude lower than gross N_2O uptake, indicating that N_2 fixation did not compensate for the N_2 emissions from these highly acidic, N-enriched forest soils. Our study generates new insights into previously unknown rates of gross N_2O emission and uptake, which are presently lacking in N budgets of forest ecosystems.

3.1. Introduction

Nitrous oxide (N_2O) is the third most important greenhouse gas, following CO_2 and CH_4, and plays a significant role in atmospheric photochemical reactions that contribute to stratospheric ozone depletion. Since there is an imbalance between the global N_2O sources and sinks, atmospheric N_2O concentration increases at a rate of ~0.25% yr^{-1} (IPCC, 2007). Soils are the major global source of atmospheric N_2O, contributing approx. 60% of total N_2O fluxes to the atmosphere (IPCC, 2013). N_2O is mainly produced via microbial nitrification and denitrification in soils, whereas denitrification can reduce N_2O further to N_2. Under certain conditions, N_2O reduction can dominate over N_2O production, leading to observations of net N_2O uptake by soils. Net N_2O uptake by soils has been reported for natural and managed ecosystems both in temperate and tropical climates (Chapuis-Lardy et al., 2007; Schlesinger, 2013). In the IPCC report of 2013, N_2O uptake by soils was included for the first time as a potentially important global N_2O sink.

Net N_2O flux at the soil surface, e.g. as measured with chamber technique, is a result of two concurrently occurring processes: gross N_2O emission and gross N_2O uptake. Gross N_2O emission, as measured by $^{15}N_2O$ pool dilution technique (Yang et al., 2011), accounts both the N_2O that is emitted from the soil to the atmosphere and the N_2O that is reduced to N_2 within the soil pores which are in active exchange with the atmosphere (Wen et al., 2016). Gross N_2O uptake also accounts not only the reduced N_2O which come from atmosphere and diffuses into soil but also the reduced N_2O within the soil pores; thus gross N_2O uptake represents the N_2 flux from the soil. Thus, net N_2O uptake from the atmosphere into the soil (i.e. net negative soil N_2O flux) can only be detected by chamber-based techniques if gross N_2O uptake exceeds gross N_2O emission (Conen and Neftel, 2007). Recent studies suggest that N_2O uptake by soils may be more important than assumed so far (Chapuis-Lardy et al., 2007; Yang and Silver, 2016a). In our earlier study, we found that substantial gross N_2O uptake occur in soils that have net positive soil N_2O fluxes, but only their gross N_2O uptake is masked by their higher gross N_2O emission (Wen et al., 2016). Other earlier studies in temperate forest soils also reported net N_2O uptake, ranging from -0.55 to -66.6 µg N m^{-2} h^{-1} (Butterbach-Bahl et al., 2002a, 1998; Dong et al., 1998; Goossens et al., 2001). Presently, little is known about gross N_2O emission and gross N_2O uptake in soil as well as their controlling factors. A crucial step for a better understanding of their underlying mechanisms is to quantify gross N_2O emission and uptake separately. This will improve our understanding of controlling factors involved in soil N_2O dynamics which, in turn, would help to predict how soil-atmosphere N_2O fluxes will response to future climatic changes.

The new method of $^{15}N_2O$ pool dilution that can simultaneously quantify in-situ gross N_2O emission and uptake in soil has been used so far in three sites in California (managed grassland, cropland and salt marsh landscape; Yang et al., 2011; Yang and Silver, 2016a; Yang and Silver, 2016b). We recently conducted a validation of this method using intact soil cores from three sites in Germany (managed grassland, cropland and unmanaged beech forest) and one site in Italy (unmanaged pine forest) (Wen et al., 2016). We found that across sites gross N_2O emission and uptake in soil are positively correlated with soil N availability, moisture content and microbial biomass. From those studies in California, soil gross N_2O emission and uptake are also regulated by soil N availability, moisture content, temperature and CO_2 emission (Yang et al., 2011; Yang et al., 2016a). There is one other method that measures directly N_2 and N_2O emissions from intact soil cores – the gas-flow soil core method – which has been used to quantify soil gross N_2O production (i.e. N_2 + N_2O fluxes) and gross N_2O consumption (i.e. N_2 fluxes) in grassland, beech and spruce forest sites in Germany (Butterbach-Bahl et al., 2002b; Chen et al., 2015; Dannenmann et al., 2008; Wen et al., 2016). This method however cannot be deployed for in-situ measurement because this instrument needs complete leak-proof setup that can only be achieved in a laboratory setting. Thus, so far only the $^{15}N_2O$ pool dilution technique is deployable for in-situ measurements.

Recent global atmospheric N_2O budget remains highly uncertain with estimates of net N_2O emission from natural (or non-agricultural) soils of 3.3-9.0 Tg N yr^{-1} and net N_2O uptake by soils of 0-1 Tg N yr^{-1} (IPCC, 2013). Forests cover roughly 31% of the land surface (FAO, 2010), and may thus substantially affect global N_2O budgets. Beech and spruce are the most common tree species in European forests (Köble and Seufert, 2000). Previous studies have shown that deciduous forests typically act as stronger net N_2O source than coniferous forests (Ambus et al., 2006). In Germany, earlier studies in forest ecosystems, which are mostly influenced by high N deposition, have shown that soil net N_2O fluxes were higher in beech than in spruce (e.g. Butterbach-Bahl et al., 1997; Schulte-Bisping et al, 2003) and that their most important controlling factors are soil or litter C/N ratios (as indicators of soil N availability) and soil moisture. To date, other than those studies conducted in California (Yang et al. 2011; Yang and Silver, 2016a; Yang and Silver, 2016b), in-situ gross N_2O emission and gross N_2O uptake in soil and their controlling factors have not been investigated in any other terrestrial ecosystems.

Furthermore, biological N_2 fixation in soil, the microbial process of converting atmospheric N_2 into bioavailable ammonia, is one of the most important processes controlling N richness of natural ecosystems (Bellenger et al., 2011), and since gaseous N losses are

commonly influenced by soil N availability, quantifying gross N_2O emission, uptake and N_2 fixation will generate new insights into the gaseous N budget of an ecosystem. Asymbiotic N_2 fixation by free-living microorganisms is an important N input in ecosystems where no or only few leguminous species are present (Keuter et al., 2014). Asymbiotic N_2 fixation is controlled by climatic factors and soil nutrient availability (Reed et al., 2011). Specifically, nitrogenase enzyme activity has been shown to be temperature dependent (Houlton et al., 2008). High soil moisture content also stimulates asymbiotic N_2 fixation as it consequently lowers the oxygen content in the soil and high oxygen content can inhibit nitrogenase activity (Limmer and Drake, 1996; Reed et al., 2011). The bioavailability of labile organic carbon is also an important controlling factor because asymbiotic N_2 fixation requires a lot of energy (Vitousek and Hobbie, 2000). Increased availability of soil nutrients has been shown to either down-regulate (mineral N) or stimulate (P, Fe, Mo) asymbiotic N_2 fixation (Jean et al., 2013; Keuter et al., 2014). No nitrogenase activity has been reported in very acidic soils possibly because of reduction of bacterial population which are intolerant to highly acidic condition (Jurgensen and Davey, 1970; Limmer and Drake, 1996) combined with low availability of nutrients (e.g. P, Mo) in acidic condition which may limit N_2 fixation (e.g. Barron et al., 2008; Reed et al., 2007; Silvester et al., 1989).

Soil gross N_2O emission, gross N_2O uptake and N_2 fixation ideally should be investigated together in an ecosystem, considering that these processes are controlled by similar factors (Reed et al., 2011; Seitzinger et al., 2006) and in view of ecosystem N input-output budget which often assumed that soil gaseous N emission (e.g. net N_2O + N_2 emission) is compensated by N_2 fixation in the soil (e.g. Brumme et al., 2009). While in aquatic ecosystem denitrification and N_2 fixation are commonly investigated together (e.g. Deutsch et al., 2007), this is rarely the case in terrestrial ecosystems.

In the present study, our objectives were to: 1) quantify gross N_2O emission and uptake and asymbiotic N_2 fixation in soils under beech and spruce forests, and 2) determine the controlling factors of these processes. Our study sites were unmanaged, old-growth spruce (*Picea abies*) and beech (*Fagus sylvatica*) forests on acidic Cambisol soil in central Germany that have been receiving high N deposition (averaging 42 and 25 kg N ha^{-1} yr^{-1} in throughfall of spruce and beech stands, respectively, since monitoring began in 1970s; Corre et al., 2003; Corre and Lamersdorf, 2004; Lower Saxony Forest Research Station, 2003; Meesenburg et al., 1995). We tested the following hypotheses: (1) the beech forest will have higher gross N_2O emission and uptake in the soil than the spruce forest; (2) gross N_2O emission and uptake in soil will be mainly regulated by soil N availability and moisture content, whereas soil

temperature and available C will additionally influence asymbiotic N_2 fixation; and (3) at both forests, with acidic soil and high N deposition, asymbiotic N_2 fixation will be lower than gross N_2O uptake (or N_2 flux from the soil). Our study provides the much needed information on the importance of spruce and beech forest ecosystems as sink of atmospheric and soil-air N_2O, and on whether asymbiotic N_2 fixation compensates for gross N_2O uptake.

3.2. Methods and materials

3.2.1. Site description and sampling design

Our study was conducted at the Solling upland (51.76° N, 9.58° E), Lower Saxony, Germany. Two adjacent stands were selected: a 132-year-old spruce stand and a 163-year-old beech stand. Both forest stands are situated at an altitude of 510 m, with a mean annual temperature of 7.3 °C and a mean annual precipitation of 1100 mm. These forest stands were on a similar soil type, formed from loess over weathered Triassic sandstone, classified as Dystric Cambisol (FAO) or Typic Dystrochrept (USDA) and has silty loam texture.

We measured soil gross N_2O emission and uptake, microbial C and N, denitrification enzyme activity (DEA), and other supporting soil parameters (see Section 2.5) from May to October in 2014. Asymbiotic N_2 fixation was measured from June to October in the same year. Measurements were carried out separately for the organic layer (combined Oi, Oe and Oa layers) and 0-5 cm mineral soil. At each site, four or five sampling points (replicates) with a minimum distance of 25 m from each other were selected. In each sampling point, intact soil samples were taken using stainless-steel cores from each layer. We measured gross N_2O fluxes and asymbiotic N_2 fixation in the field and analyzed the gas samples and supporting soil parameters in the laboratory during the same day of sampling.

3.2.2. $^{15}N_2O$ pool dilution method

At each sampling point, four intact soil cores (250 cm^3 each) were taken and placed in a glass desiccator (6.6 L) equipped with a Luer-lock stopcock which was used for incubation in the field. We used four intact soil cores based on several preliminary tests for optimizing the closure time of incubation chamber, gas sampling intervals, the concentration of the $^{15}N_2O$ label gas and the volume of the incubation chamber. To maintain a good seal, vacuum grease was applied to the flanges so that the desiccators were closed tightly. Seven mL of the $^{15}N_2O$ label gas, containing 100 ppmv of 98% single labelled ^{15}N-N_2O, 275 ppbv of sulfurhexafluoride (SF_6 as a tracer for physical loss of N_2O) and synthetic air, were injected into the chamber headspace immediately after closure. Hence, headspace concentrations

increased by approximately 106 ppbv N_2O with 12.5 atom% ^{15}N and 292 pptv SF_6. After injection of the label gas, the headspace was mixed thoroughly by pumping the inside air with the use of a syringe. 100 mL and 12 mL air samples were taken from the headspace, using syringes with Luer-lock stopcocks, at 0.5, 1, 2, and 3 h following closure and immediately stored into pre-evacuated 100 mL glass bottles and 12 mL glass vials (Exetainer; Labco Limited, Lampeter, UK) with rubber septa, respectively. The sampled air volume was then replaced with 112 mL of a gas mixture containing 80% helium and 20% oxygen to maintain the headspace under atmospheric pressure and oxygen concentration without altering the isotopic composition of the headspace N_2O. Dilution of the headspace gases caused by adding the helium-oxygen gas mixture was corrected for in our calculations (see Section 2.3). The 100 mL gas samples were used to analyze the isotopic composition using an isotope ratio mass spectrometer (IRMS) (Finnigan Delta^{plus} XP, Thermo Electron Corporation, Bremen, Germany). The 12 mL gas samples were used to measure N_2O and SF_6 concentrations using a gas chromatograph (GC 6000 Vega Series 2, Carlo Erba Instruments, Milan, Italy) equipped with an electron capture detector and an autosampler. Air temperature and barometric pressure were recorded during each sampling day.

The $^{15}N_2O$ pool dilution has an implicit assumption that the headspace-labelled $^{15}N_2O$ diffuses into the soil and results in a homogeneous mixture of $^{15}N_2O$ with soil-derived N_2O in the soil air-filled pores; this implies that these soil pores must be interconnected to the soil surface to result in homogenous mixing. Based on the modeled vertical diffusive transport of $^{15}N_2O$ label, our most conservative calculations showed that the $^{15}N_2O$ label had diffused into the 5 cm long soil cores and back to the headspace within 0.5 h. Thus, our sampling interval during the 3-hour incubation period was sufficient to allow mixing of the label gas with the soil-derived N_2O in interconnected air-filled pores.

3.2.3. Calculation of gross N_2O emission and uptake

Gross N_2O emission and uptake in soil were calculated based from Yang et al. (2011). Net flux is the result of gross N_2O emission 'E' and gross N_2O uptake 'U':

$$Net\ flux = E - U \quad (1)$$

We assume that the gross N_2O emission rate is linear over the duration of chamber closure, and diffusion of N_2O from the chamber headspace into soil and its subsequent reduction follow Michaelis-Menten-type kinetics (Vieten et al., 2009), yielding the following time-dependent equation of N_2O concentrations, $[N_2O]$:

$$Net\ flux = \frac{d[N_2O]}{dt} = E - k \times [N_2O]_t \quad (2)$$

where 'k' is the first-order rate constant for U, and '$[N_2O]_t$' is the concentration of N_2O at time t. This equation can be applied to $^{14}N_2O$ and $^{15}N_2O$ headspace concentration simultaneously:

$$\frac{d[^{14}N_2O]}{dt} = F_{14} \times \mathrm{E} - (k_{14} + k_l) \times [^{14}N_2O]_t \quad (3)$$

$$\frac{d[^{15}N_2O]}{dt} = F_{15} \times \mathrm{E} - (k_{15} + k_l) \times [^{15}N_2O]_t \quad (4)$$

Then, these equations can be transformed to the following:

$$[^{14}N_2O]_t = \frac{F_{14} \times E}{(k_{14} + k_l)} - \left\{\frac{F_{14} \times E}{(k_{14} + k_l)} - [^{14}N_2O]_0\right\} \times exp\{-(k_{14} + k_l) \times (t - t_0)\} \quad (5)$$

$$[^{15}N_2O]_t = \frac{F_{15} \times E}{(k_{15} + k_l)} - \left\{\frac{F_{15} \times E}{(k_{15} + k_l)} - [^{15}N_2O]_0\right\} \times exp\{-(k_{15} + k_l) \times (t - t_0)\} \quad (6)$$

where '$[^{14}N_2O]_t$' and '$[^{15}N_2O]_t$' represent the concentrations of $^{14}N_2O$ and $^{15}N_2O$ at time t, calculated respectively as the product of N_2O concentration and ^{14}N-N_2O atom% and ^{15}N-N_2O atom% excess (Yang et al., 2011); 'F_{14}' and 'F_{15}' represent the mole fractions of emission that is in the form of $^{14}N_2O$ (99.6569%) and $^{15}N_2O$ (0.3431%), respectively (Yang et al., 2011); and 't' represents the time of sampling from the headspace; 'k_{14}' and 'k_{15}' represent the first-order rate constant for $^{14}N_2O$ and $^{15}N_2O$ reduction to N_2, and they are related by experimentally derived stable N isotopic fractionation factors ($\alpha = k_{15}/ k_{14}$) with the average literature value of 0.9924 ± 0.0036 (Yang et al., 2011); 'k_l' represents the first-order rate constant for physical loss of the inert SF_6 tracer. We used the sum of the first-order rate constants ($k_{14} + k_l$ or $k_{15} + k_l$) in the above equations because biological consumption and physical loss simultaneously affect $^{14}N_2O$ and $^{15}N_2O$ concentrations in the chamber headspace.

We estimated E and k_{15} by simultaneously fitting the measured $[^{14}N_2O]_t$ and $[^{15}N_2O]_t$ with equations (5) and (6). The best fit of $[^{14}N_2O]_t$ and $[^{15}N_2O]_t$ was found using the least square approach and minimizing the following error function:

$$Error = \sum_{t=1}^{n} \frac{(Y_{predicted}(t) - Y_{observed}(t))^2}{SD} + \sum_{t=1}^{n} \frac{(Z_{predicted}(t) - Z_{obsserved}(t))^2}{SD} \quad (7)$$

Y, Z, n and SD indicate $[^{14}N_2O]_t$, $[^{15}N_2O]_t$, number of measurements, and standard deviation of the observed concentrations, respectively, over the duration of chamber closure (Rhew, 2011; Teh et al., 2008). Equation (7) was minimized using the 'fminsearchbnd' function in MATLAB (MathWorks, Version R2011b, USA). Finally, U was calculated by multiplying k_{14} and k_{15} by the average background atmospheric concentrations of $^{14}N_2O$ and $^{15}N_2O$ (Rhew, 2011; Yang et al., 2011), considering N_2O concentration (325.1 ppb; WMO, 2013) and ^{15}N natural abundance (0.3663 atom%).

$$U = k_{14} \times [^{14}N_2O] + k_{15} \times [^{15}N_2O] \quad (8)$$

This calculation of gross N_2O uptake represented well this flux based on the high correlation between the calculated net N_2O fluxes (*E* - *U*) and the measured net N_2O fluxes across the measurement period in both forest stands ($R^2 = 0.93$, n = 84, $P < 0.01$, slope = 1.05 ± 0.03). We reported the measured net N_2O fluxes determined from the linear increase of chamber headspace N_2O concentration.

3.2.4. Asymbiotic N_2 fixation

Asymbiotic N_2 fixation was measured using the acetylene reduction assay (Hardy et al., 1968), which we have employed in our earlier works (Keuter et al., 2014; Matson et al., 2015). We took intact soil cores (100 cm^3) from the organic layer and the 0-5 cm mineral soil from each sampling point. Soil cores were incubated immediately in the field in 1100 mL glass mason jars with lids fitted with septa for gas sampling. This incubation jars have been tested to be air-tight (Keuter et al., 2014). We replaced 10% of the headspace with acetylene (cylinder C_2H_2 with 99% purity, Westfalen AG, Münster, Germany), which was previously purified by letting it bubbled through 98% H_2SO_4 and 5 M NaOH solutions (Hyman and Arp, 1987). The jars were buried in the ground to incubate the soil cores in the same depth that they were sampled. Gas samples of 12 mL were taken from the headspace, using syringes with Luer-lock stopcocks, at 1, 3 and 24 h and immediately stored into pre-evacuated 12 mL glass vials (Exetainer; Labco Limited, Lampeter, United Kindom). Gas samples were analyzed for C_2H_4 concentration using a gas chromatograph (Shimadzu GC 14-B, Shimadzu, Duisburg, Germany) with a flame ionization detector and Hayesep T column. C_2H_4 production rates were calculated from the slope of the regression line between C_2H_4 concentration and time. C_2H_4 production rates were converted to asymbiotic N_2 fixation rates using the theoretical ratio of 3:1 (Hardy et al., 1968), which is commonly used by other N_2 fixation studies (e.g. Cusack et al., 2009 and Reed et al., 2008 [3:1]; Benner et al., 2007 and Matzek and Vitousek, 2003 [3.1:1]).

3.2.5. Supporting soil parameters

Soil biochemical characteristics were determined in the organic layer and 0-5 cm mineral soil. Soil pH was analyzed in a 1:2.5 soil-to-water ratio. Total organic C and N were measured from air-dried, ground samples using a CNS Elemental Analyzer (Elementar Vario EL, Hanau, Germany). Total Fe in the organic layer was determined by HNO_3 pressure digestion whereas exchangeable Fe in the 0-5 cm mineral soil was determined by percolation with 1M unbuffered NH_4Cl; the digest or percolate was analyzed for Fe content using inductively

coupled plasma-atomic emission spectrometer (ICP-AES; iCAP 6300 Duo VIEW ICP Spectrometer, Thermo Fischer Scientific GmbH, Dreieich, Germany). Available P and Mo were determined using resin-exchange method, as described in our earlier work (Keuter et al., 2014). Briefly, 0.5 g soil was shaken in 30 mL distilled water for 12 h together with 1 g anion exchange resin (DOWEX 41081 analytical grade, Serva Electrophoresis GmbH, Heidelberg, Germany) kept in a tea bag. Subsequently, the resin was shaken in 20 mL 0.5 M HCl for 12 h, and these extracts were analyzed for P and Mo contents using the ICP-AES.

From the same 12 mL gas samples, used to measure N_2O and SF_6 concentrations, CO_2 were also determined using the same gas chromatograph mentioned above in order to determine heterotrophic respiration rate and its relationship with gross N_2O fluxes. Following each measurement of gross and net N_2O fluxes, the four soil cores in a chamber were pooled and subsamples were used to determine moisture content, microbial C and N, extractable mineral N and organic C, and DEA. These were conducted upon arrival at the laboratory within the same day of field sampling. Gravimetric water content was determined by oven-drying the subsample soil at 105 °C for one day. Soil water content was expressed as water-filled pore space (WFPS), calculated using particle densities of 1.40 g cm^{-3} for organic layer and 2.65 g cm^{-3} for mineral soil and the measured soil bulk density at our sites. Microbial C and N were determined from another subsample soil by chloroform fumigation-extraction method (Brookes et al., 1985). About 15 g of the composite soil sample was extracted with 100 mL 0.5 M K_2SO_4 for determination of background extractable mineral N and C. Another 15 g of the paired composite soil sample was fumigated with ethanol-free chloroform for 5 days, followed by extraction with 100 mL 0.5 M K_2SO_4. Soil extraction was done by shaking the soil with K_2SO_4 for 1 h and then filtered through K_2SO_4 pre-washed filter papers. The extracts were kept frozen until analysis. The total extractable N, NH_4^+ and NO_3^- contents of the extracts were analyzed using continuous flow injection colorimetry (SEAL Analytical AA3, SEAL Analytical GmbH, Norderstadt, Germany), where total N was determined by ultraviolet-persulfate digestion followed by hydrazine sulfate reduction (Autoanalyzer Method G-157-96), NH_4^+ by salicylate and dicloroisocyanuric acid reaction (Autoanalyzer Method G-102-93) and NO_3^- by cadmium reduction method with NH_4Cl buffer (Autoanalyzer Method G-254-02). Extractable organic C was measured using ultraviolet-enhanced persulfate oxidation using a Total Organic Carbon Analyzer (TOC-Vwp, Shimadzu Europa GmbH, Duisburg, Germany). The differences in extractable C and total extractable N between the paired fumigated and unfumigated soils were assumed to indicate the C and N released from lysed soil microbes. The chloroform-labile C and N were converted to microbial biomass C

and N using a $K_C = 0.45$ and a $K_N = 0.68$, respectively (Brookes et al., 1985; Shen et al., 1984). DEA determination was based on the method described by Sutton-Grier et al. (2011). Five grams of field-moist soil were weighed into 120 mL glass incubation jars. A media with 10 mL of 1 mM KNO_3, 1 mM glucose and 1 g L^{-1} chloramphenicol in distilled-deionized water was added to each jar to ensure non-limiting substrate conditions and inhibition of protein synthesis. The jar was sealed with a lid that has a gas sampling port with a rubber septum, and an anaerobic headspace was created by repeatedly flushing the jar with N_2 gas. Acetylene (10 mL) was injected into each jar, making N_2O the final product of denitrification. Jars were placed on an orbital shaker at 120 rpm. Gas samples were collected at 2, 22, and 42 min and analyzed for N_2O concentration using the same gas chromatograph described above. DEA was calculated from the linear rates of N_2O accumulation.

3.2.6. Statistical analysis

Each parameter was first tested for normal distribution using Shapiro-Wilk's test and for equality of variance using Levene's test. Parameters with non-normal distributions or unequal variances were either logarithmically (for gross and net N_2O fluxes, mineral N , extractable organic C, microbial C and N, CO_2 emission, WFPS) or square-root transformed (for DEA). For soil biochemical characteristics measured once, analyses were conducted for each soil layer and differences between forest types (beech vs. spruce) were analyzed using independent T test at $P \leq 0.05$. For analysis of time-series data (gross and net N_2O fluxes, asymbiotic N_2 fixation, mineral N, extractable organic C, microbial C and N, DEA, CO_2 emission and WFPS), we used linear mixed effects model (LME). Analysis was conducted for each soil layer and the LME model included forest types (beech and spruce) as fixed effect whereas sampling dates and spatial replicates were included as random effects. The LME model included either 1) a variance function that allows different variances of the response variable for the fixed effect, and/or 2) a first-order temporal autoregressive process that assumes a decreasing correlation between measurements with increasing time distance, if this increased the relative goodness of the model fit (Crawley, 2007). Fixed effects were considered significant based on the analysis of variance at $P \leq 0.05$, and differences between forest types were assessed using Fisher's least significant difference test at $P \leq 0.05$. Linear regression analyses were used to explore relationships of gross and net N_2O fluxes and asymbiotic N_2 fixation with possible explanatory soil factors across the entire measurement period, conducted separately for each soil layer at each forest stand using the mean of four replicates on each sampling day. For the regression analyses, we mentioned values of $P \leq$

0.09 as marginally significant, considering that field-measured soil variables and processes have inherently high spatial variability. All statistical analyses were conducted using the open source software R (version 2.15.3).

3.3. Results

3.3.1. Gross N_2O emission, gross N_2O uptake and asymbiotic N_2 fixation

Gross N_2O emissions from the organic layer and the mineral soil were higher in the beech than the spruce stands ($P < 0.01$ for both depths; Table 1). Gross N_2O uptake did not differ between forest types either in the organic layer ($P = 0.76$) or in the mineral soil ($P = 0.53$; Table 1). As was the case with gross N_2O emissions, net N_2O fluxes were higher in the beech stand than in the spruce stand both in the organic layer ($P = 0.02$) and mineral soil ($P = 0.04$; Table 1).

Table 1. Gross N_2O emission, gross N_2O uptake, net N_2O flux, and asymbiotic N_2 fixation in the organic layer and 0-5 cm mineral soil in spruce and beech forests from May to October 2014.

Soil layer/forest type	Gross N_2O emission ($\mu g\ N\ kg^{-1}\ h^{-1}$)	Gross N_2O uptake ($\mu g\ N\ kg^{-1}\ h^{-1}$)	Net N_2O flux ($\mu g\ N\ kg^{-1}\ h^{-1}$)	Asymbiotic N_2 fixation ($\mu g\ N\ kg^{-1}\ h^{-1}$)
Organic layer				
Spruce	0.87 ± 0.12 b	0.41 ± 0.05	0.24 ± 0.04 b	0.022 ± 0.003 b
Beech	1.38 ± 0.24 a	0.22 ± 0.05	0.92 ± 0.27 a	0.035 ± 0.004 a
0-5 cm soil layer				
Spruce	0.26 ± 0.04 b	0.10 ± 0.01	0.03 ± 0.04 b	0.010 ± 0.001
Beech	0.93 ± 0.15 a	0.14 ± 0.04	0.59 ± 0.10 a	0.008 ± 0.001

At each layer, means ± standard errors (n = 4) within each column followed by the different letter indicated significant difference between forest types (linear mixed effects model with Fischer's least significant difference test at $P \leq 0.05$).

Throughout the entire measurement period of the growing season, gross N_2O emissions displayed generally large spatial and temporal variability, as shown by the large standard errors on the means (Fig. 1a), as compared to gross N_2O uptake (Fig. 1b). Net N_2O fluxes also displayed small temporal variability, with the exception of the organic layer in the spruce stand where net N_2O fluxes decreased from June to September (Fig. 1c). Both organic layer and mineral soil in the beech stand were net N_2O sources throughout the measurement period, whereas the organic layer in the spruce stand showed small net N_2O sinks in September and October (Fig. 1c).

Asymbiotic N_2 fixation rates were very low during the entire measurement period with only small increases in the beech organic layer during the summer measurements (Fig. 1d). Across the measurement period, beech organic layer had higher asymbiotic N_2 fixation than spruce organic layer ($P < 0.01$), whereas no difference was found between forest types for the mineral soil ($P = 0.99$; Table 1). Asymbiotic N_2 fixation rates were an order of magnitude lower than gross N_2O uptake in both soil layers at both forests (Table 1).

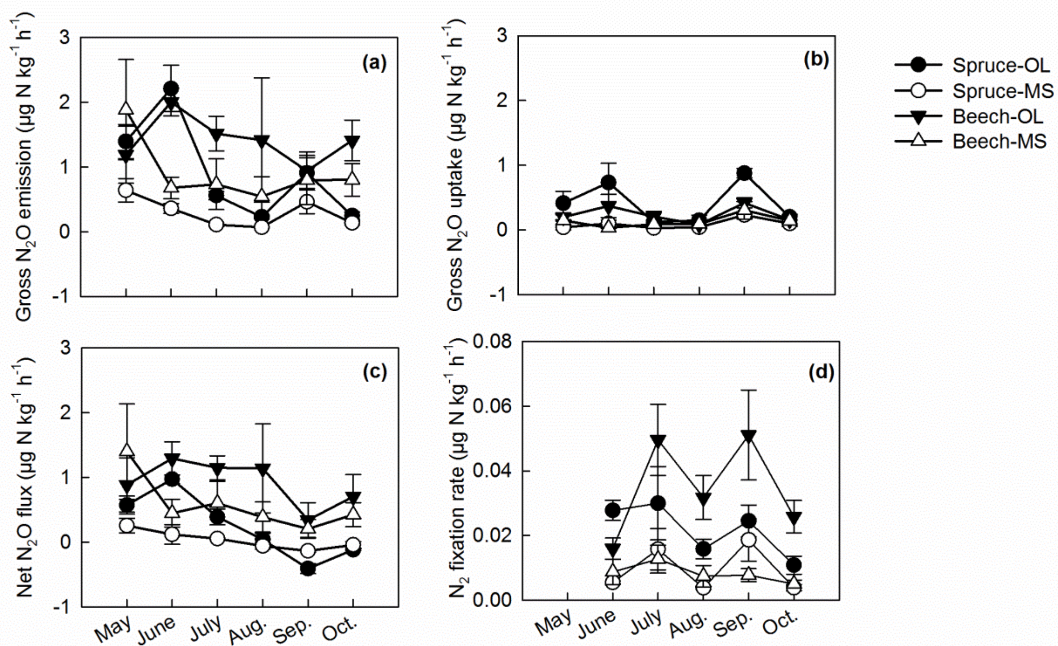

Figure 1. Temporal variability of N_2O dynamics and asymbiotic N_2 fixation (means ± standard errors, n = 4). Soil gross N_2O emission (a), gross N_2O uptake (b), net N_2O flux (c) and asymbiotic N_2 fixation (d) in the spruce organic layer (OL), spruce 0-5 cm mineral soil (MS), beech OL, and beech MS, measured from May to October 2014.

3.3.2. Soil characteristics

No differences were detected between the spruce and beech stands in pH, total organic C, total N, total or exchangeable Fe, available Mo and available P (*P* = 0.07 - 0.88; Table 2). Only the spruce stand showed a higher C:N ratio (*P* = 0.01) and a lower bulk density (*P* = 0.02) than the beech stand in the mineral soil. Of the soil factors that were measured monthly, organic layers generally showed higher soil mineral N, extractable organic C, DEA, and microbial biomass C and N, and CO_2 emission than mineral soils (Fig. 2a-g). Temporal variability of these soil properties were also more pronounced in the organic layer than in the mineral soil (Fig. 2a-g). Across the whole measurement period, NH_4^+, NO_3^-, DEA, microbial C and N did not display significant differences between spruce and beech stands within the same soil layer (Fig. 2a-b, d-f). Extractable organic C was higher in the beech organic layer than the spruce organic layer (*P* = 0.01), whereas no difference was found between forest types in the mineral soil (*P* = 0.47; Fig. 2c). Organic layers in the beech stand had larger CO_2 emission than organic layer in the spruce stand (*P* = 0.01), whereas mineral soil in the beech stand had smaller CO_2 emission than the spruce stand (*P* = 0.02; Fig. 2g). WFPS was higher for each soil layer in the beech stand compared to the spruce stand (*P* = 0.03 for both depths; Fig. 2h).

Table 2. Soil characteristics measured in the beginning of the study (May 2014).

Soil layer/ forest type	Bulk density ($g\ cm^{-3}$)	pH (1:5 H_2O)	Total organic C ($g\ C\ kg^{-1}$)	Total N ($g\ N\ kg^{-1}$)	Total C:N ratio	Fe[†] ($g\ Fe\ kg^{-1}$)	Available Mo ($\mu g\ Mo\ kg^{-1}$)	Available P ($mg\ P\ kg^{-1}$)
Organic layer								
spruce	0.16 ± 0.02	4.19 ± 0.08	439 ± 20	18.3 ± 0.2	24.1 ± 1.2	11.2 ± 4.3	79.0 ± 33.1	12.5 ± 2.0
beech	0.17 ± 0.02	4.12 ± 0.13	436 ± 25	20.7 ± 0.8	21.1 ± 0.6	3.4 ± 1.0	105.6 ± 38.3	16.3 ± 3.3
0-5 cm mineral soil								
spruce	0.57 ± 0.08b	3.67 ± 0.07	109 ± 19	5.1 ± 0.9	21.5 ± 0.4a	0.21 ± 0.02	23.6 ± 13.3	12.9 ± 4.6
beech	0.75 ± 0.06a	3.66 ± 0.07	127 ± 36	6.6 ± 1.6	18.9 ± 0.6b	0.22 ± 0.02	40.4 ± 39.1	9.6 ± 2.7

At each layer, means ± standard errors (n = 4) within each column followed by the different letter indicated significant difference between forest types (independent T test at $P \leq 0.05$). [†]Fe in the organic and mineral soil layers was determined as the total and exchangeable concentrations, respectively; available Mo and P were determined by resin-exchange method.

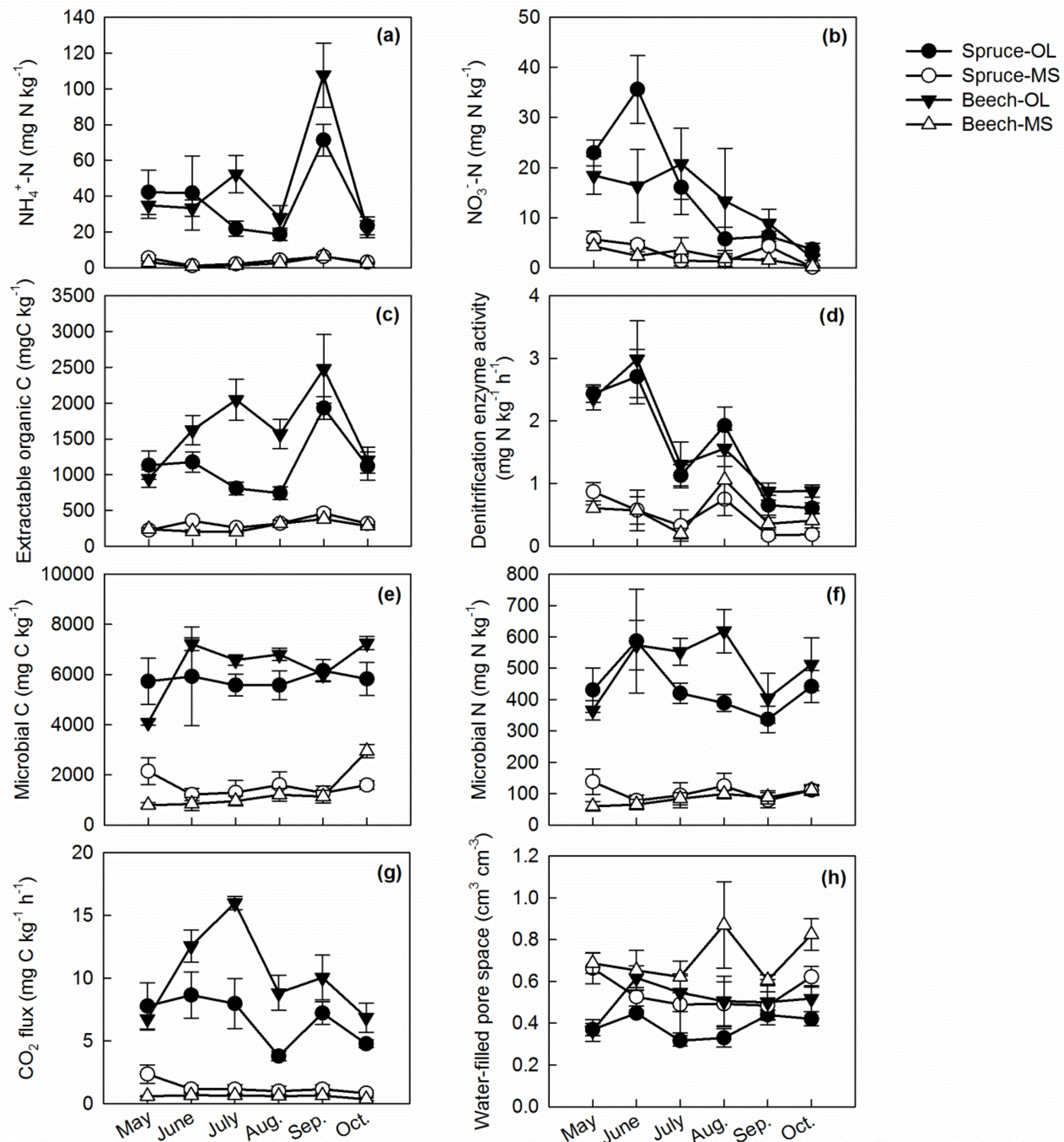

Figure 2. Temporal variability of soil properties (means ± standard errors, n = 4). NH_4^+-N (a), NO_3^--N (b), extractable organic C (c), denitrification enzyme activity (d), microbial C (e), microbial N (f), soil CO_2 flux (g), and water-filled pore space (h) in the spruce organic layer (OL), spruce 0-5 cm mineral soil (MS), beech OL, and beech MS, measured from May to October 2014.

3.3.3. Correlations of gross N_2O fluxes and asymbiotic N_2 fixation with soil factors

Gross N_2O emissions were positively correlated with soil NO_3^- concentrations in both the organic layer (Fig. 3a) and mineral soil (Fig. 3c) of the spruce stand, whereas no correlations were detected in either soil layers of the beech stand (Fig. 3b, d). Gross N_2O uptakes were positively correlated with extractable organic C contents in the organic layer (Fig. 4a) and

mineral soil (Fig. 4c) of the spruce stand and in the mineral soil of the beech stand (Fig. 4d). We also detected significant correlations of gross N_2O uptake with NH_4^+ (R^2 = 0.86, n = 6, *P* < 0.01) and microbial C (R^2 = 0.83, n = 6, *P* = 0.01) in the spruce organic layer. Net N_2O fluxes were positively correlated with gross N_2O emissions in the beech organic layer (Fig. 5b) and mineral soil (Fig. 5d), and marginally significant correlation was observed in the spruce organic layer (Fig. 5a). Asymbiotic N_2 fixation was correlated with different soil factors at each forest type. For the spruce organic layer, asymbiotic N_2 fixation was positively correlated with soil CO_2 emission (R^2 = 0.83, n = 6, *P* = 0.03) and soil temperature (R^2 = 0.90, n = 6, *P* = 0.01). For the beech stand, asymbiotic N_2 fixation was positively correlated with soil temperature in the mineral soil (R^2 = 0.94, n = 6, *P* < 0.01), and was marginally significant correlated with extractable organic C in the organic layer (R^2 = 0.66, n = 6, *P* = 0.09). Other than these, there were no other significant correlations observed.

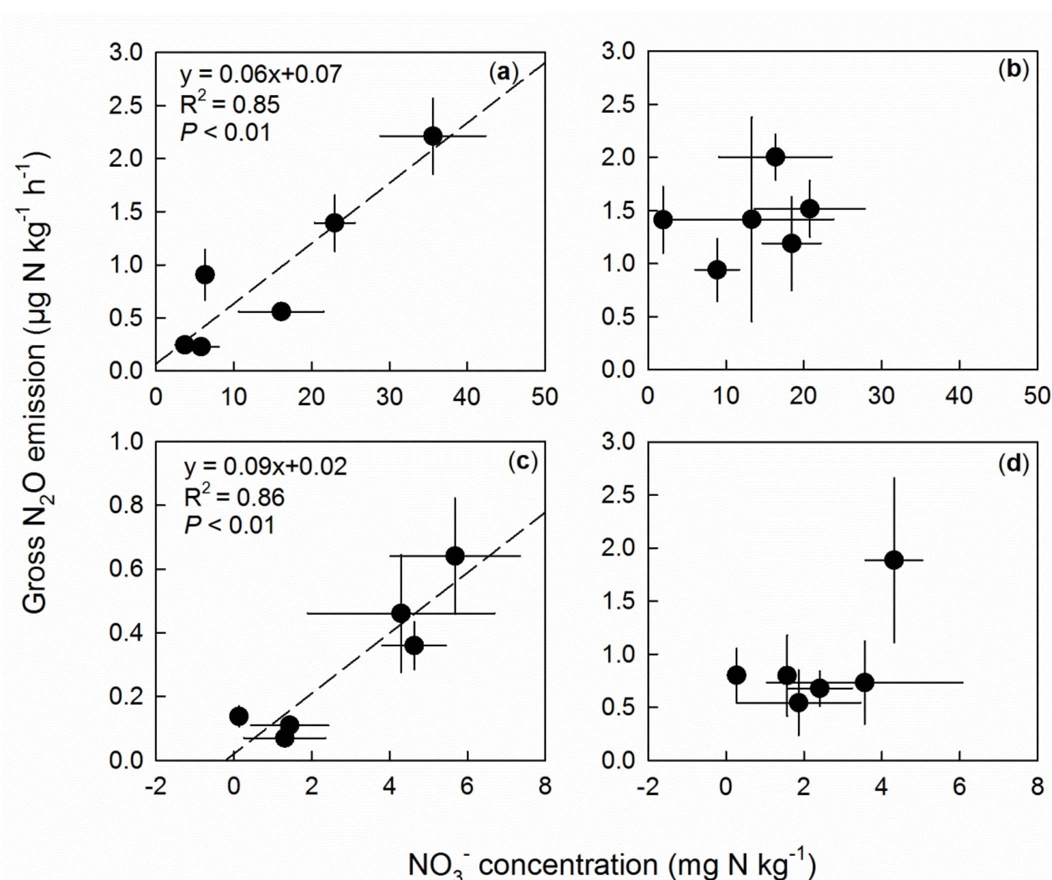

Figure 3. Relationships between gross N_2O emission and soil NO_3^- concentration in the spruce organic layer (a), beech organic layer (b), spruce mineral soil (c) and beech mineral soil (d) (n = 6, linear regression). Each point represents the mean of four replicates on each sampling day from May to October 2014, and the bars indicate standard errors.

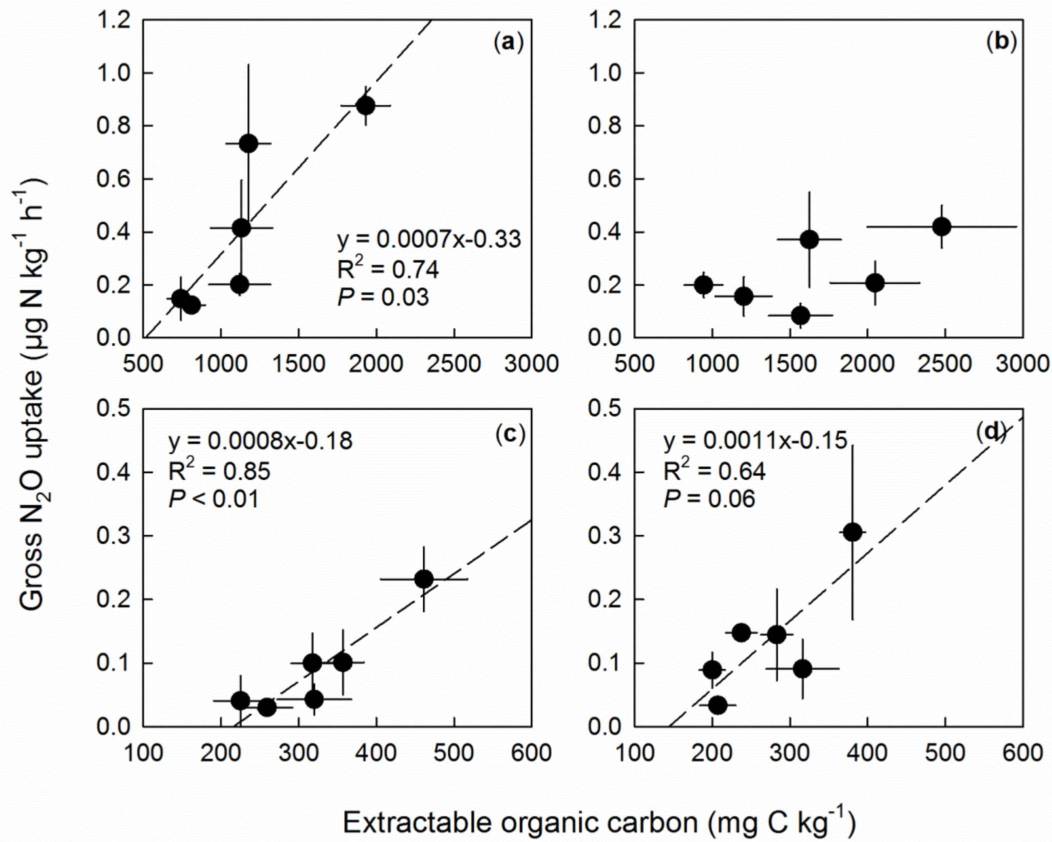

Figure 4. Relationships between gross N_2O uptake and soil extractable organic carbon concentration in the spruce organic layer (a), beech organic layer (b), spruce mineral soil (c) and beech mineral soil (d) (n = 6, linear regression). Each point represents the mean of four replicates on each sampling day from May to October 2014, and the bars indicate standard errors.

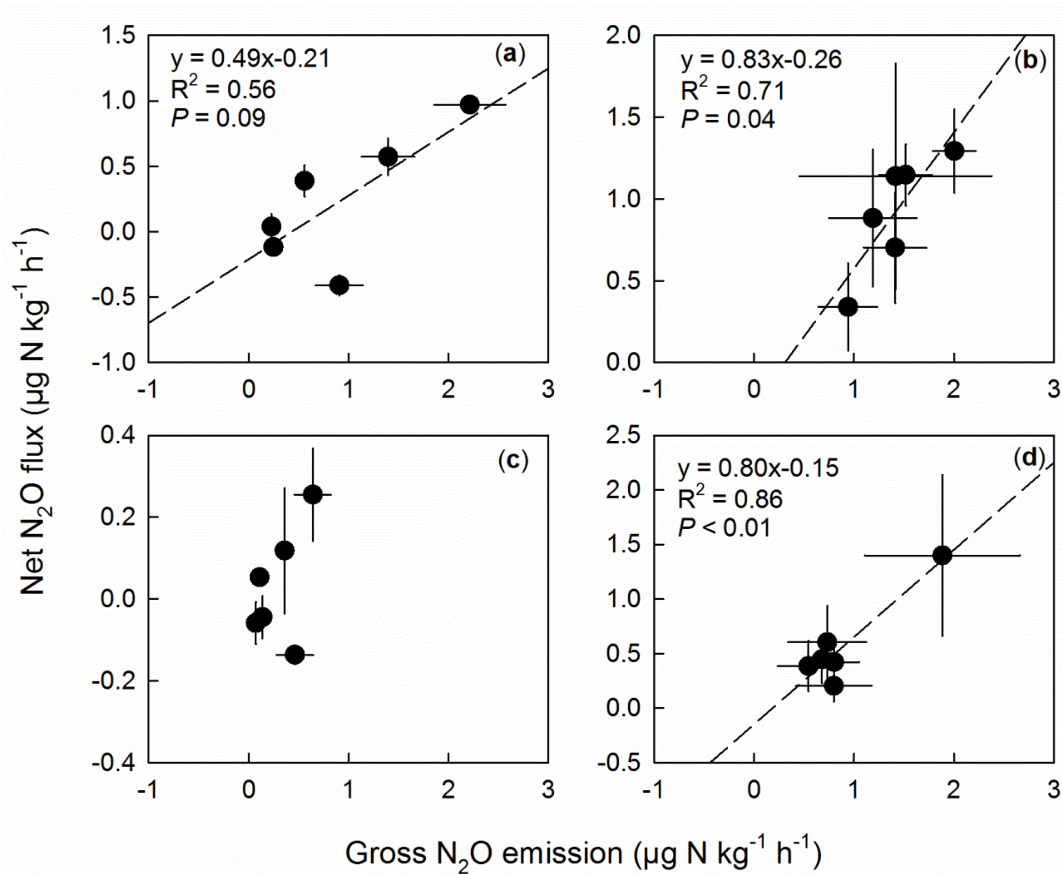

Figure 5. Relationships between net N_2O flux and gross N_2O emission in the spruce organic layer (a), beech organic layer (b), spruce mineral soil (c) and beech mineral soil (d) (n = 6, linear regression). Each point represents the mean of four replicates on each sampling day from May to October 2014, and the bars indicate standard errors.

3.4. Discussion

The higher soil gross N_2O emission, net N_2O flux and asymbiotic N_2 fixation in the beech stand compared to the spruce stand (Table 1) illustrated that tree species on the same soil type and climatic condition have a strong impact on both soil N_2O dynamics and asymbiotic N_2 fixation. These results supported our first hypothesis. Earlier studies in Germany have reported that beech forests typically act as stronger net source of N_2O than spruce forests (e.g. Butterbach-Bahl et al., 1997; Schulte-Bisping et al, 2003). Our supporting soil parameters suggest that these differences in gross and net N_2O emissions were due to higher soil N availability and more anaerobic conditions in beech than spruce stands. Our earlier studies from beech and spruce forests near to our present sites showed that gross NO_3^- production in both the organic layer and mineral soil is higher in the beech than the spruce stands (Corre et al., 2003; Corre and Lamersdorf, 2004), whereby soil NO_3^- availability was an important

factor controlling the temporal pattern of gross N_2O emission in the spruce stand (Fig. 3a, c). The higher WFPS and CO_2 fluxes in the beech than the spruce stands (Fig. 2g, h) might have also resulted in more anaerobic conditions, which may have favored not only gross and net N_2O emissions but also asymbiotic N_2 fixation. Quantifying independently the gross N_2O emission and uptake in soil offers the unique opportunity to explore separately the controlling soil factors of these concurrently occurring processes. The positive correlations of gross N_2O emission with NO_3^- concentration in the spruce stand (Fig. 3a, c) and of gross N_2O uptake with extractable organic C in both spruce and beech stands (Fig. 4a, c, d) suggest that variations in levels of electron donor and acceptor controlled the temporal patterns of gross N_2O fluxes within each stand and point to denitrification as the dominant process regulating these fluxes. Our previous study on gross N_2O emission and uptake from different sites (manured grassland, fertilized cropland and unmanaged forests), encompassing wide ranges of soil mineral N and organic C levels, also suggest denitrification as the dominant process (Wen et al., 2016). Additionally, the positive correlation of gross N_2O uptake with NH_4^+ and microbial C in the spruce organic layer reflected the similarity of their temporal patterns (Figs. 1 and 2a, e), which suggests that high gross N_2O uptake occurred during a period of high microbial activity and substrate availability (i.e. early autumn, as discussed further below). Together, our results indicate that both soil N availability (e.g. gross nitrification) and soil aeration status (i.e. WFPS, CO_2 emission) controlled the difference in gross N_2O emissions between forest stands, whereas temporal variations in electron donor (e.g. extractable organic C) and acceptor (e.g. NO_3^-) influenced gross N_2O emission and uptake within a stand. These findings supported our second hypothesis.

Since the time that net negative N_2O flux in the soil was first reported, it has been discussed whether net N_2O uptake is caused by relatively low N_2O production or high N_2O consumption (Conrad, 1994). Earlier studies frequently link net N_2O uptake in soil to low NO_3^- level and low atmospheric or fertilizer N input (Butterbach-Bahl et al., 1998; Goossens et al., 2001), suggesting that in a condition of low soil N availability net N_2O uptake may be driven by low gross N_2O emission. In our previous study, we observed net N_2O uptake in a sandy pine forest soil, which was characterized by very low soil N availability and low gross N_2O fluxes with gross N_2O uptake larger than gross N_2O emission (Wen et al., 2016). In our present study, the observed net N_2O uptake in the organic layer of the spruce stand from September to October (Fig. 1c) can also be attributed to a larger gross N_2O uptake than gross N_2O emission (Fig. 1a, b). The low gross N_2O emissions during this period (Fig. 1a) was paralleled by low NO_3^- content in the organic layer of the spruce stand (Fig. 2b), whereas the

high gross N_2O uptake (Fig 1b) was paralleled by high extractable organic C, microbial C and CO_2 emissions (Fig. 2c, e, g), suggesting high bioavailability of organic C. High organic C availability is commonly observed in temperate forests during early autumn, which has been explained by new input of easily decomposable organic materials from litterfall and still favorable temperature for decomposition (Fröberg et al., 2006; Michalzik and Matzner, 1999). On the other hand, the larger gross N_2O emissions from both soil layers in the beech than spruce stands (Table 1), which followed similar trends in gross nitrification (Corre et al., 2003; Corre and Lamersdorf, 2004), resulted to a net N_2O source throughout the measurement period (Fig. 1c). This and the positive correlation between net and gross N_2O emissions (Fig. 5b, d), but not with gross N_2O uptake, indicates that net N_2O fluxes were largely influenced by gross N_2O emissions in the beech forest stand. This result is in agreement with Yang and Silver (2016b) who reported that the spatial variability in gross N_2O emission rates among marsh zones in northern California drove their differences in net N_2O fluxes.

Asymbiotic N_2 fixation in both forest stands were very low compared to other temperate forests (0.165 g N m^{-2} yr^{-1} on average; Cleveland et al., 1999). We suspect that the low asymbiotic N_2 fixation at our sites was caused by the acidic soil pH, which also resulted in low Mo and P levels (Table 2). From extremely acidic forest soils with pH of 2.7-3.3, N_2 fixation was claimed to be absent and was attributed to intolerance of some N_2 fixers to acidic condition (Jurgensen and Davey, 1970; Limmer and Drake, 1996). In additional to the low soil pH, our study sites have been receiving high N deposition (averaging 42 and 25 kg N ha^{-1} yr^{-1} in throughfall of spruce and beech stands, respectively) accompanied with high N leaching (averaging 45% and 10% of throughfall N deposition in spruce and beech stands, respectively; Corre et al., 2003; Corre et al., 2007; Corre and Lamersdorf, 2004; Meesenburg et al., 1995). High N availability may inhibit nitrogenase activity, since the energy cost for microorganism to fix N is much greater than that to acquire mineral N from the soil (Reed et al., 2011). Moreover, the levels of available Mo and P in our present sites were comparable or an order of magnitude lower than reported values for temperate forest soils where asymbiotic N_2 fixation were limited by Mo and P (resin-exchangeable Mo of 4-75 µg Mo kg^{-1}; resin-exchangeable P:13-384 mg P kg^{-1}; Jean et al., 2013). The low levels of available Mo and P at our sites may have limited asymbiotic N_2 fixation, as P is a vital component ATP synthesis and Mo serves as a metal cofactor in nitrogenase enzyme (Barron et al., 2009; Jean et al., 2013; Reed et al., 2007; Silvester, 1989). The positive correlations of asymbiotic N_2 fixation with extractable organic C content and CO_2 flux in the organic layer of both forest stands suggest that C availability, as a source of energy, was a major factor driving the temporal

pattern of asymbiotic N_2 fixation. Free-living heterotrophic N_2-fixing microorganisms derive their energy from organic matter and maintain high respiration rates which may create anaerobic conditions that are needed for nitrogenase to fix N_2 (Hill, 1992; Knops et al., 2002; Reed et al., 2011). The positive correlations between asymbiotic N_2 fixation and soil temperature, especially in spruce organic layer and beech mineral soil that had generally low N_2 fixation rates (Table 1), suggest that under conditions of low N_2 fixation activity temporal variation in temperature additionally limited the enzymatic process of asymbiotic N_2 fixation (Houlton et al., 2008; Reed et al., 2011). These results were in line with our second hypothesis that, in addition to soil N availability, soil C availability and temperature influenced asymbiotic N_2 fixation.

Although previous studies have reported a coupling between free-living N_2 fixation and denitrification (Reed et al., 2011; Seitzinger et al., 2006) and similar controlling factors (e.g. organic C availability and aeration status), we did not detect a correlation between asymbiotic N_2 fixation and gross N_2O uptake or emission. In both beech and spruce stands, asymbiotic N_2 fixation rates (Fig. 2d; Table 1) were an order of magnitude lower than either the soil net N_2O fluxes or gross N_2O uptake (i.e. N_2O reduce to N_2), indicating that asymbiotic N_2 fixation did not compensate the gaseous N losses from these highly acidic and N-enriched temperate forest soils. This result was in agreement with our third hypothesis.

3.5. Conclusions

Our findings show that tree species had a large influence on gross N_2O emission, net N_2O flux and asymbiotic N_2 fixation, and thus large-scale field quantification under similar soil types and climatic conditions can be based on tree-species stratification as a promising basis to scale up these rates. The tree species effects on gross N_2O emission were largely through soil N availability (e.g. gross nitrification) and soil aeration status (i.e. WFPS, CO_2 emission), whereas temporal variations of gross N_2O emission and uptake were mainly driven by soil NO_3^- and organic C availability. Therefore, extrapolation of gross N_2O fluxes with soil depths and seasons in these stands can be based on the regression relationships with these soil explanatory variables. Gross N_2O emission played an important role in controlling the direction and magnitude of net N_2O flux, and their regression relationships (indicating ratios of net to gross N_2O emission of 0.5-0.8 across the measurement period) also open the possibility of making estimates of soil gross N_2O emissions based on measured soil net N_2O emissions. Our study offers new insights into gross N_2O fluxes and asymbiotic N_2 fixation,

which are not concurrently investigated in any other ecosystems so far, and provides hitherto unknown gaseous N fluxes which can improve N budgets of forest ecosystems.

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Chapter 4

Nitrous oxide fluxes from tree stems of temperate forests

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Abstract

Plants are important to regulate the physical and chemical state of the atmosphere through the exchange of soil-generated N_2O. Presently, little is known about N_2O fluxes from mature trees under field conditions as well as their contributions to total forest (soil + stem) N_2O fluxes. We quantified *in situ* stem and soil N_2O fluxes from mature alder (*Alnus glutinosa*) trees on poorly-drained soil and mature beech (*Fagus sylvatica*) and spruce (*Picea abies*) trees on well-drained soils in central Germany during March-October 2015. Alder, beech and spruce consistently emitted N_2O via stems and all displayed clear seasonal patterns. Soil factors (e.g. temperature, water content, N_2O concentration) and climatic factors (air temperature, vapor pressure deficit) influenced the temporal variability in stem N_2O fluxes. Stem and soil N_2O fluxes from the alder stand were higher ($P < 0.01$ for both) than beech and spruce stands. Stem N_2O fluxes represented 8-11% of the total N_2O fluxes in the spruce and beech stands but only 1% in the alder stand. Our study highlights the importance to conduct long-term, field-based measurements of stem N_2O fluxes on mature trees and suggests that relative contribution of tree-mediated N_2O fluxes is more important in upland trees than in wetland trees.

4.1. Introduction

Atmospheric nitrous oxide (N_2O) is a trace gas of environmental concern since it has a high global warming potential and is predicted to be the main ozone depleting substance in the 21st century (Ravishankara *et al.*, 2009). Soils are the dominant source of N_2O, and microbial nitrification and denitrification are the major N_2O producing processes (Davidson *et al.*, 2000). Three different pathways of soil-atmosphere exchange of N_2O have been described: (1) direct diffusion from soil to the atmosphere, what is commonly measured by chamber-based method; (2) ebullition of gas bubbles, which can occur under water-logged conditions (Clough *et al.*, 2005); and (3) plant-mediated transport, which is a common pathway in rice paddies but has also been described for trees and even bromeliads (Yu *et al.*, 1997; Rusch & Rennenberg, 1998; Yan *et al.*, 2000; Martinson *et al.*, 2010).

Several mechanisms have been described to explain how plants serve as an effective conduit for soil-atmosphere gas exchange. Wetland plants typically develop aerenchyma tissue, i.e. internal lacunae formed by cell separation or cell breakdown in their roots, culms and stems, to facilitate transport of atmospheric oxygen downwards to the anoxic rhizosphere (Armstrong, 1979); it can also enable transport of soil-borne N_2O upwards through the plant, followed by its release to the atmosphere (Rusch and Rennenberg, 1998). Unlike wetland plants, most upland plants lack aerenchyma tissue, and hence gaseous N_2O diffusion through their roots and stems is typically considered to be only of minor importance. However, recent studies demonstrate that some upland plants without aerenchyma tissue consistently emit N_2O (Díaz-Pinés *et al.*, 2016; Machacova *et al.*, 2016). Since considerable amounts of N_2O can dissolve in water, N_2O is thought to move preferentially in dissolved form via the transpiration stream (Yu *et al.*, 1997; Chang *et al.*, 1998; Pihlatie *et al.*, 2005; Díaz-Pinés *et al.*, 2016). Ultimately, this N_2O will be released to the atmosphere through leaf stomata (Zou *et al.*, 2005), stem surface (Rusch & Rennenberg, 1998) and stem lenticels (Díaz-Pinés *et al.*, 2016).

Plant-mediated N_2O fluxes are influenced by soil physico-chemical characteristics, climatic factors, and plant-specific properties. Correlations of plant-mediated N_2O fluxes with various soil characteristics have been observed including soil temperature (Machacova *et al.*, 2013), soil water content (Chang *et al.*, 1998; Rusch & Rennenberg, 1998; Yan *et al.*, 2000; Machacova *et al.*, 2013), soil nutrient availability (Smart & Bloom, 2001; Chen *et al.*, 2002; Pihlatie *et al.*, 2005; Díaz-Pinés *et al.*, 2016), gas mixing ratio (Rusch & Rennenberg, 1998), N_2O concentration in soil solution (Pihlatie *et al.*, 2005), and soil N_2O fluxes (Díaz-Pinés *et al.*, 2016; Machacova *et al.*, 2016). In addition, light conditions have been linked to plant-

mediated N_2O fluxes, suggesting a possible light-dependent gas transport or N_2O production mechanism in the plant (Jørgensen *et al.*, 2012). Plant-specific properties (e.g. leaf area index) and plant age have also been shown to affect plant-mediated N_2O fluxes (Smart & Bloom, 2001; Pihlatie *et al.*, 2005; Díaz-Pinés *et al.*, 2016).

Although plant-mediated N_2O fluxes have been studied for about two decades, research has almost exclusively focused on herbaceous plants (especially crops, such as rice, wheat, soybean, maize). Investigations of tree-mediated N_2O fluxes are rare and mostly restricted to seedlings and/or saplings under laboratory conditions (e.g. Rusch and Rennenberg, 1998; Pihlatie et al., 2005; Machacova et al., 2013). Laboratory studies usually included flooding and fertilization manipulations at rates which are often out of range for field conditions. Presently, very little is known on the processes responsible as well as the environmental controls of N_2O fluxes from mature trees under field conditions. No information has been published on the seasonal variation of tree-mediated N_2O fluxes. Moreover, current estimates of trace gas emissions from forest ecosystems are based on chamber-based measurements of soil N_2O fluxes and do not include tree-mediated N_2O fluxes. Exclusion of the contribution of trees to N_2O exchange with the atmosphere may lead to a systematic underestimation of total ecosystem fluxes (Machacova *et al.*, 2016). A better understanding of tree-mediated N_2O fluxes is thus crucial to further constrain estimates of forest N_2O emissions and to improve prediction of forest ecosystem responses to future climatic change.

In the present study, our aims were to 1) quantify *in situ* tree-mediated N_2O emissions and their seasonal patterns, and 2) assess their controlling factors in order to infer the mechanisms responsible for tree-mediated N_2O emissions. We conducted simultaneous *in situ* measurements of stem and soil N_2O fluxes from mature alder (*Alnus glutinosa*), beech (*Fagus sylvatica*), and spruce (*Picea abies*) stands. Alder was selected as it is a typical wetland tree species, which facilitates oxygen supply to its roots through aerenchyma and lenticels. Beech and spruce were selected as they are the most common upland tree species in Europe and do not have aerenchyma tissue. We hypothesized that: (1) tree-mediated N_2O fluxes will be higher in alder than in beech and spruce stands as the former is a wetland and N_2-fxing tree species, of which anaerobic and high soil N conditions may promote high soil N_2O production, whereas the latter are upland and non N_2-fixing tree species; (2) N_2O transport in alder stem will be dominated by N_2O diffusion from the soil to the aerenchyma tissue and lenticels, and thus alder stem N_2O emission will be influenced by the amount of N_2O produced in the soil; (3) N_2O transport in beech and spruce stems will be mainly through dissolved form via xylem sap flow and thus will be limited by the sap flow rate which, in turn, will be influenced by air

temperature, vapor pressure deficit and soil water content. Our study provides new insights into the effect of trees on atmospheric N_2O concentration and the temporal controls of tree-mediated N_2O emissions, and thus obtains a better constraint of terrestrial N_2O dynamics.

4.2. Materials and methods

4.2.1. Site description and experimental design

Our study was conducted at Solling uplands (51.72° N, 9.73° E) in central Germany. We selected three adjacent forest stands: a 39-year-old alder, an 81-year-old beech and a 68-year-old spruce. Average tree heights were 32 m in the alder, 27 m in the beech and 33 m in the spruce stand, and average diameters at breast heights were 0.18 m in the alder, 0.20 m in the beech and 0.23 m in the spruce stand. All three stands were located at an elevation of 310 m, mean annual temperature was 8.9 °C and mean annual precipitation was 791 mm yr^{-1} (period 2006-2015; German Weather Service station at Moringen-Lutterbech). Soils were classified as Histosols for the alder stand and Gleysols for the beech and spruce stands (FAO classification). Soil texture was dominated by silty clay in the alder stand, clay silt in the beech stand, and silty loam in the spruce stand.

In each stand, we selected 6 trees with a minimum distance of 25 m from each other. We measured stem N_2O fluxes within a 0.2-m length of stem section at a breast height, taken at 1.3 m from the ground; hence, the measured stem section was between 1.2-1.4 m stem height. Soil N_2O fluxes were measured from chamber bases, which were installed at 1-m distance from the measured trees. In the center between the sampled tree stems and the soil chambers, we also installed stainless steel soil gas samplers at 0.4-m depth to measure soil N_2O concentrations. From March 26 to October 28, 2015, we conducted 11 measurement periods during which we measured stem N_2O fluxes, soil N_2O fluxes, soil N_2O concentrations and climatic and soil variables for potential controlling factors of stem N_2O fluxes. Measurements were performed bi-weekly during spring and summer and monthly during autumn. In June and July 2015, we conducted additional measurements of stem N_2O fluxes at 0.2-0.4 m and at 2.2-2.4 m stem heights from the ground in order to assess whether stem N_2O fluxes showed trends with stem height.

4.2.2. Measurements of stem and soil N_2O fluxes and soil N_2O concentrations

We used flexible plastic chambers made of polyethylene-terephthalate, used normally as oven bags (hereafter called ‘oven bag chambers’), to measure *in situ* stem N_2O fluxes (Fig. 1). For a gas sampling port, a hole was punched on the oven bag, then fitted with a Teflon bulkhead

union and closed with a Luer-lock stopcock (Fig. 1). This method has originally been developed to sample volatile organic compounds emitted from tree stems (Rachow *et al.*, 2012), and was tested by our group through preliminary works for measurements of N_2O fluxes from tree stems. One week before the first measurement, we applied a 1-cm wide strips of silicone sealant on the surface of the tree stems (at 1.2 m and 1.4 m heights from the ground) to smooth out any irregularities of the bark in order to ensure an air-tight seal with the oven bag chambers during measurement. The silicone sealant also served as a mark so that we sampled the same 0.2-m length stem section every measurement period. The silicone sealant we used (Otto Seal ® S110) did not contain acetic acid since this may damage trees (Bernhard Schuldt, pers. comm.). On every measurement period, we wrapped the oven bag chambers (approximately 0.6 m length with the custom-made sampling port in the middle) around the stem section marked by the silicone sealant strips, and closed the side-ends of the bag with a medical adhesive tape. Once fixed onto the stem, we used a gas-powered heat-gun (E4500, HellermannTyton GmbH, Tornesch, Germany) on the top and bottom parts of the oven bag to 'shrink' it onto the stem so that it fitted snugly onto the silicone strips. Then, strips of polyethylene foam were wrapped around the stem at the top and bottom parts of the oven bag, leaving a length of 20 cm in the middle for gas sample collection. These strips of foam was tighten around the entire stem using lashing straps with ratchet tensioners, resulting the straps to fix the oven bags tightly onto the silicone sealant strips (Fig. 1). With the lashing straps tightly fixed, the strips of foam and the oven bag adjusted to any irregularities on the bark, ensuring an air-tight seal (Fig. 1). Since this installation of the oven bag chambers is quick, new chambers were attached onto the stem every measurement period and permanent chamber installation was not necessary. In contrast to permanently installed chambers, this reduced the risk of damaged oven bag chambers affecting flux measurements. Moreover, the foam protected the stem from damage during chamber installation and measurements. Following installation, the oven bag chamber was first completely evacuated by attaching a syringe with Luer-lock one-way check valve onto the sampling port and pumping the air out the oven bag repeatedly until it is visibly shrinking onto stem from being empty. The oven bag chamber was then refilled with a known volume of ambient air (i.e. 2 L) using a manual pump. Immediately following chamber filling, a gas sample of 20 mL was removed at 0, 20, 40, 60 minutes by attaching a syringe into the Luer-lock sampling port, and injecting the gas sample immediately into a pre-evacuated 12 mL exetainers with rubber septa (Labco Limited, Lampeter, UK), keeping an overpressure.

Figure 1. Oven bag chamber method, used to measure *in situ* stem N_2O fluxes.

Soil N_2O fluxes were measured using the standard method that our group has employed in our earlier studies (see for a detailed description: Corre et al., 2014; Veldkamp et al., 2013). Round chamber bases made of polyvinyl chloride (area 450 cm^2, height 13 cm) were inserted ~2 cm into the soil at least a week before the first measurement period and installed permanently for the entire measurement period. On each measurement period, chamber covers equipped with a Luer-lock sampling port, were attached tightly onto the chamber bases (25 cm total chamber height and approx. 11 L total volume). Using a plastic syringe, we removed gas samples of 20 mL each at 1, 21, 41, and 61 min following chamber closure. Gas samples were stored into pre-evacuated 12 mL exetainers with rubber septa.

Soil N_2O concentrations were sampled at 0.4-m depth using stainless-steel probes (1 mm inner diameter), where one end was perforated with small holes to extract soil air. This method had been successfully used in our earlier studies (e.g. van Straaten *et al.*, 2011; Koehler *et al.*, 2012; Corre *et al.*, 2014). The stainless-steel probes were inserted into the soil prior to the first measurement period and were left permanently on the ground. Before taking a gas sample, 5 mL of air was removed and discarded to clear the probes of the 'dead' air

volume. We took 20 mL gas samples using a plastic syringe, attached the top end of the probes, and stored the samples into pre-evacuated 12 mL exetainers with rubber septa.

4.2.3. N_2O analysis and flux rate calculations

All gas samples were analyzed one day after the field sampling using a gas chromatograph (GC 6000 Vega Series 2, Carlo Erba Instruments, Milan, Italy) with an autosampler (Gilson SAS, Villiers, Le Bel, France), equipped an electron capture detector. N_2O fluxes were calculated from the linear change of N_2O concentrations in the chamber versus time and were adjusted with the field-measured air temperature and atmospheric pressure at the time of sampling. Stem N_2O fluxes were expressed on a stem-area basis, and soil N_2O fluxes were on soil-area basis.

Flux rates of N_2O were further estimated for the entire stem using the total area of stem surface, which was calculated as the lateral surface area of a circular cone calculated from the stem diameter at breast height and the stem height of the tree (Machacova *et al.*, 2016). For the alder trees, we used the observed decreases in N_2O fluxes with stem height to calculate the fractions of stem N_2O fluxes at 0.2-0.4 m and at 2.2-2.4 m heights above the ground, measured in June and July 2015, in relation to the regular measurements at 1.2-1.4 m height. These calculated fractions were then used to weight the regularly measured stem N_2O flux at 1.2-1.4 m height for the entire stem height. For the beech and spruce trees, stem N_2O fluxes at 0.2-0.4 m and at 2.2-2.4 m heights above the ground did not differ to those at 1.2-1.4 m height, and hence we extrapolated the regularly measured stem N_2O flux at 1.2-1.4 m height for the whole stem height. Since stem N_2O fluxes in all tree species were influenced by temperature and vapor pressure deficit that display a clear diurnal variation (Hogg *et al.*, 1997; O'Brien *et al.*, 2004; Saveyn *et al.*, 2008), we assumed that measured stem N_2O fluxes were representative for 12 effective hours per day (the average daytime across a year).

Annual stem N_2O fluxes and soil N_2O fluxes were calculated by applying the trapezoidal rule (linear interpolation of measured rates) over the sampling time intervals. Since no measurements were conducted during winter, the N_2O fluxes measured in March and October were assumed to represent the value over the winter that we interpolated. The contribution of stem N_2O fluxes to the total forest N_2O flux (soil N_2O flux + stem N_2O flux) was calculated using the following equation:

$$Contribution\ (\%) = \frac{N_2O_{stem} \times N}{N_2O_{stem} \times N + N_2O_{soil}} \times 100 \qquad \text{Eqn 1}$$

where N_2O_{stem} is the annual N_2O flux from one stem in g N tree^{-1} yr^{-1}; N is the number of tree stems per ha; N_2O_{soil} is the annual N_2O flux from soil surface in g N ha^{-1} yr^{-1}.

4.2.4. Auxiliary measurements

General soil characteristics for the organic layer and the underlying top 5 cm mineral soil were determined once in March 2015. Soil pH was analyzed in a 1:2.5 soil-to-water ratio. Total organic C and N were measured from air-dried, ground samples using a CN Elemental Analyzer (Vario EL Cube, Elementar Analysis Systems GmbH, Hanau, Germany). Total Ca, Mg, Al, and K contents in the organic layers were determined from air-dried ground samples (using pressure digestion in concentrated HNO_3), and exchangeable Ca, Mg, Al, and K contents and effective cation exchange capacity (ECEC) in the mineral soil were determined from air-dried, 2-mm sieved samples (using cation exchange method by percolation with 1 M NH_4C1 solution), using inductively coupled plasma-atomic emission spectrometer (iCAP 6300 Duo VIEW ICP Spectrometer, Thermo Fischer Scientific GmbH, Dreieich, Germany).

Soil temperature, moisture, mineral N and extractable organic C contents were measured during each sampling period. Soil temperature was determined in the top 5-cm depth using a digital thermometer (GMH 3210, Greisinger electronic GmbH, Regenstauf, Germany). All the rest of the soil variables were measured from samples taken within the top 10-cm depth. Gravimetric moisture content was measured by drying 20 g of freshly sampled soil to constant weight at 105 ℃. About 15 g of soil sample was extracted with 100 mL 0.5 M K_2SO_4 by shaking (1 h) and filtering through K_2SO_4 pre-washed filter papers. Soil extracts were kept frozen until analysis. Total extractable N and mineral N contents of the soil extracts were analyzed using continuous flow injection colorimetry (SEAL Analytical AA3, SEAL Analytical GmbH, Norderstadt, Germany), where total extractable N was determined by ultraviolet-persulfate digestion followed by hydrazine sulfate reduction (Autoanalyzer Method G-157-96), NH_4^+ by salicylate and dicloroisocyanuric acid reaction (Autoanalyzer Method G-102-93), and NO_3^- by cadmium reduction method with NH_4Cl buffer (Autoanalyzer Method G-254-02). Extractable organic C contents of the soil extracts was measured using UV-enhanced persulfate oxidation with a Total Organic Carbon Analyzer (TOC-Vwp, Shimadzu Europa GmbH, Duisburg, Germany). Soil microbial N and C were determined by the chloroform fumigation-extraction method, and measured three times during the entire period of measurement (once in spring, summer and autumn). About 15 g of fresh soil was extracted with 100 mL 0.5 M K_2SO_4. Another 15 g of soil sample was placed in a desiccator and fumigated with ethanol-free chloroform for 5 days. Afterwards, the fumigated samples were extracted with 100 mL 0.5 M K_2SO_4. The total extractable N and extractable organic C contents of the extracts were analyzed as above. The differences in total extractable N and extractable organic C between the fumigated and unfumigated samples were assumed

to indicate the N and C released from lysed soil microbes. The N and C were converted to microbial biomass N and C using a K_N=0.68 and Kc=0.45, respectively (Shen *et al.*, 1984). Data of hourly air temperature and relative humidity during the entire measurement period were obtained from the weather station of the German Weather Service at Moringen-Lutterbech, 7 km to our study site. Vapor pressure deficit (VPD) was calculated from the difference between saturation vapor pressure and actual vapor pressure, which derived from air temperature and relative humidity data (Allen *et al.*, 1998).

4.2.5. Statistical analysis

Data sets were first tested for normal distribution (Shapiro-Wilk's test) and equality of variance (Levene's test). We assessed the differences in soil characteristics among stands using one-way analysis of variance (ANOVA) with Fisher's least significant difference test. When the data were not able to attain normal distribution and equality of variance, differences among sites were tested using the Kruskal-Wallis ANOVA with multiple comparisons test. Linear mixed effects (LME) models were used for analysis of time-series data (stem N_2O flux, soil N_2O flux, and other parameters). The LME model included tree species (alder, beech and spruce) as fixed effects whereas sampling dates and spatial replicates were included as random effects. The LME model included either 1) a variance function that allows different variances of the response variable for the fixed effects, and/or 2) a first-order temporal autoregressive process that assumes a decreasing correlation between measurements with increasing time distance if this improved the relative goodness of the model fit (Crawley, 2007). Residuals were checked for normality and homoscedasticity, and data were log-transformed in case of non-normal distribution and/or heteroscedastic residuals. Fixed effects were considered significant based on the analysis of variance at $P \leq 0.05$, and differences between species were assessed using Fisher's least significant difference (LSD) test. Pearson correlation tests were used to explore relationships of stem N_2O fluxes with possible explanatory variables, and data were log-transformed to fit the assumption of normality. For all tests, the level of statistical significance was set at $P \leq 0.05$. Since all measurements were conducted in the field with considerable spatial variability, we also discuss a few specified parameters with values of $P \leq 0.07$ that we considered marginally significant. When we plotted VPD against stem N_2O flux, we excluded VPD data that were above 1.5 kPa in the beech and spruce stands (Fig. 5h,i) since water use by these trees will decrease under high VPD, due to reduced stomatal conductance (Hogg *et al.*, 1997; O'Brien *et al.*, 2004). All statistical analyses were conducted using open source software R (version 2.15.3).

4.3. Results

4.3.1. Soil properties

The alder stand showed higher total organic C and N as well as lower total C:N ratios in both the organic layer and mineral soil compared to the beech and spruce stands ($P < 0.01$ for all; Table 1). In the organic layer, differences among stands were only detected for total Ca ($P = 0.04$; Table 1) and total Al ($P < 0.01$; Table 1). In the 0-0.05 m mineral soil, exchangeable Ca, Mg, K and ECEC were higher in the alder stand than the beech and spruce stands ($P < 0.01$-0.03; Table 1). Across the entire sampling period, all soil factors known to influence soil N_2O fluxes displayed differences among stands. The alder stand showed higher gravimetric water content, extractable NO_3^-, extractable organic C, soil N_2O concentration, microbial N and C compared to the beech and spruce stands ($P < 0.01$-0.05; Table 2). Extractable NH_4^+ was higher in the alder and spruce stands than the beech stand ($P < 0.01$; Table 2).

4.3.2. Temporal variations in stem N_2O fluxes and soil N_2O fluxes

Alder, beech and spruce consistently emitted N_2O via stems throughout the sampling period and displayed clear temporal variation (Fig. 2). Stem N_2O fluxes of alder increased from 0.6 ± 0.3 μg N m^{-2} stem h^{-1} in March to 8.0 ± 1.7 μg N m^{-2} stem h^{-1} in July, and then decreased to 0.9 ± 0.2 μg N m^{-2} stem h^{-1} in October (Fig. 2a). Stem N_2O fluxes of beech increased from spring to summer, levelled off between August and September (1.5 ± 0.5 μg N m^{-2} stem h^{-1}) and decreased thereafter (Fig. 2b). Stem N_2O fluxes of spruce were low from March to late June, increased to a peak of 1.9 ± 0.4 μg N m^{-2} stem h^{-1} in August and thereafter decreased to a similar level as in spring (Fig. 2c). Across the entire measurement period, stem N_2O fluxes of alder (2.2 ± 0.5 μg N m^{-2} stem h^{-1}) were higher than stem N_2O fluxes of beech and spruce (0.7 ± 0.1 μg N m^{-2} stem h^{-1} for beech and 0.7 ± 0.1 μg N m^{-2} stem h^{-1} for spruce; $P < 0.01$).

Soil N_2O fluxes displayed considerable spatial variability, as shown by the large standard errors (Fig. 3a-c). Soil N_2O fluxes from the alder stand increased from March to July and decreased sharply thereafter (Fig. 3a). However, irregular temporal patterns of soil N_2O fluxes were observed in the beech and spruce stands; in the beech stand, soil N_2O flux increased from early to late spring, decreased in early summer and remained low till fall (Fig. 3b). In the spruce stand, there was net N_2O uptake in the soil (negative flux) in the late spring and in July (Fig. 3c). Across the entire period of measurements, the alder stand showed higher ($P < 0.01$) soil N_2O flux rates (106 ± 27 μg N m^{-2} h^{-1}) than the beech stand (2.9 ± 1.1 μg N m^{-2} soil h^{-1}) and spruce stand (4.0 ± 1.0 μg N m^{-2} h^{-1}).

Table 1. Soil characteristics measured in March 2015.

Stands	pH 1:2.5 H_2O	Total organic C (g C kg^{-1})	Total N (g N kg^{-1})	Total C:N ratio	Ca (g kg^{-1})	Mg (g kg^{-1})	Al (g kg^{-1})	K (g kg^{-1})	ECEC (mmol charge kg^{-1})
Organic layer									
Alder	na	536 ± 4 a	30.0 ± 1.2 a	18.0 ± 0.8 b	10.8 ± 0.8 a	1.6 ± 0.3	0.7 ± 0.2 b	1.3 ± 0.1	na
Beech	na	419 ± 37 b	17.6 ± 1.3 b	23.8 ± 1.3 a	7.6 ± 0.9 b	1.0 ± 0.1	4.1 ± 1.1 a	1.4 ± 0.2	na
Spruce	na	500 ± 3 ab	19.5 ± 0.8 b	25.8 ± 1.0 a	9.4 ± 0.6 ab	1.1 ± 0.2	1.5 ± 0.2 ab	1.0 ± 0.0	na
0-5 cm mineral soil									
Alder	4.2 ± 0.3	275 ± 72 a	16.7 ± 4.1 a	16.7 ± 1.2 b	2.10 ± 0.79 a	0.21 ± 0.08 a	0.27 ± 0.09	0.06 ± 0.02 a	252 ± 58 a
Beech	4.3 ± 0.1	36 ± 5 b	1.9 ± 0.3 b	18.8 ± 0.6 b	0.09 ± 0.03 b	0.01 ± 0.00 b	0.39 ± 0.02	0.01 ± 0.00 b	59 ± 4 b
Spruce	4.0 ± 0.1	35 ± 4 b	1.5 ± 0.2 b	24.1 ± 0.9 a	0.06 ± 0.01 b	0.01 ± 0.00 b	0.47 ± 0.05	0.01 ± 0.00 b	76 ± 9 b

Mean ± standard errors (n = 6) within each column followed by different letter indicate significant differences among stands (one-way ANOVA with Fisher's LSD tests at $P \leq 0.05$ or Kruskal-Wallis ANOVA with multiple comparisons of mean ranks at $P \leq 0.05$). na indicates not applicable. Cation concentrations in the organic layer are total amount whereas in the mineral soil are exchangeable amount.

Table 2. Soil factors measured in the top 10-cm depth, except for soil N_2O concentration measured at 40-cm depth, in the alder, beech and spruce stands.

Parameters	Alder	Beech	Spruce
Gravimetric water content (g g^{-1})	2.31 ± 0.28 a	0.57 ± 0.09 b	0.54 ± 0.05 b
NH_4^+ (mg N kg^{-1})	10.0 ± 1.3 a	4.5 ± 0.5 b	14.3 ± 3.8 a
NO_3^- (mg N kg^{-1})	27.4 ± 3.5 a	4.5 ± 1.7 b	6.4 ± 1.7 b
Extractable organic C (mg C kg^{-1})	517± 41 a	335 ± 51 b	388 ± 59 b
Soil N_2O concentration (ppm)	7.33 ± 1.19 a	0.51 ± 0.04 b	0.72 ± 0.12 b
Microbial N (mg N kg^{-1})	308 ± 40 a	127 ± 9 b	126 ± 13 b
Microbial C (mg C kg^{-1})	3361 ± 415 a	1216 ± 81 b	1731 ± 194 b

Mean ± standard errors (n = 6) within each row followed by different letter indicate significant differences among stands (linear mixed model at $P \leq 0.05$). Gravimetric water content, extractable NH_4^+, NO_3^- and organic C, and soil N_2O concentration were measured on the same sampling period as stem and soil N_2O fluxes from March to October 2015; microbial N and C were measured once in spring, summer and autumn of 2015.

Table 3. Estimation of N_2O emitted from stems of trees in relation to the total forest N_2O flux (stem N_2O flux + soil N_2O flux).

Parameters	Alder	Beech	Spruce
Tree density (stem ha^{-1})	1308	803	1015
Factor of stem N_2O flux at 0.2-0.4 m [a]	1.5 ± 0.1	1.1 ± 0.4	0.8 ± 0.2
Factor of stem N_2O flux at 2.2-2.4 m [a]	0.9 ± 0.1	1.0 ± 0.4	0.8 ± 0.3
Annual stem N_2O flux (g N ha^{-1} yr^{-1})	64.6 ± 16.2	18.9 ± 4.4	31.0 ± 4.5
Annual soil N_2O flux (g N ha^{-1} yr^{-1})	6396 ± 1395	161 ± 110	346 ± 59
Contribution of stem to forest N_2O flux (%)	1.0	10.5	8.2

Mean ± standard errors (n = 4 for stem N_2O flux at 0.2-0.4 m and 2.2-2.4 m heights, or n = 6 for the regular measurements at 1.2-1.4 m height).

[a] Fraction of stem N_2O flux at 0.2-0.4 m or 2.2-2.4 m height above the ground in relation to stem N_2O flux at 1.2-1.4 m height.

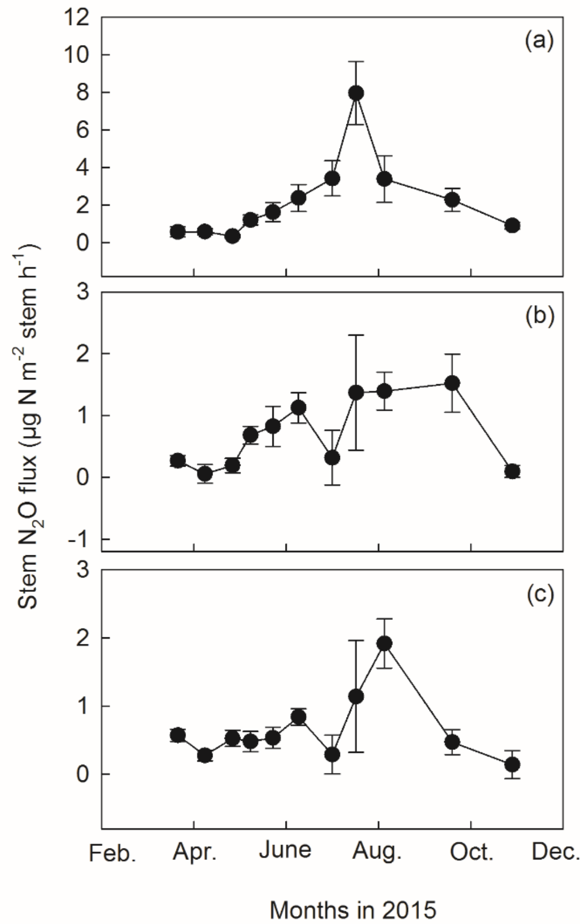

Figure 2. Temporal variations of N_2O fluxes (mean ± standard errors, n = 6) from the stems of alder (a), beech (b), and spruce (c) measured at breast height (between 1.2 m and 1.4 m heights above the ground) from March to October 2015.

Annual stem N_2O flux from the alder stand was approximately 2- to 3-folds higher than the beech and spruce stands (Table 3). However, since annual soil N_2O flux in the alder stand was 40 times higher than the beech stand and 18 times higher than the spruce stand (Table 3), the relative contribution of stem N_2O fluxes of upland beech and spruce stands to total forest N_2O fluxes was higher than wetland alder trees (Table 3).

4.3.3. Relationships between stem N_2O fluxes and controlling factors

In the alder stand, stem N_2O flux was positively correlated with soil N_2O flux (Fig. 4a) and soil N_2O concentration (Fig. 4b), while in the beech and spruce stands, we did not detect significant correlations of stem N_2O flux with either soil N_2O flux or soil N_2O concentration. Stem N_2O flux was positively correlated with soil temperature in the top 5-cm depth in the alder (Fig. 5a) and beech (Fig. 5b) stands. Positive correlations between stem N_2O flux and air temperature were detected in the alder (Fig. 5d) and beech (Fig. 5e) stands, while a marginally

significant correlation was detected in the spruce stand (Fig. 5f). Stem N_2O flux displayed positive correlations with VPD in the alder (Fig. 5g) and spruce (Fig. 5i) stands. In the alder stand, we also found positive correlations of soil temperature with soil N_2O flux ($R = 0.77$; $P < 0.01$), and soil N_2O concentration ($R = 0.67$; $P = 0.02$).

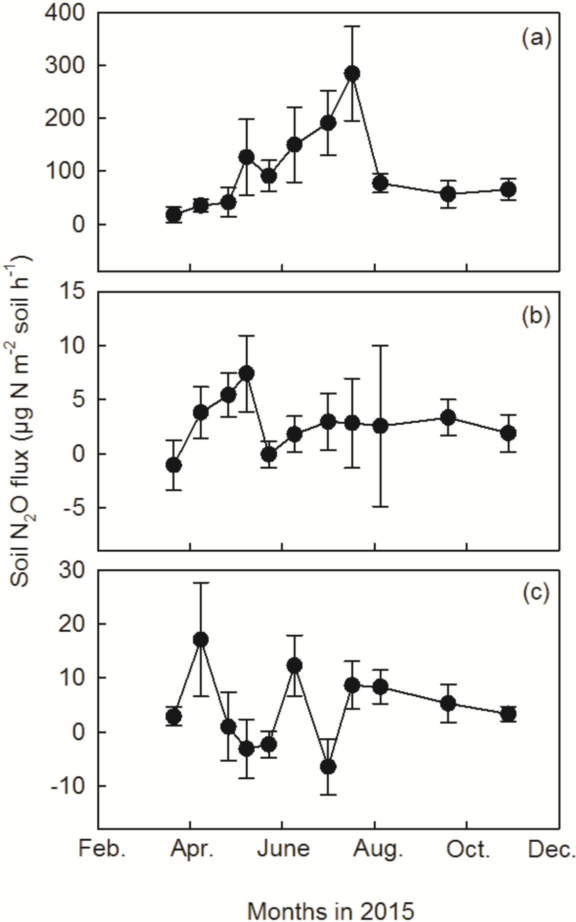

Figure 3. Temporal variations of soil N_2O fluxes (mean ± standard errors, $n = 6$) from alder (a), beech (b), and spruce (c) stands, measured from March to October 2015.

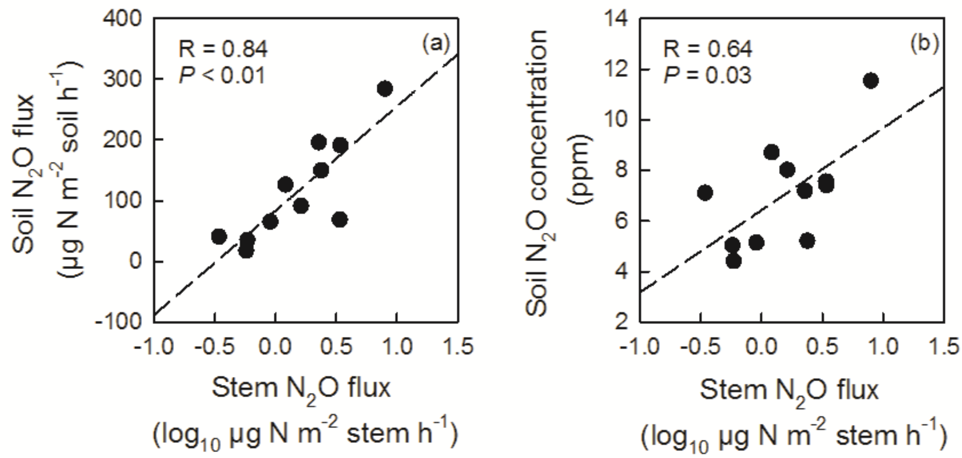

Figure 4. Pearson correlation tests between stem N_2O flux and soil N_2O flux (a), soil N_2O concentration (b) in the alder stand. Each data point represents the mean of six replicates on each measurement period from March to October 2015 (n = 11). Data of stem N_2O fluxes from alder were log_{10} transformed to fit normal distribution.

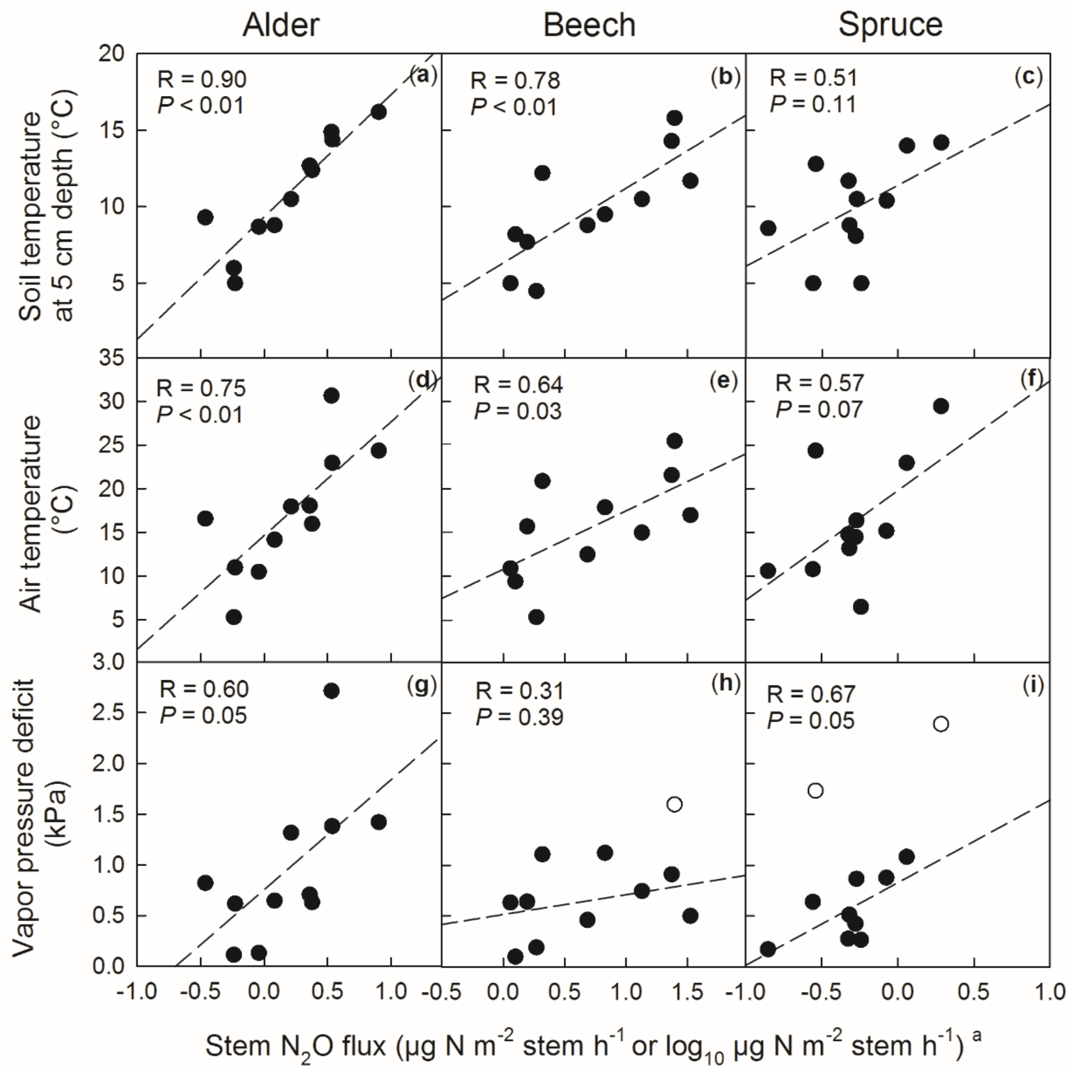

Figure 5. Pearson correlation tests between stem N_2O flux and soil temperature in the top 5-cm depth (a, b, c), air temperature (d, e, f), and vapor pressure deficit (g, h, i) in the alder, beech and spruce stands. Each data point represents the mean of six replicates on each measurement period from March to October 2015 (n = 11). Correlation excluded values of vapor pressure deficit higher than 1.5 kPa (circle without fill) in the beech and spruce stands.

[a] Stem N_2O fluxes from alder and spruce were $\log_{10}$ transformed to fit normal distribution.

4.4. Discussion

4.4.1. Temporal variability of stem N_2O fluxes in different tree species

The consistent emissions of N_2O throughout the measurement period from stems of adult alder, beech and spruce (Fig. 2 a-c) exemplified that not only mature wetland trees but also mature upland trees can serve as conduits for N_2O. Our mean stem N_2O fluxes were in the range of those reported by previous studies, which did not involve manipulations such as flooding or fertilization (0.014 µg N m^{-2} stem h^{-1} to 5.01 µg N m^{-2} stem h^{-1}; Machacova et al., 2013, 2016; Díaz-Pinés et al., 2016). However, only the fluxes we measured in August (1.4 µg N m^{-2} stem h^{-1}) were comparable with published stem N_2O fluxes from beech measured in the same month (12-year-old beech: 2.2 µg N m^{-2} stem h^{-1}; Díaz-Pinés *et al.*, 2016). Compared to the fluxes that we measured in other months, published values were up to 37 times higher, illustrating the considerable seasonal variability in stem N_2O fluxes (Fig. 2 a-c). Since young beech trees have been reported to emit larger stem N_2O fluxes than old beech trees (Díaz-Pinés *et al.*, 2016), annual stem N_2O fluxes can easily be overestimated if extrapolation is based on fluxes measured during the summer or on fluxes measured from young trees.

The higher N_2O fluxes that we observed from alder stems compared to beech and spruce (Fig. 2) were consistent with published results that stem N_2O fluxes from alder stems were 25 times higher compared to beech stems (Rusch & Rennenberg, 1998; Machacova *et al.,* 2013; Díaz-Pinés *et al.*, 2016) and support our first hypothesis that wetland trees emit more N_2O than upland trees. The factors that contributed to the high stem N_2O fluxes were related to levels of electron acceptor, donor and aearation status; alder in symbiosis with N_2-fxing bacteria can fix atmospheric N_2 into available N form , enriching the ecosystem with N (Dick *et al.*, 2006), as was shown by the high soil NO_3^- levels (electron acceptor) in alder stand. This, in combination with high extractable organic C (as index of C availability to microbial activity) and high soil water content (Table 2), made the alder stand a hot spot for heterotrophic denitrification, the dominant microbial process that produces N_2O in soils (Butterbach-Bahl et al., 2013). Moreover, N_2O diffusion from alder stem is mainly through aerenchyma, while in beech and spruce, N_2O is transported in dissolved form via sap flow (Machacova *et al.*, 2013). Passive gas diffusion in air-filled aerenchyma can lead to considerable stem N_2O fluxes, since the diffusion coefficient of N_2O in air is several orders of magnitude higher than in xylem sap which consists mainly of water (Heincke &

Kaupenjohann, 1999). Also the presence of lenticels in alder stems accelerates the gas exchange between stem and the atmosphere (Pangala *et al.*, 2014).

Tree species not only influenced the rate but also the vertical pattern of N_2O fluxes along the tree stem. For alder, the observed decrease in stem N_2O fluxes with increasing stem height (Table 3) was probably also due to the presence of aerenchyma, causing a rapid diffusion of N_2O from the stem to the atmosphere and resulting in steep decreases in stem N_2O emissions higher up the stem (Díaz-Pinés *et al.*, 2016). In contrast, for beech and spruce N_2O fluxes at different stem heights were nearly identical (Table 3), probably because diffusion of dissolved N_2O from xylem sap to the atmosphere is a slow process relative to the sap flow rate.

4.4.2. Factors controlling stem N_2O fluxes

In the alder stand, strong correlations of stem N_2O fluxes with soil N_2O fluxes and soil N_2O concentrations (Fig. 4a,b) suggest that N_2O emitted from alder stems originated in the soil and the temporal variation in stem N_2O fluxes was driven by the amount of N_2O produced in the soil. This supports our second hypothesis. Elevated soil N_2O concentrations were probably caused by high soil N_2O production combined with impeded diffusion out of the soil, both resulting from high soil water and substrate (NO_3^- and extractable organic C) contents (Table 2). The elevated N_2O concentrations in soil air may have stimulated diffusion of soil-borne N_2O into aerenchyma of tree roots, subsequently transported upward in the stem through aerenchyma, and ultimately led to N_2O emission from tree stem. High N_2O emissions from alder stems, of which roots were exposed to high N_2O concentrations, have also been reported in an earlier laboratory study (Rusch & Rennenberg, 1998). Additionally, the positive correlation of alder stem N_2O fluxes with soil and air temperatures (i.e. increasing towards the summer and decreasing thereafter; Figs. 2a, 5a,d) and the positive correlations of soil temperature with soil N_2O fluxes and soil N_2O concentrations in the alder stand elucidated that temperature additionally drove the temporal variations in alder stem N_2O emissions, where substrate level and aeration status were conducive to N_2O production, as enzymes involved in denitrification are temperature-regulated (e.g. Knowles, 1982). Furthermore, an increase in soil temperature typically stimulates soil respiration, which will increase oxygen consumption and thus enhances anaerobic condition (Butterbach-Bahl *et al.,* 2013).

For alder, N_2O transport in stems as dissolved form via sap flow has been thought to be of minor importance compared to transport as gaseous form via aerenchyma, since diffusion in gas is several orders of magnitude faster than diffusion in water. Gas transport via sap flow

in xylem tissue is an active process, whereas gas transport via aerenchyma is caused by passive gas diffusion (Pangala *et al.*, 2015). If sap flow rate is high, the contribution of sap flow gas transport could be substantial than previously thought. Indeed, correlations of alder stem N_2O flux with air temperature and VPD (Fig. 5d,g) suggest that N_2O transport via sap flow was also occurring. Higher air temperature and VPD typically stimulate sap flow rates (Hogg et al., 1997; O'Brien et al., 2004) and thus also the transport of dissolved N_2O. Since alder leaves have no mechanism to reduce transpiration (Braun, 1974), considerable amounts of water are transpired (Herbst *et al.*, 1999), and it appears that this pathway of N_2O transport through alder stems is more important than previously assumed.

Beech and spruce are typical upland trees, which do not have aerenchyma. The positive correlations of stem N_2O fluxes with soil and air temperatures and VPD (Fig. 5) suggest that dissolved N_2O transport via xylem sap was the major mechanism for N_2O transport in upland trees. Substantial amounts of N_2O can dissolve into water (Koehler *et al.*, 2012) which can be taken up by beech and spruce roots, conveyed through xylem sap flow to the stem and eventually diffused out of the xylem sap into the atmosphere. Since increasing air temperature and VPD enhanced sap flow rates if soil water is sufficient (Fig. 5e, f, i), these findings support our third hypothesis.

4.4.3. Contributions of stem N_2O fluxes to total forest N_2O fluxes

For the three tree species, the contributions of stem N_2O fluxes to total forest N_2O fluxes (1-11%; Table 3) were within the range of previous studies (1-18%; Díaz-Pinés *et al.*, 2016; Machacova *et al.*, 2016), although previous estimates were based on extrapolations of short-term measurements in summer. In contrast to our expectations, the lower contribution of stem N_2O flux to total forest N_2O flux in the alder stand than in the beech and spruce stands was because of the very high soil N_2O fluxes from the alder stand, minimizing the relative contribution of the stem N_2O emissions. In our study, we did not include N_2O emissions from branches and leaves, which have also been shown to emit N_2O under laboratory, greenhouse and field conditions (Smart & Bloom, 2001; Pihlatie *et al.*, 2005; Machacova *et al.*, 2013, 2016). Quantifying branch and leaf N_2O emission from mature trees in the field will be logistically challenging. Nonetheless, our estimates of contribution of tree-mediated N_2O may still be conservative.

The contribution of tree-mediated N_2O to total ecosystem N_2O fluxes was low compared to published estimates of fertilized crops, where plant-related emissions ranged from 11 to 87% (Yan *et al.*, 2000; Chen *et al.*, 2002; Zou *et al.*, 2005). However, our study

shows that N_2O estimates from forest ecosystems solely based on the measurements of soil N_2O fluxes are probably conservative, since tree-mediated fluxes are not included. If our observation of the relative contribution of tree-mediated N_2O fluxes in upland trees will be further corroborated by other studies, tree-mediated N_2O fluxes may be more important in upland trees than in wetland trees. However, it should be kept in mind that inclusion of tree-mediated N_2O fluxes will not make significant changes to global N_2O budgets since even a 10% increase would easily fall within the standard errors of present global estimates. Finally, our study highlights the importance to conduct long-term, field-based measurements since the complexity of ecosystems cannot be simulated under laboratory conditions.

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Author contributions

M.D.C and E.V designed the experiment. Y.W. performed field and lab experiments. Y.W. analyzed data and all authors interpreted the results. Y.W., M.D.C, C.R. and E.V wrote the manuscript.

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Chapter 5

Synthesis

Several key conclusions can be drawn from this work:

(1) **Limitation of the $^{15}N_2O$ pool dilution technique.** The $^{15}N_2O$ pool dilution ($^{15}N_2OPD$) technique was first proposed as a robust method to separate gross N_2O production and consumption in soil (Yang et al., 2011), but our study demonstrates that this technique is not able to measure N_2O produced in soil but consumed immediately. It is confirmed by the large disparity between $^{15}N_2OPD$ and GFSC measurements that gross N_2O production and gross N_2O consumption measured by the $^{15}N_2OPD$ technique were only 10% and 6% of the values measured by the GFSC method. The low fractions are mainly because the produced N_2O is consumed immediately without mixing the $^{15}N_2O$ labeled gas, as assumed by Well & Butterbach-Bahl (2013). Hereby, we proposed a conceptual model to illustrate different pathways of N_2O dynamics. This model provides a new insight into various N_2O pathways either in soil or at the soil-atmosphere interface. Furthermore, to avoid misinterpretations of terminologies we proposed that the terms 'gross N_2O emission and uptake' should be used for gross N_2O fluxes measured with the $^{15}N_2OPD$ technique and 'gross N_2O production and consumption' should be used for gross N_2O fluxes measured with the GFSC method. Additionally, we gave clear definitions of the processes measure by the $^{15}N_2OPD$ technique. Gross N_2O emission accounts both the N_2O that is emitted from the soil to the atmosphere and the N_2O that is reduced to N_2 within the soil pores which are in active exchange with the atmosphere; while gross N_2O uptake accounts not only the reduced N_2O which comes from atmosphere and diffuses into soil but also the reduced N_2O within the soil pores.

(2) **Advantages of the $^{15}N_2O$ pool dilution technique.** Although the $^{15}N_2OPD$ technique is not able to measure gross N_2O production and gross N_2O consumption in soil, it still has significant advantages. Since gross N_2O emission and uptake occur simultaneously, these two processes have not been measured due to the methodological challenge. Only with the $^{15}N_2OPD$ technique, we are able to separate the net N_2O fluxes into gross N_2O emission and uptake. Moreover, this technique can be used in the field, and thus it allows us to investigate the underlying mechanisms and controlling factors of N_2O fluxes under actual field conditions.

Measuring gross N_2O emission and uptake improves our ability to predict N_2O dynamics across the soil-atmosphere interface and understand the future response of N_2O dynamics to climate change.

(3) **Relationships among gross N_2O production, consumption, emission and uptake.** Although previous studies have showed that substantial N_2O produced in subsoil could be consumed along the diffusion pathway towards soil surface, as indicated by various ^{15}N-N_2O enrichment along soil depth (Conen and Neftel, 2007; Koehler et al., 2012), our study gave a direct proof of N_2O consumption in soil. The significantly lower gross N_2O emission at the soil surface than gross N_2O production in soil suggested that considerable amount of N_2O were reduced to N_2 during diffusion along 5 cm length soil core. N_2O produced in soil may be consumed within the same denitrifier cell (Knowles, 1982), or consumed by other microorganism, which may have N_2O reductase but cannot act on the preceding substrate of the denitrification pathway (Sanford et al., 2012). Although atmospheric N_2O can diffuse into soil and subsequently be reduced to N_2, it only accounts 6% of gross N_2O consumption in soil.

(4) Net N_2O flux & gross N_2O emission. Our studies provide a new perspective on the mechanisms that control net N_2O fluxes at the soil surface. We found that variations in gross N_2O emission rather than gross N_2O uptake drove spatio-temporal patterns in both net N_2O emission and net N_2O uptake. Net N_2O uptake was observed in the pine (Chapter 2) and spruce forest soils (Chapter 3) when gross N_2O emission rates dropped below gross N_2O uptake rates. The ratios of net to gross N_2O emissions (63 – 79% in mineral soils under grassland, cropland , beech and spruce forests; Chapter 2 and 3) were similar to the values reported by Yang et al. (2011) and Yang and Silver (2016) from managed grassland and cropland in California (net to gross N_2O emission ratio of 68 – 70%). These generally comparable ratios may open the possibility of making estimates of gross N_2O emissions and uptake based on measured net N_2O emissions.

(5) Environmental factors controlling gross N_2O emission and gross N_2O uptake. Independently quantifying gross N_2O emission and gross N_2O uptake offers the unique opportunity to explore the soil factors controlling these concurrently occurring processes. Soil N availability, aeration status and microbial activity controlled the variations in gross N_2O emission and uptake among different sites (Chapter 2), whereas the availabilities of

extractable organic C (electron donor) and NO_3^- (electron acceptor) influence the temporal variations in gross N_2O emission and uptake within a stand (Chapter 3).

(6) Temporal variability of stem N_2O fluxes. Our results showed for the first time that both wetland and upland trees could consistently emit N_2O across the whole growing season. The clear seasonal patterns of tree-mediated N_2O fluxes were related to the temporal variability in soil factors (e.g. temperature, water content, N_2O concentration) and climatic factors (e.g. air temperature, vapor pressure deficit). Hereby, our study highlights the importance to conduct long-term, field-based measurements since the complexity of ecosystems cannot be simulated under laboratory conditions.

(7) Tree-mediated N_2O fluxes as a 'missing' N_2O source. If our observation of the relative contribution of tree-mediated N_2O fluxes in upland trees (8-11%) will be further corroborated by other studies, trees have to be considered as a significant source of N_2O in the upland forest ecosystems. Omission of this pathway from process models may result in an underestimation of total N_2O emissions from global forest ecosystems. Hence, our findings highlight the important, but often neglected role of upland trees in N_2O exchange between the biosphere and the atmosphere and the importance of including tree N_2O emissions to the total N_2O budget. However, it also should be kept in mind that even a 10% increase would easily fall within the standard errors of present global estimates.

(8) Outlook. This research highlights that the need for further work to accurately characterize gross N_2O fluxes and more detailed measurements of spatio-temporal variability are necessary. This can be crucial for the future estimate of N_2O source and sink in terrestrial ecosystem. For future gross N_2O fluxes studies, clear definitions of processes that can be measured by the $^{15}N_2O$PD technique as well as correct usage of terminologies can avoid confusing. To clarify all pathways of N_2O emission from soil to the atmosphere, more field studies with adult trees are urgently needed to quantify the extent of this release under natural conditions. Moreover, branches and leaves, which have also been shown to emit N_2O under laboratory, greenhouse and field conditions (Machacova et al., 2016, 2013; Pihlatie et al., 2005; Smart and Bloom, 2001), should be included into future emission studies allowing estimation of a complete ecosystem budget of N_2O fluxes from forests into the atmosphere.

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DECLARATION OF ORIGINALITY AND CERTIFICATE OF AUTHORSHIP

I, Yuan Wen, hereby declare that I am the sole author of this dissertation entitled "Nitrogen fluxes in temperate forest ecosystems", and that all references and data sources have been appropriately acknowledged. I furthermore declare that this work has not been submitted elsewhere in any form as part of another dissertation procedure. I certify that the manuscripts presented in chapters 2, 3 and 4 have been written by me as first author.

Göttingen, May, 2017 ______________________________

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PUBLICATIONS

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