The soil N cycle: new insights and key challenges

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Abbreviations: BNF: Biological Nitrogen Fixation, **S-BNF**: Symbiotic Biological Nitrogen Fixation; **F-BNF**: free-living Biological Nitrogen Fixation, **DNRA**: Dissimilatory Nitrate Reduction to Ammonia.

Abstract

The study of soil N cycling processes has been, is, and will be at the center of attention in soil science research. The importance of N as a nutrient for all biota; the ever increasing rates of its anthropogenic input in terrestrial (agro)ecosystems; its resultant losses to the environment; and the complexity of the biological, physical, and chemical factors that regulate N cycling processes all contribute to the necessity of further understanding, measuring and altering the soil N cycle. Here, we review important insights with respect to the soil N cycle that have been made over the last decade, and present a personal view on the key challenges for future research. We identified three key challenges with respect to basic N cycling processes producing gaseous emissions:

1. Quantifying the importance of nitrifier denitrification and its main controlling factors;
2. Characterizing the greenhouse gas mitigation potential and microbiological basis for N_2O consumption;
3. Characterizing hot-spots and hot-moments of denitrification;

Furthermore, we identified a key challenge with respect to modelling:

1. Disentangling gross N transformation rates using advanced $^{15}N/^{18}O$ tracing models;

Finally, we propose four key challenges related to how ecological interactions control N cycling processes:

1. Linking functional diversity of soil fauna to N cycling processes beyond mineralization;
2. Determining the functional relationship between root traits and soil N cycling;
3. Characterizing the control that different types of mycorrhizal symbioses exert on N cycling;
4. Quantifying the contribution of non-symbiotic pathways to total N fixation fluxes in natural systems;

We postulate that addressing these challenges will constitute a comprehensive research agenda with respect to the N cycle for the next decade. Such an agenda would help us to meet future challenges on food and energy security, biodiversity conservation, water and air quality and climate stability.

1. Introduction

Humankind's relationship with soil nitrogen (N) has been a long and troubled one. For most of agricultural history, farmers have struggled to maintain soil fertility levels in their fields, relying mostly on biological N fixation (BNF), decomposition of soil organic matter and redistribution of organic materials to provide N to their crops. With the onset of large-scale application of mineral fertilizers in the 1950's, the main focus in large parts of the world has gradually shifted towards minimizing harmful losses to the environment resulting from the large amounts of N entering the global food production system (Galloway et al., 2013).

The history of research on the soil N cycle reflects this shift. The study of N cycling processes started after Carl Sprengel's discovery (popularized by Justus Von Liebig) of the importance of N as a factor limiting the growth of crop plants in the mid-19th century (Gorham, 1991). More than 150 years of research has demonstrated that this element limits ecosystem productivity over large areas of the globe and is highly sensitive to changes in temperature, precipitation, atmospheric CO_2 and disturbance regimes (Galloway et al., 2008). Since the 1960s, following the realization that excess N has negative effects on water, air and ecosystem and human health (Compton et al., 2011; Davidson et al., 2012), the study of the N cycle has intensified, focusing on N loss pathways next to the more traditional study topics such as plant N uptake. Most recently, the realization that the response of ecosystems to global environmental change would to a large extent depend on N dynamics (Van Groenigen et al., 2006; Luo et al., 2011) has generated further interest in the soil N cycle.

The need for more information on soil N cycling process rates is highlighted by large amounts of ‘missing N’ that dominate N balances at all scales. Inputs of N through fertilization, BNF, atmospheric deposition and human- and animal waste have been found to be substantially

higher than hydrological outputs of N in many studies, at many scales (Howarth et al., 1996; Boyer et al., 2002; Groffman, 2008). There is much uncertainty about the fate of this excess N (Van Breemen et al., 2002). Is it stored in soils or vegetation? Is it converted to gas, and if so, in which forms? This uncertainty is particularly compelling in agricultural systems which receive high rates of N input. The air and water quality impacts of the N exports in these systems are a cause for great concern (Davidson et al., 2012). In other ecosystems, on the other hand, there is concern about missing N inputs. Unexplained accumulation of N in aggrading forests (Bernal et al., 2012; Yanai et al., 2013) or in vegetation exposed to elevated levels of atmospheric CO_2 (Zak et al., 2003; Finzi et al., 2007) suggest unmeasured inputs of N via BNF (Cleveland et al., 2010) or uncharacterized mechanisms of soil N turnover and mineralization (Drake et al., 2011; Phillips et al., 2011; Phillips et al., 2012).

A particularly pressing need in N cycling research has been in the area of gaseous emissions, especially of those that contribute to global warming. The role of soil biogeochemists is to generate field data on terrestrial greenhouse emissions, but high uncertainties in soil N_2O and N_2 budgets still exist. Much of this uncertainty arises from a lack of information about the importance of the variety of N gas forming processes occurring in the soil and the methodological constraints on flux measurements (Ambus et al., 2006). Evidence is emerging that processes, other than nitrification and denitrification, are far more important than previously assumed for gaseous N production from soils. Processes such as nitrifier denitrification (Wrage et al., 2001), in-situ N_2O reduction (Schlesinger, 2013), anammox (Mulder et al., 1995), feammox (Sawayama, 2006), dissimilatory nitrate reduction to ammonium (DNRA) (Tiedje, 1988), and codenitrification (Spott et al., 2011) have all been hypothesized to play a role in the gaseous N cycle. Novel and fascinating efforts to extract DNA and RNA and to define microbial

98 communities have recently produced new information on the agents that carry out many of these
99 processes (Isobe and Ohte, 2014). Yet, information on process rates and their dynamics in
100 response to a myriad of environmental factors are clearly lacking. Such information is vital
101 however, as gene presence is a proxy for *potential* activity, but is not a final proof of the
102 occurrence of ecologically significant process rates.

103 One of the reasons that it has been so difficult to quantify and characterize N cycling
104 processes is that they are to a large extent controlled by indirect, biotic interactions. It is
105 becoming increasingly clear that ecological interactions play a major role in the terrestrial N
106 cycle. The realization that global change may alter the nature and timing of biotic interactions
107 and thereby their effects on the N cycle only increases the need for their study (Díaz et al., 1998;
108 Chapin et al., 2000). In some ecosystems, N inputs to terrestrial ecosystems are dominantly
109 mediated by mutualistic associations between plants and specific N-fixing microbial groups
110 (Batterman et al., 2013a). More generally, plant species have an overarching impact on soil N
111 cycling by directly mediating energy and material fluxes to soil microbial communities and/or by
112 altering abiotic conditions that regulate microbial activity. For example, the type of mycorrhizal
113 fungi that colonizes the plant root has been shown to correlate with organic N depolymerisation
114 as fungal groups produce a specific set of enzymes. Also soil fauna have both a direct and
115 indirect role in the soil N cycle as grazing may strongly affect microbial N release as well as alter
116 soil physical properties. All these ecological interactions have a high degree of specificity and
117 sensitivity to global change, which increases the probability that a change in plant-, microbial- or
118 faunal- community composition will have cascading effects on the rest of the system and on the
119 overall soil N cycle (Chapin et al., 2000).

Here, we review important insights with respect to the soil N cycle that have been made over the last decade, and present our view on the key challenges for future soil research (Fig. 1). The approach adopted in this paper is three-fold:

1. To identify and critically review specific N transformation pathways related to the production of N_2O and N_2. We focus on nitrifier denitrification (Section 2.1), which is a potentially important source of N_2O; and N_2O reduction (Section 2.2), the important but little-understood final step of denitrification. We focus on these two processes as we believe that sufficient literature information is available to demonstrate that these processes are key unknowns with respect to the emission rates of gaseous N forms. Additionally, we discuss challenges with respect to measuring hot-spots and hot-moments of denitrification (Section 2.3);

2. To present methodological developments on ^{15}N tracing models that should further aid studies on the production of gaseous N forms in soils (Section 3); and

3. To review mechanisms on how ecological interactions impact soil N cycling. Specifically, we focus on soil faunal effects (Section 4.1), plant root controls (Section 4.2), mycorrhizal symbioses (Section 4.3), and biological N fixation (Section 4.4). Although other nutrient cycles can have strong effects on all aspects of the N cycle (e.g. Baral et al., 2014), we consider stoichiometric relations to be mostly outside the scope of this paper and do not exhaustively review them.

Although all authors agree with the contents of the final paper, some freedom has been given to express a somewhat personal view on developments within our respective fields of expertise (see Author Contribution section). This paper is not meant as a comprehensive literature review of soil N cycling research in the past. Instead, we have tried to be judicious with respect to referencing older studies, only citing some key papers and focusing instead on more

143 recent work. As such, we hope that our paper will spark discussion and inspire further research
144 on the elusive aspect of soil N cycling.
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147 **2. Emerging insights on gaseous nitrogenous emissions**
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149 **2.1. Nitrifier denitrification**
150 The study of nitrifier denitrification as a significant biogeochemical N_2O-producing process in
151 soils has been severely hampered by two persistent problems: one related to *terminology*, the
152 other to *methodology*.
153 With respect to *terminology*, it took a landmark paper (Wrage et al., 2001) to clearly
154 identify nitrifier denitrification as a distinct pathway for N_2O production, as it was often
155 confused- or combined with two other N_2O production pathways: nitrifier nitrification and
156 nitrification coupled denitrification (which is actually a combination of two classical processes
157 rather than a novel one: nitrifier nitrification followed by classical denitrification; Fig. 2).
158 Nitrifier denitrification is the production of N_2O by autotrophic ammonia oxidizing bacteria by
159 reduction of NO_2^-. The process is therefore carried out by the same organisms that can produce
160 N_2O through nitrification. However, the two N_2O producing pathways are fundamentally
161 different; during nitrification N_2O is formed as a byproduct of a chemical process: the
162 spontaneous oxidation of one of the intermediate N species (hydroxylamine). Nitrifier
163 denitrification, on the other hand, is a stepwise reduction controlled by enzymes during which
164 N_2O is one of the intermediate products that might escape to the atmosphere. In fact, the
165 enzymes responsible for this stepwise reduction during nitrifier denitrification are remarkably

similar to those of canonical denitrification (possibly due to lateral gene transfer); they do not appear to differ phylogenetically from NiR and NOR found in denitrifying organisms (Casciotti and Ward, 2001; Garbeva et al., 2007).

Despite the similarity with classical denitrification, there are good reasons to assume that nitrifier denitrification is controlled by different factors and should therefore be considered a distinct source of N_2O emissions from soil. The main reason for this is that denitrifiers are heterotrophic, whereas ammonia oxidizing bacteria are chemo-autotrophic. It is not entirely clear yet why ammonia-oxidizing bacteria perform nitrifier denitrification. One hypothesis is that it is a response to NO_2^- toxicity under marginally aerobic conditions (Shaw et al., 2006). Alternatively, the energetic gain from coupling NH_4^+ oxidization to NO_2^- reduction is similar to that from using O_2, making nitrifier denitrification energetically attractive under marginally aerobic conditions (Shaw et al., 2006).

The process was described by early pure culture studies in the 1960s and 1970s (Hooper, 1968; Ritchie and Nicholas, 1972). Since then, it has been reported several times (e.g. Poth and Focht, 1985; Schmidt et al., 2004), but always in pure cultures. Despite suggestions that nitrifier denitrification could be an important contributor to soil N_2O emissions (Granli and Bøckman, 1994; Webster and Hopkins, 1996), and that conventional methods of 'nitrification N_2O' measurements such as ^{15}N tracing or inhibition with O_2 or acetylene might actually include nitrifier denitrification (Granli and Bøckman, 1994; Mosier et al., 1998), proof of its occurrence in actual soils has remained elusive.

The main challenge to evaluating the importance of nitrifier denitrification in soils is *methodology*. As the N in N_2O produced from both nitrification and nitrifier denitrification originates from the same NH_4^+ pool, it is impossible to distinguish between these two processes

189 with conventional ^{15}N tracing methods (Stevens et al., 1997) alone. Methods using inhibition of
190 specific steps of (de)nitrification were proposed as a method to quantify nitrifier denitrification
191 (Webster and Hopkins, 1996), but a series of studies showed that inhibition was unreliable due to
192 problems with effectiveness and selectivity (Tilsner et al., 2003; Beaumont et al., 2004; Wrage et
193 al., 2004a; Wrage et al., 2004b).

194 Various efforts have been undertaken to employ advanced stable isotope analysis to
195 determine the contribution of nitrifier denitrification as an N_2O source. Sutka et al. (2006)
196 suggested that the intramolecular distribution of ^{15}N within the asymmetrical N_2O molecule (site
197 preference) might be employed. In pure culture studies, they showed that the site preference
198 signature of nitrifier denitrification and denitrification differed significantly from that of classical
199 nitrification (Sutka et al., 2006) as well as fungal denitrification (Ostrom and Ostrom, 2011).
200 However, in a recent assessment Decock and Six (2013) concluded that huge challenges remain
201 (related to process rates, heterogeneity, unaccounted-for processes, among others) before such an
202 analysis can be reliably applied to soils. They conclude that analysis of site preference will likely
203 remain a qualitative indicator of mechanisms underlying N_2O emissions, and recommend more
204 studies to systematically characterize variation in site preference as a function of ecosystem, soil
205 parameters as well as biogeochemical processes. Such studies are currently being conducted (e.g.
206 Koster et al., 2013; Lewicka-Szczebak et al., 2014; Yano et al., 2014).

207 Wrage et al. (2005) proposed an alternative method based on artificially enriched stable
208 isotope tracing. They combined ^{15}N with ^{18}O tracing to isolate nitrifier denitrification, utilizing
209 the fact that all O in nitrifier-derived N_2O originates from O_2, but half of the O from nitrifier
210 denitrification is derived from H_2O. However, their method, employing ^{18}O-enriched H_2O as
211 well as $^{15}N\text{-}NO_3^-$ and $^{15}N\text{-}NH_4^+$, did not take into account O exchange between H_2O and

intermediates of the (de)nitrification pathways (Kool et al., 2007; Kool et al., 2009). This exchange can be quantified using ^{18}O labelled NO_3^- (Kool et al., 2010; Kool et al., 2011b). With the help of a revised method, Kool et al. (2011a) showed that nitrifier denitrification exceeded 'classical nitrification' as a dominant source of NH_4^+-derived N_2O emission, and was a dominant pathway of total N_2O production at low and intermediate soil moisture contents. Other studies using this method have confirmed that nitrifier denitrification was indeed the dominant pathway for NH_4^+ derived N_2O emissions (Zhu et al., 2013). With terminology established and a method developed, nitrifier denitrification is now ready to be studied in detail in soils. However, methodological constraints still exist, as the dual isotope method is elaborate and includes a relatively large number of assumptions.

2.2. Nitrous oxide consumption

Both net atmospheric and *in situ* N_2O consumption occur in the soil, reducing both atmospheric lifetimes of N_2O and net N_2O effluxes. Consumption of N_2O is enzymatically and energetically feasible. Net atmospheric consumption of N_2O has been sporadically reported for several terrestrial ecosystems, but mostly for wetlands and peatlands. A recent review by Schlesinger (2013) reports a net N_2O uptake range of <1 – 207 μg N m^{-2} h^{-1}, but almost all uptake fluxes fall between 1 and 10 μg N m^{-2} h^{-1}, with a median of 4 μg N m^{-2} h^{-1}. The latest IPCC report (Stocker et al., 2013) mentions a global surface N_2O sink of 0 – 1 Tg N_2O-N yr^{-1}. Another recent review (Majumdar, 2013) reported *in situ* N_2O consumption rates in rice fields ranging from 0.13 - 191 μg N m^{-2} h^{-1}. For that purpose, Yang et al. (2011) developed an $^{15}N_2O$ isotope dilution method that allows for calculation of gross N_2O production and consumption rates. These authors observed a relative N_2O yield of 0.84, indicating that 16% of the gross N_2O production was

consumed *in situ*. However, Well and Butterbach-Bahl (2013) question the validity of the latter experimental approach. Understanding the role of *in situ* N_2O reduction for attenuation of the net soil N_2O release warrants careful attention because of a recently identified microbial guild capable of N_2O reduction (Sanford et al., 2012).

Based on recent evidence from the literature we have identified three possible routes for N_2O consumption. First, in addition to the 'typical' nitrous oxide reductase (nosZ I) that reduces N_2O during denitrification, a recently identified microbial guild is suggested to mediate the soil N_2O sink (Sanford et al., 2012; Jones et al., 2014). Newly discovered non-denitrifier, 'atypical' N_2O reductase (nosZ II) gene diversity and abundance potentially play a significant role in N_2O consumption in soil. Orellana et al. (2014) indicated that 'atypical' nosZ outnumber typical nosZ in soil.

Second, some bacteria that perform dissimilatory nitrate reduction to ammonia (DNRA) are capable of N_2O reduction to N_2 as they carry a *nos* gene encoding for N_2O reductase (N_2OR) (Simon et al., 2004). Mania et al. (2014) indicated that, depending on the environmental conditions, these bacteria may reduce N_2O that is provided by other bacteria or that they produced themselves as a by-product during DNRA.

Third, there is evidence that both direct assimilatory N_2O fixation via nitrogenase (Vieten et al., 2008; Ishii et al., 2011; Farías et al., 2013) or indirect N_2O fixation via a combination of N_2O reduction and N_2 fixation can account for N_2O consumption. Itakura et al. (2013) showed that inoculation of soil grown with soybean with a non-genetically modified mutant of *Bradyrhizobium japonicum* with a higher N_2O reductase activity (nosZ++) reduced N_2O emission. In farm-scale experiments on an Andosol, an N_2O mitigation of ca. 55% was achieved with such inoculation. Desloover et al. (2014) identified a *Pseudomonas stutzeri* strain that was

able to grow on N_2O as the only source of N and electron acceptor. *Pseudomonas stuzeri* is known to possess both nitrogenase and nitrous oxide reductase (nosZ I) (Pomowski et al., 2011). A ^{15}N labelling study showed that N_2O is immobilized into microbial biomass via N_2O reduction to N_2 followed by re-uptake of the released N_2 and subsequent fixation into NH_4^+ via nitrogenase (Desloover et al., 2014).

In conclusion, five possible pathways for N_2O consumption have been identified (Fig. 3): (1) dissimilatory N_2O reduction to N_2 via typical, denitrifier nosZ I, (2) atypical, non-denitrifier nos Z II, (3) DNRA that produces N_2O as a by-product, (4) direct assimilatory N_2O fixation via nitrogenase to NH_3, and (5) indirect assimilatory N_2O fixation (N_2O reduction to N_2 followed by N_2 fixation). Clearly, NO_3^- reduction in soil is handled by a network of actors (Kraft et al., 2011) and has a more modular character than the classical linear presentation of denitrifying enzymes suggests (Simon and Klotz, 2013). Moreover, a high degree of metabolic versatility is observed for many organisms; genes encoding for denitrification, DNRA, and atmospheric N fixation have, for instance, been found in a single bacterial species (Simon, 2002; Mania et al., 2014). Finally, Verbaendert et al. (2014) showed that molecular tools that have been developed to identify denitrifying bacteria are biased towards Gram-negative denitrifiers. Hence, we propose that the analysis of expression of novel, recently discovered genes involved in N_2O consumption in conjunction with quantification of N_2O fluxes in various soil types is required to advance our understanding of microbial and physicochemical controls on N_2O consumption, and ultimately to develop improved biogeochemical models of soil N_2O sink function.

2.3. Denitrification

Denitrification, the anaerobic microbial conversion of the nitrate (NO_3^-) and nitrite (NO_2^-) to the gases nitric oxide (NO), nitrous oxide (N_2O) and dinitrogen (N_2) (Seitzinger et al., 2006; Groffman, 2012) is an extremely challenging process to measure. This process is of great interest because it can significantly reduce pools of reactive N (and thus productivity) in ecosystems and because NO_3^-, NO and N_2O cause diverse air and water pollution problems (Davidson et al., 2012). Denitrification is difficult to quantify because of problematic measurement techniques (especially for its end product N_2), high spatial and temporal variability, and a lack of methods for scaling point measurements to larger areas (e.g. Groffman et al., 2006). A particular challenge is the fact that small areas (hotspots) and brief periods (hot moments) frequently account for a high percentage of N gas flux activity, and that it is increasingly recognized that denitrification is in many ways a modular rather than a singular process. This presents a variety of problems related to measurement, modelling and scaling (Groffman et al., 2009). Global mass balance analyses (Seitzinger et al., 2006) suggest that the biggest global sink for anthropogenic N must be terrestrial denitrification, yet there are few direct measurements to support these results. Modelling efforts estimate that global N_2 production from denitrification may increase from 96 Tg yr^{-1} in 2000 to 142 Tg yr^{-1} in 2050 due to increased N inputs in the global agricultural system (Bouwman et al., 2013). Questions about 'missing N' and denitrification are particularly dramatic and compelling in agricultural ecosystems, landscapes and regions, where most industrially derived N is applied and the opportunity for large terrestrial denitrification fluxes exists.

Addressing the challenge of denitrification requires advances in three main areas; 1) improved methods for quantifying N gas fluxes (see also section 2.2); 2) experimental designs

302 that incorporate hotspot and hot moment phenomena; and 3) approaches for temporal and spatial
303 scaling that account for hotspot and hot moment phenomena at multiple scales.

304 Denitrification has always been a challenging process to measure (Groffman et al., 2006),
305 primarily due to the difficulty of quantifying the flux of N_2 from soil against the high natural
306 atmospheric background of this gas (Yang and Silver, 2012; Yang et al., 2014). Most
307 denitrification methods therefore involve alteration of physical or chemical conditions through
308 the use of inhibitors (e.g., acetylene) or amendments (e.g., ^{15}N) that produce inaccurate or
309 unrealistic estimates of rates. However, there have been recent advances in methods for
310 quantifying N_2 flux and in isotope-based methods that provide area and time-integrated
311 denitrification estimates that are more relevant to ecosystem-scale questions.

312 Our understanding of the N_2 flux associated with denitrification has been improved at
313 least somewhat by the development of soil core-based gas recirculation systems that involve
314 replacement of the natural soil N_2/O_2 atmosphere with a He/O_2 atmosphere, followed by direct
315 measurement of N_2 and N_2O production as well as their ratio (Swerts et al., 1995; e.g.Wang et al.,
316 2011; Kulkarni et al., 2014). It is important to note that these new methods are based on
317 extracted soil cores, incubated over extended periods, which can create artificial conditions
318 (Frank and Groffman, 2009). However, some confidence in the flux estimates from cores can be
319 developed by comparing estimates of CO_2 and N_2O fluxes in the cores and *in situ* field chambers.

320 The new soil core incubation systems, along with new soil O_2 sensors, have also
321 advanced our understanding of hot moments of denitrification. Because it is possible to vary the
322 O_2 concentration of the recirculation stream in the new incubation systems, denitrification versus
323 O_2 relationships can be established and linked with continuous estimates of soil O_2 from the new
324 sensors to produce continuous estimates of flux (Burgin and Groffman, 2012; Duncan et al.,

2013). Recent studies have shown that these relationships are more complex than previously thought. For example, in northern hardwood forests in north-eastern North America, denitrification rates have been found to be higher at 5% or 10% O_2 than under completely anaerobic conditions, suggesting that there is tight coupling between NO_3^- production by nitrification and denitrification in these soils (Morse et al., 2014a).

As our ability to quantify denitrification has improved, our understanding of the factors that control the occurrence of hotspots and hot moments of activity has also increased. Riparian zones have been studied in this regard for several decades (e.g. Lowrance et al., 1997; Mayer et al., 2007). This has resulted in efforts to protect and restore riparian zones to decrease N delivery to receiving waters in many locations. Still, there is great uncertainty about just how much N is denitrified in riparian zones and through other N control practices, and how much N remains in the soils and vegetation of these areas where it is susceptible to later conversion back to NO_3^- or N_2O (Woli et al., 2010).

There has long been recognition of the potential for hotspots and hot moments denitrification to occur within crop fields or pastures. Periods of transient saturation low in the soil profile can support significant amounts of denitrification that are missed in sampling programs that focus on surface soils (Werner et al., 2011; Morse et al., 2014b). Areas of wet soil, low soil O_2 and possibly high denitrification are also common at the transition between fall and winter and between winter and spring (Walter et al., 2000). Animal grazing and excretion can create hotspots of N deposition, mineralization, nitrification, denitrification and N_2O flux (de Klein et al., 2014).

Experiments incorporating new ideas about hotspots and hot moments can benefit from recent studies that have characterized diversity in denitrifying phenotypes that reflect adaptation

to prevailing environmental conditions with consequences for denitrification activity (Bergaust et al., 2011). These ideas have the potential to improve these experiments by allowing for more mechanistic, hypothesis-driven approaches that underlie more 'black-box' ideas based on proximal drivers of denitrification.

Estimates of denitrification produced by direct measurement in soil cores can be validated using isotope measurements and models. Shifts in $^{15}N\text{-}NO_3^-$ have been used to indicate denitrification in soils, riparian zones, agricultural streams, and large rivers (e.g. Kellman and Hillaire-Marcel, 1998; Vidon and Hill, 2004). Dual natural isotope ($\delta^{18}O$- and $\delta^{15}NO_3^-$) analysis has been used to estimate denitrification in aquifers (Wassenaar, 1995), agricultural (Burns et al., 2009) and urban (Kaushal et al., 2011) catchments as well as in tropical forest soils (Houlton et al. 2006).

The time is thus ripe for ecosystem, landscape and regional-scale studies of denitrification. We have new methods capable of producing well constrained estimates of denitrification at the ecosystem scale and new ideas about the occurrence of hotspots and hot moments at ecosystem and landscape scales. In combination with independent approaches for validation of denitrification estimates, our estimates of this important process are likely to improve markedly over the next decade.

3. ^{15}N tracing modelling for understanding N cycling processes

This section will focus on how ^{15}N enrichment in combination with process oriented modeling (Rütting et al., 2011b; Huygens et al., 2013) has helped to advance our understanding of N cycling dynamics in soils, and will be able to do so further in the future.

371 The stable isotope ^{15}N has been used as a tracer for the quantification of gross N
372 transformation rates for 60 years. In their two seminal papers Kirkham and Bartholomew (1954,
373 1955) developed the isotope pool dilution technique, enabling for the first time the quantification
374 of gross transformation rates of N cycling processes. Quantification of gross rates has deepened
375 our understanding of the terrestrial N cycle tremendously. For example, Davidson et al. (1992)
376 showed that old-growth forests exhibit high gross mineralization rates, challenging the paradigm
377 (based on net mineralization rate measurements) that these ecosystems have low mineralization
378 activity. The isotope pool dilution technique is still widely used, even though it has some
379 important limitations. The most crucial disadvantage is that only total production and
380 consumption rates of a labelled N pool can be quantified, which may be the result of several
381 simultaneously occurring N processes (Schimel, 1996). For example, gross nitrification as
382 quantified by the isotope pool dilution technique can be comprised of two separate processes,
383 autotrophic (NH_4^+ oxidation) and heterotrophic (the oxidation of organic N to NO_3^-) nitrification.
384 To overcome this limitation, ^{15}N labelling can be done in conjunction with numerical ^{15}N tracing
385 models (Rütting et al., 2011b). These models describe the flow of N and ^{15}N though the various
386 soil N pools (e.g. NH_4^+, NO_3^- and organic N), whereby transformations are represented by
387 kinetic equations (e.g. zero- or first-order kinetics). The first ^{15}N tracing model which could
388 separate autotrophic from heterotrophic nitrification was presented by Myrold and Tiedje (1986).
389 Subsequent studies using ^{15}N tracing models have shown that heterotrophic nitrification can be a
390 significant or even the dominant NO_3^- production pathway in forest and grassland soils
391 (Barraclough and Puri, 1995; Rütting et al., 2008; Taylor et al., 2013). In addition, ^{15}N tracing
392 models have been shown to be useful for investigating the importance of DNRA in various soils
393 (Rütting et al., 2011a). Moreover, they can be used to distinguish DNRA from alternative

394 pathways such as remineralization and plant efflux (Burger and Jackson, 2004). Recently an ^{15}N
395 amino acid pool dilution approach has been developed (Wanek et al., 2010), which can be a
396 useful tool for investigating whether depolymerization or N mineralization is the rate limiting
397 step of the terrestrial N cycle (Schimel and Bennett, 2004), particularly if incorporated in
398 numerical ^{15}N tracing models.

399 In addition to quantification of gross N transformation rates, ^{15}N enrichment has proven
400 useful for partitioning nitrous oxide (N_2O) emission sources. Using a two-source mixing model,
401 Stevens et al. (1997) investigated the contribution of NO_3^- reduction (i.e. denitrification) and
402 NH_4^+ oxidation (i.e. autotrophic nitrification) to N_2O emission. Subsequent work, however,
403 suggested that organic N can be a third substrate for N_2O production. Indeed, ^{15}N studies using a
404 triplet tracer approach and either analytical (Stange et al., 2009) or numerical (Stange et al., 2013;
405 Müller et al., 2014) ^{15}N tracing models showed a significant or even dominant contribution of
406 oxidation of organic N (heterotrophic nitrification) to N_2O production in soils. The numerical
407 models have the additional advantage that gross N_2O production rates can be quantified. Using
408 oxygen isotopes (^{18}O) as an additional tracer allows the separation of NH_4^+ derived N_2O
409 emission between NH_4^+ oxidation and nitrifier-denitrification (Kool et al., 2011a). The
410 limitations and opportunities of this approach are discussed in Section 2.1. A further step for
411 understanding sources of N_2O emission from soil would be to incorporate ^{18}O into numerical
412 tracing models, i.e. development of a combined ^{15}N-^{18}O-tracer model. Overall, stable isotope
413 labeling approaches (^{15}N and ^{18}O) have greatly increased our understanding of the diverse N
414 cycle processes contributing to N_2O production in soils. Moreover, these studies have confirmed
415 the importance of NO_2^- dynamics for N_2O production (Stange et al., 2013; Müller et al., 2014)
416 and for the soil N cycle in general (Rütting and Müller, 2008; Isobe et al., 2012).

4. Ecological interactions and N cycling processes

4.1. Soil fauna

Until recently, the influence of soil fauna on the soil N cycle in agroecosystems has been mostly neglected. Nitrogen transformation processes and -loss pathways have almost exclusively been related to the interplay between microbial dynamics in the soil and abiotic factors. At first glance this seems logical: micro-organisms dominate the biomass of soil life to a large degree, and many conversions in the N cycle (e.g. nitrification, denitrification, nitrifier-denitrification, N fixation, DNRA) are the exclusive domain of micro-organisms. Biochemical as well as physical processes, such as nitrification and N leaching are controlled by abiotic factors (e.g. pH, porosity and temperature). In turn, both microbial dynamics and abiotic factors can be changed by human influences such as N deposition in natural systems and fertilization, liming, soil tillage and animal husbandry in agricultural systems (Fig. 4a).

What important role do soil fauna then have in the N cycle? Like the effect of humans, their role can be dramatic but is essentially indirect: through trophic interactions and burrowing activities they may strongly affect microbial dynamics in the soil as well as soil physical properties (Fig. 4b).

The only part of the soil N cycle where the role of soil fauna has been reasonably well established is N mineralization and subsequent plant uptake. Soil fauna affects N mineralization by a combination of activities, including trophic interactions (grazing on micro-organisms, predation) as well as fragmentation of organic matter, mixing organic matter into the soil,

excreting nutrient-rich compounds and dispersing microbial propagules (Bardgett and Chan, 1999).

In a literature study across natural and agricultural systems, Verhoef and Brussaard (1990) found a relatively stable faunal contribution to N mineralization of around 30%. Different functional groups of soil fauna, however, contribute to N mineralization differently, with the largest contributions provided by bacterial-feeding micro-fauna (nematodes and amoeba), followed by earthworms and potworms, and minor contributions by fungal-feeding nematodes and micro-arthropods (De Ruiter et al., 1993). Among meso- and macro-fauna, the role of earthworms has been most extensively studied (e.g. Postma-Blaauw et al., 2006; Van Groenigen et al., 2014). As 'ecosystem engineers', they are well-known to affect soil structure and litter redistribution, thereby affecting many aspects of the N cycle as well as other soil processes (Shipitalo and Le Bayon, 2004; Blouin et al., 2013). In a recent meta-analysis, Van Groenigen et al. (2014) showed that in agricultural systems earthworms increase crop yield on average by 25%. This effect was consistent between different functional groups of earthworms, but increased with earthworm density and crop residue application rates. Because this beneficial effect disappeared with adequate N fertilization, it was mainly ascribed to increased N mineralization from crop residue and soil organic matter. In tropical ecosystems soil-feeding termites are known to have a similarly large impact on N mineralization (Ji and Brune, 2006). Termites are also able to volatilize ammonia from their gut as well as from their faeces. However, this has only been shown to lead to high NH_3 concentrations in their nest atmosphere. It is not yet clear whether the NH_3 accumulating in the internal nest atmosphere can escape into the ambient air (Ji and Brune, 2006).

462 The effect of faunal diversity rather than single faunal groups is complex. Combinations
463 of functionally dissimilar soil fauna can increase the N-mineralization rate due to facilitative
464 interactions (Heemsbergen et al., 2004). These include one group benefitting from the activity of
465 another group, for example through changes in soil structure or litter shredding by isopods
466 promoting microbial growth (Wardle, 2006). Yet, competitive interactions may also positively
467 influence mineralization rates (Loreau, 1998). For instance, predatory mites in the soil feed on
468 fungivorous mites and potworms as well as springtails and nematodes (De Ruiter et al., 1995),
469 and can thereby influence microbial activities through trophic cascades (induced positive effects
470 on microbes by feeding on microbial feeders). Even though empirical evidence of such trophic
471 cascades in soil food webs is scarce (Mikola and Setälä, 1998; Bardgett and Wardle, 2010), the
472 presence of predatory mites can potentially influence the behavior of fungivorous mites and
473 potworms in terms of their feeding rate and spatial distribution. Such interactions (both
474 facilitative and competitive), within and across trophic levels, have not yet been explored for
475 most N cycling processes, including N loss pathways.
476 Among the relatively few studies that have focused on processes other than N
477 mineralization, earthworms are again by far the most studied group. They have been shown to
478 affect microbial N immobilization (Brown et al., 1998) as well as nitrification and denitrification
479 (e.g. Parkin and Berry, 1999; Rizhiya et al., 2007). A growing body of literature shows that
480 earthworms can considerably increase N_2O emissions (Lubbers et al., 2013). A recent meta-
481 analysis on the effect of earthworms on soil greenhouse gas emissions reported an average
482 earthworm-induced increase in N_2O emissions of 42% (Lubbers et al., 2013). This was
483 hypothesized to be the result of effects on the denitrifier community as well as changes in soil
484 structure affecting gas diffusivity and anaerobicity (Drake and Horn, 2006; Drake and Horn,

2007; Nebert et al., 2011). Further work on soil microbiology and soil structure is needed to determine what the exact effects are of earthworm activity on microbial producers and consumers of N_2O as well as on net soil N_2O emission. Molecular microbial analysis and soil X-ray tomography are state-of-the-art experimental techniques that may shed more light on the mechanisms behind earthworm effects on N_2O emission.

Evidence for involvement of other faunal groups in these processes is scarce. Potworms, phylogenetically related to earthworms and with similar foraging and burrowing habits (albeit at a smaller scale), have been recognized as vectors for microbial colonization (Rantalainen et al., 2004) and may influence both nitrification and denitrification processes (Van Vliet et al., 2004). High soil NO_3 levels in the presence of potworms have been linked to increased nitrification potential (Liiri et al., 2007). Recent work has shown that trophic interactions involving springtails, fungivorous mites and predatory mites can strongly affect N_2O emissions (Kuiper et al., 2013; Thakur et al., 2014), although the exact pathways remain unclear - both 'real' trophic relations as well as altered behavior due to sensing of the presence of predators may play a role.

Changes in soil structure (porosity, aggregation) by faunal activity can affect soil physical processes as well. Burrowing activities of earthworms may create preferential flow pathways that increase leachate volume and consequently the total leaching loss of inorganic N and dissolved organic N (e.g. Dominguez et al., 2004). Interactions between other soil faunal species have received little attention with regard to their effects on soil physical properties. Smaller fauna such as potworms, springtails, mites and nematodes are often assumed to have negligible direct effects on larger-scale soil structure, because they are usually confined to pre-existing voids in litter or soil (Lee and Foster, 1991; Whalen and Sampedro, 2010). However, these small fauna can significantly alter soil microstructure by producing faecal pellets, and

potworms can also increase soil porosity and pore continuity by their burrowing activity (Topoliantz et al., 2000; Van Vliet et al., 2004).

Overall, soil biota are essential for maintaining healthy soils and providing ecosystem services, such as N mineralization and plant uptake for food, fuel and fiber production. However, it is not clear whether they are able to do so without creating detrimental effects on N loss pathways such as N leaching and N_2O emissions. Understanding the role of soil fauna in soil N research should therefore focus on potential trade-offs between the need to produce enough food, fuel and fiber on the one hand, and the need to mitigate global warming and avoid biodiversity loss due to eutrophication on the other. So far, mechanistic knowledge on the controlling factors for possible mitigation options is largely lacking. Addressing the question of how to reap the benefits of a diverse soil community while avoiding the drawbacks will provide fundamental insights that can be used to design future sustainable agricultural systems.

4.2. Rhizodeposition and plant traits

Soil microbial communities depend almost exclusively on plant derived resources for their energy and nutrient supply. For a long time, it was presumed that plant litter was the most relevant organic matter input for the soil food web, and that plant effects on soil biogeochemistry were mainly mediated via the indirect impacts of plant inputs on relatively inert soil properties. Therefore, most of our initial understanding of soil biogeochemistry was based on experiments with root-free soils.

The impact of spatially and temporarily dynamic processes occurring in the rhizosphere on N cycling has rarely been considered (Frank and Groffman, 2009; Rütting et al., 2011b). Nevertheless, an important share of the energy for microbial metabolism is delivered by

belowground plant parts through root exudation, cell sloughing, and root and mycorrhizal fungal turnover (Nguyen, 2003). Healthy growing roots pass a large proportion of the C they receive to the soil as root exudates. This includes a range of materials, but soluble compounds, consisting of organic acids, carbohydrates and amino acids comprise the largest component (Farrar et al., 2003). The total amount and composition of root exudates varies between plant species and genotypes, and is influenced by plant phenology and environmental conditions (Nguyen, 2003). Moreover, fine root turnover, caused by the production, mortality and decay of short-lived C-rich roots, is another key pathway of significant nutrient flux in terrestrial ecosystems that may equal or even exceed that of above-ground litter fall in certain ecosystems (Gill and Jackson, 2000; Yuan and Chen, 2010).

There are several mechanisms through which plant roots can affect rhizosphere N cycling (reviewed in Paterson, 2003; Dijkstra et al., 2013; Cheng et al., 2014). Rhizodeposition may enhance microbial growth and activity and stimulates production of microbial exoenzymes that mine for more complex soil organic N compounds, a process often referred to as 'priming' (Paterson, 2003). Nitrogen immobilized by the microbial community may temporarily reduce soil N availability, but immobilized N can become available in the rhizosphere due to microbial turnover and the grazing of rhizosphere microorganisms by soil micro-fauna (See Section 4.1). The quality of rhizodeposition is an important determinant for soil microbial communities; any shifts in their composition may affect decomposition processes through the production of distinct sets of extracellular enzymes (Dennis et al., 2010; Kaiser et al., 2010). Nevertheless, under conditions of low N availability, plant N uptake may limit microbial substrate N availability and reduce microbial growth and decomposition activity (Dijkstra et al., 2010; Blagodatskaya et al., 2014). Moreover, the production of specific metabolites that act as signaling molecules could

554 accelerate or retard soil N cycling if they act upon certain functional microbial taxa (De-la-Pena
555 and Vivanco, 2010). Finally, specific N cycling processes, such as denitrification or N fixation
556 could be altered in the rhizosphere due to altered microbial substrate conditions, encompassing C,
557 O_2 and NO_3^- availabilities (Philippot et al., 2009). Altogether, rhizodeposition mostly causes an
558 increase in microbial activity and soil N decomposition compared to bulk soils. Nevertheless,
559 nutrient availability in the rhizosphere and competitive interactions between plant and microbial
560 communities may shift the magnitude and direction of N cycling processes. This holds especially
561 true for those processes that are performed by phylogenetically less diverse microbial functional
562 groups; processes such as nitrification and methane uptake should therefore be much more
563 sensitive to shifts than N mineralization (Philippot et al., 2009; Dijkstra et al., 2013).

564 Although the quality and quantity of rhizodeposits clearly influence rhizosphere N
565 cycling, a major challenge lies in determining to what extent plant community characteristics
566 explain the observed variations of rhizosphere impacts (Cheng et al., 2014). Considering the
567 great difficulties in assessing rhizodeposition under field conditions (Pausch et al., 2013a), a
568 prospective approach may involve measuring 'soft' plant traits that are relatively easy to observe
569 and quantify (Fry et al., 2014). There are several traits that are good candidates due to their
570 putative intimate relationship with rhizodeposition. For example, root exudation is linked to the
571 intensity of canopy photosynthetic activity and photo-assimilate supply (Kuzyakov and Cheng,
572 2001). Fast-growing, acquisitive plants with high specific leaf area and short life span are thus
573 thought to be associated with a larger rhizosphere effect (Wardle et al., 2004). Because root
574 exudation is concentrated at the apices of the roots and at the nodes where lateral roots emerge
575 (Jaeger et al., 1999), root architectural traits determine the expansion of the rhizosphere and
576 exudate fluxes per unit of root biomass. A densely branched root system with high biomass and a

577 rapid turnover thus contributes large quantities of exudates (Van der Krift et al., 2001). The
578 chemistry of rhizodeposits is a key controlling variable of rhizosphere dynamics, as microbial
579 communities may shift their N use efficiency in response to substrate stoichiometry, leading to
580 changes in soil N cycling fluxes (Moorshammer et al., 2014).

581 Several studies have examined presumed relationships between N cycling parameters and
582 plant traits, especially of aboveground plant organs (e.g. Wedin and Tilman, 1990; Orwin et al.,
583 2010; Garcia-Palacios et al., 2013; Grigulis et al., 2013). Soil N cycling processes appear to be
584 primarily driven by traits of the most abundant species (the biomass ratio hypotheses; Grime,
585 1998), although complex effects may arise due to interspecies interactions and non-additive
586 species effects (Grigulis et al., 2013; Pausch et al., 2013b). These studies confirm that plant
587 characteristics, including under-investigated root traits, exert a key control over soil microbial
588 communities, and modify the fundamental physiologies that drive soil N cycling. Nevertheless,
589 the lack of clear-cut relationships between specific plant traits and N cycling parameters
590 indicates the necessity for more research on plant communities to establish consistent links
591 between plant traits and N cycling variables, especially under field conditions.

592

593 **4.3. Mycorrhizal associations**

594 This section will focus on the extent to which the main types of mycorrhizal symbioses,
595 arbuscular mycorrhiza and ectomycorrhiza, differentially affect the soil N cycle. Early
596 conceptual models linked the replacement of arbuscular mycorrhizal plants by ectomycorrhizal
597 plants to succession (Read, 1991) or to latitudinal and altitudinal gradients from warmer to
598 colder climates (Read and Perez-Moreno, 2003). This was considered to be driven by shifts from
599 P to N limitation, where simultaneously an increasing fraction of the N and P was present in

600 organic forms to which ectomycorrhizal fungi were supposed to have better access than
601 arbuscular mycorrhizal fungi. However, Dickie et al. (2013) noted a poor fit between these
602 models and actual data on primary succession and suggested that nutrient limitation shifts from
603 N- to P-limitation in retrogressive succession. Although a new model of general applicability has
604 not yet been proposed, the underlying idea of a fundamental difference between arbuscular
605 mycorrhiza-dominated ecosystems with more open, inorganic nutrient cycles and
606 ectomycorrhiza-dominated ecosystems with more closed, organic nutrient cycles has persisted,
607 especially for forests in temperate regions (Phillips et al., 2013; Bradford, 2014). We note that
608 the same distinction was proposed between bacterial- and fungal-dominated agro-ecosystems by
609 De Vries and Bardgett (2012). Their conceptual model is apparently not applicable for the
610 tropics, where both arbuscular mycorrhizal and ectomycorrhizal forests are characterized by an
611 open N cycle (Kuyper, 2012; Tedersoo et al., 2012). This geographical contrast raises the
612 question to what extent the nature of the mycorrhizal symbiosis is causally relevant for
613 differences in forest ecosystem functioning, or whether plant traits other than the mycorrhizal
614 symbiosis cause these differences. Arguments that the mycorrhizal symbiosis is causally relevant
615 for soil N cycling are connected to the claim that ectomycorrhizal fungi, contrary to arbuscular
616 mycorrhizal fungi, possess extensive saprotrophic activity are therefore able to make N available
617 in the soil (‘mining’) (Koide et al., 2008; Talbot et al., 2008), and therefore could access organic
618 sources of N and phosphorus.

619 Several authors have compared uptake of various amino acids by arbuscular and
620 ectomycorrhizal plants. The ability to depolymerize large N-containing molecules (proteins) into
621 smaller fragments that can be taken up (Schimel and Bennett, 2004) and the ability to increase
622 access to these large molecules, which are often bound to phenolics and other recalcitrant

compounds, have been mainly studied for ectomycorrhizal fungi. Talbot and Treseder (2010) demonstrated widespread ability among ectomycorrhizal fungi to take up amino acids and noted that the relative benefit of the symbiosis was largest for the most common amino acids. Arbuscular mycorrhizal fungi also have widespread ability to take up amino acids (Whiteside et al., 2012). Arbuscular mycorrhizal plants took up significantly larger amounts of eight amino acids (phenylalanine, lysine, asparagine, arginine, histidine, methionine, tryptophan, and cysteine) than non-mycorrhizal plants and significantly smaller amounts in the case of aspartic acid. Contrary to the hypothesis by (Talbot and Treseder, 2010) for ectomycorrhizal plants, the authors noted that the mycorrhizal effect on uptake was inversely related to the abundance of that amino acid in the database of all known proteins. The authors speculated that preferential use of rare amino acids by arbuscular mycorrhizal plants may reduce competition with ectomycorrhizal plants for amino acids. However, the extent to which this form of niche differentiation would reduce competition depends on the rate at which amino acids become available in the soil solution and hence to what extent the two preceding steps (increased access to protein - phenolic complexes; depolymerization of proteins) are rate-limiting. It is therefore necessary to assess the mycorrhizal role in those two steps.

Lindahl et al. (2007) showed an increased C:N ratio in deeper humus layers, and this effect was attributed to selective N mining by ectomycorrhizal fungi. Several studies have provided explicit support that ectomycorrhizal fungi can mine humus layers for N and have identified the relevant ectomycorrhizal fungi (Hobbie et al., 2013; Rineau et al., 2013; Bödeker et al., 2014). Wu (2011) on the other hand claimed that direct access by ectomycorrhizal fungi to N from the protein – polyphenol complex is likely limited and attributed a major role for interactions between saprotrophic and ectomycorrhizal fungi. Current evidence suggests that

arbuscular mycorrhizal fungi have neither the ability to degrade humus for N-rich compounds nor the ability to depolymerize proteins into amino acids. The widespread ability of arbuscular mycorrhizal fungi to take up amino acids may therefore not be related to closed nutrient cycles with a major role for uptake of organic nutrients, but may rather function as a scavenging mechanism to re-absorb exudates, including amino acids. More information about the role of arbuscular mycorrhiza in the uptake of organic N is provided in recent reviews by Veresoglou et al. (2012) and Hodge and Storer (2014).

The stable isotope ^{15}N has been used to study the role of mycorrhizal symbioses in accessing different N pools. Whereas early studies had examined the congruence between the ^{15}N signal of a potential N source and that of mycorrhizal fungi as evidence for uptake from that source, recent studies have emphasized the importance of N partitioning between fungus and plant (fractionation of N-depleted chitin or enriched proteins that are transferred to the plant) as a major control of isotopic composition (Hobbie and Högberg, 2012). Both the ability to take up N from organic sources (proteolytic fungi) and a relatively large transfer from fungus to plant are consistent with ^{15}N enrichment of ectomycorrhizal fungi. Both mechanisms are likely correlated as fungi in more N-limited sites transfer relatively more N per unit C at the symbiotic interface. Further study of both traits is needed to better understand ectomycorrhizal fungal isotopic signatures, and especially cases of extreme enrichment (up to 20‰) where the nature of the N source is unknown.

A corollary of the conceptual model of Phillips et al. (2013) and of earlier models is that arbuscular mycorrhizal and ectomycorrhizal plants differ in their carbon and nutrient cycling traits (decomposability and nutrient release). Data by Cornelissen et al. (2001) were consistent with that prediction, showing that the mycorrhizal trait is a predictor for the so-called ‘fast –

669 slow' spectrum (Reich, 2014). However, the comparison involved plant species that are not only
670 different with regard to the mycorrhizal trait but also with regard to a number of other traits.
671 Koele et al. (2012) applied phylogenetic correction, by comparing sister clades that differed only
672 in their mycorrhizal habit. Their data, based on 17 pairs of taxa, indicate no differences in leaf N
673 or phosphorus status after phylogenetic correction and imply that the mycorrhizal trait is
674 correlated rather than causally related with these functional differences. Other claims about
675 differences in N cycling between arbuscular mycorrhizal and ectomycorrhizal forests in the
676 northern temperate zone may similarly indicate problems of establishing whether mycorrhizal
677 status is a causally relevant or only a correlated trait. Thomas et al. (2010) showed a larger
678 positive response to N deposition by arbuscular mycorrhizal than ectomycorrhizal trees,
679 suggesting that the ability of the latter group to acquire organic N was traded off against the
680 possibility of benefitting from increased inorganic N. Midgley and Phillips (2014) reported
681 higher NO_3^- leaching in arbuscular mycorrhizal forests than in ectomycorrhizal forests, but as
682 most of the data on arbuscular mycorrhizal forests pertain to maple (*Acer saccharum*) forests, the
683 generality of that pattern needs further study.

684 Averill et al. (2014) reported that competition between ectomycorrhizal fungi / plants and
685 decomposer microbiota results in N-limitation for the latter group, which retards litter
686 breakdown and hence results in increased C storage. They noted 70% more C storage per unit N
687 in ectomycorrhizal forests than in forests dominated by arbuscular mycorrhizal trees and
688 suggested that mycorrhizal status exerts a much larger control over soil C than climatic variables
689 at the global scale. However, this effect appears to be mainly driven by boreal trees (there is a
690 dominance in the database of ectomycorrhizal trees belonging to the Pinales and Fagales, both
691 orders that are characteristic for nutrient-poor soils) and the effect is only marginally significant

692 when the analysis is performed on temperate and tropical forests (Averill et al., 2014). Therefore,
693 plant traits that are inherently associated to mycorrhizal status should further be considered when
694 assessing the key drivers of the differential C:N stoichiometry and C storage.

695 Nitrogen immobilization in the mycorrhizal mycelium may also have a large impact on
696 the N cycle by reducing mineral N availability for plants. The general claim that mycorrhizal
697 symbioses are beneficial for the plant and that cases of a negative plant performance in the
698 mycorrhizal condition are explained by C costs of the symbiosis was refuted by Côrrea et al.
699 (2012), who concluded that smaller plant size was caused by lower N uptake. Lower N content
700 of the ectomycorrhizal plant could be due to mycorrhiza-driven progressive N limitation (Luo et
701 al., 2004). Alberton et al. (2007) showed this to be the case as plant N content was significantly
702 negatively correlated with hyphal length. Näsholm et al. (2013) showed that immobilization of N
703 in the ectomycorrhizal mycelium can aggravate plant N limitation. They modelled competition
704 between plant and fungus for N in a market model, and concluded that at N limitation the
705 symbiosis does not alleviate plant N limitation but in fact even reduces plant growth (Franklin et
706 al., 2014; Kuyper and Kiers, 2014). Yet, despite this negative effect on plant performance, a
707 non-mycorrhizal strategy is competitively inferior, and therefore trees are trapped as they cannot
708 terminate the association. Because the biomass of the arbuscular mycelium is usually one or two
709 orders of magnitude smaller than that of the ectomycorrhizal mycelium, the amount of N
710 immobilized by the arbuscular mycorrhizal mycelium is sometimes hypothesized to be
711 quantitatively unimportant from the plant's perspective. However, recent studies (Hodge and
712 Fitter, 2010; Grman and Robinson, 2013) indicate that N uptake and immobilization by
713 arbuscular mycorrhizal fungi can also reduce plant performance.

Other pathways through which the mycorrhizal symbiosis may affect soil N cycling are modification of root exudation, root architecture, and fine root turnover (Churchland and Grayston, 2014). It is important to determine which of these differences are caused by the symbiosis and which by other root trait differences among species. For example, Comas et al. (2014) found that, after accounting for phylogenetic relations, ectomycorrhizal plants have thinner roots and greater branching intensity than arbuscular mycorrhizal plants. It is therefore still a matter of debate whether differences with respect to the mycorrhiza-associated nutrient economy (Phillips et al., 2013) are controlled by the mycorrhizal trait, or whether the mycorrhizal trait is instead correlated with causally relevant plant and climate traits.

4.4. N_2 fixation

An important share of bioavailable N enters the biosphere via biological fixation of atmospheric N_2 (BNF) (Vitousek et al., 2013). Biological N fixation can be natural (e.g. N_2 fixing trees that are present in forest ecosystems) or anthropogenic (e.g. N_2 fixation by leguminous agricultural crops). Two types of BNF, both using the nitrogenase enzyme, are present in nature: symbiotic N_2 fixation (S-BNF) and free-living N_2 fixation (F-BNF). Symbiotic N_2 fixation is here defined via the infection of plant roots by bacteria - such as *Rhizobia*, *Bradyrhizobia* or actinomycetes - followed by the formation of nodules. All other forms of BNF are regarded as free-living N_2 fixation (including e.g. fixation by bacteria in soil and litter, but also N-fixation in lichens) (Reed et al., 2011). Here we highlight the importance of N_2 fixation for N budgets in pristine tropical forest, peatlands and cryptogamic soil crusts, as well as for sustainable production of biofuels.

Nitrogen demand in young successional tropical forest is high. The large fraction of leguminous plant species that forms symbiosis with N_2-fixing bacteria has recently been identified as a key element of functional diversity to overcome ecosystem-scale N deficiencies in tropical forest successions (Batterman et al., 2013a). Symbiotic fixation is thus considered to relieve N limitations and safeguard forest regrowth and CO_2-accrual as an ecosystem service. Nevertheless, S-BNF has also been postulated as the reason why mature tropical forest, having a lower N-demand than early succession stands, become relatively rich in N and as a consequence loses (sometimes large amounts of) bioavailable N (Hedin et al., 2009) via NO_3^- leaching (e.g. Brookshire et al., 2012) or gaseous N loss (e.g. Werner et al., 2007).

However, a plant-level physiological perspective counters this assumption, as numerous experiments have shown that symbiotic S-BNF by leguminous species is mostly facultative and down-regulated when located in an N-rich environment. Tropical leguminous species thus have the potential to fix atmospheric N_2, but it is likely that they only do so actively in young forest successions or disturbed ecosystems, and far less in mature forests. Secondly, only a part of the *Fabaceae* family has nodule-forming capacities (mainly belonging to the *Mimosoideae* and *Papilionoideae* subfamilies). This consideration decreases the omnipresence and abundance of potential N-fixers in tropical forests, making their role as a vital chain in the tropical N-cycle less credible. Therefore, Hedin et al. (2009) have suggested a possible mechanism for explaining this tropical N paradox via a 'leaky nitrostat model' (Fig. 5). This concept brings forward the importance of F-BNF, which is hypothesized to take place, even in N-rich ecosystems, in localized N-poor microsites, such as litter layers, topsoil, canopy leaves, lichens or bryophytes on stems, etc. Combined, these free-living N_2 fixers would bring high amounts of N in the system, resulting in high N availability. However, spatially explicit data are virtually absent and

759 largely based on geographically biased, indirect measurements using the acetylene reduction
760 assay rather than direct $^{15}N_2$ incubation measurements.

761 A recent spatial sampling method to assess total BNF indicated that tropical forest BNF is
762 likely much lower than previously assumed (Sullivan et al., 2014). These authors reported mean
763 rates of total BNF in primary tropical forests of 1.2 kg N ha^{-1} yr^{-1}, while previous empirical or
764 modeled data ranged between 11.7 and 31.9 kg N ha^{-1} yr^{-1}. Secondary successional forests, as
765 mentioned above, had higher total BNF than primary forest (6.2 – 14.4 kg N ha^{-1} yr^{-1}). Sullivan
766 et al. (2014) proposed a time-integrated total BNF rate of 5.7 kg N ha^{-1} yr^{-1} for primary forest in
767 Costa Rica, of which 20-50% is attributed to S-BNF. It remains to be shown whether this BNF
768 rate from primary tropical forest and proportions between S-BNF and F-BNF are valid for the
769 pan-tropics. But if total BNF in tropical forests is indeed much lower than previously thought,
770 this will fundamentally alter our assessment of tropical forest N cycles and the relative
771 contribution of anthropogenic inputs (Sullivan et al., 2014). There is indeed emerging evidence
772 that anthropogenic N deposition in tropical ecosystems is more substantial than assumed, as a
773 result of biomass burning, dust and biogenic deposition (Chen et al., 2010; European
774 Commission-Joint Research Center, 2014; Cizungu et al., unpublished data). Hence, the relative
775 contribution of human perturbation (e.g. wild fire, livestock fossil fuel combustion) to the
776 tropical N cycle is likely much larger and warrants careful attention, e.g., by increasing N
777 deposition measurement networks in tropical forests (Matson et al., 1999). Moreover, there is
778 only limited understanding of the effects of proximate (N-, P- and Mo-availability) controls
779 (Barron et al., 2009; Wurzburger et al., 2012; Batterman et al., 2013b), and the impact of global
780 change factors (temperature, moisture, N-deposition) on F-BNF.

781 In boreal forests, symbiosis between cyanobacteria and feather mosses provides an

important N-input (DeLuca et al., 2002; Gundale et al., 2012). In peatlands, which contain approximately 30% of global soil carbon, *Sphagnum* mosses living in close association with methanotrophic bacteria, which can stimulate BNF and constitutes an important mechanism for N accumulation in peatlands (Larmola et al., 2014). These authors found N_2 fixation rates between 1 and 29 kg N ha^{-1} yr^{-1}, up to 10 times larger than current atmospheric N deposition rates. This also shows that N_2 fixation contributes considerably to the N budget of peatlands. Cyptogamic covers that consist of cyanobacteria, algae, fungi, lichens and bryophytes are suggested to account for ca. half (49 Tg N) of the biological N_2 fixation on land (Elbert et al., 2012). From a sustainable agronomic management point of view, associative N_2 fixation could be promoted in certain crops. For example, field experiments with sugar cane and *Miscanthus* with little N input showed that a substantial portion of new plant N was derived from N_2 fixation (Keymer and Kent, 2014).

While large uncertainties exist regarding the temporal and spatial variability, dominant determinants, and the magnitude and impact of BNF on terrestrial ecosystems functions and services; even less is known regarding its future trajectories in view of global change.

5. Conclusions

This is an exciting time to study the soil N cycle. Years of surprising findings on unanticipated pathways and mechanisms have expanded the horizons of researchers. These findings have stimulated efforts to develop and test new methods for quantifying these processes. This has resulted in a better understanding of the complexity of soil N cycling processes as well as powerful tools for future exploration.

Critical challenges remain. Many processes are still difficult to quantify and variability and heterogeneity hamper our ability to provide well constrained estimates relevant to water and air quality issues. We postulate that addressing the issues formulated above would constitute a comprehensive research agenda with respect to the N cycle for the next decade. Particularly, we urge the following blueprint for action:

1. Abandoning the long-disproven but persistent assumption that gaseous N production in soils is the exclusive result of the interplay between nitrification and denitrification, and to focus on a better assessment of alternative pathways;

2. Dedicating scientific efforts to the continuing development of improved techniques for the characterization, quantification, and modelling of alternative N transformation pathways, eventually in conjunction with state-of-the-art molecular techniques to determine the functional microbial communities involved; and

3. Consider ecological interactions and trophic cascades as indirect but essential drivers of soil N cycling, in particular in response to global change.

Success will require interactions between soil science and other disciplines that address both smaller (e.g., molecular and microbial) and larger (ecosystem, landscape and regional) scales. We believe that such an agenda would help us meet future challenges on food and energy security, biodiversity conservation as well as climate stability.

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Author contributions

All authors contributed to selecting the topics addressed in this manuscript. P.B. wrote the sections on BNF and N_2O consumption; T.R. wrote the section on ^{15}N models; D.H. and Th.W.K co-wrote the section on mycorrhizal associations; D.H. wrote the section on rhizodeposition and plant traits; I.M.L and J.W.V.G. co-wrote the section on soil fauna; J.W.V.G wrote the section on nitrifier denitrification; P.G. wrote the section on denitrification. J.W.V.G., D.H. and P.G. co-wrote the remaining sections. All authors commented on the final draft.

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Figure captions

Figure 1. New insights and key challenges with respect to the soil N cycle, as identified in this paper. These include three N cycling processes (Sections 2.1 - 2.3), a modelling challenge (Section 3) and four pathways through which ecological interactions might affect N cycling processes (Sections 4.1 - 4.4).

Figure 2. Different pathways of N_2O production in soil. Classical nitrification by autotrophic bacteria or archaea (nitrifier nitrification); nitrifier denitrification by the same group of autotrophic bacteria; nitrification followed by denitrification (nitrification-coupled denitrification) and direct denitrification of applied nitrogen fertilizer (fertilizer denitrification). Reproduced from Kool et al. (2011a).

Figure 3. The N_2O production and consumption network showing five pathways for N_2O consumption. Dissimilatory N_2O reduction to N_2 via typical, denitrifier nosZ I (1), atypical, non-denitrifier nos Z II (2), dissimilatory NO_3^- reduction to NH_3 (DNRA) (3), direct assimilatory N_2O fixation via nitrogenase to NH_3 (4), and indirect assimilatory N_2O fixation (N_2O reduction to N_2 followed by N_2 fixation) (5); abiotic pathways that produce gaseous N (Feammox and chemo-denitrification are not shown).

Figure 4. The influence of soil fauna on soil N processes and loss pathways. Conventionally (a), these processes and loss pathways were often considered as the result of interactions between microbes and soil structure. More recently (b), it is recognized that many microbial and physical

properties are influenced by faunal diversity through trophic relations and through changes in the soil structure by ecosystem engineers.

Figure 5. The 'leaky nitrostat' model adapted from Hedin et al. (2009). This model indicates the importance of symbiotic (S-BNF) and free-living (F-BNF) biological N_2 fixation along a forest successional gradient, from young (green) to mature (red) forest stands. At the initial stages of ecosystem succession, the N supply via S-BNF, F-BNF and N deposition supports high ecosystem N demands. In mature forest stands with a lower N demand, S-BNF is down-regulated, but N inputs via F-BNF and N deposition lead to ecosystem N losses via N leaching and gaseous N production.