Potential effects of arbuscular mycorrhiza fungi on denitrification potential activity and nitrous oxide (N_2O) emissions from a fertile agricultural soil

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Foreword

This dissertation is a cumulative work of manuscripts from my publication list, either published or to be submitted.

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Summary

This doctoral thesis reports on the relevance of arbuscular mycorrhizal fungi (AMF) on denitrification potential activity and nitrous oxide (N_2O) emissions from a native fertile agricultural soil. Agricultural soils are the main source of N_2O, a powerful greenhouse gas contributing to on-going climate change and destruction of our stratospheric ozone layer. There is a growing recent scientific interest in understanding of the significance of AMF, widespread symbiotic soil fungi, on denitrification and N_2O emissions. However, most of the existing studies have performed reductionist experiments with single plant host systems grown under artificial soil substrates and have highlighted that the composite effect of AMF reduces denitrification and N_2O emissions.

In Chapter 2, we reviewed the role of AMF on environmental controls of denitrification and N_2O emissions. In Chapter 3, to address direct vs indirect effects of AMF on denitrification potential opposed to composite effects, we performed a realistic experiment where we combined a manipulation of two factors: AMF and soil aggregation (state which represents the native microhabitats of denitrifying microbes) through either crushing mechanically soil aggregates or using agricultural soil with intact soil aggregates. Furthermore, we added AMF inoculum or not in the form of *Rhizophagus irregularis* into the indigenous AMF community in both soil aggregate treatments. We then grew a single host plant *Zea mays* in compartmentalized mesocosms for 4 months. Our results were inconsistent with the existing literature, highlighting that AMF reduced denitrification and N_2O emissions. In contrast, using a denitrification enzyme activity (DEA) assay, we found that potential N_2O emission rates and the denitrification potential ratio of potential N_2O emission rates over total potential denitrification activity were higher in the undisturbed soil and in the mesocosms with higher AM hyphal densities (direct effect of AMF). Further, AMF-promoted soil aggregation increased both denitrification potential parameters (indirect effect of AMF). In Chapter 4, we explored how AMF might behave in a typical European grassland plant community experimental setting with a realized gradient in plant diversity. A gradient in plant diversity emerged from stochastic differences in plant seedling establishment across the sixteen replicates, irrespective of AMF treatment. Furthermore, plant diversity mitigated potential emission rates of N_2O while AMF reduced microbial denitrification potential activity and the denitrification potential ratio. Our results were incongruent to the existing literature, indicating that the interactive effects between AMF and plant diversity mask their

independent contributions to the denitrification ecosystem process. We presented a novel holistic picture of AMF functioning on denitrification potential in natural grassland systems.

Finally, in Chapter 5 we tested whether AMF also mitigates current N_2O emissions when there are no differences in nitrate (NO_3^-) availability between AMF treatments at harvest using a gas-flow-soil-core incubation system. We found that AMF reduced the convexity of the cumulative N_2O emission curves in inoculated agricultural soil cores with AMF compared to noninoculated incubated in oxic and anoxic conditions. A reduction in the convexity meant a decrease in microbial denitrification activity rates and N_2O emissions from soil cores by AMF. We found here the first compelling evidence that AMF hyphae abundance mitigates current cumulative N_2O emissions originating probably from nitrification and denitrification under native fertile agricultural soils, irrespective of initial soil NO_3^- concentrations.

By performing realistic experiments and using unsterilized arable soil with intact soil aggregates inoculated or not with a model AMF species, *R. irregularis*, we increased the ecological relevance of our results by demonstrating that AMF strongly influence N_2O emission rates, but also denitrification potential activity and denitrification ratio compared to the existing studies. The results presented here point out that managing the AM plant symbiosis could have significant implications for better management of N_2O emissions from agricultural soils.

Zusammenfassung

Diese Doktorarbeit berichtet über die Relevanz von arbuskulären Mykorrhizapilzen (AM-Pilze) für das Denitrifikationspotenzial und Emissionen von Distickstoffmonoxid (N_2O) aus natürlichen, fruchtbaren Ackerböden. Ackerböden sind eine bedeutende Quelle an N_2O, einem äußerst wirksamen und schädlichen Treibhausgas, welches zum Klimawandel und der Zerstörung der Ozonschicht beiträgt. Es gibt in den letzten Jahren ein steigendes wissenschaftliches Interesse daran, zu verstehen welche Bedeutung AM-Pilze, eine Gruppe weit verbreiteter symbiotischer Pilze, für den Denitrifikationsprozess und für N_2O-Emissionen haben. Die meisten Studien zu diesem Thema haben bisher mit stark vereinfachten Experimenten gearbeitet, z. B. mit einzelnen Wirtspflanzen, die in künstlichen Bodensubstraten wuchsen. Es konnte hierdurch jedoch bereits herausgestellt werden, dass der Gesamt-Effekt von AM-Pilzen die Denitrifikation und dadurch N_2O-Emissionen reduziert.

In **Kapitel 2** haben wir die Rolle von AM-Pilzen bei der Denitrifikation und N_2O-Emissionen in einem Review zusammengefasst.

Um die direkten versus die indirekten Effekte von AM-Pilzen auf das Denitrifikationspotenzial im Vergleich zu gesamtheitlichen Effekten zu untersuchen, haben wir in **Kapitel 3** ein realistisches Experiment durchgeführt, bei dem wir zwei Faktoren manipuliert haben: AM-Pilze und den Zustand der Bodenstruktur. Die Bodenstruktur stellt das natürliche Mikrohabitat für denitrifizierende Mikroorganismen dar. Wir veränderten die Bodenstruktur eines Ackerbodens entweder durch mechanische Zerkleinerung der Bodenaggregate oder beließen die Bodenaggregate intakt. Außerdem haben wir zu einem Teil der Versuchseinheiten ein AM-Pilz-Inokulum in Form von *Rhizophagus irregularis* Sporen hinzugefügt zur bereits vorhandenen AM-Pilz-Gemeinschaft. Für 4 Monate ließen wir als Wirtspflanze Mais (*Zea mays*) in kompartimentierten Töpfen wachsen. Unsere Ergebnisse stimmten nicht mit der vorhandenen Literatur überein, welche bisher gezeigt hat, dass AM-Pilze Denitrifikation und N_2O-Emissionen reduzieren können. Im Gegensatz dazu haben wir durch die Untersuchung der Denitrifikations-Enzym-Aktivität (DEA) herausgefunden, dass die potenziellen N_2O-Emissionsraten und das Denitrifikationspotenzial-Verhältnis (potenzielle N_2O-Emissionsraten zu gesamter Denitrifikationsaktivität) höher waren im ungestörten Boden und in den Böden mit einem dichten AM-Pilz Myzelnetzwerk (direkter Effekt der AM-Pilze). Darüber hinaus hat die durch AM-Pilze verbesserte Bodenaggregation beide Denitrifikationspotenzial-Parameter erhöht (indirekter Effekt der AM-Pilze).

In **Kapitel 4** haben wir untersucht wie AM-Pilze sich in einem Versuchsaufbau mit europäischen, natürlichen Grasland-Pflanzengemeinschaften mit einem Pflanzendiversitäts-Gradienten verhalten könnten. Wir haben gefunden, dass AM-Pilze nicht mit der Pflanzendiversität zusammenhingen. Jedoch ist - unabhängig von der Behandlung mit AM-Pilzen - ein Gradient bei der Pflanzendiversität durch stochastische Unterschiede in der Etablierung der Setzlinge über alle 16 Replikate aufgetreten. Darüber hinaus hat die Pflanzendiversität die potenziellen Emissionsraten an N_2O reduziert, während die AM-Pilze die gesamte mikrobielle Denitrifikationspotenzial-Aktivität und das Denitrifikationspotenzial-Verhältnis reduzierten. Unsere Ergebnisse stimmten nicht mit der vorhandenen Literatur überein, was darauf hinweist, dass die interaktiven Effekte zwischen AM-Pilzen und Pflanzendiversität ihre unabhängigen Anteile am Ökosystemprozess Denitrifikation überdecken. Wir zeigten ein neues holistisches Bild der Funktionen von AM-Pilzen auf das Denitrifikationspotenzial in natürlichen Grasland-Systemen.

Schließlich haben wir in **Kapitel 5** mit einem neuartigen Inkubationssystem (Gas-Fluss-System gekoppelt an ein Bodenbehältnis) getestet, ob AM-Pilze N_2O-Emissionen mindern können, wenn es keine Unterschiede in der Verfügbarkeit von NO_3^- zwischen den AM-Pilz-Behandlungen gibt. Die AM-Pilze konnten - sowohl unter oxischen als auch anoxischen Bedingungen - in den mit AM-Pilzen inokulierten Behandlungen die Konvexität der kumulativen N_2O-Emissionskurven verringern, im Gegensatz zu den Behandlungen ohne Inokulation. Eine verringerte Konvexität der Kurve bedeutet eine Reduzierung der mikrobiellen Denitrifikationsaktivitätsrate und N_2O-Emissionen vom Boden durch AM-Pilze. Wir fanden hier die ersten überzeugenden Belege dafür, dass die Hyphen der AM-Pilze gegenwärtige kumulative N_2O-Emissionen vermindern können, die wahrscheinlich durch Nitrifikation und Denitrifikation aus natürlichen fruchtbaren Ackerböden stammen, unabhängig von der Ausgangskonzentration an Nitrat im Boden.

Unsere Ergebnisse sind von hoher ökologischer Relevanz, weil sie deutlich zeigen, dass AM-Pilze einen starken Einfluss haben auf reelle Denitrifikationsraten, die Denitrifikationspotenzial-Aktivität und N_2O-Emissionsraten unter realistischen experimentellen Bedingungen, die vergleichbar sein könnten mit einer Situation im Feld. Die hier gezeigten Ergebnisse verdeutlichen, dass eine Steuerung der AM-Pilz-Symbiose signifikante Implikationen haben könnte für ein verbessertes Management von N_2O-Emssionen aus Ackerböden.

Chapter 1 :

General introduction

Global nitrous oxide, Agro-ecosystems and Climate change

Between 2010 and 2050, we expect food demand to increase by 50-90% (Springmann et al., 2018). Though agriculture significantly contributes to sustain food security, it represents one of the largest drivers of global warming and climate change, globally. Agriculture, forestry and land use changes represent the second largest source of anthropogenic gas emissions (Carbone dioxide (CO_2), Methane (CH_4) and Nitrous oxide (N_2O)) after electricity and heat production accounting to approximately 24 % of total global greenhouse gases (GHG) emissions (IPCC, 2014). A major GHG which is responsible for approximately 10 % of the realized global warming is N_2O (IPCC, 2014). A 70 % of world's N_2O originates from the agricultural sector (IPCC, 2014). Global N_2O emissions and mean surface temperature are on the rise and expected to increase further (IPCC, 2018). Specifically, global soil N_2O emissions have strongly increased from 6.3 ± 1.1 Tg N_2O-N yr^{-1} in the pre-industrial period (the 1860s) to 10.0 ± 2.0 Tg N_2O-N yr^{-1} in the recent decade (2007-2016) and cropland soil N_2O emissions account for 82 % of the total increase (Tian et al., 2018).

Significance of N_2O

N_2O is a powerful greenhouse gas. On a molecular basis, N_2O has 310 and 16 times higher global warming potentials than that of CO_2 and CH_4, respectively, over a 100-year period (Forster et al., 2007). Besides, it has the longest atmospheric lifetime of about 150 years (IAEA, 1992). Furthermore, the breakdown of N_2O to NO in the stratosphere results in the depletion of the ozone layer (Ravishankara et al., 2009). Currently, N_2O is the single most important ozone-depleting substance, expected to remain the largest throughout the 21st century and doubling its concentration in the atmosphere would result in a 10 % decrease of the ozone layer (Forster et al., 2007; Ravishankara et al., 2009). Increased N_2O emissions represent therefore, not only a national potential threat, but also a global threat to global warming, food production and human health.

State-of-the art methods for quantification of soil potentials for denitrifying activity and N_2O production

The most widely used technique to assay denitrification is the denitrifying enzyme activity (DEA) technique. This technique was proposed by Yoshinary and Knowles (1976) as a way of assessing the potential optimum activity of existing denitrifying enzymes in soil. We understand by microbial DEA, the total potential denitrification rates which develop from the soil slurries under idealized anaerobic conditions with abundant available C and N in the presence of C_2H_2. Total denitrification potential rate is thus a proxy of maximal DEA in soils (Tiedje et al., 1989; Pell et al., 1996). The measurement principle of total denitrification potential activity (the sum of N_2O + N_2), in C_2H_2-treated soil samples, is based on the assumption that every N_2 molecule which would normally escape from the soil system, remains in the form of N_2O and is detected by gas chromatograph (Pell et al., 1996). DEA is considered to be an important factor in identifying the main driver of denitrification and N_2O production in soils (Tiedje et al., 1989). DEA provides a "snap-shot" picture of the microbial denitrifying potentials in the soil at the time of sampling (Tiedje et al., 1989; Pell et al., 1996). Furthermore, it allows for the possibility of running a significant number of soil samples at the same time and it is cost-effective compared to the other approaches e.g., isotopic methods. The DEA assay (see Chapter 3 and 4) was used in this thesis alongside novel gas-flow-soil-core incubation technique (see Chapter 5) because of some drawbacks of DEA like C_2H_2 inhibition of nitrification and the absence of significant differences between experimental treatments in NO_3^- availability in soils (Felber et al., 2012; Butterbach-Bahl et al., 2013). This newly developed system for simultaneous measurements of cumulative N_2 and N_2O total emissions from intact soil cores has been validated successfully (Wang et al., 2011; Butterbach-Bahl et al., 2013).

Factors affecting denitrification

Environmental factors that can affect denitrification have been categorised as proximal and distal regulators (Groffman et al., 1988; Wallenstein et al., 2006). Although it is well established that proximal factors (e.g., NO^-_3 and C availability, O_2, pH) affect denitrification, we are still struggling to understand the role of distal factors (e.g., soil moisture, soil structure, plant community) (Groffman et al., 1988; Wallenstein et al., 2006; Senbayram et al., 2011; Morales et al., 2015). Moreover, there are substantial knowledge gaps regarding the ecological interactions between soil biota and potential denitrification activities.

Biology of arbuscular mycorrhiza fungi (AMF)

A key biotic factor that can strongly interact with denitrifiers and influence denitrification in soil is soil biota. However, existing studies addressing how soil biota influence denitrification rates and N_2O emissions are to a large degree limited to earthworms and nematodes (Djigal et al., 2010; Bradley et al., 2011). Overall, the significance of symbiotic microorganisms on the regulation of microbial denitrification potential and related potential N_2O emissions has not been specifically addressed. A key player in the rhizosphere of plants that could potentially influence denitrification activity and N_2O production is AMF.

AMF are crucial for the functioning of terrestrial ecosystems, they form symbiotic interactions with terrestrial plants and colonize the majority of plant species including many grassland plants and crops (Smith and Read, 2008). AMF are a monophyletic group (Schüßler et al., 2001), recently moved to the subphylum Glomeromycotina in the phylum Mucoromycota (Spatafora et al., 2016). The characteristic structures of the symbiosis are the arbuscules, tree like structures that are located in the apoplast of the root (Bonfante and Genre, 2010). Arbuscules have a large surface area and are thought to be sites of nutrient exchange between the AMF and plant host and this could have strong implication for nutrient cycling processes, e.g., the cycling of N (Bonfante and Genre, 2010; Veresoglou et al., 2012). AMF also produce an extraradical mycelium which are hyphae that extend out into the soil and beyond the plant root system (Bonfante and Genre, 2010). AM hyphal length densities can be 13 times higher than root lengths in soils (Camenzind and Rillig, 2013). Length is a common parameter used in quantifying fungal hyphae densities and AMF functions are related to them.

Effects of AMF on denitrification and N_2O emissions

AMF have been reported to modulate potential nitrification rates; but the significance of mycorrhiza on potential denitrification rates had been overlooked (Veresoglou et al., 2011). Few recent advances in mycorrhizal research demonstrated that AMF reduced N_2O flux from soils (Bender et al., 2014; Lazcano et al., 2014; Zhang et al., 2015; Bender et al., 2015; Storer et al., 2017). These previous studies only measured N_2O fluxes which because they depend strongly on substrate availability vary considerably in space and in time. This is particularly the case for controlled experiments where exchangeable nutrients often depleted at the late stages of plant growth. Furthermore, quantifying N_2O fluxes is not, per se, proof that denitrification is occurring. The reason for this is that the absence of N_2O flux from a

denitrifying environment may simply suggest that denitrification has gone to completion and instead formed N_2. DEA assay has been successfully used in this respect to correct this, because this technique forces all N reduced to accumulate as N_2O due to a specific inhibition of nitrous oxide reductase (*Nos*) by C_2H_2 (Pell et al., 1996; Philippot et al., 2011). Thus, a major limitation is that these abovementioned studies did not assay DEA which represents the potential maximum activity of existing microbial denitrifying enzymes which catalyse N_2O production and consumption in soils and reflects the field denitrification potential (Morales et al., 2015; Krause et al., 2017).

In a grassland experiment with *Lolium multiflorum* var. Oryx plant monoculture, Bender et al. (2015) found that the N_2O flux was lower following the application of NO_3^- in the AM plants compared to nonAM plants. Storer et al. (2017) reported that AMF hyphae alone were sufficient to reduce N_2O production. This reduction was linked to AMF-reduced soil NH_4^+ concentration from soil. Storer et al. (2017), however, found no effect of AMF hyphae on N_2O flux after NO_3^- concentrations addition and Bender et al. (2015) after NH_4^+ addition. Furthermore, Lazcano et al. (2015) showed that a reduction in N_2O fluxes was linked to the higher use of soil water by AMF rather than increased N uptake. These results clearly indicate that the effects of AMF on N_2O emissions are not fully understood.

It is feasible that other AMF mechanisms than the abovementioned could potentially alter denitrification and related N_2O production from soils (Bender et al., 2014). Most of these previous studies have performed reductionist experiments with a single AM plant species host planted on sterilized and reinoculated artificial soil substrates which do not resemble the native habitat of microbial denitrifiers and AMF community. These specific growth settings could have changed the relative importance of N_2O consumption and reduction and thus yield a biased view on the role mycorrhiza plays in denitrification and N_2O formation from soil. Likewise, these studies have been limited to describe direct effect of AMF (i.e effect of AM hyphal growth) on actual denitrification rates and related N_2O emissions. Two major factors by which AMF can exert a strong control on denitrification are soil aggregation and plant community. The effects of AMF on the state of soil aggregation and plant community and how these effects are indirectly related to DEA and N_2O potential emissions might provide advance new insights in the cycling of N.

Interaction between AMF and soil aggregation on DEA and N_2O emission rates.

Soil aggregates play a pivotal role in the development of soil structure and consist of soil particles which are held together by binding agents (Tisdall and Oades, 1982). The effect of soil aggregation on microbial DEA and related N_2O potential emissions has not been deeply explored. Recently, soil aggregates have been conceptualized as massively concurrent evolutionary incubators (Rillig et al., 2017). Soil aggregates also represent the natural microhabitats of denitrifiers where most of the denitrification takes place (Sexstone et al., 1985; Schlüter et al., 2018; Wang et al., 2018). The size of soil aggregate is one of the most important factors that affect strongly microbial activity and N_2O production and consumption (Drury et al., 2004; Khalil et al., 2005; Diba et al., 2011; Wang et al., 2018). Available C and N and oxygen concentration profiles within and outside of soil aggregates can significantly affect DEA and N_2O production (Sexstone et al., 1985; Diba et al., 2011; Schlüter et al., 2018). Some studies found higher DEA and N_2O production in larger aggregates or macroaggregates (> 0.25 mm) compared to smaller aggregates or microaggregates (< 0.25 mm) (Drury et al., 2004; Khalil et al., 2005; Diba et al., 2011). Macroaggregates have been associated with larger concentrations of soil organic C and N compared to microaggregates (Khalil et al., 2005; Diba et al., 2011). Schlüter et al. (2018) found that smaller aggregates were better supplied in O_2 concentration compared to larger aggregates so that the onset of N_2O emission was earlier in small aggregates, but delayed in larger aggregates which finally produced larger N_2O emission on the long term. In contrast, other studies reported higher DEA and N_2O production in small aggregates harbouring more anaerobic microsites than macroaggregates (Seech and Beauchamp, 1988; Khalil et al., 2005; Uchida et al., 2008; Sey et al., 2008). These authors found that labile C rather than NO_3 was the limiting factor in DEA in larger aggregates. Some recent studies demonstrated that microaggregates may hold more diverse microbial communities than macroaggregates (Rillig et al., 2017; Bach et al., 2018). For e.g., more denitrifiers have been found in microaggregates than in macroaggregates (Sey et al., 2008). Wang et al. (2018) reported that the majority of compiled studies supported an overall positive relationship with aggregate size, of which more than half found more N_2O production from macro-aggregates than micro-aggregates. These results are thus contrasting and demonstrate that the effect of soil aggregation size on DEA and N_2O production warrants to be deeply understood.

Most of these studies have been focusing on the effects of aggregate size while very few studies reported on the impact of soil disaggregation-aggregation (aggregate turnover), a

process that occurs in managed agricultural systems, for example following a disturbance, such as tillage. It has been shown that soil biota including AMF can strongly influence the state of soil aggregation (Lehmann et al., 2017). Soil aggregation represents a dynamic equilibrium state of two processes: formation of soil aggregates by soil biota and disaggregation resulting from the gradual degradation of the binding agents in the aggregate (Caruso et al., 2011). Any change in the state of soil aggregation by AMF might have strong implications for denitrification and related N_2O production (Wang et al., 2018). To the best of our appreciation, the role of AMF on DEA and N_2O potential emissions in relation to soil aggregation formation is still unknown. The existing literature on AMF and denitrification has largely overlooked to segregate between direct (i.e effect of AMF hyphae growth) and indirect effects (i.e effect of AMF on soil aggregation promotion) on denitrification potential activity and N_2O emissions.

AMF hyphae have been reported to promote soil aggregation by enmeshing and entangling soil particles and binding agents together and thereby facilitating macroaggregate formation and increasing the volume of pore space between aggregate particles (Rillig and Mummey, 2006; Rillig et al., 2010; Leifheit et al., 2014; Lehmann et al., 2017). Promotion of macroaggregation formation might be a potential distal AMF mechanism which could affect denitrification and related N_2O emissions via soil aeration alteration (Zumft, 1997; Burgin and Groffman, 2012). Moreover, it is now largely acknowledged that AMF play a key role in the nutrient cycling of N (Veresoglou et al., 2012; Hodge and Storer, 2015).

Interactive effects of AMF and plant community on DEA and N_2O emission rates.

The effects of plant species diversity and composition on DEA and potential N_2O emissions remain largely unexplored (Abalos et al., 2014; Niklaus et al., 2016). As a result, there is a growing recent interest in understanding how changes in plant diversity and composition regulate denitrification potential and N_2O emissions, particularly in grassland systems where the manipulation of soil organisms to promote reliance on soil biological processes of nutrient turnover is central to sustainable production (Niklaus et al., 2016; Han et al., 2016; Abalos et al., 2017). Plant diversity (i.e., the number of different species present) and/or community composition (i.e., the particular species present) may potentially affect the functional capacity of denitrifier and N_2O emissions by influencing abiotic and biotic soil factors (Bremer et al., 2007; Han et al., 2016; Niklaus et al., 2016). These factors include mineral N availability in

soil, and root C exudates sources for denitrifiers, soil pH, soil structure and microbial denitrifier community (Bremer et al., 2007; Abalos et al., 2017). Some recent studies reported that plant diversity reduced DEA and N_2O production via enhanced N removal efficiency and below-ground plant complementary trait effects e.g., differences in root C exudates and morphologies in soils (Niklaus et al., 2016; Han et al., 2016; Abalos et al., 2017) while others reported the opposite (Bremer et al., 2007; Sutton-Grier et al. 2011). The mechanisms underlying these responses remain largely obscure. For example, Abalos et al. (2014) established monocultures and two- and four species mixtures of common grass species with diverging functional traits. They found lower N_2O emissions in plant species identity of monocultures and two-species mixtures while four species mixtures of common grass species increased N_2O emissions.

Most of these studies have investigated the role of aboveground plant traits on denitrification process with single plant species, several plant species belonging to the same functional group or plant community with a low diversity. Moreover, these studies have provided a limited mechanistic understanding in the role of belowground symbiotic microorganisms on denitrification. Understanding the functional interaction between aboveground plant community and belowground plant associated rhizospheric microbiota such AMF in the plant-soil systems could provide advance new insights into the microbial denitrification process. However, the existing literature on AMF and denitrification has performed reductionist experiments with single plant species systems which are relevant for agricultural settings, but not for native grassland systems with a more diverse plant community.

It is well established that AMF promote plant diversity (Van der Heijden et al., 2002, Lin et al., 2015), but our understanding on how this effect relates to denitrification activity and N_2O production in experimental grassland plant diversity settings remains largely unknown. By promoting plant diversity, AMF could be enhancing the N uptake of subordinated plants compared to dominant plants (Lin et al., 2015) and the depletion of soil C and N availability (Govindarajulu et al., 2005; Tanaka et Yano, 2005; Atul-Nayyar et al., 2009; Veresoglou et al., 2012; Hodge et Storer, 2015) and thereby lowering the magnitude of N_2O production and functional activity of denitrifiers because plants affect denitrification by providing C as an energy source for denitrifier communities through root C exudates and/or by mediating soil N availability via N uptake (Niklaus et al., 2016; Abalos et al., 2017; Han et al., 2016, but see Zak et al., 2003). We can hypothesize that highly AM dependent plants may have more access to soil nutrients and may deplete soil more efficiently from nutrients compared with plants

that are less dependent on AMF and this would have strong impact on plant diversity and denitrification potential activity. Finally, the efficiency with which AMF and plant derive benefits (e.g., soil resources, plant productivity) from the symbiosis could differ considerably with more than one symbiotic plant partner (Selosse and Rousset, 2011) and this could strongly alter the direction and magnitude of denitrification potential and N_2O emissions.

Introduction to chapters and aims

This thesis reports on the relevance of AMF on denitrification potential activity and N_2O emissions from a native fertile agricultural soil. In **Chapter 2,** we review the role of AMF in environmental controls of denitrification and soil N_2O emissions. Likewise, we perform three mesocosm experiments to unravel how AMF influence potential denitrification activities and N_2O emissions from an arable soil. We address the substantial knowledge gaps regarding the mechanisms of AMF modification of soil potential denitrification activities and N_2O production through the alteration of keystone ecosystem processes such as the turnover of soil aggregates, promotion of plant community structure and mineral N cycling.

In **Chapter 3** the direct effects of AMF via hyphal growth on denitrification potential parameters will be disentangled from those resulting from indirect AMF-induced changes in soil aggregation using a realistic mesocosm experiment where we combine a manipulation of two factors: AMF and soil aggregation. We address the following two hypotheses: (1) Changes in soil conditions that develop in response to the addition of AMF inoculum would reduce potential denitrification activity and potential N_2O production rates (Bender et al., 2014); (2) Differences in soil aggregation status, simulated through mechanical disturbance of the soil and affected by the presence of AMF (Rillig et al., 2010), would reduce potential denitrification activity and potential N_2O production rates as well.

Most of the existing studies have performed reductionist experiments with single host systems relevant for agricultural settings, but not for natural systems with a more diverse plant community. Therefore, in **Chapter 4** compared to **Chapter 3,** we explore how AMF might behave under a typical European grassland plant community to gain insights into the microbial denitrification process. We perform a realistic grassland experimental setting where we will establish a full randomized monofactorial design with AMF under a realized gradient in plant diversity. We hypothesize that in our seed mix which is dominated by grasses which do not depend strongly on mycorrhiza, (1) AMF would promote plant diversity (Bergelson

and Crawley 1988); and (2) that higher plant diversity would reduce denitrification potential activity and N_2O potential emission rates (Niklaus et al., 2016; Abalos et al., 2018); (3) we would also observe a detrimental effect of AMF inoculation on denitrification potential activity and N_2O potential emission rates in agreement with Bender et al. (2014) and Storer et al. (2017).

In C**hapter 5** we test whether AMF influence real denitrification rates and related cumulative N_2O emissions in oxic and anoxic conditions when there are no differences in NO_3^- availability between AMF treatments at harvest using a gas-flow-soil-core incubation system. We hypothesize that intact fertile agricultural soil sample cores originating from mycorrhiza-exclusion cultures would display a higher convexity which would represent evidence that, irrespective of its original availability, NO_3^- is denitrified at higher affinity than in samples originating from mycorrhizal cultures and that the exclusion of mycorrhiza increases total N_2O emissions independent of NO_3^- concentrations.

The discussion (**Chapter 6**) presents the results of the previous chapters in the framework of the significance of AMF in microbial ecology of denitrification potential and related N_2O production rates, and provides outlook for future potential research questions and applications.

Chapter 2 :

Understanding the role of arbuscular mycorrhizal fungi in environmental controls of denitrification and soil N_2O emissions: a review

Abstract

Nitrous oxide (N_2O) is a powerful greenhouse gas representing a threat to the stratospheric ozone layer and global warming. Recent advances in mycorrhizal research reported that arbuscular mycorrhizal fungi (AMF) can reduce denitrification rates and the magnitude of N_2O emissions from soils. However, potential AMF-mediated mechanisms controlling denitrification has not yet been addressed in detail. In this review, we explore for the first time how AMF-mediated mechanisms could generate changes in proximal (direct) and distal (indirect) controls of denitrification with prominent effects on the magnitude of N_2O emissions. We demonstrate how proximal AMF mechanisms may contribute to the reduction of N_2O emissions through the depletion of available soil N and C, metal ions or increased soil pH and C and N immobilization or via the alteration of denitrifying community, *nirK* and *nosZ* key genes involved in denitrification. Likewise, we outline the effects of distal AMF mechanisms on denitrification, such as the regulation of soil moisture, organic matter decomposition, as well as the promotion of soil aggregation, plant diversity and productivity. The performance of AMF under the influence of elevated CO_2, temperature and drought stress is briefly discussed and its influence on N_2O production described. We suggest a number of AMF mechanisms that could be tested in future experiments. We come to the conclusion that proximal and distal AMF mechanisms may control the magnitude of N_2O production and lead to an overall mitigation of N_2O emissions. This review shows that harnessing and managing AMF would be a potential biotechnological tool for the development of climate-smart agriculture.

Keywords: Arbuscular mycorrhiza, proximal and distal controls, denitrification rates, N_2O emissions.

Introduction

Nitrous oxide (N_2O) is a potent greenhouse gas contributing to global warming. N_2O has a 300 times higher global warming potential compared to carbon dioxide (CO_2) and has been labelled the "dominant ozone-depleting substance in the 21st century", responsible for the destruction of the stratospheric ozone layer (Forster et al., 2007; Ravishankara et al., 2009). An estimate of 60% of the global soil N_2O emissions originates from agricultural soils (Syakila and Kroeze, 2011; Reay et al., 2012). Grasslands account for two thirds of agricultural land across the world and are responsible for approximately 18% of global N_2O emissions (Lee et al., 1997). Grass and cropland soils are of special interest for this review, as arbuscular mycorrhizal fungi (AMF) are the dominant mycorrhizal type in these ecosystems (Smith and Read, 2008). Global N_2O emissions and temperature are on the rise and expected to increase further (IPCC, 2018). Increases in population and income levels will lead to an intensification of the food production system, like a heavier use of nitrate (N) fertilizers, which will amplify environmental effects such as N-leaching (Springmann et al., 2018). If no technological changes or dedicated mitigation measures are implemented, these environmental effects could increase by 50-90% between 2010 and 2050, as estimated by Springmann et al. (2018). The challenge now and in the future is to balance the trade-off between the reduction of N_2O emissions from agricultural soils and an increasing food demand.

N_2O is produced in soils in the course of two contrasting processes: denitrification and nitrification (Butterbach-Bahl et al., 2013). However, over two thirds of global soil N_2O emissions originate from denitrification (Thompson et al., 2012). Denitrification is a biological process in which soil nitrate (NO^-_3) is reduced to dinitrogen (N_2) under anaerobic conditions with the intermediate formation of N_2O. During nitrification, transformation of soil ammonium ions (NH_4^+) to NO^-_3 under aerobic conditions may also generate N_2O and the process is called nitrifier-denitrification (Butterbach-Bahl et al., 2013). It is therefore relevant to have an in-depth understanding of the environmental factors controlling denitrification.

Denitrification is a microbial respiratory process depending on a multitude of environmental factors (physical, chemical and biological) controlling soil N_2O production and reduction. The process is carried out by microbes under anaerobic conditions, and in cases of incomplete reactions, the end product is N_2O instead of N_2 (Robertson and Groffman, 2015). The environmental factors controlling denitrification rates (N_2O and N_2 emissions) have been

grouped into proximal and distal categories (Groffman et al., 1988; Wallenstein et al., 2006; Robertson and Groffman, 2015, see section 1.1, Table 1; Fig 2).

Understanding the microbial ecology of denitrification process and mitigating N_2O emissions requires a profound ecological knowledge of the functioning of soil biota involved in the process. AMF are a widespread group of soil fungi living in symbiosis with the roots of 80 % of terrestrial plant species, including agricultural plants (Smith and Read, 2008). AMF often contribute positively to plant nitrogen (N) and phosphorus (P) uptake and plant yield and simultaneously promote soil aggregation (van der Heijden et al., 2006; Nwaga et al., 2010; Okiobe et al., 2015, Tisdall and Oades, 1982; Rillig and Mummey, 2006; Leifheit et al., 2014). The direct and indirect AMF effects on terrestrial ecosystem processes and carbon cycling have been discussed, for instance in Rillig, (2004), but the potential role of AMF on N-cycling and denitrification processes has been less explored.

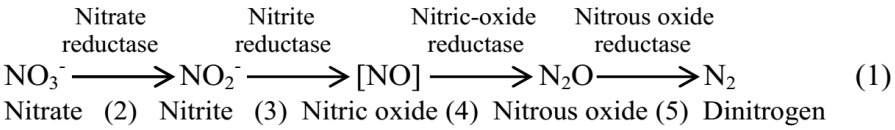
The potential influence of AMF on N_2O emissions was first studied by Cavagnaro et al. (2012). However, the authors did not observe a significant effect of AMF on N_2O fluxes. There have been several reviews addressing the role of AMF in N related processes (Veresoglou et al., 2012a; Hodge and Storer, 2015; Cavagnaro et al., 2015), but none of these studies has addressed AMF regulated controls of denitrification and related N_2O emissions in detail. Recent advances in mycorrhizal research demonstrate that AMF reduce N_2O emissions from soils (e.g Bender et al., 2014; Lazcano et al., 2014; Zhang et al., 2015; Bender et al., 2015; Storer et al., 2017; Teutscherova et al., 2018). These new findings are of great relevance owing to the fact that agricultural management practices, such as conventional tillage, have been reported to reduce AMF colonization and abundance in crop systems, which might contribute to increased N_2O emissions (Bender et al., 2014; Bowles et al., 2016). Managing agricultural soils for increased AMF abundance may not only contribute to a greater sustainability in crop production (Rillig et al., 2018), but also to a reduction of N_2O emissions. A comprehensive understanding of AMF-mediated mechanisms on environmental controls of denitrification is therefore particularly desirable.

Here, we present a comprehensive review with new insights into how AMF can potentially alter proximal and distal controls of denitrification rates and how they could impact the magnitude of N_2O emissions. First, we illustrate the difference between proximal and distal controls and their potential effect on denitrification rates and related N_2O emissions. Second, we explain how AMF can influence proximal environmental controls of denitrification like the denitrifier community structure, soil nitrate, oxygen and metal concentrations, available

carbon and soil pH as well as distal environmental controls like organic matter and C/N ratio, nitrification, soil moisture and structure and plant diversity and how this relates to the magnitude of N_2O emissions. We briefly discuss the potential of AMF to mitigate climate change via their influence on denitrification rates. We conclude with suggestions for future studies on AMF-mediated mechanisms on denitrification and N_2O emissions.

1. Environmental controls of denitrification and implications of AMF presence for denitrification and N_2O formation

During biological denitrification, soil bacteria, but also some fungi convert NO_3^- to NO_2^-, then to nitric oxide (NO) gas, and to N_2O and finally to N_2 in a sequence of reactions (see Fig. 1) (Zumft, 1997; Saggar et al., 2012). These reactions are catalysed by several enzymatic complexes which are: nitrate reductase (Nar); nitrite reductase (Nir); nitric oxide reductase (Nor) and nitrous oxide reductase (Nos), respectively (Zumft, 1997). The complete process can be expressed as a net balanced redox reaction, where NO_3^- gets fully reduced to N_2 (see global reaction 6, Fig 1).



$$NO_3^- + 2e^- + 2H^+ \longrightarrow NO_2^- + H_2O \quad (2)$$

$$NO_2^- + e^- + 2H^+ \longrightarrow NO + H_2O \quad (3)$$

$$2NO + 2e^- + 2H^+ \longrightarrow N_2O + H_2O \quad (4)$$

$$N_2O + 2e^- + 2H^+ \longrightarrow N_2 + H_2O \quad (5)$$

$$2NO_3^- + 10e^- + 12H^+ \longrightarrow N_2 + 6H_2O \quad (6)$$

Fig. 1 Simplified equations of denitrification, a microbially mediated process of dissimilatory reduction of nitrate that may ultimately produce dinitrogen through a series of gaseous N oxide products (Zumft, 1997).

The analyses of the complete genomes of sequenced denitrifying bacteria revealed that approximately 1/3 have a truncated denitrification pathway ((Philippot et al., 2011; Saggar et al., 2012). The complete denitrification of NO_3^- to N_2 predominantly occurs through the synergistic interactions of microbial enzyme consortia (Zumft, 1997; Saggar et al., 2012). In order for denitrification to proceed, N must be present in the form of NO_3^-. The NO_3^- can be

present either through the direct addition of ammonium nitrate fertilizer (NH_4NO_3) or because fertilizer NH_4^+ has been converted to NO_3^- via nitrification.

Denitrification in soil is primarily controlled by three main factors in a direct way: i) NO^-_3 availability, ii) O_2 concentration and iii) soil pH; but also labile soil carbon (C) and temperature have been reported to be direct controls (Firestone et al., 1979, Davidson and Swank 1986, Weier et al., 1993, Thomas et al., 1994). These factors are described as 'proximal controls' of denitrification because they can instantaneously or quickly affect N_2O emissions over short periods of time (minutes/ hours) (Groffman et al., 1988; Wallenstein et al., 2006; Robertson and Groffman, 2015). The proximal factors can indirectly be affected by many physical and biological factors. We describe these factors as 'distal controls' of denitrification because they can regulate the composition and diversity of denitrifying communities via the alteration of proximal factors at larger spatial and temporal scales (e.g. months) (Wallenstein et al., 2006). The summary of proximal and distal controls of denitrification and their effect on denitrification rates and N_2O emissions is shown in Table 1.

Table. 1 Overview of the effects of proximal and distal factors on denitrification rates

Factors	Type of factors	Potential effects on denitrification rates and related N_2O emissions	Refs
Proximal	Nitrate concentration (NO^-_3)	Denitrification rates and N_2O emissions strongly increase along with soil NO^-_3.	Groffman et al., 1988, Senbayram et al., 2012, Krause et al., 2017.
	Oxygen concentration (O_2)	Denitrification rates and N_2O emissions strongly decrease with an increasing soil oxygen concentration. High oxygen concentration inhibits both, the synthesis and activity, of denitrifying enzymes.	Burgin et al., 2012, Robertson and Groffman, 2015, Sexstone et al., 1985.
	Carbon availability (C)	Denitrification rates and N_2O emissions were significantly enhanced by the increased availability of soil soluble organic carbon.	Chen et al., 2017, Groffman et al., 1988, Senbayram et al., 2011.
	pH	Denitrification rates and N_2O emissions generally decrease with increasing soil pH..	Šimek & Cooper, 2002; Čuhel & Šimek, 2011; Samad et al., 2016, Dannenmann et al., 2008.
	Temperature	Denitrification rates and N_2O emissions increase with increasing air temperature.	Butterbach-Bahl et al. 2013., Butterbach-Bahl and Dannenmann, 2011.
	Nitrification	Increased soil NH_4^+ concentration generally enhances denitrification rates	Hodge et al., 2001, Veresoglou et al., 2011, Robertson and Groffman, 2015.
	Moisture	Denitrification rates and N_2O emissions have their optimum in the range of 70–80% water-filled pore space depending on soil	Davidson et al., 2000, Butterbach-Bahl et al., 2013.

Table 1. continues

Distal		type.	
	Organic matter	Decomposition and mineralization of organic matter can increase nitrification as well as denitrification rates.	Krause et al., 2017, Chen et al., 2012, 2017.
	Plant identity and diversity	Soil N_2O emissions decreased with increasing plant diversity, but could be variable with plant species identity	McGill et al., 2010, Niklaus et al., 2016.
	Soil structure	Denitrification rates and N_2O emissions were found to be generally higher in larger aggregates than in smaller while an increased soil structure improves aeration and thus can reduce N_2O production.	Drury et al., 2004, Robinson et al., 2014, Khalil et al., 2005, Ball 2013, Smith et al., 2003.
	Soil texture and water drainage	Denitrification rates were found to be higher in clayey and poorly drained soils compared to sandy ones.	Groffman et al., 1988, Groffman et al., 1990.
	Climate change	Denitrification rates could increase as a direct effect of climate change, caused by changes in temperature and precipitation, but also by indirect effects, e.g. changes in CO_2 atmospheric composition.	Butterbach-Bahl et al., 2013, Butterbach-Bahl et al., 2011.

The concept of proximal and distal controls can be highly useful to guide future research to more clearly describe the multifaceted mechanisms that lead to changes in N_2O production as we do here for AMF-mediated processes and mechanisms. Most of the existing studies demonstrated that AMF reduce denitrification and N_2O production (see Table 2, Fig 2). Actually, there is no a direct evidence for the effects of AMF on denitrification and N_2 formation because of its high atmospheric background and difficulty to quantify it (Bender et al., 2014; Butterbach-Bahl et al., 2013). Thus, this review reports on the effects of AMF on denitrification and related N_2O production rates.

Table. 2 Overview of some studies investigating the effects of AMF on N_2O emissions from soils. GC = gas chromatograph

Host-experimen-tal systems	Methodology for N_2O collection and quantification	Manipulated proximal or/and distal factors	AMF treatments	AMF effects on N_2O emissions denitrification	Refs
Tomato greenhouse experiment	Static chamber technique for gas collection and GC for analysis.	Soil N concentrations	Mycorrhiza defective tomato mutant and its mycorrhizal wildtype progenitor	No significant effects on N_2O emissions, but low N_2O production in AMF root-colonized plants.	Cavagnaro et al., 2012

Table 2. continues

Grass greenhouse experiment	Gas collected from the headspace of microcosms connected to an automated N_2O analyzer.	Soil N concentrations	Mixture of three species : *Funneliformis mosseae, Rhizophagus irregulare and Claroideoglomus claroideum*,	Soil N_2O emissionswere reduced by 42 % in the microcosms with high AMF abundance.	Bender et al., 2014.
Tomato greenhouse experiment	Gas collected from the headspace of microcosms connected to an TEI 46c-automated N2O analyser	Soil N Concentrations	Tomato wildtype and AMF defective mutant	Soil N_2O emissions were reduced by 33% in the microcosms with high AMF abundance.	Bender et al., 2014.
Tomato experiment greenhouse	Static chamber technique for gas collection and GC for analysis .	Soil N concentrations and Soil moisture	Mycorrhiza defective tomato mutant "rmc" and "76R" its mycorrhizal wildtype progenitor	A significant reduction in N_2O fluxes at high soil moisture conditions by AMF tomato compared to non-AMF tomato was observed.	Lazcano et al., 2014.
Grassland microcosm	Gas collected from the headspace of microcosms connected to an automated N_2O analyzer.	Soil N and P concentrations and soil types	Mixture of : *Funneliformis mosseae, Rhizophagus irregularis and Claroideoglomus claroideum*	Approximately 2.3 and 3 times reduction in N_2O fluxes in AMF treatment compared to non-AMF	Bender et al., 2015.
Field rice experiment	Static chamber technique for gas collection and GC for analysis .	Soil N concentrations and Soil moisture	*Rhizophagus Irregularis*	AMF inoculation reduced N_2O fluxes by 58.8 and 10.9 %, compared to the non-AMF treatment	Zhang et al., 2015
Compartmented-maize mesocosm units	Gas probe and continuous flow loop sampling with an attached Isotopic N_2O analyser	Soil N concentrations and Organic matter	*Rhizophagus Irregularis*	Approximately 3 to 2.3 fold reduction in N_2O concentration from organic patches in the AMF treatment compared to non-AMF control. About 1.5 times reduction in N_2O fluxes with AMF addition after addition of NH_4^+.	Storer et al., 2017.

2. AMF-mediated mechanisms affect proximal controls of denitrification rates

2.1 AMF-mediated mechanisms on available mineral soil N concentrations

AMF root colonization and hyphal length have been reported to be negatively correlated with N_2O emissions (Bender et al., 2014, Storer et al, 2017, see Table 2). N_2O emission rates could increase with an increase in inorganic soil N (Table 1 and Fig.2). However, the presence of AMF can affect available soil N concentrations and decrease denitrification and the relative rates of N_2O emissions via the following mechanisms: uptake, assimilation, immobilization in fungal tissue and translocation of N to the host plant (Govindarajulu et al., 2005, Tanaka and Yano, 2005; Atul-Nayyar et al., 2009, Hodge and Storer, 2015). This implies that AMF could play an important role in the modulation of soil N and could thereby regulate N_2O emission rates. Interestingly, genes encoding for glutamine synthetases and some nitrate reductases have been identified in AMF hyphae, confirming that extraradical hyphae of AMF have the ability to assimilate N in the forms of NH_4^+ and/or NO_3^- (Govindarajulu et al., 2005; Tian et al., 2010). Moreover, it has been shown that AMF can deplete soil inorganic N pools from, e.g., inorganic fertilizers and transfer N from soil to plant (Johansen et al., 1992; Hodge and Storer, 2015; Atul-Nayyar et al., 2009; Cavagnaro et al., 2012). NH_4^+ is the dominant form of N absorbed by AMF whereas NO_3^- seems to play a minor role in the AMF mediated transfer of N (Govindarajulu et al., 2005; Tanaka and Yano, 2005; Johansen et al., 1993). As a result, AMF can reduce the amount of N-compounds available to the denitrifying community, thus reducing their activity and the amount of N_2O produced and possibly the absolute proportion of N_2O/N_2 which remains to be adequately quantified (Bender et al., 2015; Zhang et al., 2015, Fig. 2). This reduction in N_2O emissions could also be explained by AMF-mediated increase of N immobilization into plant tissue and microbial biomass, thereby reducing concentrations of mineral soil N as a substrate for N_2O emission (Bender et al., 2014). Likewise, the positive correlation of AMF extraradical hyphal length to soil microbial biomass N in an experiment with tomato plant and the increased soil microbial biomass in the mycorrhized plants in an experimental grassland contributed to the reduction of N_2O emissions (Bender et al., 2014). The effects of AMF on N_2O emissions are not always related to NO_3^- (Storer et al., 2017; Lazcano et al., 2014). In another experiment with tomato plants, AMF colonization of tomato roots did not result in any detectable differences in N_2O emissions from soils, despite the fact that 76R (mycorrhizal tomato) had higher ^{15}N-KNO_3 content compared to rmc (non-mycorrhizal mutant) (Cavagnaro et al., 2012). In this case, it is possible that N additions may have inhibited the growth and functioning of AMF (Camenzind et al., 2016), thereby resulting

in less N uptake from soils and no significant effect on N_2O production. Conversely, Lazcano et al., (2014) showed for example that a reduction in N_2O fluxes was linked to the higher use of soil water by AMF rather than increased N uptake. Storer et al. (2017) stated that reduced N_2O emissions by AMF were likely due to reduced NH_4^+ concentrations from soil. This could be partly due to the fact that AMF preferentially assimilate immobile NH^+_4 compared to highly mobile NO^-_3, owing to the higher assimilation cost of NO^-_3 compared to NH^+_4 (Raven et al., 1992; Veresoglou et al., 2012b). We can postulate that AMF would reduce the mineral availability of N subjected to denitrification and this would make the soil to become a N_2O sink by stimulating the conversion of N_2O to unreactive N_2 gas (Bender et al., 2014; Teutscherova et al., 2018).

2.2 AMF-mediated mechanisms on soil carbon availability

Heterotrophic denitrification is often limited by labile C in agricultural soils and denitrifiers require a readily available C source before denitrification starts (Saggar et al., 2012). Any AMF mechanism that influences the amount of available C in soils can therefore have a major impact on the magnitude of N_2O emissions. AMF have been shown to be a major C sink and alter the composition of rhizodeposition (Graham, 1981; Jones et al., 2004). Between 4 and 30 % of photoassimilated plant C is transferred to AMF hyphae (Drigo and Donn, 2017), providing an essential C source for the survival of the fungus and therefore limiting C allocation to the soil. Via this mechanism AMF may reduce the amount of energy source for denitrifiers thereby delaying and reducing N_2O emissions (Fig. 2). For example, denitrification rates and N_2O production have been found to be enhanced by the increased availability of glutamate, the more abundant soil soluble organic compound from plant residues (Chen et al., 2017). AMF-reduced C input into the mycorrhizosphere (the volume of soil influenced by AMF and roots) or hyphosphere (the volume of soil influenced by AMF hyphae) was linked to decreased bacteria community composition, see section 2.5 for more details, Amora-Lazcano et al., 1998; Nuccio et al., 2013). On the other hand, AMF can also be a source of C in soil, by exuding C from their hyphae, and through turnover of AM fungal hyphae (Kaiser et al., 2015, Jones, 2004), but, microbial derived carbon is efficiently being incorporated into mineral-stabilized soil organic matter, thereby mitigating possible stimulating effects on the microbial community (Sokol and Bradford, 2019). Overall, these studies indicate the need to better understand the complex interactions between AMF and the soil C cycle in order to draw meaningful generalizations.

2.3 AMF-mediated mechanisms on soil pH

Soil pH plays an important role in the activity of denitrifying enzymes and the regulation of N_2O emissions. Several studies have demonstrated that N_2O emissions and the denitrification potential ratio [$N_2O/(N_2O + N_2)$] decrease with increasing pH values (Šimek and Cooper, 2002; Čuhel and Šimek, 2011; Samad et al., 2016). The activity of N_2O reductase, a relevant enzyme responsible for the reduction of N_2O to N_2 in soils, could be repressed by acidic pH and thus increase N_2O emissions (Dannenmann et al., 2008; Čuhel and Šimek, 2011). Bago et al. (1996) reported that the presence of AMF hyphae increased the pH of a growth medium enriched with NO^-_3 by enhanced NO^-_3 uptake and releasing OH^- into or removing H^+ from the medium. Increase in soil pH by AMF had also been observed in the rhizosphere of onion (*Allium cepa* L.) (Bago and Azcón-Aguilar, 1997). Therefore, any increase in the soil pH could reduce N_2O production. Bender et al., (2014) found significant differences between AMF treatments for soil pH in the mycorrhizal tomato plant compared to non-mycorrhizal mutant grown in a calcareous sandy loam soil, but no AMF effects in the grass experiment using a slightly acidic sandy loam soil. The increased in soil pH in tomato experiment was also associated to an increase in copy numbers of *nos*Z genes which encode for N_2O reductase enzyme responsible for the reduction of N_2O to N_2 (Zumft, 1997). By contrast, in a compartmentalised pot system Li *et al.* (1991) found that AMF also reduced the pH of both mycorrhizosphere and hyphosphere soils by up to 1 pH unit in both Luvisol and Cambisol which are alkaline and acidic soils, respectively. It has been proposed that the observed decreases in pH were a result of H^+ release during NH_4 uptake (Li et al., 1991; Villegas and Fortin, 2001).

In summary, the effect of AMF on soil pH and N_2O emissions could be soil pH status context dependent and cannot be simply generalized. Most of the existing studies which relate AMF to denitrification have addressed the composite effect of AMF on denitrification in relation to soil pH (e.g Bender et al. 2014). We don't really understand yet how AMF may affect denitrification through manipulation in soil pH in pot culture or under field conditions.

2.4 AMF-mediated mechanisms on soil oxygen concentration

Overall, a larger number of studies has shown an increased soil respiration by AMF (Langley et al., 2005; Hughes et al., 2008; Cavagnaro et al., 2008; Shi et al., 2010; Nottingham et al., 2010; Cavagnaro et al., 2012). This increase was ascribed to heterotrophic respiration of external AMF hyphae (Heinemeyer et al., 2006). For example, Nottingham et al. (2010) found

that AMF mycelia respired carbon at a rate of 1.4 $t.ha^{-1}$ yr^{-1}, which accounted for about 14 % of total soil respiration and 26 % of root-derived respiration in the forest soil. A greater AMF mycelia respiration may deplete oxygen concentration, which, in turn, decreases soil O_2 availability and creates more suitable denitrifying conditions for N_2O production (Sextone et al., 1985). This effect could stimulate denitrifying N_2O producers that are known to thrive under poor oxygen conditions (Groffman et al., 1988). Higher level of oxygen concentration either represses the formation of the enzyme nitrate reductase or acts just as an electron acceptor, thereby preventing the reduction of NO^-_3. However, the exact control mechanism exerted by oxygen on denitrifying enzyme synthesis, has not been clearly demonstrated yet, and may very well vary among species of denitrifiers (Zumft, 1997). Low soil oxygen concentrations could therefore increase denitrifying enzyme activities while high oxygen concentrations would inhibit this activity (Zumft, 1997; Burgin et Groffmann, 2012). Therefore, the presence of abundant AMF hyphae may stimulate a higher soil respiration rate contributing to the increase in soil oxygen consumption and thereby increasing the rates of denitrification and N_2O emissions. However, in similar experimental conditions, AMF strongly enhanced soil CO_2 fluxes while N_2O fluxes were significantly reduced (Bender et al., 2014, Lazcano et al., 2014). This may mean that the amount of respiration that is increased by AMF is not enough to produce sufficient anaerobic conditions in the soil. This may also imply that distal AMF mechanisms are involved in such conditions as we suggested earlier and could have masked the subsequent effect of AMF-promoted soil respiration on N_2O production. For example AMF are known to promote soil aggregation, a potential indirect regulator of soil aeration and oxygen concentration, which will be discussed in section 3.4 (Rillig and Mummey, 2006).

Most of the existing studies have been conducted with soil forests. Given that soil respiration could have significant implications for soil C turnover and denitrification in agricultural soils and AMF colonization and abundance being significantly reduced (Bender et al., 2014; Bowles et al., 2016), we are currently lacking studies which address the influence of AMF on denitrification and N_2O emissions via AMF-induced change in soil oxygen concentration under agricultural soils.

2.5 AMF-mediated mechanisms on denitrifying community structure and function

The role of AMF on denitrification community structure and how this relates to environmental controls, denitrification enzyme activity and denitrification products is a key

for understanding denitrification process. However, this research question remains largely understudied. Few studies have shown that AMF may alter denitrifying community composition as well as nitrifying microbial community (Amora-Lazcano et al., 1998; Veresoglou et al., 2011; Veresoglou et al., 2012 b; Nuccio et al., 2013). AMF have been suggested to reduce the activity of ammonia oxidisers (e.g., ammonia oxidising bacteria-AOB) and thereby reducing potential nitrification rates (Veresoglou et al., 2011). This indicates that they may be capable of outcompeting nitrifiers for NH^{+}_{4}. A reduction in the number of denitrifiers present in the mycorrhizosphere soil was reported by Amora-Lazcano et al. (1998), and was linked to AMF-reduced soil C input (Toljander et al., 2007). A reduction in potential nitrification rates and abundance of denitrifiers by AMF could then potentially reduce denitrification rates and N_2O production rate (Veresoglou et al., 2011; Veresoglou et al., 2012 a). Additionally, the presence of AMF significantly increased the relative abundance of two out of four denitrifying bacterial groups (Gemmatimonadetes and Deltaproteobacteria) possessing *nosZ* genes that have the ability to use N_2O as an electron acceptor and to reduce it into N_2 (Nuccio et al., 2013). AMF hyphae have also been reported to alter the abundance of denitrifying key genes *nirK* and *nirS* which are involved in the reduction of NO_3^- into NO_2^- and *nosZ* which is involved in the reduction of N_2O into N_2 (Bender et al., 2014), but also the community structure of denitrifying *nirK* gene (Veresoglou et al., 2012b). The abundance of denitrifying genes has been reported to correlate well with assayed denitrification rates and N_2O production (as well as denitrifying microbial diversity, but not for all cases (Philippot et al., 2013; Philippot et al., 2009). Bender et al. (2014) found that the abundance of key genes responsible for N_2O production (nir*K*) was negatively and for N_2O reduction (*nosZ*) positively correlated to AMF abundance, indicating that N_2O reduction was mediated by AMF-induced changes in the soil microbial denitrifying community composition. The effect of AMF on N_2O emissions rates via microbial community shift is poorly understood. It would be very relevant for the N_2O mitigation technology to increase understanding in which conditions AMF might increase *nos*Z gene activity, because this would stimulate the N_2O-reducing ability of the denitrifiers and accelerate the conversion of N_2O to N_2 and therefore reduce the magnitude of N_2O emissions.

On the other hand, some studies have shown inverse relationship between the abundance of ammonia oxidizers and N_2O emission rates in the presence of AMF (Cavagnaro et al., 2007; Teutscherova et al., 2018). It is also possible that AMF-mediated effects on the denitrifying community shift and gene alteration induce no changes in denitrification rates. The modification of denitrifying key genes and soil microbial denitrifying community do not

necessarily mean that genes will be expressed in the environment (for example in the presence of AMF) or that the gene products will function equivalently and correlate with assayed denitrification rates (Wallenstein et al., 2006; Philippot et al., 2011). Most of denitrifiers lack the nosZ gene encoding the nitrous oxide reductase and do not have the capacity to denitrify, even though some denitrifiers lacking these genes have been reported to influence denitrification rates from soils (Philippot et al., 2011; Saggar et al., 2012). Additionally, we do not know whether changes in AMF abundance would also affect the rate and kinetic of denitrification activity or whether AMF-induced changes in denitrification community structure affect denitrification activity (i.e., rate of potential denitrification enzyme activity and N_2O formation). For example, no correlations were found between the abundance of most denitrification genes and potential denitrification activity or potential N_2O production (Philippot et al., 2009). This result uncovers the significance of nondenitrifiers such fungi, nitrifiers which have been shown to affect N_2O formation (Philippot et al., 2011; Saggar et al., 2012, Butterbach-Bahl et al., 2013; Hallin et al., 2017).

In summary, the role of AMF on denitrification community structure and function (enzyme activity) is central and represents a key soil component to understand denitrification (Fig 2). AMF would directly affect denitrification and N_2O formation through changes in denitrification community structure which acts as a transducer (reduces NO^-_3 to nitrogen gas products) or in other cases, indirectly through changes in the other environmental controls of denitrification. Any changes in the rates of denitrification and N_2O production through proximal or distal AMF mechanisms would be mainly modulated by denitrifier community and/or denitrification activity. The activity of the enzymes involved in denitrification would be also affected directly by AMF or/and AMF-induced changes in denitrifier composition (Bender et al., 2014), but in other cases, changes in physical and biochemical environmental factors of denitrification by AMF would be the dominant determinants of denitrification activity and N_2O production (Zhang et al., 2015).

2.6 AMF-mediated mechanisms on soil metal concentration

Soil metals have been overlooked as proximal controls of denitrification (Wang et al., 2016). Denitrifying enzymes require cofactors to operate which can be soil metal ions such as copper (Cu), iron (Fe) and molybdenum (Mo) (Zumft, 1997). A cofactor is a non-protein chemical compound or metallic ion that is required for an enzyme's activity. Cofactors are "helper molecules" that assist in biochemical transformations, for example denitrification (Zumft, 1997). Mo acts as a cofactor for Nar, Fe for both Nar and Nir while Cu acts as a cofactor for

Nir and specifically for Nos because it is the only enzyme with no alternative Cu-cofactor (Saggar et al., 2012; Zumft, 1997). Although, AMF are usually considered important primarily for N and P uptake (Smith and Read, 2008), they can also increase plant acquisition of zinc (Zn), Mo, Fe and Cu. A meta-analysis reported overall positive effect of AMF on Cu, manganese (Mn) and Fe uptake by crops (Lehmann and Rillig, 2015). Interestingly, Cu and Fe protein transporters have been identified in the mycorrhizal structures of *R. irregularis*, a model AMF (Tamayo et al., 2014). We postulate that changes in soil metal concentration by AMF will have potential effects on denitrification enzymes and N_2O emission rates. The Nir in some organisms can contain Cu and could therefore be limited by Cu availability (Zumft, 1997; Suzuki et al., 2000; Stein, 2011). Thus, if AMF are taking up Cu, they may reduce the magnitude of N_2O production by repressing the activity of the Cu-based Nir. However, a reduction in the availability of Cu can also increase N_2O production as the activity of the Nos, which also has a Cu-cofactor, is reduced (Zumft, 1997). AMF reduced soil Cu^{2+} availability via uptake would therefore leave most of the denitrified N in the form of N_2O (Lehmann and Rillig, 2015). On the other hand, when the availability of soil Fe (III) was high, N_2O production has been shown to increase (Bengtsson et al., 2002). Recent studies found that the availability of Fe in soils can be positively linked to N_2O production (Zhu et al., 2013, Wang et al., 2016). Since AMF can reduce the availability of Fe by taking up Fe^{2+} and transfer it to their host plants, they have the potential to reduce denitrification rates by altering the activity of Nar and Nir. However, Wang et al. (2016) reported a high N_2O production when soil Fe^{2+} concentration was decreased. This study indicated that the increased production of N_2O was regulated by oxido-reduction reactions and the donation of electrons to NO^-_3 via microbial oxidation of Fe (II). The presence of AMF may therefore alter oxydo-reactions between nitrates and metal ions (Fig. 2). We presume that changes in N_2O production could be dependent on the regulation of soil Cu (II)/NO_3^- or Fe (II)/ NO_3^- via proximal AMF-reduced soil metal concentration in soils.

In summary, it is possible that the limitation of trace metals by AMF in soils can lead to the reduction in microbial community and N_2O production. This assumption needs to be tested with mechanistic studies focusing on the direct effect of the presence of AMF on metal concentrations in soil and the relation to N_2O release.

3. AMF-mediated mechanisms on distal controls of denitrification rates

3.1 AMF-mediated mechanisms on organic matter and C/N ratio

The C/N ratio of decomposed organic matter (OM) has been reported to be an important distal control of denitrification rates because it can influence the availability of soil N (Table 1 and Fig. 2). AMF have been shown to enhance decomposition and mineralization of mostly high quality plant litter (with low C/N ratios), and thus possibly increasing the release of mineral N, but at the same time they increased the uptake of released mineral N by their host plants (Hodge, 2001; Hodge et al., 2001; Hodge and Fitter, 2010; Hodge and Storer, 2015). Amounts of mineralized N captured by AMF can be high: Leigh *et al.* (2009) reported that up to one-third of the N contained in organic patches (C:N 9.5-27:1) was removed by AM fungi and transferred to the plant with up to 20% of the plant N being patch derived. During decomposition the C/N ratio in organic patches decreases more in the presence of AMF compared to AMF free soil, resulting in higher amounts of mineralized N (Atul-Nayyar et al., 2009). In this study up to 25 % of the mineralized N were recovered and translocated to the host plants. Additional to the transport of N to plants, AMF can influence the fate of N in the soil via their influence on other soil microorganisms, with consequences for N_2O emissions: Storer et al. (2017) showed that AM-plants with access to an organic patch contained more N compared to non-inoculated plants and that N_2O emissions were strongly reduced in pots with AMF hyphae present. The authors hypothesized that AMF hyphae outcompete slow-growing nitrifiers for NH^+_4 and thereby reduce N_2O emissions mainly from nitrification, but not from denitrification. On the other hand, Bender et al. (2014) found that the presence of AM hyphae significantly increased microbial biomass N and thereby lowered N_2O emissions via immobilization of N in microbial biomass.

In summary, AMF can firstly influence the decomposition of OM and secondly influence the fate of the mineralized substances which would affect the rates of N_2O production by influencing denitrifier community and denitrification activity. We can see that distal mechanisms, such as AMF effects on OM decomposition, are linked to proximal mechanisms. Obviously, we do not yet fully understand the mechanisms by which AMF influence the mineralization of organic matter or the mechanisms which have an impact on mineralized compounds. Most of the studies looking at the effects of AMF on decomposition of organic matter were conducted in the lab or greenhouse and were for a short-term only. Field scale studies are rare and the relevance of lab studies for the field is unclear, especially when we

think about quantities of N that might be transported or immobilized in the presence of a natural community as opposed to inoculated single species.

3.2 AMF-mediated mechanisms on soil moisture

One of the most important parameters which can be used to quantify the effect of soil moisture on denitrification rates is water filled spore space (WFPS) (Table 1). N_2O emissions rates have been shown to increase with increasing WFPS (Fig. 2) (Groffman et al., 1988, Butterbach-Bahl et al., 2013). Several studies have reported that the AM symbiosis with plant roots has the potential to increase plant water uptake and nutrient use efficiency, thereby changing soil moisture conditions (Augé; 2001; Augé, 2004). Lazcano et al. (2014) performed a greenhouse experiment to compare the effect of mycorrhizal tomato (76R MYC) and its non-mycorrhizal mutant (rmc) on the N_2O emissions under different soil moisture conditions (drought and watered). They found that plant genotype affected the relationship between N_2O and WFPS: soil N_2O emissions in the 76R MYC treatment were significantly reduced at high soil moisture with WFPS (> 50 %) compared to rmc. They reported that the reduction of N_2O production was related to an increased photosynthesis rate and stomatal conductance at high soil moisture with 76R MYC, but also to increased plant water use efficiency by AMF colonized roots. This study supports findings from Bender et al. (2014), indicating a reduction in WFPS in response to AMF inoculation. They presumed that the fast water removal in the 76R MYC treatment increased the oxygen availability in the soil and therefore reduced N_2O emissions, as denitrifying enzymes are expressed under low oxygen conditions to maintain respiration (Burgin et al., 2012). By contrast, Zhang et al. (2015) found that the WFPS of the non-inoculated soil was slightly greater than that of the AMF inoculated soil and there were no differences between the control and AMF treatment during the flooding and draining stages in the rice paddy field. Nevertheless, N_2O emissions were significantly reduced in the inoculated soils compared to non-inoculated because AMF reduced NH_4^+ and NO_3^- concentrations in the flooded and drained stages, respectively. Generally, the influence of AMF on soil water transport seems to be higher in dryer soils, as Augé et al. (2015) found in their meta-analysis of 460 studies that the promotion of stomatal conductance to water vapour by AMF increased with increasing drought conditions. Hence, AMF functions vary with water table conditions, but seem to have an overall positive effect on the removal of water from soil pores, thereby reducing anaerobic conditions that favour denitrification and related N_2O production.

3.3 AMF-mediated mechanisms on plant diversity and productivity

Few studies reported that increased plant diversity reduced N_2O production via enhanced N removal efficiency and below-ground plant complementary trait effects in soils or changes in denitrifying communities (Niklaus et al., 2016; Abalos et al., 2017; Wenjuan et al., 2017 but see Bremer et al., 2007). AMF are known to promote plant productivity and diversity (van der Heijden et al., 2015; Lin et al., 2015). The authors found that mycorrhizal responsiveness (difference in plant biomass between AMF and nonAMF treatments) was the main mechanism driving plant biodiversity and productivity. It has been shown that AMF enhance plant productivity through increased plant N uptake (Nwaga et al., 2010; Veresoglou et al., 2011; Okiobe et al., 2015). Increased AMF abundance in the plant community has been reported to lead to a higher competition between plants for soil nutrients, especially N and P and water, possibly limiting the growth of denitrifiers (van der Heijden et al., 2006; van der Heijden et al., 2015). These distal AMF effects could therefore reduce the amount of denitrified N_2O. Furthermore, AMF promoted plant diversity could also increase plant complementary traits effects (Van der Heijden et al., 2002; Lin et al., 2015). Decrease in N_2O emissions have been previously related to increased grassland plant functional traits (specific leaf area and root length density) as well as to grassland plants that produced higher plant biomass and showed larger N uptake (Abalos et al., 2017). Plant functional group and plant identity could also play an important role in denitrification process. Leguminous plants are known to enhance N_2O production via atmospheric fixation of N_2 and increased soil NH^+_4 availability while grasses generally reduce N_2O production (Abolos et al., 2014, 2017; Niklaus et al., 2016). Plant species identity and AMF status have been reported to be the most significant independent variables explaining the reduction in potential nitrification rates in nitrogen-limited grassland soils (Veresoglou et al., 2012b). Moreover, plant species identity strongly reduced N_2O emissions from grassland and surpassed species richness which is considered as a key driver of N_2O production (Abalos et al., 2014). To our knowledge there is no study reporting on AMF-promoted effects on plant community structure and productivity in relation to denitrification rates.

The existing literature on AMF and denitrification has performed reductionist experiments with single plant species systems which are relevant for industrial agricultural settings, but not for native grassland systems with large plant diversity. We may postulate that AMF-promoted increases in plant productivity and diversity will reduce N_2O emissions. The magnitude of reduction in N_2O release would differ considerably with the degree of mutualism between

AMF and their individual host (importance of plant species identity) and the availability of symbiotic partners (importance of plant community).

3.4 AMF-mediated mechanisms on soil structure

Numerous studies have reported positive effects of AMF on soil structure and aggregation (Tisdall and Oades, 1982; Rillig and Mummey, 2006; van der Heijden et al., 2006; Leifheit et al., 2014). Improved soil structure and the resulting improved soil aeration have been reported to strongly influence microbial activity and N_2O emissions, as denitrification represents a process that depends on oxygen availability and diffusivity (Sexstone et al., 1985; Khalil et al.; 2005; Ball, 2013; Balaine et al., 2016). AMF hyphae serve to enmesh and entangle soil primary particles, organic materials and small aggregates, facilitating macroaggregate formation thereby increasing the volume of pore space and air porosity between soil particles (Rillig and Mummey, 2006). This represents a potent distal AMF mechanism, which could strongly decrease denitrification and N_2O production via augmenting oxygen concentrations in the soil.

AMF mycelial networks could also improve soil water holding capacity and soil water repellency through enhanced soil aggregation (Veresoglou et al., 2012b; Rillig et al., 2010). AMF-improved soil water holding capacity may therefore increase soil moisture and thus create conditions favourable for denitrification. However, Lazcano et al. (2014) showed that AMF reduced N_2O fluxes at high soil moisture by increasing plant water use efficiency. It is possible that the AMF-induced effect of increased plant water use efficiency dominates over increasing the water holding capacity, so that even at higher soil moisture conditions, N_2O emissions are reduced.

Additional to the effects on soil aeration, improved soil aggregation by AMF can increase microbial heterogeneity in the soil matrix, which can in turn affect denitrification. A large number of different microenvironments, such as those formed in and between soil aggregates, can promote diverse microbial communities (Rillig and Mummey, 2006, Bach et al., 2018).
This AMF-mediated high spatial soil heterogeneity may induce aggregated point patterns in many microbial denitrifiers, and an uneven distribution of microbial N_2O producers and N_2O reducers. Benjamin et al. (2008) found that N_2O production is different between micro- and macroaggregates. These are two microhabitats, of which the macroaggregates are much more influenced by AMF than microaggregates (Rillig and Mummey, 2006). This could result in

spatial variability of denitrification where some aggregated soil microhabitats host incomplete denitrification and others complete.

The interaction between soil aggregation and denitrification rates and the role of AMF therein have not yet been adequately addressed. The existing literature on AMF and denitrification has largely overlooked to segregate between direct (i.e effect of AMF hyphae growth on mineral N for e.g) and indirect effects (i.e effect of AMF on soil aggregation) on denitrification and N_2O emissions (see Table 2).

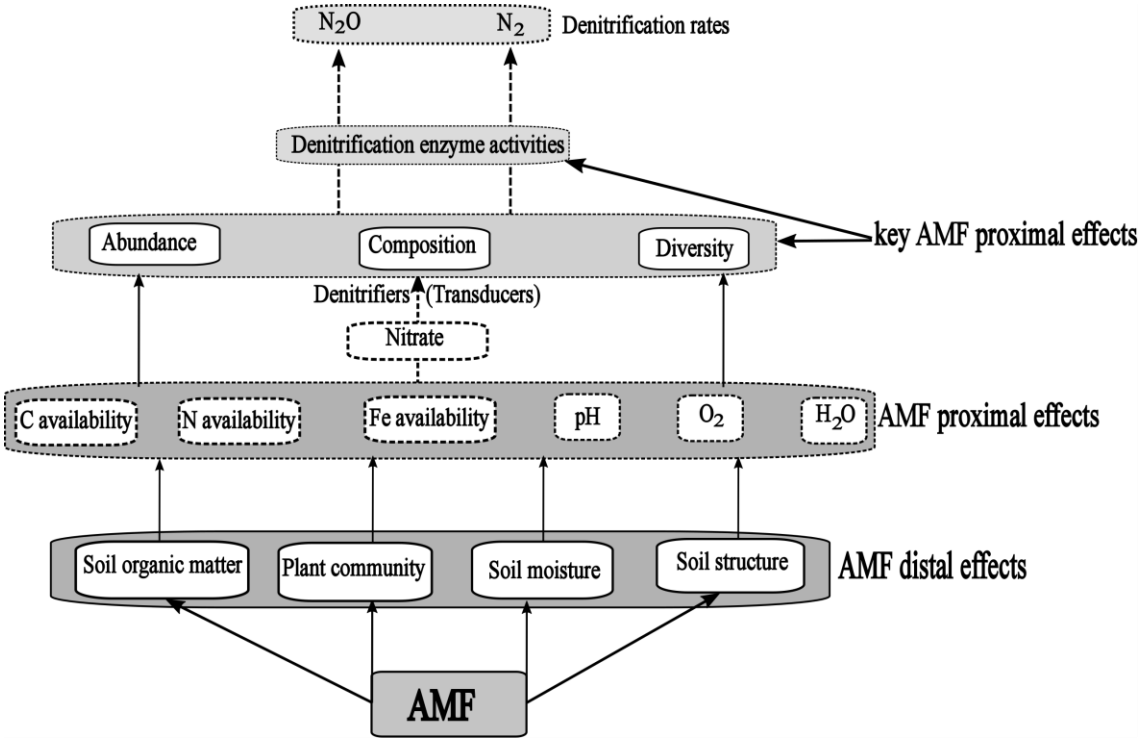


Fig. 2 A conceptual framework of detailed potential proximal and distal arbuscular mycorrhizal fungi (AMF) mechanisms on denitrification rates: nitrous oxide (N_2O) and dinitrogen (N_2). Changes in the rates of denitrification through proximal or distal AMF mechanisms would be mostly modulated by denitrifier community structure and/or denitrification activity. Nitrate is the main substrate for denitrification. The three dotted lines indicate simplified denitrification process. This conceptual diagram has been designed based on Rillig, (2004); Groffman et al. (1988); Wallenstein et al. (2006); Bender et al. (2014); Lazcano et al. (2014); Zhang et al. (2015); Bender et al. (2015) and Storer et al. (2017).

4. The role of AMF on denitrification rates in a changing climate

Climate change will directly affect denitrification via changes in temperature and soil moisture regimes (e.g. drought). Indirect effects include rising atmospheric CO_2

concentrations and associated changes in plant water use efficiency, plant biomass production and rhizodeposition of C substrates (Butterbach-Bahl and Dannenmann, 2011). Overall, AMF hyphal growth and root colonization have been reported to positively respond to increased temperature and atmospheric CO_2 levels while negatively to drought stress (Augé, 2001; 2004; Rillig et al., 1999; Rillig et al., 2000; Fitter et al., 2000; Rillig et al., 2002; Compant et al., 2010). In this section, we elaborate why we think that AMF may reduce N_2O emissions from soils at increased temperatures and atmospheric CO_2 concentration.

Increased air temperature can result in increased plant photosynthesis rates which lead to a greater allocation of C to AMF and thereby fostering AMF root colonization and hyphal growth (Rillig et al., 2002; Heinemeyer and Fitter, 2004). We may postulate that an increase in temperature will increase AM hyphal densities thereby reduce N_2O emissions through soil N and C limitation in soils (see paragraph 2.4, 3.2).

Similarly, increased levels of atmospheric CO_2 increased AMF mycelium growth and soil microbial activity, induced higher rates of plant photosynthesis and oxygen consumption in the rhizosphere (Anderson et al., 2011; Staddon and Fitter, 1998; Staddon et al., 1999; Rillig et al., 1999, 2000). Cheng et al. (2012) found that AMF increased organic carbon decomposition under elevated CO_2, but optimized NH_4^+ acquisition from soil while reducing nitrification rates. We assume that reduction in nitrification rates by AMF (see Veresoglou et al., 2011, Storer et al., 2017) would also reduce denitrification under elevated CO_2 enrichment.

Drought stress, which may happen more frequently with climate change, is often responsible for the reduction in plant growth where both below- and above-ground plant biomass may be impaired. This may lead to changes in the allocation of photosynthates in the rhizosphere as well as in extraradical AM mycelium. The density of extraradical mycorrhizal hyphae and AMF root colonization has been reduced or altered by drought, even though some ubiquitous AMF species, like *Glomus sp*., seem to tolerate it (Augé, 2001; Staddon et al., 2004). N_2O emissions from soil of mycorrhizal plants (76R MYC) were higher than that of non-mycorrhizal mutants (rmc) in a drought treatment (low WFPS <50 %), but decreased in a watering treatment (high WFPS > 50 %), therefore showing lower emissions at high soil moisture and WFPS (Lazcano et al., 2014). This was explained by the fact that photosynthesis rate and stomatal conductance of 76R MYC were reduced by drought stress compared to the watering treatment. However, water use efficiency increased in 76R MYC compared with rmc plants during drought (Lazcano et al., 2014). Zhang et al. (2015) showed that the proximal AMF-mediated effects on soil N concentrations showing reduction in N_2O emissions were

dependent on the flooding and draining seasons. While drought stress is expected to affect AMF growth, flooding conditions could be more tolerated by AMF plant symbiosis. Thus, inoculation with AMF may condition a different ecophysiological response of plants in different stress conditions and this would impact N_2O production. The proximal and distal AMF-mediated effects on denitrification rates could be therefore regulated differently in arid regions compared to temperate or tropical areas, because of variation in climate drivers accompanied by a spatial and temporal heterogeneity of N_2O production. There is a need to further investigate the role of AMF on denitrification rates under a changing climate (i.e. changes in temperature, CO_2 and soil moisture regimes).

Research studying effects of elevated temperatures or CO_2 have often applied an abrupt change in conditions, producing larger (and thus overestimated) effects that do not occur under a gradual change or would level off in the long-term. For microorganisms like AMF it is important to consider that there might be some degree of adaptation to gradual changes of temperature and CO_2 levels, keeping observed effects at the same level, .i.e. increased photosynthesis by plants leads to increased interception of C assimilates and reduced provision of C substrate to the denitrifying community and N_2O emissions. On the contrary, drought events often happen abruptly and will continue to in the future, but there might still be some degree of adaptation via prior drought events that prime microorganisms for the reactions to droughts (Augé, 2001; Lazcano et al., 2014). Nevertheless, the positive effects of AMF on plant growth, especially the provision of water, might become more relevant in the future during sudden changes in soil moisture.

Future prospects

Most studies relating AMF and denitrification have addressed composite effects of AMF on N_2O emissions. They have lacked to segregate between direct and indirect AMF effects and to detect precise mechanisms by which AMF may affect N_2O emissions (e.g Bender et al., 2014). Further research efforts should be redirected towards the mechanistic investigations of direct (i.e. effect of AMF growth on proximal controls) and indirect AMF effects (i.e effect of AMF on distal controls) on N_2O production at the scale of greenhouse using mesocosms or in the laboratory conditions using compartmentalized petri-dishes. Distal AMF mechanisms should be preferentially tested with realistic experimental settings or in field conditions and N_2O collected via static chamber for e.g (Butterbach-Bahl et al., 2013). Field studies can be overcome using a genotypic approach (e.g. tomato mutant) to control for the formation of

AMF and should also be complemented with isotope labelling and molecular techniques (Butterbach-Bahl et al., 2013).

Actually, there is no a direct evidence for the effects of AMF on denitrification and N_2 formation. Further research studies should determine whether AMF increase total N_2 emissions and mitigate denitrification ratio N_2O:N_2. Denitrification enzyme activity and stable isotopes techniques can be used to measure N_2O potential emission rates and total denitrification activity, estimate denitrification ratio, but also follow the amount of N incorporated by AMF and N_2O released, respectively (Philippot et al., 2011; Saggar et al., 2012; Butterbach-Bahl et al., 2013). The study of microbial interactions with AMF (like the potential competition with denitrifies and nondenitrifiers such nitrifiers and saprophytic fungi), the potential stimulation of microbial growth with consequent incorporation and immobilization of N and the AMF influence on the fate of N are pivotal to uncover the presence or lack of interactions between denitrification community and denitrification rates.

It is critically relevant to unravel how AMF influence N_2O emissions by investigating the functional relationship between AMF and N_2O production via the alteration of molecular markers (*nosZ* and *nirK and nirS*) under different soil conditions. A functional gene approach would shed light on microbial origins of N_2O in relation to AMF communities in soils because of the phylogenetic spread of denitrifying microbes (bacteria and recently also fungi) and the variation in structural gene composition of microbes that do or do not possess the *nosZ* gene. The recent discovery of a previously unknown clade of *nosZII* ecophysiologically different to *nosZI* offers new perspectives to deepen understand denitrification process (Hallin et al., 2017). Further studies should be done to determine whether AMF affect this new clade. The role of ectomycorrhiza on N_2O emissions from drained soil forests deserves also further attention (Ernfors et al., 2010).

In addition, it would be relevant to investigate if AMF-promoted effects on physical soil properties such soil structure relate to changes in N_2O emissions as well as to changes in microbial denitrifying community and denitrifying key genes. Moreover, the role of AMF-associated microbiota on soil aggregation and denitrification requires further research attention (Rillig et al., 2005). The study of the functional interaction between plant community and AMF in the plant-soil systems are really scarce and could provide new insights into the microbial denitrification in natural ecosystems.

Finally, it is very critical to design climate change experiments to unravel how AMF might behave or affect denitrification under the change of climate drivers. For example it is still unknown how warming and drought affect the response of AMF on denitrification and associated N_2O production pathways. Moreover, the influence of gradual or abrupt temperatures on AMF and N_2O emissions is unknown.

Conclusion

The specific objective of this review was to explore the influence of AMF mechanisms in environtmental controls of denitrification and N_2O production. We show that AMF can influence many biogeochemical and physical controls of denitrification in soil with subsequent effects on the regulation of N_2O emissions. AMF mechanisms can be categorized as proximal, if they transiently and simultaneously induce changes in denitrification and N_2O production or reduction in the short-term and as distal, if they slowly modify proximal factors and induce changes in denitrification in the long-term. The rate of denitrification and the relative proportion of N_2O and N_2 produced depend strongly on the complex interactions between plant, soil and microbiota: therefore it is pivotal to understand the role of symbiotic microbes such as AMF which interact with several controls of denitrification. Recent advances in modelling, genomics and metabolomics, N_2O collection and quantification techniques provide a big potential to gain new insights into the implications of AMF for denitrification and N_2O production and reduction (Butterbach-Bahl et al., 2013; Hallin et al., 2017). Altogether, this would help leveraging beneficial effects of the mycorrhizal plant symbiosis for climate-smart agriculture.

Conflict of interest

The authors declare no conflict of interest.

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Chapter 3 :

Disentangling direct and indirect effects of mycorrhiza on nitrous oxide activity and denitrification

Abstract

Nitrous oxide (N_2O) is a potent greenhouse gas emitted at considerable rates from agricultural soils. A few recent studies have indicated the potential to reduce N_2O emission rates in agricultural systems through management of soil microbiota, such as mycorrhizal fungi. Our limited understanding of how mycorrhiza influences N_2O emission rates, however, forestalls such management practices. To address the knowledge gap regarding the mechanism of mycorrhizal modification of N_2O formation and denitrification, we disentangle the direct effects of arbuscular mycorrhizal fungi (AMF) via hyphal growth on potential denitrification rates from those resulting from indirect AMF-induced changes in soil aggregation. We manipulated AMF status in a factorial experiment through adding or not AMF inoculum in the form of *Rhizophagus irregularis* and soil aggregation state through either crushing soil aggregates or using agricultural soil with intact soil aggregates. We then grew *Zea mays* (maize) in compartmentalized mesocosms for 4 months. At harvest, we observed approximately three-fold higher AMF root colonization and hyphal densities in the treatments where we had added *Rhizophagus irregularis*. Potential N_2O activity rates and the ratio of potential N_2O activity over total potential denitrification were higher in the undisturbed soil and in the mesocosms with a dense AMF hyphal network (direct effect of mycorrhiza). Further, AM-hyphae promoted soil aggregation while also altering denitrification potential (indirect effect of mycorrhiza). Overall, we present here a comprehensive case study on how mycorrhiza alters potential denitrification rates and related N_2O activity from agricultural soils.

Keywords: Arbuscular mycorrhizal fungi, soil aggregation, potential denitrification rates, potential nitrous oxide activity, denitrification end-products ratio, N-cycling biogeochemistry

Introduction

Agriculture, forestry and other land uses contribute an average of 24 % to total emissions of global greenhouse gases, making them the second most important source after electricity and heat production (IPCC, 2014). Nitrous oxide (N_2O) is a potent greenhouse gas which has a global warming potential 300 times higher per molecule than CO_2 (Forster et al., 2007; Ravishankara et al., 2009). Over 40% of the global soil N_2O emissions originate from agricultural soils (Syakila and Kroeze, 2011; Reay et al., 2012). N_2O emissions are on the rise (Ciais et al., 2013) and are further projected to increase 35-60% by 2030 due to heavier use of nitrogen fertilizers and animal manure production (FAO, 2003). It is thus highly relevant to explore ways to mitigate soil N_2O emissions.

Over two thirds of global N_2O emissions originate from denitrification (Thompson et al., 2012). Biological denitrification is a process where soil nitrate (NO^-_3) is reduced to N_2 with the intermediate formation of gaseous N_2O. The process takes place under anaerobic conditions (Robertson and Groffman, 2015) and in some cases is incomplete, meaning that the end product is N_2O. There are numerous factors that influence denitrification rates, including substrate and carbon availability and temperature (Senbayram et al., 2012). Because of the requirement of anaerobic conditions, soil aggregation is a key factor that should influence denitrification and emissions of N_2O from the soil (Diba et al., 2011; Sexstone et al., 1985; Drury et al., 2004; Khalil et al., 2005; Robinson et al., 2014). Soil aggregates consist of soil particles that are held together by binding agents (Tisdall and Oades, 1982). Soil aggregation is a good indicator of soil fertility and represents a dynamic equilibrium state of two processes: formation of soil aggregates by soil biota and disaggregation resulting from the gradual degradation of the binding agents in the aggregate (Caruso et al., 2011). Several studies have addressed how different fractions of soil aggregates influence N_2O emission fluxes and have yielded contrasting results. For example, Robinson et al. (2014) found that temporal patterns of N_2O emissions were affected by aggregate size with higher peak emissions in the large (4-5.6 mm) and medium (2-4 mm) aggregates. However, the total emissions were the same due to a 'switch' in emissions at day 66, after which smaller (1-2 mm) aggregates produced higher N_2O emissions. The authors hypothesized that the cause of the switch was an increase in aggregate disruption in the small aggregates. By contrast, Drury et al. (2004) reported more N_2O lost through denitrification in dry-sieved macroaggregates than microaggregates. Most studies focused on the effects of aggregate size while few studies

reported on the impact of soil disaggregation, a process that occurs in managed agricultural systems, for example following a disturbance, such as tillage.

Existing studies addressing how soil biota influence denitrification rates and N_2O emissions are to a large degree limited to earthworms and nematodes (Djigal et al., 2010; Bradley et al., 2011; Lubbers et al., 2013). Given that arbuscular mycorrhizal fungi (AMF) can alter the community structure of denitrifiers (Veresoglou et al. 2012a), it is surprising that there has been so little research addressing their impact on denitrification rates and potential activity. AMF form symbioses with the roots of most terrestrial plant species including many agricultural crops (Smith and Read, 2008) and often contribute positively to their biomass, N and P uptake and yield (van der Heijden et al. 2006; Nwaga et al., 2010; Okiobe et al., 2015). Even though AMF hyphae simultaneously promote soil aggregation (Tisdall and Oades, 1982; Miller and Jastrow, 2002; Rillig and Mummey, 2006; Leifheit et al., 2014), these increases in soil aggregation so far have not been considered in mycorrhizal studies quantifying N_2O production (Fig 1). We believe that this represents a major gap in the existing literature.

Veresoglou et al. (2012b) outlined three mechanisms via which AMF could potentially affect denitrification rates and reduce potential nitrification rates, namely competition, allelopathy and altered nutrient availability. Some more recent studies under typical experimental settings, consisting of a previous sterilized soil substrate, showed that AMF reduce overall N_2O emissions by inducing changes in soil N concentrations (Zhang et al. 2015) and the microbial denitrifying community (Bender et al., 2014) or via improving water use efficiency (Lazcano et al., 2014). Furthermore, Storer et al. (2017) reported that AMF hyphae reduced soil N_2O production from N_2O hotspots (*Fig. 1: direct mechanisms*). The changes in denitrification rates and N_2O emissions reported in Storer et al. (2017), Bender et al. (2014) and Zhang et al. (2015) describe effects of AMF on actual denitrification rates via nutrient assimilation, proliferation and mycodeposition that occur relatively quickly. We describe these collectively - as direct mechanisms via which AMF impact denitrification potential and N_2O activity. A limitation of the above-mentioned studies is that they did not assay soil aggregation, even though AMF substantially influences soil aggregation (Leifheit et al., 2014). By doing so, AMF alter soil aeration, soil water-holding capacity and soil water repellency (Veresoglou et al., 2012b; Rillig et al., 2010) but also induce heterogeneity in the way soil microbes are distributed inside and outside soil aggregates (*Fig. 1: indirect mechanisms*).

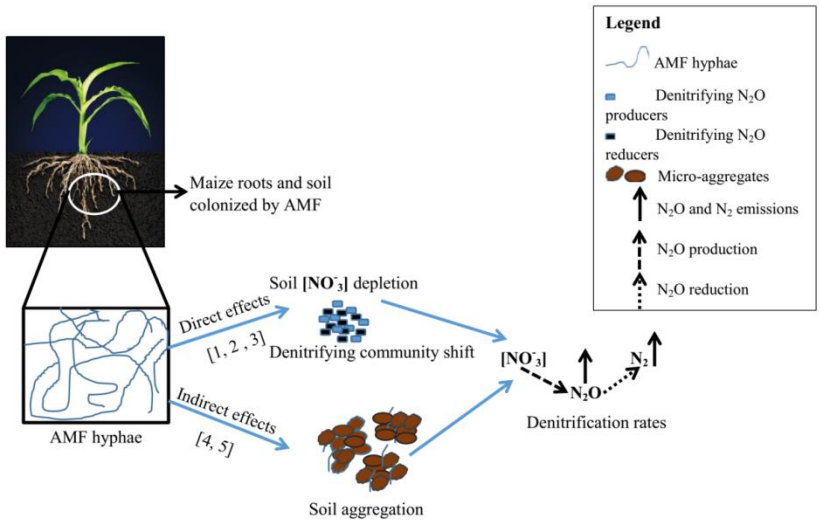

Fig. 1. Summarized potential effects of arbuscular mycorrhizal fungal (AMF) hyphae on denitrification rates. AMF direct effects describe changes in denitrification via depleting soil nitrate and modifying denitrifying microbial community [1.Bender et al., 2014; 2. Veresoglou et al., 2012a; 3. Storer et al., 2017]. AMF also promote the formation of macroaggregates [4. Rillig and Mummey, 2006; 5. Rillig et al., 2010] which should then stimulate denitrification (Drury et al., 2004). We describe the ways mycorrhiza influences denitrification via promoting soil aggregation as indirect mechanisms. Plant root diagram with Corn downloaded from http://ebode.co/plant-root-diagram-with-corn.html.

We refer to these mechanisms that should collectively induce pronounced effects on denitrification and N_2O potential activity - as indirect mechanisms, and differentiate them from direct mechanisms in that they require a longer time to develop. There are still many open questions related to the segregation of direct and indirect AMF effects, such as increased plant growth and promotion of soil aggregates.

Here we report on a controlled experiment in which we disentangled direct and indirect effects of AMF on potential denitrification activity. We manipulated two parameters, the quantity of AMF propagules and the state of soil aggregation in our mesocosms at set-up. The strength of the two manipulations differed: AMF propagule additions aimed at altering mycorrhizal colonization of the plant roots to a degree comparable to what we commonly observe in nature. Our manipulation of soil aggregation had the goal to induce low rates of soil aggregation. Hence, we cannot compare the relative strength of direct vs indirect mechanisms; but we can disentangle between the two mechanisms and assess their overall contribution to denitrification parameters.

We addressed the following two hypotheses: (1) Changes in soil conditions that develop in response to the addition of AMF inoculum reduce potential denitrification and potential N_2O

production rates.; (2) Differences in soil aggregation status, simulated here through mechanical disturbance of the soil and affected by the presence of AMF (Rillig et al., 2010), induce differences in soil conditions that impact potential denitrification and potential N_2O production rates. To test these hypotheses, we (1) quantify the response of AMF inoculation on the recovery of water stable aggregates (WSA) after a mechanical soil disaggregation; (2) investigate the direct effect of AMF community on the relationships between AMF hyphae length and potential denitrification rates; and (3) disentangle the indirect effect of AMF community on the relationships between WSA and potential denitrification rates.

2. Materials and Methods

2.1 Description of the study area and soil characteristics

The experiment was carried out in a climate chamber at the Institute of Biology of Freie Universität Berlin. The soil was a sandy loam collected in October 2016 from a field where maize was grown at 53°19'2.44"N and 13°51'48.03"E in northern Brandenburg/Uckermark, Germany. Physico-chemical characteristics of this soil were evaluated in the central laboratory of the former Institute of Landscape Biogeochemistry (now Research Area 1 "Landscape Functioning"), ZALF, Müncheberg, Germany. The soil chemical properties were as follows: pH-water 5.6, 30 mg NH^+_4-N. g^{-1} soil, 138 mg NO^-_3-N. g^{-1} soil, 469 mg total P. g^{-1} soil, 379 mg available P. g^{-1} soil, 1313 mg available K. g^{-1} soil, 86.30 µS electrical conductivity.cm^{-1}, 0.54 % total organic matter, 0.02 % total inorganic carbon, 0.06 % total nitrogen (Table A1). The soil had the following texture: 69.8 % sand; 20.7 % silt and 9.5 % clay. The soil that we used for the experiment was not sterilized and thus contained an indigenous AM fungal and microbial community. Because the soil originated from an agricultural site, we expected mycorrhizal infection potential to be low.

2.2 Climate chamber experiment

2.2.1 Mesocosm design

We used 16 compartmentalized acrylic mesocosms as experimental units. Each acrylic mesocosm consisted of two compartments (Veresoglou et al., 2011). Two half cylindrical plastic containers each with a radius of 5 cm, a height of 20 cm, and a volume of 1 L were sealed together with a cable binder. Before sealing, a 38 µm mesh was placed between the cylindrical containers resulting in a compartmentalized mesocosm. This mesh size is sufficient for AMF hyphae to cross, but prevent roots from passing, therefore creating one

root-containing (rhizosphere) and one root-free (hyphosphere) compartment. This design allowed us to separately study the effects of roots and hyphae on denitrification rates.

2.2.2 Experimental approach

The experiment was a 2 x 2 factorial complete randomized design. The two factors were the addition (or not) of propagules of an AM fungus (*Rhizophagus irregularis*) and the manipulation of the original state of soil aggregation (crushed or intact). Each treatment combination was replicated 4 times for a total of 16 compartmentalized plastic mesocosms.

To disaggregate the soil (low aggregation status) vs. maintain it intact (high aggregation status), sandy loamy soil was sieved using a 2 mm sterilized sieve and the sieved soil was divided into two parts. One part was left intact (undisturbed soil with aggregates), while the second part was mechanically crushed for two hours to destroy and reduce soil aggregates using a small-scale concrete mixer machine (Limex 125LP, Croatia) which contained three large rocks. We then determined the percentage of water stable soil aggregates (WSA) (Kemper and Rosenau, 1986). Crushed soil contained 2.65% WSA, while undisturbed soil had 11.34 %. Each mesocosm compartment was filled with 500 g of undisturbed or crushed soil, respective to treatment. Prior to the experiment, we ran a two-month preliminary experiment to identify a suitable AMF isolate for our experiment. *R. irregularis* performed considerably better than *Gigaspora margarita* and *Scutellospora gregaria*, which was also found by Stockinger et al. (2009). We made a 2 cm deep hole in the plant compartment of each pot and inserted one seed of *Zea mays* (the seeds were previously surface sterilized using 10 % bleach for 10 min and 70 % ethanol for 30 s, rinsed with deionized water after each step); we added 300 mg of AMF inoculum and the hole was covered by a fine layer of soil. This inoculum was from SYMPLANTA Company, Germany, and composed of clay powder containing 10.000 spores.$g^{-1}$of *R. irregularis*.

2.2.3 Growth settings and harvest

The average day/night temperature in the climate chamber was 20/16 °C, respectively. The relative air humidity was 60 %. Pots were watered equally by volume and randomized regularly throughout the experiment. Maize plant roots and soil were harvested 4 months after inoculation and sowing, which is roughly equivalent to the four month growth period typical in agricultural settings. The mesocosms were gently emptied and the roots were removed thoroughly from the soil and rinsed with water, a portion of the root biomass from each pot was cut into pieces of 2 cm and stored in 70 % ethanol for AMF root colonization. Fresh soil

was collected in the middle of each pot; a portion of this was stored at -20°C for AMF hyphal length, whereas soil for WSA and potential denitrification rates assessments was stored on a short-term basis (i.e. potential denitrification was assayed less than a week after the harvest).

3. Lab analyses

3.1 Quantification of AMF structures

We stained a representative root fraction per mesocosm with 0.05% Trypan Blue according to a modified staining protocol (Phillips and Hayman, 1970). Root pieces were cleared in 10% KOH at 80 °C for 30 min, washed with tap water three times, acidified in 1 % HCl at room temperature and stained for 15 min in 0.05% Trypan Blue at 80 °C. AMF root colonization was quantified at 200 X magnification using the magnified intersections method (200 intersects per sample) (McGonigle et al., 1990). AMF structures were recorded separately; arbuscules, hyphae and vesicles and the total percentage of AMF structures in maize roots was calculated. AMF hyphal length in soil was quantified according to Rillig et al. (1999) following an aqueous extraction with sodium hexametaphosphate.

3.2 Quantification of WSA percentage and residual NO_3^- concentrations

The total WSA percentage in the 1-2 mm size range was quantified from each mesocosm compartment by a wet sieving technique modified from Kemper and Rosenau, (1986). We used the wet sieving apparatus code 08.13 from Eijkelkamp, The Netherlands. Residual NO_3^- was measured according to Miranda et al. (2001).

3.3 Quantification of potential denitrification rates

3.3.1 Denitrification enzyme activity protocol

To quantify potential N_2O activity, potential denitrification activity and estimate the denitrification ratio, we used a DEA (denitrification enzyme activity) assay modified from Tiedje et al. (1989) and Groffman et al. (2006). The assay yields estimates of denitrification rates under idealized conditions and yields a proxy of total denitrifying enzymes in soil. To inhibit the final step of denitrification (i.e. block nitrous oxide reductases) we used acetylene. N_2O production was quantified from 64 soil subsamples with no acetylene addition (4 treatments x 2 compartments x 4 replicate pots x 2 replicates without acetylene) and 64 with acetylene (4 treatments x 2 compartments x 4 replicate pots x 2 replicates with acetylene). Briefly, 25 g of field-moist soil from each soil subsample were weighed into 125 mL

incubation bottles for a total of 128 incubation bottles and supplied with 15 ml C&N solution containing 1.07 mM KNO_3 as N-source +1 mM glucose as easily available C source and 0.7 mM chloramphenicol (CAP); a broad-spectrum bacteriostatic agent, inhibiting ribosomal de novo protein synthesis (Philippot et al., 2013). Anaerobic conditions were established by evacuating and flushing all the bottles with Helium (He) gas for 5 min. Thirteen mL of acetylene corresponding to 10 % (v/v) was added into each of 64 bottles and overpressure was subsequently released to bring the internal bottle pressure to atmospheric pressure. Ten ml gas samples were taken from the headspace of each incubation bottle at 45, 90, 150 and 210 min (from the time acetylene was added) using a 15 mL syringe. 10 mL of gas mixture (9:1 of He:acetylene) were immediately replaced in the bottles with acetylene, and 10 mL He gas in the bottles without acetylene. A total of 512 pre-evacuated 50 ml Exetainers (Supelco®, Bellefonte, USA) (128 incubation bottles x 4 gas sampling times) were used for sample collection; each was filled with 10 ml gas sample.

3.3.2 Gas analysis and potential denitrification parameters

Gas samples were analysed using an Agilent GC -14A Shimadzu, equipped with an electron capture detector and flame ionisation detector (Agilent Technologies Inc., Santa Clara, CA, USA) in the Leibniz Centre for Agricultural Landscape Research (ZALF), Müncheberg, Germany (Loftfield et al., 1997). A total of 512 greenhouse gas concentration values from N_2O were obtained. Potential N_2O production (N_2O) was defined as soil N_2O rates in samples without acetylene addition, while potential denitrification activity (N_2O+N_2 production) was accumulated soil N_2O rates in the presence of acetylene. The ratio between potential N_2O activity and total potential denitrification activity was defined as the denitrification ratio [$(N_2O/(N_2O+N_2)$] (Philippot et al., 2011; Domeignoz-Horta et al., 2015).

4. Statistical analysis

After correction for sample dilution, gas concentrations were expressed in ng $N_2O\text{-}N.h^{-1}.g^{-1}$soil dry using the Ideal Gas Law (Baker et al., 2003). Gas concentrations were then regressed against time (Hutchinson and Mosier, 1981); values were accepted when the linear regression coefficient was above 0.75. N_2O concentrations were corrected with consideration for the amount of N_2O that may remain in the aqueous phase using the Bunsen absorption coefficient (Groffman et al., 1999). To correct for the compartmentalized nature of the mesocosms in our statistical analysis, we used a nested design (i.e. rhizosphere and hyphosphere compartments were nested within the variable mesocosm) as implemented in repeated-measurements

designs. Because we used unsterilized field soil for our experiment, the strength of the resulting mycorrhizal colonization varied considerably across samples. We summarize our modelling approach below:

We first present results to establish the efficacy of our treatments, which in this case translates to higher root colonization in the mesocosms inoculated with mycorrhiza and higher WSA in the treatment with intact soil aggregates. We also report on the two most conspicuous differences in denitrification rates which were both due to differences in soil aggregation.

We then report on the nested models. Each model contained a nested factor which was the compartments within each mesocosm. All competing models included at least one AMF-related parameter (either the categorical parameter of AMF manipulation or the continuous variable hyphal densities at harvest) and a soil aggregation related parameter (either the categorical parameter of soil aggregation manipulation or the continuous parameter water-stable-aggregates (WSA) at harvest). We carried out our model selection based on the Akaike information criterion (AIC) values of all competing models (Appendices 1 & 2). There is no straight-forward way to extract AIC values from repeated-measures models in R, thus we refitted the models in the form of mixed-effects linear models with the random-effects factor "subject" and extracted AIC values from these equivalent models. Such mixed-effects models are often used in the literature to address nested designs but they are more liberal than their repeated-measures equivalents. We report model performance statistics for the more conservative (Haverkamp & Beauducel, 2017) repeated-measures models.

Because of our particular modelling approach, which used continuous variables as predictors, we do not present our data in the traditional form of bar plots. We provide instead scatter plots with the raw data and overlaid best-fit lines.

R by default uses Type-I sum of squares. This means that the partitioning of the variance in ANOVA models depends on the order in which the predictors are entered in the model. To address possible concerns associated with this we used the following rules. We fitted continuous predictors before categorical ones. We additionally interchangeably fitted parameters (either categorical or continuous) which were competing in the same model. Because of the simple structure of the model we did not carry out a path analysis to assess the strength of direct and indirect pathways, which in this case would have been equivalent to simple partial regressions. Instead, we report the effect sizes of each of these Type-I sum of squares models. Moreover, to the degree that inoculation with AMF could have also increased

soil aggregation towards the end of the experiment and induced indirect effects on denitrification, we overestimated the contribution of direct effects. The soil aggregation manipulation that we included in our experimental design and we classify as a source of *indirect* effects, unlike *direct effects*, described strong differences in soil aggregation at the beginning of the experiment and was remained continuously present and influencing actual denitrification rates and denitrifiers until the end of the experiment (i.e. endpoint assay of soil aggregation). As a result, we expected the effect sizes relating to soil aggregation to have been considerably smaller when we calculated *direct* pathways, compared to the case of *indirect* pathways.

Model parameters were appropriately transformed to meet the assumptions of the analyses. All analyses were carried out in R version 3.0.2 (R Core Team., 2013).

5. Results

Treatment effectiveness on AMF root colonization, WSA and potential denitrification rates (Fig. 2). *R. irregularis* successfully colonized the maize plant roots: an average of 32 % root colonization was observed in the AMF treatment compared to 9 % in the non-inoculated treatment (Fig.2a) after 4 months of the experiment. The densities of hyphae that we observed were comparable to those in Storer et al. 2017 and reflected the high fertility of the soil (A1). Differences in hyphal length across the two mycorrhizal treatments were subtler and there were mesocosms that did not receive additional inoculum which had higher hyphal length than mesocosms that did. The percentage of WSA was higher in undisturbed soil ($p < 0.005$) compared to crushed soil (Fig.2b). This percentage increased in both crushed and undisturbed soil after 4 months of the experiment compared to the percentage in the beginning of this experiment.

We detected differences in potential N_2O production and the denitrification ratio resulting from the manipulation of soil aggregation ($p < 0.0001$) (Model 2. in Supplementary material). These two denitrification parameters were higher in undisturbed soil with higher WSA compared to crushed soil with lower WSA (Fig. 2c, d; Model 2). Potential N_2O activity was approximately 8 times higher in undisturbed compared to crushed soil, while the ratio of denitrification ($N_2O/[N_2O + N_2]$) was approximately 6 times higher in undisturbed compared to crushed soil (Fig. 2d).

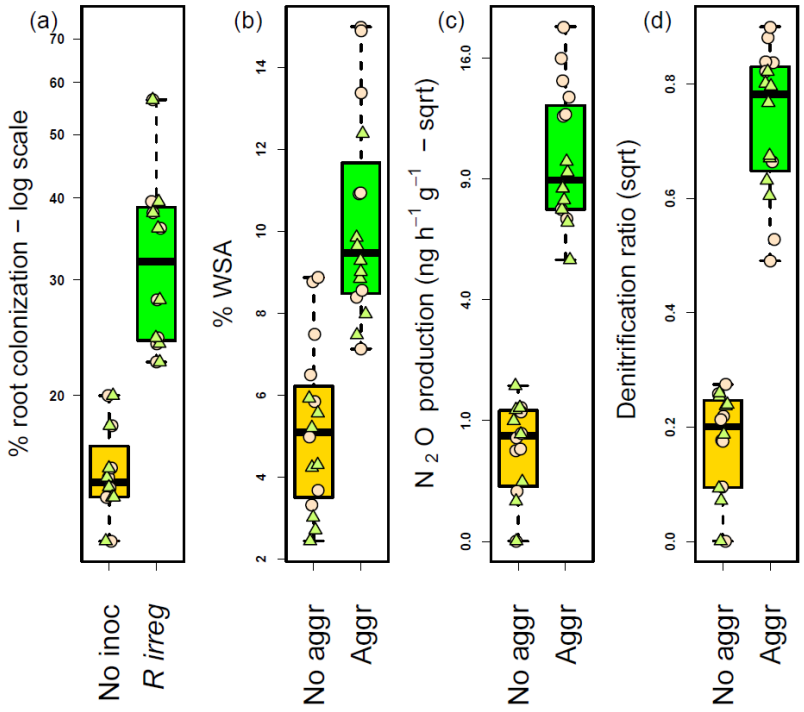

Fig. 2. Overview of the treatment effectiveness on water stable soil aggregate (WSA), AMF root colonization and potential denitrification rates, 4 months after sowing. (a) AMF maize root colonization (% - log scale) as affected by the addition of *R. irregularis* spores; (b) WSA (%) as influenced by manipulation of the soil aggregation state; (c) potential N_2O activity (ng N_2O-N.h^{-1}.g^{-1}dry soil) as influenced by manipulations of soil aggregation state; (d) denitrification [$(N_2O)/(N_2O+N_2)$] ratio as affected by manipulations of soil aggregation state. Abbreviations are as follows: No aggr: No aggregates (crushed soil), Aggr: Aggregates (undisturbed soil agggregate), No inoc: No inoculation with *Rhizophagus irregularis*, R irreg: Addition of *Rhizophagus irregularis* spores. Jittered points are individual measurements - *n* in all cases equals 16; circles in hive plots stand for rhizosphere samples whereas triangles for hyphosphere ones. Model statistics are reported in Appendix 2.

Across-samples variation in WSA and NO_3^- concentrations. The optimal model for WSA included the parameters AMF treatment, soil aggregation treatment, their interaction compartment and the density of AMF hyphae (Table S1). There were more WSA in samples with a relatively dense AMF mycelial network at harvest (F=11.8, P=0.006) as well as in samples where we had left the soil aggregates intact (F=12.5, P=0.004) – Fig. 3. There were also differences depending on the AM treatment and sample compartment (F=10.6, P=0.008 and F=17.3, P<0.0001, respectively). The interaction term between AMF and soil aggregation treatment was not significant (Optimal model 1). NO_3^- was lower in the mesocosms that received crushed soil and in the rhizosphere compared to the hyphosphere (Model 5).

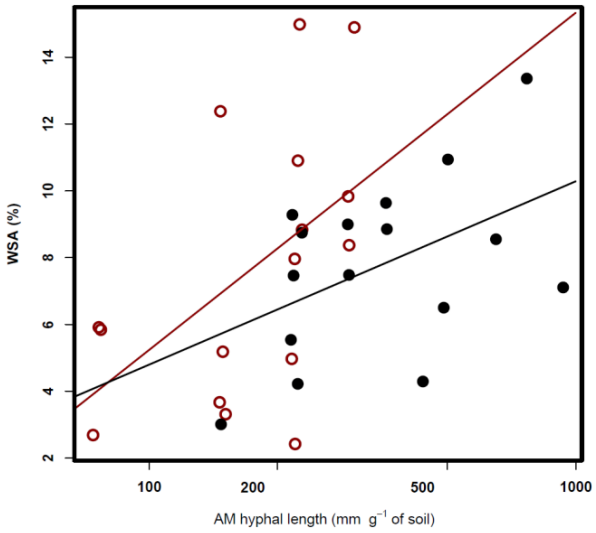

Fig. 3. Relationship between the estimates of WSA and AM hyphal densities in soil at harvest. Red open circles represent non-inoculated soil samples while black filled circles represent soil samples inoculated with *R. irregularis*. Hyphal length at harvest (F=11.8), addition of AM propagules (F=10.6) and soil-aggregate manipulation treatment (F=12.5) had significant effects on WSA. We overlay the first order best-fit lines for the groups of non-inoculated and inoculated soil samples. Model statistics are presented in Appendix 2.

Variance in potential nitrous oxide activity rates. The optimal model included all parameters other than WSA (Table S2). Potential nitrous oxide activity rates were higher in samples with a denser mycelial network (F=43.14, P<0.001 – Fig. 4a) and in the intact soil aggregation treatment (F=59.88, P<0.001 – Fig. 5a). There were differences across mycorrhizal treatments (rates were lower per unit hyphae following the additional of propagules, F=31.17, P<0.001) but there was no significant interaction with soil aggregation treatment (Optimal model 2).

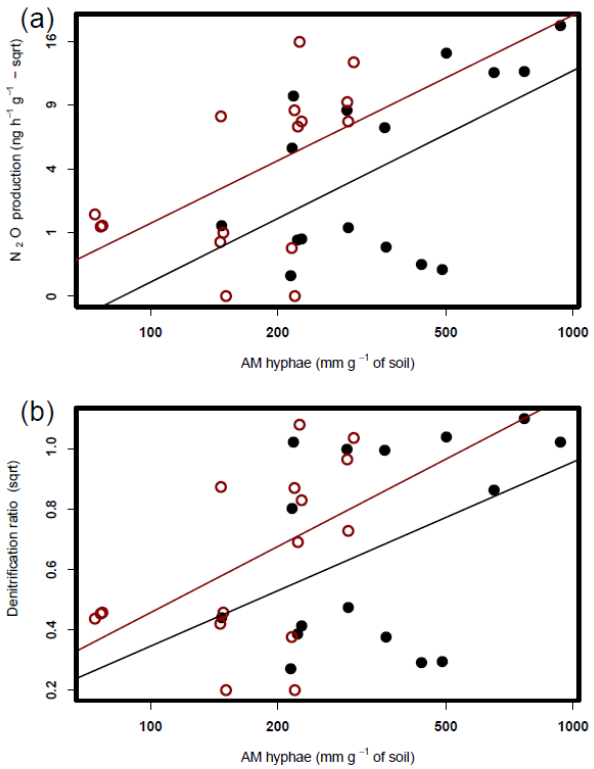

Fig. 4. Relationship between AM hyphal densities in soil samples and the resulting nitrous oxide potential activity (a) and the denitrification ratio of end-point activity (i.e. nitrous oxide over potential denitrification activity) (b). We present non-inoculated soil samples in the form of red open circles and soil samples that had been inoculated with *R. irregularis* as black filled circles. We overlay the first order best-fit lines for the groups of non-inoculated and inoculated soil samples. Hyphal length at harvest (F=241.9), addition of AM propagules (F=174.1) and soil aggregate manipulation treatment (F=334.5) influenced N_2O activity, whereas hyphal length at harvest (F=53.6) and soil-aggregate manipulation treatment (F=109.0) influenced denitrification ratio. Model statistics are presented in Appendix 2.

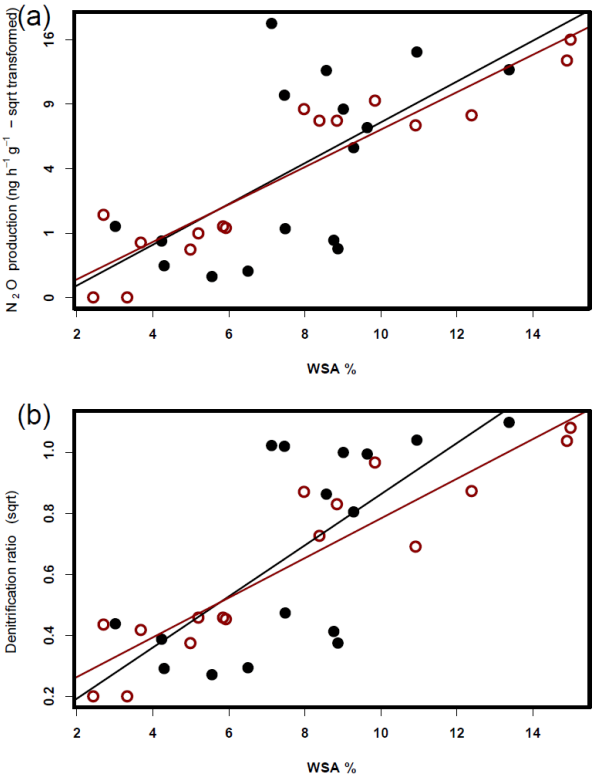

Fig. 5. Relationship between water stable soil aggregates and nitrous oxide potential activity (a) and the response ratio of nitrous oxide over total denitrification (b) across the soil samples in the experiment. We present non-inoculated soil samples in the form of open circles and soil samples that were inoculated with *R. irregularis* as black circles. We overlay the first order best-fit lines for the groups of non-inoculated and inoculated soil samples. Model statistics are presented at Fig. 4 and in Appendix 2.

Variance in potential denitrification activity. The optimal model included all possible predictors other than hyphal densities (Table S3). The only significant predictor, however, was the interaction between AM treatment and soil aggregate manipulation treatment (Optimal model 3 - Fig. 6).

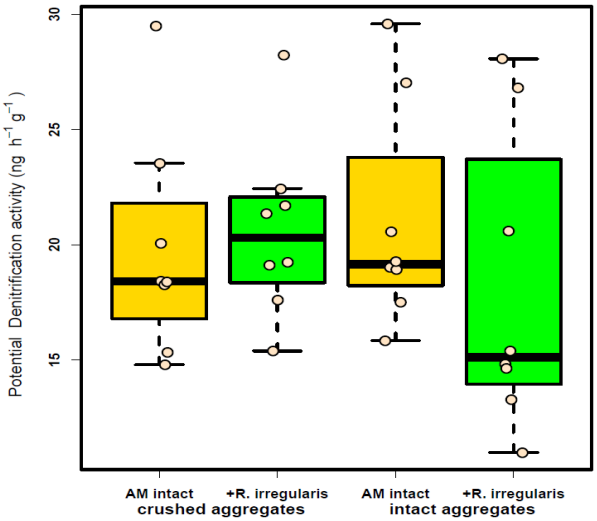

Fig. 6. Variance in total denitrification potential activity across the different treatments in our experiment. The specific parameters were deemed influencial through a model selection procedure which we present in Table S3. The only significant predictor was the interaction between the AMF treatment and that of soil aggregates. AM intact: indigenous AMF community. We present the exact statistics of the model in Appendix 2.

Variance in the response ratio of potential denitrification end-products. The optimal model included the parameters soil aggregation treatment and hyphal densities (Table S4). Nitrous oxide comprised a higher proportion of the denitrification end-products in the soil samples in which a relatively dense mycelial network had established (F=53.62, P<0.001 – Fig. 4b) and in mesocosms where we had not crushed soil aggregates (F=109.03, P<0.001 – Fig. 5b) (Optimal model 4). We observed differences in the slope of the relationship between the ratio of denitrification and WSA between the inoculated and non-inoculated soils (Fig. 5).

Addressing confounding effects of root growth on denitrification. Plant roots in the rhizosphere compartment could mask the influence of mycorrhiza on denitrification. To address whether this was the case, we added interaction terms between the compartment and AM parameters to the mixed effects versions of our optimal models (Model 6 – Appendix 2). None of these terms were significant meaning that mycorrhiza in the hyphosphere and rhizosphere compartments similarly influenced denitrification.

6. Discussion

We show here that mycorrhiza strongly influences potential N_2O activity rates and the denitrification ratio. A unique feature of our analysis is that we can segregate the influence of mycorrhiza into direct effects which occur via hyphal growth in the soil and indirect effects

which occur via improving soil structure (Fig. 1). We structure our discussion in sections specifically addressing these mechanisms.

AMF significantly increased WSA

Several studies have reported positive effects of arbuscular mycorrhiza on soil aggregation (Tisdall and Oades, 1982; Rillig and Mummey, 2006; van der Heijden et al. 2006; Leifheit et al., 2014; Lehmann et al. 2017). We found this effect here as well, even though we used an agricultural soil, rich in nutrients; and with a background of mycorrhiza, together with other soil animals and microbes, since we used non-sterile soil. We show that the mean slope between log transformed hyphal densities and WSA exceeds the value of 2.2 (Fig. 3), suggesting that the influence of AMF on soil aggregation has been strong. Our study thus adds to the existing body of literature showing that AM fungal hyphae, particularly in agriculture, contribute to a beneficial ecosystem process, the formation of soil aggregates.

AMF hyphal density effects on potential N_2O activity.

We found higher potential N_2O activity rates when AMF were relatively abundant (Fig 4a) but also dependencies on soil aggregation status (Fig. 5a). These results indicate a clear influence of mycorrhiza in promoting N_2O emissions (i.e. influence of AMF-hyphae – direct effect). Our original expectation (Hypothesis One) was that AMF hyphal densities would induce lower N_2O potential activity by altering soil conditions in various ways, such as transporting exchangeable N in the form of NH_4^+ to the plant host, thus reducing its relative availability in soil (Bender et al., 2014; Zhang et al., 2015). Recently, Storer et al. (2017) reported that the presence of AMF was sufficient to lower N_2O production by reducing soil NH_4^+ concentrations. The apparent mismatch between the above-mentioned studies and our experiment might have resulted from our use of a high-fertility agricultural soil or because we assayed potential denitrification and not actual denitrification rates. Higher densities of AMF hyphae in the treatments where we added AMF inoculum could thus have led to a decline in soil N concentrations.

AMF can alter the community structure of the denitrifying community (Veresoglou et al., 2012 a; Veresoglou et al., 2012 b). AMF hyphae, for example, significantly increased the relative abundance of two of four denitrifying bacterial phyla (Gemmatimonadetes and Deltaproteobacteria) possessing *nosZ* genes with the ability to use N_2O as an electron acceptor (Nuccio et al., 2013). In our study, inoculation with *R irregularis* and the presence of high

AMF hyphal densities may have decreased the relative abundance of denitrifiers capable of reducing N_2O, leading to the observed higher potential N_2O production in the AM treatment.
AMF hyphae have also been reported to alter densities and the community structure of key denitrifying genes *nirK* and *nirS* in soil, which are involved in the reduction of NO_3^- into NO_2^-, and *nosZ* in soil, which is involved in the reduction of N_2O into N_2 (Veresoglou et al. 2012a; Bender et al. 2014). Bender et al. (2014) found that the abundance of one of the key genes responsible for N_2O production (nir*K*) was negatively and for N_2O consumption (*nosZ*) positively correlated to AMF abundance, indicating that N_2O regulation was mediated by AMF-induced changes in the soil microbial community. Many experiments that include manipulations of AMF have been carried out in soil that has been sterilized (to remove existing AMF propagules) and re-inoculated. This comes at the cost of risking the establishment of the nitrifying and denitrifying communities (Amora-Lazcano et al., 1998). A unique feature of our study was that we had not sterilized our soil. As a result, we had a more realistic system with regards to bacterial community structure and soil aggregate state in the intact water stable aggregates treatment. Abundance of denitrifying genes correlates well with assayed denitrification rates (e.g. Henderson et al., 2010). We believe that in our system the denitrification community did not respond to mycorrhiza in the same way as in Bender et al. (2014) and that this was due to us not having sterilized the soil.
In our experiment, we observed the strongest effect sizes when we used potential N_2O activity rates as a response variable (Appendix 2). By contrast, we found weak effect sizes when using total potential denitrification rates as a response variable and the influence of both WSA and hyphal densities in the soil were no longer detectible (Fig. 6). This was an interesting point which we believe may have occurred because nitrous-oxide reductases are produced by a narrower range of organisms in the soil than nitrate reductases (Henry et al., 2006).

AMF-mediated increases in soil aggregation promote potential N_2O production.

We demonstrate a strong relationship between WSA and potential N_2O activity (i.e. influence through promoting WSA – indirect effect) (Fig 2a and 5a.). Because we did not measure actual denitrification rates, this relationship obviously reflects the influence of soil conditions during the experiment. This was partially attributable to the lower availability of NO_3^- when the soil aggregate state in the mesocosms was kept intact (Model 5 in Appendix 2). We observed differences in the slope of the relationship between the rate of potential N_2O activity and WSA between the inoculated and non-inoculated soils. Any AMF-mediated changes in WSA should, therefore, in the long-term also influence potential denitrification rates.

The higher fraction of WSA that we observed as a result of AMF additions in our study may have benefited soil aeration, which could affect denitrification and alter potential N_2O production, because denitrification strongly depends on a low oxygen concentration (Sexstone et al., 1985; Smith et al., 2003; Balaine et al., 2016). AMF hyphae in the soil could increase diffusion rates of O_2 towards the interiors of soil aggregates and the soil matrix in intact soil. Soil aeration could have thus inactivated nitrous oxide reductase (Robertson and Groffman, 2015) and enhanced over the course of the pot experiment potential N_2O production. By contrast, the low O_2 concentration and diffusion in crushed soil could have favored conditions where denitrification was complete, resulting in lower N_2O production and being expressed as low potential N_2O activity compared to undisturbed soil.

Moreover, through the formation of macroaggregates (Rillig and Mummey, 2006), over the course of the experiment, AMF could have generated anaerobic zones inside the aggregate interiors of these soils, thereby favouring denitrification and stimulating the growth of denitrifiers.

Effects of AMF symbiosis on the denitrification ratio

Technologies aiming at mitigating greenhouse gas emissions often endeavor to reduce total denitrification rates in agricultural soils. An alternative way to address greenhouse gas emissions is through reducing the proportion of N_2O as an end-product of denitrification. Our study showed that arbuscular mycorrhiza changes both directly and indirectly (i.e. via changes in soil aggregate state) the denitrification ratio in agreement with hypotheses 1 and 2 (Fig. 4b and Fig. 5b, Fig. 1; Optimal model 4). However, we observed increases in the ratio in response to AMF abundance, instead of a decline, which we had predicted in the undisturbed soil.

Some studies report that the denitrification ratio positively correlates with N_2O emission rates (Senbayram et al., 2011; Krause et al., 2017). We also found support for this relationship implying that the denitrification ratio depended mainly on potential N_2O activity rates (Fig. 4 and Fig. 5). The addition of arbuscular mycorrhiza in our systems may have altered potential N_2O activity rates and consequently the denitrification ratio via altering the structure of the denitrifying microbial community and the potential activity of N_2O reductase.

The denitrification ratio depends strongly on the abundance of denitrifying N_2O reducer bacteria possessing *nosZ* gene encoding for N_2O reductase activity (Philippot et al., 2009, Philippot et al., 2011; Krause et al., 2017). We showed that the proportion increased in soil samples with high AMF hyphal densities and when we kept the soil aggregates intact (Fig. 4b;

Fig. 5b). We found significant differences in our two sets of analyses, the first consisting of the manipulation factor AMF and soil aggregation manipulation and the second of the continuous variables WSA and hyphal density in N_2O potential activity rates. We conclude that the potential activity of denitrifying N_2O reducers was lower at high AMF hyphal densities and in intact soil. By contrast, at low AMF abundance and in crushed soil the activity of denitrifying N_2O reducers was higher, thereby lowering potential N_2O activity as well as the ratio of denitrification.

The changes in the potential activity and therefore the denitrification ratio might be explained by shifts in denitrifying microbial community in response to AMF abundance (Veresoglou et al., 2012a) and soil aggregation modification. Bender et al. (2014) showed lower cumulative N_2O fluxes and *nos*Z genes copies in the presence of high AMF hyphal densities in their controlled experiment where they had used presterilized soils. This contrasting result can be explained by the fact that we did not sterilize our soils and we quantified the soil potential for N_2O production using DEA assay at an endpoint harvest.

It is also likely that AMF and soil aggregates altered the activity of the N_2O reductase in soil resulting in differences in the denitrification ratio. N_2O reductase activity is often limited by the availability of Cu^{2+} in soil (e.g. Zumft, 1997). In our controlled set-up, AMF could have reduced the availability of Cu^{2+} via uptake (Lehmann and Rillig 2015) so that most of the denitrified N was in the form of N_2O. We are unsure whether high aggregate state could have comparable effects via increasing the spatial heterogeneity in the availability of Cu^{2+}. Variances in this ratio have been also in the past linked to changes in the abundance and diversity of N_2O reductase gene *nos*Z and in the soil properties (Domeignoz-Horta et al., 2015; Krause et al., 2017). AMF may drive the ratio of denitrification end-products through causing a shift in the denitrifying microbial community and altering potential N_2O reductase activity in the short term or via changes in soil aggregation in the longer term.

Conclusion

We hypothesized that AMF mediate potential N_2O production and the denitrification end-product ratio through the AM hyphal network or via soil aggregation modification in the undisturbed soil. We found that the AM symbiosis increases potential denitrification rates when we kept the soil intact through direct effects (e.g., AMF hyphae effects on soil N concentrations) or via indirect effects (AMF hyphae effects on soil aggregation promotion). The influence of AMF on potential N_2O activity from soils could be seen as an aggregate ecosystem process with distinct proximal (direct) and distal (indirect) steps (Fig. 1). Any modification of AMF abundance and soil aggregation as caused by intensive

agricultural management practices and high fertilizer additions could thus cascade to below-ground interactions and impact N_2O emissions rates.

Conflict of interest.

The authors declare no conflict of interest.

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Chapter 4 :

Plant diversity masks arbuscular mycorrhiza-induced changes in N_2O potential emissions in experimental plant communities

Abstract

Land use induced emission of nitrous oxide (N_2O) into the atmosphere is linked to ongoing climate change, because N_2O is one of the most effective greenhouse gases. To date the literature covers mostly how aboveground (i.e. plant community structure) and belowground (i.e plant associated soil microbes) biota separately influence denitrification in isolation of each other. We address the role of arbuscular mycorrhiza fungi (AMF) on denitrification enzyme activity and N_2O formation in temperate grassland plant diversity. We here perform a mesocosm experiment where we combine a manipulation of belowground biota (i.e. addition of a model AMF species; *Rhizophagus irregularis* propagules to the indigenous mycorrhizal community) with a realized gradient in plant diversity. We used a seed mix typical of European vascular grassland species and by stochastic differences in species establishment across the sixteen replicates per manipulation induced a gradient in plant diversity. We show that unlike denitrification potential activity, N_2O potential emissions do not change with AMF and depend instead on realized plant diversity. By linking mycorrhizal ecology and denitrification potential activity, we present a comprehensive picture to date of denitrification dynamics under natural conditions, which should be generalizable for grassland settings.

Keywords: Arbuscular mycorrhizal fungi, European grassland plant diversity, nitrous oxide potential emissions, denitrification enzyme activity, plant-soil-microbial interactions

Introduction

Nitrous oxide (N_2O) is a powerful greenhouse gas and the single most important ozone-depleting substance responsible for 10 % annual global warming and thus climate change (Forster et al. 2007; Bates et al. 2008; Ravishankara, Daniel and Portmann 2009; IPCC 2014). N_2O is mainly produced during two N-cycling microbial processes, namely nitrification and denitrification, with denitrification accounting for over 70% of N_2O emissions (Bateman and

Baggs et al. 2005; Carter 2007; Saggar et al. 2013). Denitrification is a biological process where nitrate (NO_3^-) and nitrite (NO_2^-) are converted to the gaseous end-products N_2O (incomplete denitrification) and dinitrogen (N_2-complete denitrification) (Groffman et al. 1999). The two nutrients that most often limit denitrifier activity are nitrogen (N) and carbon (C) (Philippot et al. 2011; Morales et al. 2015; Krause et al. 2017) and in soil their availability is maximum in the proximity of plant roots, the rhizosphere (Philippot et al. 2013; Kuzyakov and Xu, 2013).

Some recent studies have showed that plant species identity surpasses species richness as a key driver of N_2O emissions from grassland (e.g Abalos et al. 2014) while others reported the opposite (Niklaus et al. 2016; Abalos et al. 2017; Han et al. 2017). However, the mechanisms underlying these responses remain largely obscure likely because most of these studies have investigated the role of aboveground plant traits with a limited mechanistic understanding of the role of soil microbiota. Plant-soil-microbe interactions play a particularly important role in denitrification by promoting but also in some cases suppressing denitrification (Bardon et al. 2014; Guyonnet et al. 2017); we currently do not sufficiently understand these interactions.

A key player in the rhizosphere that can modify denitrification and N_2O emissions are arbuscular mycorrhiza fungi (AMF; Veresoglou et al. 2012; Bender et al. 2014; Storer et al. 2017). AMF have a ubiquitous distribution across terrestrial biomes and form nutritional symbioses with the majority of terrestrial plant species (Treseder and Cross 2006; Öpik et al. 2010) with implications for plant community composition (Rillig et al. 2014; van der Heijden et al. 2015; Lin, Cormack and Guo 2015), plant C economy (Jakobsen and Rosendahl 1990), nutrient cycling (Rillig 2004; Veresoglou, Chen and Rillig 2012, Bender et al. 2015) and soil aggregation (Leifheit et al. 2014; Lehmann et al. 2017).

To date, most studies addressing the role of AMF on denitrification and denitrification potential activity have used plant-soil systems with one or more individuals belonging to a single plant species (e.g. Bender et al. 2014; Lazcano et al. 2014; Storer et al. 2017). These settings represent well agricultural practices but fall short of capturing more realistic settings with higher plant diversity. For example, in plant monocultures, experimental settings mask the strong changes that AMF induce to plant diversity (e.g. Klironomos et al. 2011) which can promote microbial denitrification potential and N_2O potential emissions (Sutton-Grier et al. 2011; Niklaus et al. 2016 but see Niklaus et al. 2006; Han et al. 2016; Abalos et al. 2017). Moreover, AMF through altering plant diversity could be influencing soil C decomposition

and the mineralization of N (e.g. Zak et al. 2003; Meier and Bowman 2008; Ebeling et al. 2014) increasing substrate availability to denitrifiers. Finally, the efficiency with which plant and AMF derive benefits from the symbiosis could differ considerably with more than one symbiotic partner (Selosse and Rousset 2011).

We here addressed the implications to denitrification-related parameters of supplementing existing AMF propagules of a high fertility agricultural soil, most-likely maintaining a low AMF propagule availability, with a common AMF inoculant, *Rhizophagus irregularis* and growing a seed mix. We hypothesized here that in our seed mix which was dominated by grasses which do not depend strongly on mycorrhiza, AMF would promote plant diversity (Bergelson and Crawley 1988; *Hypothesis One*); AMF would reduce potential denitrification enzyme activity and N_2O emissions in agreement with Bender et al. (2014) and Storer et al. (2017) – (*Hypothesis Two*); and that mesocosms supporting higher plant diversity would induce higher rates of potential denitrification activity and N_2O emissions than those of lower plant diversity (Niklaus et al., 2006) – (*Hypothesis Three*).

Materials and methods

Experimental design

The experimental design was a fully randomized one factor experiment with 16 replicates. The manipulation factor was the addition of AMF propagules (*Rhizophagus irregularis*, SYMPLANTA, Germany at a rate of approximately 5,000 spores per mesocosm) to boost the indigenous mycorrhizal inoculum already present. The soil was a sandy loam collected from northern Brandenburg/Uckermark, Germany 53°19'2.44"N and 13°51'48.03"E in November 2017, from a wheat field (Okiobe et al. 2019). Prior to use, the physico-chemical properties of the soil (Table 1a) were analyzed at the central laboratory of the Institute of Landscape Biogeochemistry, ZALF, Müncheberg, Germany. The soil was air dried and sieved (<2 mm) for homogenization purposes.

We used a seed mix consisting of 90% grasses, 10 % of forbs including 5% of legumes (Seed mix Nr. 20, *PR 3 UG 5*, Rieger-Hoffmann Company, Raboldshausen -Table 1b) which resembled the plant community structure of European old fields. Seeds were added at the recommended density of 8 g/m^2 as follows:

We mixed for each mescocosm approximately 0.156 g of the seed mix with 300 g of soil and 500 mg of carrier material with *R. irregularis* (for the subset of mesocosms receiving

additional mycorrhizal propagules) and we added the resulting mix to the top of 1800 g of soil for each mesocosm. The pots had a 2 L capacity (17 cm height x 14 cm maximum diameter). The plants were allowed to grow for four months in a climate chamber at the Institute of Biology of Freie Universität Berlin. The average day/night temperature and photoperiodicity in the climate chamber were 20/16 °C and 14/10 h respectively, with a relative air humidity of 60 %. Mesocosms were watered gravimetrically three times a week and randomized regularly throughout the growth phase of the experiment.

Plant community structure and biochemical measurements

At harvest we visually identified plant individuals to species and separately oven-dried them at 70°C to a constant weight. At identification we assayed root material (~10% root wet weight) from the individual and stored it at 4^{o}C to later assess mycorrhizal root colonization. The rest of the root material was washed and was used to assay total root dry weight. We corrected for the root material that we had removed for mycorrhizal root colonization on the assumption that the fresh weight ratio of the two fractions was equivalent to that of dry weight. Soil was stored at 4°C to quantify potential denitrification enzyme activity (DEA) and N_2O potential emissions rate.

Representative pieces up to 2 mm from the root per species from each mesocosm were stained with 0.05% Trypan Blue according to a modified staining protocol (Phillips and Hayman 1970). The percentage of AMF root colonization was quantified at 200 X magnification using the magnified intersections method (100 intersects per sample) (McGonigle et al. 1990). AMF structures were recorded separately; arbuscules, hyphae and vesicles and the total percentage of AMF structures in each mesocosm was calculated. AMF hyphal length in soil of each mesocosm was quantified according to Rillig et al. (1999) following an aqueous extraction with sodium hexametaphosphate.

To assess the relative weight of water stable aggregates (WSA) we used a wet sieving technique modified from Kemper and Rosenau (1986). We agitated a soil sample of 2 g per mesocosm in an insert with a 0.25 mm mesh on the bottom for 5 minutes in water in an Eijkelkamp wet sieving apparatus (Eijkelkamp, 08.13, The Netherlands).

To assay DEA we used a modified (Pell et al. 1996; Philippot et al. 2011) version of the DEA technique. Soil samples (25g) were incubated anaerobically under idealized conditions of nutrient availability in 125 mL flasks. Half of the flasks received acetylene (C_2H_2) which is a N_2O reductase activity inhibitor which allowed us to discriminate between total potential denitrification (with the inhibitor) and potential N_2O emissions rates (without the inhibitor).

Chloramphenicol (CAP) was used at the concentration of 0.7 mM to inhibit synthesis of new enzymes (Pell et al. 1996). We maintained two technical replicates per sample with C_2H_2 and two without. Gas samples were taken 45, 90, 150 and 210 min (from the time C_2H_2 was added) using a 15 mL syringe. We used the slope of the reactions to assess total potential denitrification and N_2O potential emissions in the samples with C_2H_2 and without, respectively (Hutchinson and Mosier 1981). Gas samples were analysed using an Agilent Gas Chromatograph (GC) -14A Shimadzu, equipped with an electron capture detector and flame ionisation detector (Agilent Technologies Inc., Santa Clara, CA, USA) at the Leibniz Centre for Agricultural Landscape Research (ZALF), Müncheberg, Germany (Loftfield et al. 1997). The ratio between potential N_2O emission rate and total potential denitrification rate was defined as the denitrification potential ratio [$(N_2O/(N_2O+N_2)$] (Philippot et al. 2011).

Statistical analyses

To assess the influence of AMF on plant community dynamics we first carried out a series of *t*-tests with mycorrhizal treatment as a grouping factor and Shannon diversity of the plant community, Pielou's evenness and total plant biomass (i.e. equivalent to net primary productivity – NPP) as response variables. We also used correlation tests between the above mentioned response variables and the continuous predictor hyphal length as an alternative way to assess the impact of mycorrhiza and discriminate between AMF inoculum presence/absence and "dose" dependent effects. To visualize how plant community structure related to the parameters that were assayed in the experiment (predictors), we carried out a redundancy analysis on the plant community matrix following a Hellinger transformation. To assess significance of the predictors we implemented a per term significance test following 9999 permutation, as implemented in the routine *anova.cca* in the R package *vegan* (Legendre, Oksanen and terBraak 2011).

To assess the influence of AMF on denitrification potential parameters we fitted a series of linear models. The linear models were fitted sequentially to the response variables (i) N_2O potential emissions; (ii) total denitrification potential; (iii) denitrification ratio. For each response we fitted two alternative models, one with Shannon diversity as a diversity metric and one with Pielou's evenness as a diversity metric. These models consisted of four predictors: NPP (i.e. to correct for differences in biomass across mesocosms; R by default uses Type I Sums of Squares and this is why we fitted this predictor first), AMF treatment, the diversity metric and the interaction between AMF treatment and the diversity metric. We also

fitted versions of the models where we replaced the predictor AMF treatment with the assayed predictor AMF hyphae in soil.

To partially address indirect effects of mycorrhiza as for example promoting soil aggregation and visualize relationships between productivity and denitrification potential parameters, we finally produced a correlogram of the parameters assayed in the study. We plotted any significant correlations between those parameters. All analyses were performed in R version 3.0.2 (R Core Team 2013).

Results

AMF effects on plant community metrics

The influence of AMF on plant community metrics was not significant. In contrast, we found a significant (t = 5.91, P < 0.001) effect of AMF on NPP of the plant community. Shannon diversity varied between 0.21 and 0.82 (interquartile range 0.45 – 0.75) in the non-inoculated mesocosms and between 0.08 and 1.06 (interquartile range 0.44 – 0.78) in the AM-inoculated mesocosms and did not differ between the two treatments (t = 0.26, P = 0.795). Evenness varied between 0.14 and 0.50 (interquartile range 0.24 – 0.42) in the non-inoculated mesocosms and between 0.07 and 0.44 (interquartile range 0.24 – 0.38) in the AM-inoculated mesocosms and did not differ between the two treatments (t = 0.87, P = 0.393). ANOVA results were similar when AM hyphal densities or AMF treatment were fitted in the models.

The influence of AMF on denitrification potential parameters

The added *R. irregularis* successfully colonized the soil and increased three-fold the AM fungal hyphal density in the inoculated soil compared to the non-inoculated soil at harvest (Fig. 1). The influence of AMF on denitrification potential activity was significant whether AM hyphal densities or AMF treatments were fitted in the models. Total potential denitrification activity rate was significantly (F= 18.89; P = 0.0001; Fig. 1, Models 3 and 4 in Supplementary material) reduced in the inoculated soil with AMF compared to the control. Strikingly, we observed an abatement of about 31 % in the magnitude of total potential denitrification activity rate compared to non-inoculated soil. We observed that total potential denitrification activity rate significantly decreased with an increased AM hyphal density stimulated by the addition of *R. irregularis* (Fig.1). The interaction term between AMF and plant evenness was significant (F= 5.48; P = 0.02) while the interaction between AMF and Shannon diversity was marginal (F= 3.12; P = 0.08).

There was no significant difference between AMF treatments in N_2O potential emission rates and they were left unaffected by the addition of *R. irregularis.* However, potential emission rate of N_2O was significantly affected by plant Shannon diversity (F= 5.55; P = 0.02) and evenness (F= 8.56; P = 0.007; Fig. 1, Models 1 and 2 in Supplementary material) independently of *R. irregularis* addition (F= 0.02; P = 0.88) and plant productivity (F= 0.45; P = 0.50).

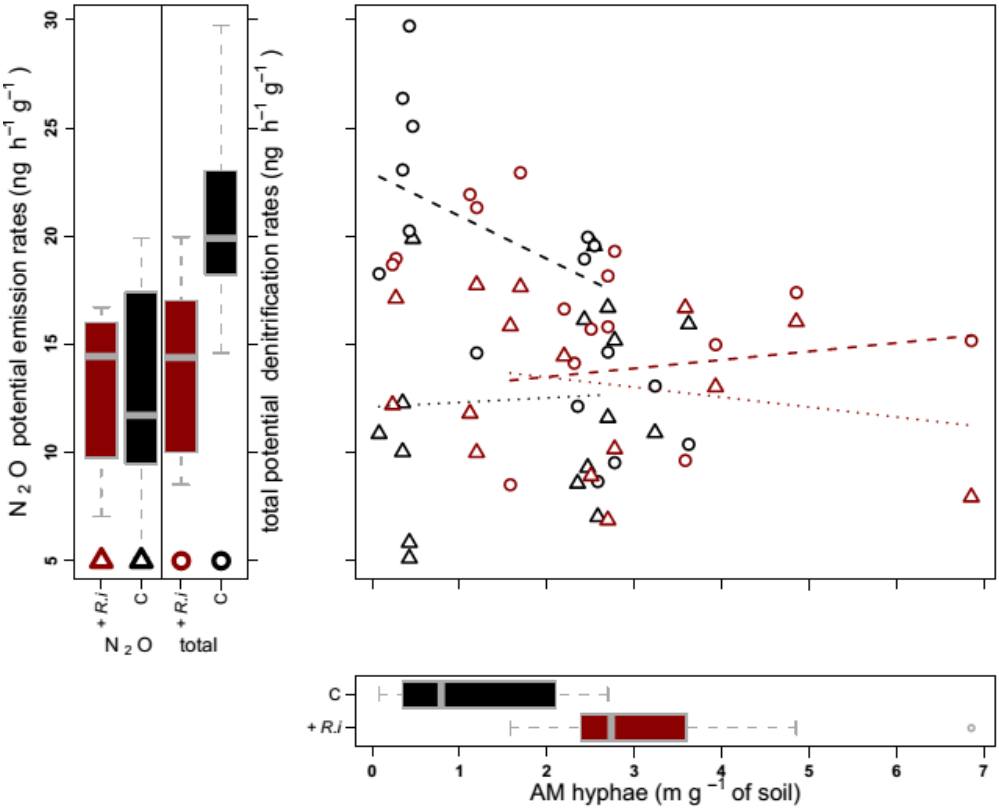

Fig 1. Relationships between AM fungal hyphae (*x*-axis), N_2O potential emission rates (triangles) and potential denitrification rates (cycles) (*y*-axis) across mesocosms without (in black) or with (in red) the addition of *Rhizophagus irregularis* to the indigenous AMF community (*n*=16). Associated boxplots highlight the differences between the two mycorrhizal treatments (significant for total potential denitrification rates and AM fungal hyphae). The relationship between AM fungal hyphae and N_2O potential emissions was non-significant (F = 0.11, P = 0.74) but the relationship with total potential denitrification rates was (F = 5.89, P = 0.02 – full statistics on the models with AMF treatment as a categorical parameter can be found in Appendix 1). Dotted lines describe N_2O potential emissions, and discontinuous lines potential denitrification rates.

Overall, denitrification potential ratio depended on all explanatory variables at the end of the experiment. This ratio was significantly affected by the addition of AMF (F= 5.50; P = 0.02, Models 5 and 6 in Supplementary material). Plant Shannon diversity (F= 5.34; P = 0.02) and

evenness (F= 6.02; P = 0.02) as well as increases in total plant biomass (F= 4.30; P = 0.04) contributed also to the variation in the denitrification ratio. However, the interaction terms between AMF and Shannon or evenness were not significant.

Variation in plant community metrics relates to denitrification potential parameters

Redundancy analysis (RDA) revealed that the x-axis (RDA1, 37.5%) and y-axis (RDA2, 8.3%) together explained 45.8% of the variation in plant community (see Model RDA in Supplementary material). Depending on the order with which we fitted the different parameters, the significant predictors in the RDA were denitrification-related parameters other than the denitrification ratio, plant productivity and plant diversity metrics. Total denitrification potential was the significant predictor and accounted for the major variability in plant diversity and productivity. Plant communities were dominated by grasses. *Bromus secalinus* and *B. hordeaceus* were the most abundant grass species, but *B. hordeaceus* the most productive and dominant in all the mesocosms. Legumes were mostly represented by *Trifolium pratense* which positively closely correlated to plant evenness and productivity and negatively with N_2O potential emissions, whereas *B. hordeaceus* correlated negatively with Shannon diversity index and positively with N_2O potential emissions. Non-legume forbs were very rare in the plant community and less productive. Overall, evenness and Shannon diversity of plant species negatively correlated with potential emissions of N_2O (Fig 2).

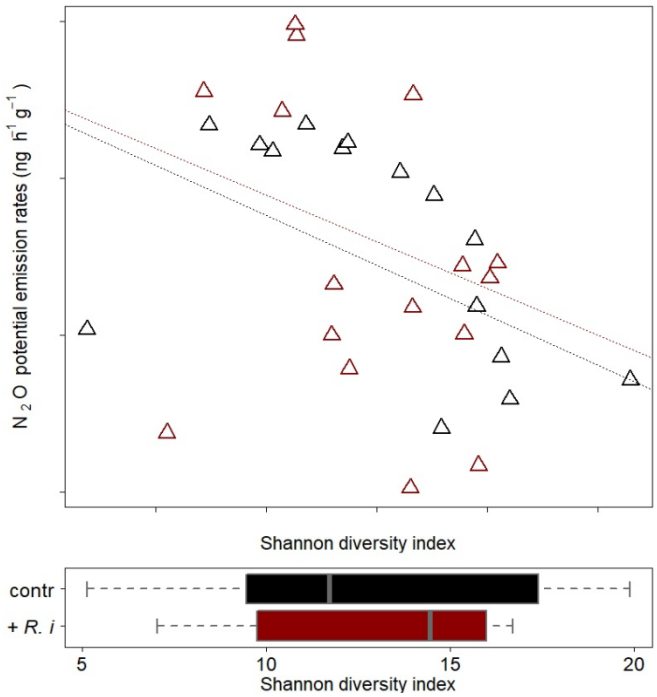

Fig 2. Relationship between N_2O potential emission rates (*y*-axis) and Shannon-diversity index of the plant community (*x*-axis) across mesocosms without (in black) or with (in red) the addition of *Rhizophagus irregularis* to the indigenous AMF community (*n*=16). The relationship was significant (F = 5.99, *P* = 0.02). The associated boxplots highlights the lack of differences in Shannon diversity between the two mycorrhizal treatments (*t* = 0.26, *P* = 0.795). Lines are best fit lines.

Potential links between AMF and plant community structure on denitrification potential parameters

We used a corellogram to explore potential functional relationships between our response variables and denitrification potential parameters. Total biomass was negatively related to AM hyphal production and total potential denitrification in the mesocosms (Fig. 3). There was a positive relationship between total potential denitrification activity with the denitrification ratio (Fig. 3), while no significant relationship between total potential denitrification activity and potential N_2O emission rate was found. There was a significant negative relationship of plant evenness and Shannon diversity with potential emissions of N_2O (Fig 2; Fig. 3). Total potential denitrification activity and denitrification ratio were negatively related to AMF and soil aggregation formation, while no significant relationships between N_2O potential emission rates and AMF or soil aggregation were found (Fig. 3 and 4). Relationships between AMF hyphae, soil aggregation, NPP, denitrification ratio, total denitrification potential with either plant evenness or Shannon diversity were not significant.

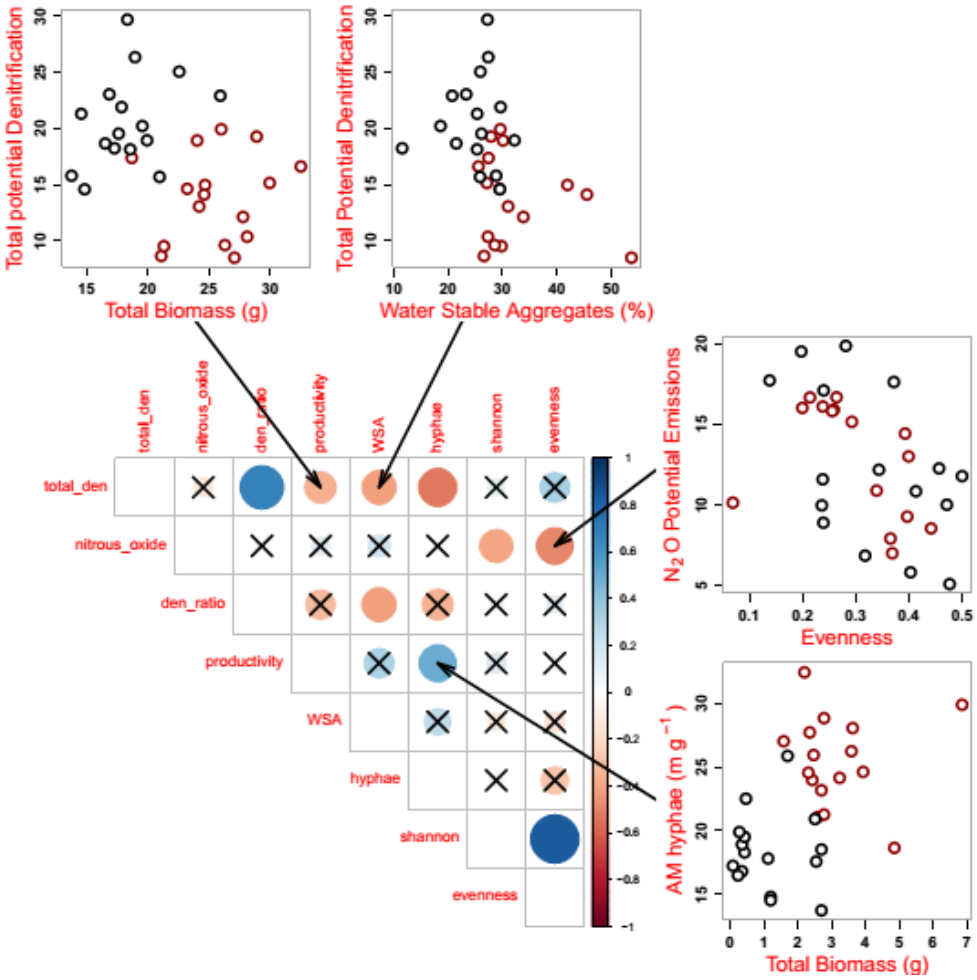

Fig 3. Correlogram for the eight continuous parameters that were assayed in the experiment. A subset of four significant relationships are presented in detail (in black mesocosms with no *R. irregularis* inocculation; in red with *R. irregularis* additions). The size of the cycles in the correlogram is representative of the strength of the correlation; relationships with an "x" are non-significant.

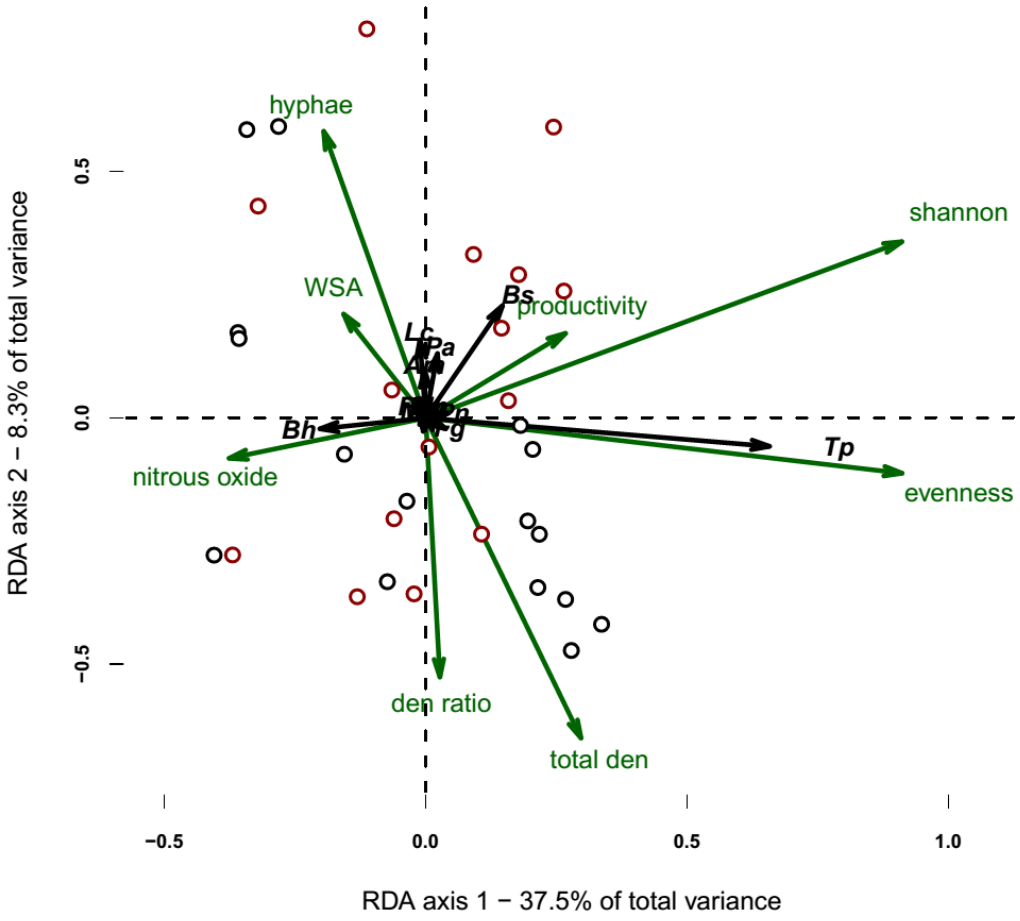

Fig. 4. Redundancy analysis (RDA) of the mechanistic correlations between potential denitrification parameters (total den: total potential denitrification activity, nitrous oxide: N_2O potential emission rates and den ratio: denitrification potential ratio) and predictors (total biomass productivity, plant evenness and Shannon diversity, hyphae: AM hyphal densities, WSA: water stable soil aggregate) at harvest after inoculation with *R. irregularis*. Black open circles represent non-inoculated soil samples while red open circles represent soil samples inoculated with *R. irregularis*. *Bromus secalinus (Bs), Bromus hordeaceus (Bh), Poa angustifolia (Pa), Trifolium pratense (Tp)* and *Lotus corniculatus (Lc).* The length of arrows refers to the strength relative to other variables. The intersection angle between vectors represents the affinity degree of their relationships (smaller angle with closer correlation). For all vectors, values on the x and y axes represent the percentage change explained by RDA 1 and RDA 2 respectively. Model RDA statistics are presented in the supplementary material.

Discussion

We tested here the influence of mycorrhiza on parameters related to denitrification potential in the presence of a diverse plant community. In the literature, the role of mycorrhiza in

denitrification has been addressed in several studies (e.g. Bender et al. 2014; Lazcano et al. 2014; Storer et al. 2017) but this was in all cases done with a single plant species and the results might not be representative of natural plant communities. All three abovementioned studies found that mycorrhiza lowers N_2O emissions from soil. Some of these effects were linked to gene abundance (e.g. Bender et al. 2014) and are thus generalizable for N_2O potential emissions. By contrast, in our experiment we observed no effect of mycorrhiza on N_2O potential emissions, and any relationships might have been masked by the mitigating action of plant diversity (Fig. 2). Mycorrhiza mitigated, however, total denitrification potential and altered the denitrification ratio. These results were robust to the measures of plant diversity we used and to the across-mesocosms corrections for net primary productivity.

It is rare that grasslands experience limitations of mycorrhizal propagules (Veresoglou et al. 2012). However, the relative availability of AMF propagules varies a lot in space (Richter, Tiller and Stutz 2002; Treseder and Cross 2006; Martinez and Johnson, 2010) and, even though the exact implications of this source of variability have received little attention (but see Horn et al. 2014), they could induce differences in the way AM hyphae colonize the soil. We here observed three-fold higher AM hyphal densities at harvest in mesocosms with *R. irregularis* (Fig.1) which might have been the driver that reduced denitrification potential. Alternatively, such differences in the availability of AMF propagules could induce changes in the structure of the plant and fungal community as well as differences in NPP. Further research studies should be directed to assess the influence of AMF on denitrification potential via manipulation of single plant species and plant diversity with the specific goal to disentangle how mycorrhiza associated with a diverse plant community alters denitrification than single host systems.

We did not observe a significant effect of AMF on plant diversity and composition, which was inconsistent with *Hypothesis One*. We had expected that in our seed mix, which is dominated by grasses, which do not depend strongly on mycorrhiza, AMF would promote plant diversity. Although the existing literature addressing plant community shifts following inoculation with mycorrhiza is limited, it is apparent that mycorrhiza plays a pivotal role in shaping plant community structure and diversity (Klironomos et al. 2011). However, studies addressing mycorrhiza are usually carried out under conditions of low fertility which might exaggerate mycorrhizal effects. The soil here was an agricultural soil that had received fertilizers at regular intervals (i.e. in the year preceding harvest it was 80, 60 and 60 kg N/ha at the first stem, last leaf and flowering phase, respectively). Even though the soil in the

experiment had been colonized extensively with hyphae (i.e. the AMF hyphal length was above 2 m.g $^{-1}$ of soil; Fig 1), the mycorrhizal community most likely contributed little to the nutrition of the plants and as a result had little influence on plant community structure. Moreover, we observed a relatively low evenness in our systems (Pielou's evenness was below 0.5 and in some cases as low as 0.05). This suggests that the plant community was dominated by certain plant species, such as *Bromus hordeaceus*, which on average accounted for over 83% of total biomass in the mesocosms. Possibly the high degree of dominance in the mesocosms masked any mycorrhizal-mediated effects on plant community structure.

Grassland ecosystems worldwide provide vital goods such as forage material, recreation and drinking water, which might be under threat from global change. Grassland soils account for 18 % of N_2O global emissions (Lee et al. 1997). Potential N_2O emission rates in our studies were mitigated by plant diversity but not by mycorrhiza (Fig 2). This was inconsistent with *Hypothesis Two* that AMF would reduce both microbial denitrification enzyme activity and N_2O emissions. This result, however, agrees with some recent studies demonstrating that plant diversity reduces N_2O production via enhanced N removal efficiency and below-ground plant complementary trait effects such as diverging root morphology (e.g., Niklaus et al. 2016; Han et al. 2016; Abalos et al. 2017, but see Abalos et al. 2014; Kravchenko et al. 2018). Most of these studies have been carried out, again, with single plant species or a mixture of a single plant functional group. Abalos et al. (2014) reported lower N_2O emissions in plant species identity of monocultures (e.g., *Lolium perenne L.(Lp)* or *Festuca arundinacea Schreb (Fa)*) and two-species mixtures (e,g., *Lp + Fa*) while four species mixtures of common grass species increased N_2O emissions. In contrast, our plant community was composed of different plant functional groups represented by grasses, forbs and legumes at harvest. Niklaus et al. (2006, 2016) demonstrated that even though including legumes in experimental plant communities induces increases in N_2O emissions, N_2O emissions from soil decline with plant richness. The negative relationships of plant evenness and Shannon diversity with potential emissions of N_2O we observed in this study clearly support that plant diversity strongly reduces N_2O emissions (Fig. 2). This result is in disagreement with *Hypothesis Three* that higher plant diversity would induce higher rates of potential denitrification and N_2O production and that these effects would be smaller in magnitude than those of mycorrhiza. We observed that certain mesocosms with high plant evenness and diversity released lower N_2O potential emissions while others with low plant evenness and diversity produced higher N_2O potential emissions, irrespective of AMF treatments (Fig. 2 & Fig. 3).

Conclusion

This study investigated the implications of AMF additions to a grassland plant community under high soil fertility settings. We present compelling evidence to date that increased densities of AMF in a typical European grassland soil with diverse plants reduce the denitrification ratio and microbial denitrification enzyme activity of N_2O producers. We also show that the AM fungi symbiosis influences mainly denitrification potential activity, whereas plant diversity decreases N_2O potential emissions. This study uncovers the ecological relevance of AM plant symbiosis for better management of N_2O in temperate grassland systems.

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Conflict of interest.

The authors declare no conflict of interest.

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Chapter 5:

Arbuscular mycorrhiza induces no changes in nitrate availability, but mitigates nitrous oxide emissions from a native fertile agricultural soil using a gas flow system

Abstract

Arbuscular mycorrhizal fungi (AMF) have been shown to reduce emissions of the powerful greenhouse gas, nitrous oxide (N_2O), through reducing the availability of nitrate (NO_3^-) in the soil. From an agricultural point of view, this mechanism is of little importance because of continuous fertilization events. We assayed here N_2O emissions and denitrification rates from an untreated agricultural soil in which we had grown maize with or without the further inoculation of AMF in the form of *Rizophagus irregularis* and used an approach that enabled us to assess N_2O emissions and denitrification independently of soil NO_3^- availability. We show that soils with a low density of AMF hyphae emit higher rates of N_2O compared to soils that are more extensively colonized with mycorrhiza and of equivalent NO_3^- concentrations. This adds to the arguments for managing agricultural land in favour of AMF.

Keywords: arbuscular mycorrhiza fungi, N_2O emissions, nitrification, denitrification, Gas-flow incubation system.

Introduction

Agroecosystems represent major sources of global nitrous oxide (N_2O) emissions (Tian et al. 2018). The booming organic food market necessitates that farmers turn indigenous soil biota to their advantage. A major group of soil biota to manage in organic farming is arbuscular mycorrhizal fungi (AMF), representing ubiquitous mutualists of terrestrial plants (Smith and Read 2008). Several experimental studies have addressed the possibility that AMF alter N_2O emission rates in agroecosystems, reporting higher N_2O emissions when mycorrhiza is prevented from establishing (Bender et al. 2014; Lazcano et al. 2014; Zhang et al. 2015; Bender et al. 2015; Storer et al. 2017).

Denitrification is an anaerobic microbial process that is commonly limited by substrate availability. Most assays of denitrification and N_2O emissions are subject to the availability of residual nitrate (NO_3^-, i.e. the substrate for denitrification) in the soil. It is next to impossible to eliminate AMF in the field, and thus most mycorrhizal experiments are carried out under controlled conditions, following soil sterilization with plants subsequently being grown aseptically in small or larger containers (e.g. Bender et al. 2015; Storer et al. 2017). Plant growth promotion and improved N nutrition of mycorrhiza in partially closed systems (Smith and Read 2008) induces unrealistic imbalances in N availability in soil between mycorrhizal and non-mycorrhizal treatments which could be the actual reason why there are differences in N_2O emissions. In many cases, authors have noted that the pathway through which AMF suppress N_2O emissions was by more effectively depleting NO_3^- in soil (Zhang et al. 2015; Storer et al. 2017), whereas in other cases estimates of N_2O emissions were carried out following pulses of fertilizer additions (Bender et al. 2014; Bender et al. 2015), which might have represented major disturbances to the plant-soil systems.

Here we tested a novel continuous flow measurement technique to assess N_2O emissions from soil (Eickenscheidt et al. 2014), with the ultimate purpose of addressing whether AMF also mitigate N_2O emissions when there are no differences in NO_3^- availability between inoculated soil with mycorrhiza and non-inoculated. We monitored emission rates of N_2O (and N_2) over a 62 h period and assessed the convexity (i.e. the rate of change of the slope between N_2O production and time or by how much does N_2O production change in response to a change in time ?) of the cumulative emission N_2O curves. We hypothesized that samples originating from mycorrhiza-exclusion pots display a higher convexity which would represent evidence that, irrespective of its original availability, NO_3^- is denitrified at higher affinity than in samples originating from mycorrhizal soil and that the exclusion of mycorrhiza increases N_2O emissions independent of NO_3^- concentrations.

Materials and Methods

Experimental Design

The experimental procedure has been described in detail in Okiobe et al. (2019). In brief, soil was collected from an intensively managed agricultural site sowed with maize in Brandenburg/Uckermark, Germany (53°19'2.44"N and 13°51'48.03"E). The soil (see Appendix B for soil characteristics) was used as a substrate for a bifactorial experiment (factor A: addition of *Rhizophagus irregularis* propagules; factor B: destruction of soil aggregates via mechanically crushing them) in compartmentalized mesocosms with four

replicates per treatment. In Okiobe et al. (2019) we report on AMF induced increases in denitrification enzyme activity. For the present study, we report on the results from incubating six soil core samples (300 g of soil each) in a continuous flow measurement system (Eickenscheidt et al. 2014), shortly (i.e. 1 days) after harvesting the experiment.

Lab analyses

AMF hyphal length in soil was quantified according to Rillig et al. (1999) following an aqueous extraction with sodium hexametaphosphate. Residual NO_3^- was measured according to Miranda et al. (2001). N_2O and N_2 analyses were conducted in the laboratory of the Institute for Landscape Biogeochemistry, Leibniz Centre for Agricultural Landscape Research (ZALF), Müncheberg, Germany. The measurements of N_2 and N_2O fluxes were conducted in two types of atmosphere: Diamond and pure He which represent oxic and anoxic conditions respectively, following the gas-flow-soil-core incubation technique (Eickenscheidt et al. 2014). The calculations of cumulative gas fluxes were done according to Fiedler et al. (2017) (Appendix C in the supplementary material).

Statistical analysis

We estimated convexity of cumulative N_2O emissions and denitrification rates separately for the two types of atmospheres. We first divided time into five (to the degree possible) equidistant intervals. To calculate convexity we approximated calculation of the second derivative of the curves at the three intermediate points. For the first derivative we used the following formula:

$$PDF'_{(i1,i2)} = \frac{PDF_{i2} - PDF_{i1}}{t_{i2} - t_{i1}} \qquad (\ldots 1)$$

Where i1 and i2 are two consecutive time points (in minutes), PDF the cumulative function and PDF´ the first derivative of it. The second derivative was then defined as:

$$PDF''_i = \frac{PDF'_{(i-1,i)} - PDF'_{(i,i+1)}}{t_{i+1} - t_{i-1}} \qquad (\ldots 2)$$

where t_{i+1} and t_{i-1} are the time points before and after the time point *i*, PDF´´$_i$ the convexity at time point *i*.

We then calculated convexity (PDF´´) as the mean of the three second derivatives:

$$PDF'' = \overline{PDF_i''} \qquad (\ldots 3)$$

To assess whether there were differences in the convexity between samples that received AMF inoculum and those that did not we used a repeated measures ANOVA with convexity as response variable, AMF treatment as predictor and the two types of atmospheres as within-groups factor. To address whether our results were driven by substrate availability we carried out a *t*-test with NO_3- concentrations as response variable and our treatment as grouping factor. Moreover, we used a *t*-test to compare AMF hyphae across the two AMF treatments (inoculated soil with AMF and noninoculated). Analyses were carried out in R version 3.0.2 (R Core Team 2013).

Results and discussion

Cumulative N_2O and (N_2) fluxes were higher in Diamond compared to pure He atmosphere, but there were no differences between AMF inoculated and non-AMF inoculated soil samples (Fig. 1). Most of the N lost from the system was in the form of N_2O (*y*-axes - Fig. 1). Convexity for total denitrification did not differ between samples that had been inoculated with AMF and those that had not. We found, however, a higher convexity for N_2O emissions in the samples that had not received AMF inoculum (Fig. 2- F= 26.33; P= 0.007; Supplementary material-Appendix **A**). There was no difference between AMF treatments in NO_3^- concentrations (F= 3.89; P= 0.12; Supplementary material-Appendix A). Hyphal densities were three-fold higher in the samples that received AMF inoculation compared to noninoculated and were similar to Okiobe et al. (2019).

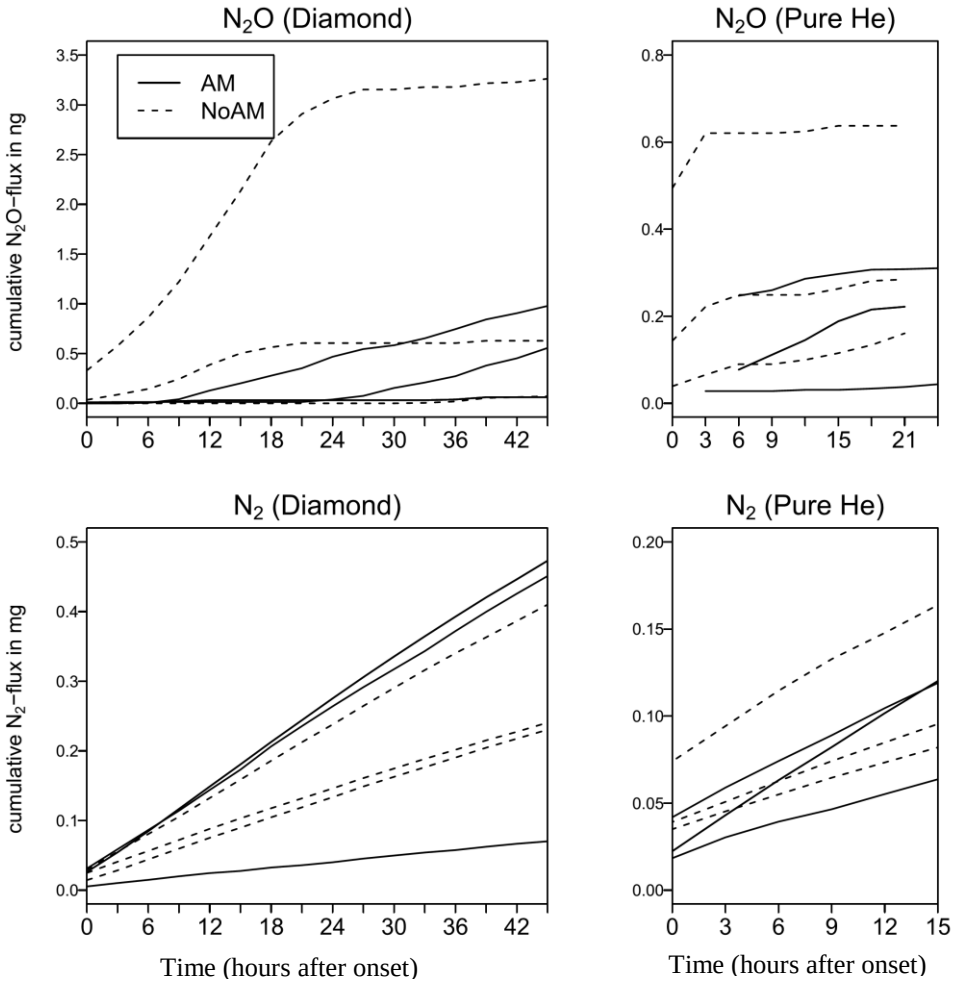

Fig.1 Cumulative N_2O and N_2 fluxes from soils inoculated with arbuscular mycorrhizal (AM) fungi in the form of *Rizophagus irregularis* propagules or not (three replicates of inoculated soil cores with AMF and three for noninoculated-noAM) incubated in artificial atmosphere (Diamond) and anaerobic conditions (pure Helium) respectively.

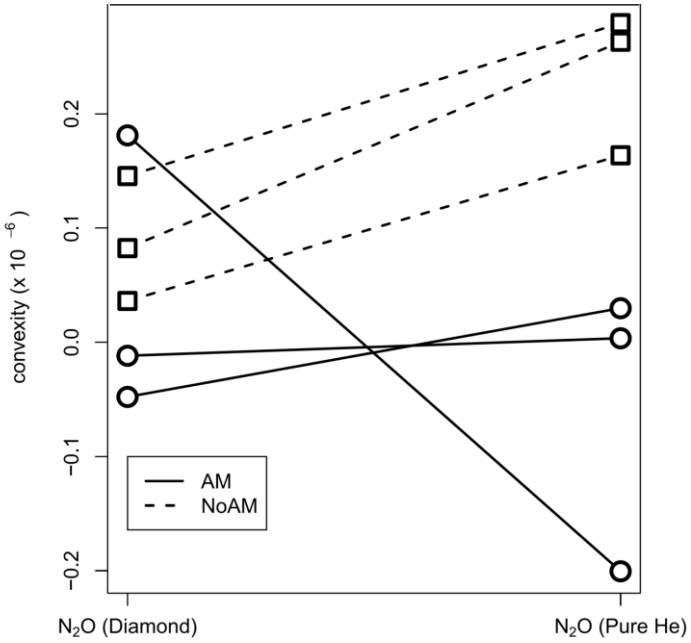

Fig. 2 Convexity of cumulative N_2O fluxes from each soil inoculated with arbuscular mycorrhizal (AM) fungi or not (three replicates of soil samples for AMF and 3 without AMF inoculation-noAM) incubated in artificial atmosphere condition (Diamond) and anaerobic condition (pure Helium) respectively. AMF addition significantly (F = 26.33; P= 0.00683) affected the convexity of cumulative N_2O fluxes.

Denitrification is an ecosystem process that is commonly limited by substrate availability. In the technique we used here, unlike the bulk of the literature and most of the studies involving AMF, we did not spike the soils with N before the measurements, but we used a high fertility soil. This implies that the measurements depended strongly on the residual NO_3^- in the soil. A higher convexity in N_2O emissions (given that this represented the predominant form of N loss) implied that a site with low densities of AMF should deplete N faster than another site with similar environmental conditions and NO_3^- availability but a higher density of AMF hyphae, resulting in higher rates of N_2O emissions. This is congruent with the existing literature showing that AMF reduce N_2O emission rates (Bender et al. 2014; Bender et al. 2015; Zhang et al. 2015; Storer et al. 2017).

It is well established that among the multifaceted effects that AMF have on N cycling dynamics, they lower the availability of N in soil (Zhang et al. 2015; Storer et al. 2017), which has been proposed to be the mechanism through which N_2O emissions from soils with high AMF densities decline (Storer et al. 2017). By using the specific technique, here, we

show that AMF-induced declines in N_2O emissions can be independent of substrate availability. This is important in systems like organic farming, where the soil intermittently receives organic fertilizer and the availability of N remains naturally high. There has been a recent debate on whether managing mycorrhiza in agricultural systems is helpful for agriculture (Ryan and Graham 2018, but see Rillig et al. 2019). We here present the argument, that through AMF it might be possible to mitigate N_2O emissions from agricultural soils.

We could not here fully discriminate between the two main pathways that generate N_2O, nitrification and denitrification (Khalil et al. 2004). Having used an agricultural soil, it is likely that nitrification rates were higher than in most other soils and that the relative importance of nitrification-induced N_2O in our results is high. In the literature it has been proposed that AMF suppress nitrification (Veresoglou et al. 2011). Some recent studies have further demonstrated that AMF reduce N_2O emissions from nitrification, although results remain inconsistent (Storer et al. 2017; Teutscherova et al. 2018). Teutscherova et al. (2018), for example, found that AMF increased the abundance of nitrifiers while reducing N_2O emissions from nitrification. These authors suggested that immobilization of N and changes in the activity of ammonia-oxidizing bacteria by AMF, rather than abundance have been potential AMF mechanisms which contributed to reduced N_2O emissions. More recently, Veresoglou et al. (2018) demonstrated that AMF altered the community structure of ammonia oxidizers at high fertility via competition for soil NH_4^+.

Studies on the topic are subject to diverse shortcomings such as experimental procedures like shifts in the microbial community through a prior sterilization of the growth substrate and spiking the soil with N-substrate, which could justify the lack of a consensus. We offer here a report that could facilitate future syntheses on the topic by addressing some of the shortcomings of earlier studies that have been carried out at larger experimental scales.

In conclusion, we presented in the introduction two hypotheses, that samples originating from mycorrhiza-exclusion cultures display a higher convexity and that the exclusion of mycorrhiza increases N_2O emissions independent of NO_3^- concentrations. We obtained support for both hypotheses and argued that managing arable soil to increase the availability of mycorrhiza, irrespective of any increases in crop yield, can contribute towards mitigating N_2O emissions. Given that N_2O emissions from agriculture account for a substantial proportion of global N_2O emissions (Tian et al. 2018), this means that mycorrhiza could represent a powerful tool in reaching the targets that have been set for global warming.

Conflict of interest

The authors declare no conflict of interest.

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Chapter 6:

General Discussion

The role of AMF on the emissions of the greenhouse gas N_2O and the way mycorrhiza could increase the sustainability of plant soil systems has been in general terms overlooked in the literature. Some representative knowledge gaps relate to the mechanisms through which AMF alter denitrification and N_2O production. In this PhD thesis, I addressed whether AMF alter actual denitrification rates, denitrification potential activity and related N_2O emissions through manipulation of keystone ecosystem functions such soil aggregation and plant community structure.

The limited existing literature on the topic has only quantified composite effects of AMF on in-situ denitrification and related N_2O flux from soils (Bender et al., 2014; Lazcano et al., 2014; Zhang et al., 2015; Bender et al., 2015; Storer et al., 2017). They showed that a combination of multiple single AMF effects reduces N_2O production by manipulating only AMF presence or absence. Moreover, most experiments with AMF, however, were carried out under unnatural growth conditions such as autoclaved (and thus potentially partially de-aggregated) soil and low fertility settings. These specific growth settings could change the relative importance of the underlying mechanisms producing (or consuming) N_2O and thus yield a biased view on the role mycorrhiza plays in total N_2O emissions from soil. To fill this gap, we here disentangled mechanistic effects of AMF on N_2O potential emission rates, but also on key denitrification parameters: denitrification potential activity and estimated denitrification ratio. In two of the chapters, Chapter 3 and 4 we assayed ex-situ potential denitrification activity, N_2O production and estimated denitrification potential ratio with the DEA assay, representing the commonest technique to assay denitrification which maximized our potential to compare the data to the existing literature (Pell et al., 1996; Philippot et al., 2011). By contrast, in Chapter 5 we addressed the influence of AMF on in-situ denitrification rates and related cumulative N_2O total emissions which was more comparable to the five above-mentioned studies on the interaction between AMF and N_2O emissions.

In Chapter 3 we found that potential N_2O emission rates and the denitrification potential ratio of potential N_2O emission rates over total potential denitrification activity were promoted by mycorrhizal hyphae (i.e. here expressed as AMF hyphal density in the soil), indicating that

AM hyphal production directly increases N_2O potential emission rates. Furthermore, AMF-induced increases in soil aggregation promoted both denitrification potential parameters (indirect effect of AMF). We showed clearly here that the effects of AMF are not composite but mechanistic by disentangling between direct vs indirect effects of AMF and detecting precise mechanisms by which AMF promoted N_2O potential emissions. Our results were inconsistent with the previous studies indicating that a combination of AMF effects reduced denitrification and N_2O emissions (Bender et al., 2014; Lazcano et al., 2014; Zhang et al., 2015; Bender et al., 2015; Storer et al., 2017; Teutscherova et al., 2018). This inconsistency could be explained by the fact that here we measured DEA which is less sensitive to substrate availability than actual denitrification measurements and that the detrimental effect of AMF on N_2O emissions could be less pronounced under optimal conditions for denitrification. Higher rates of N_2O potential emissions have previously been found in macroaggregates compared to microaggregates (Drury et al., 2004; Khalil et al., 2005; Diba et al., 2011) and AMF are known to promote macroaggregate formation (Rillig and Mummey, 2006). However, further biochemical and molecular studies are needed to assess the activity and abundance of microbes associated to N_2O production and to draw firm conclusions on these results.

Most of these previous studies have performed reductionist experiments with single host system relevant for agricultural settings, but not for natural systems with a diverse plant community. Therefore, in Chapter 4 compared to Chapter 3**,** we explored how AMF might behave under a typical European grassland plant community. We showed that the influence of AMF on N_2O emissions might not be consistent between agricultural settings (i.e. plant monocultures - Chapter 3) and native grassland settings (i.e. a plant community with a higher plant diversity - Chapter 4). Mycorrhizal network densities were lower in our experiment setting with maize plant monoculture in Chapter 3 compared to Chapter 4 with grassland plant community diversity and were three fold higher in AMF treatments in experiment 1 -Chapter 3 compared to Experiment 2-Chapter 4. Experimental settings with plant monoculture could have prevented the establishment of AMF plant symbiosis in the high fertility agricultural soil and the efficiency with which plant and AMF derive benefits (e.g., soil nutrient resources) from the symbiosis (Selosse and Rousset, 2011) while the diverse plant community in Chapter 4 could have fostered better AM sysmbiotic associations which was evidenced this by a power relationship between NNP and AM hyphal densities in soils and this strongly reduced total denitrification potential activity (Chapter 4). This could also suggest that some minimum

threshold level of AMF propagules in soils may be required for successful reduction in denitrification potential activity. We do not know whether the level of AM hyphae density in similar soil conditions could differently affect denitrification and resulting N_2O production. The results from Chapter 4 revealed also the ecological significance of plant diversity for the sustainability of plant soil systems.

In Chapter 4, using a subset of soil samples originating from inoculated soil with AMF and noninoculated soils from the realistic experiment 1- Chapter 3 and which did not differ in nitrateNO^-_3 concentration, we found that AMF reduced real denitrification rates and related cumulative N_2O emissions **in** aerobic (Diamond) and anaerobic (pure He) conditions **using** a gas-flow-soil-core incubation system. This result was incongruent with the existing studies highlighting that AMF reduce N_2O emissions through decreasing the availability of NO_3^- in the soil (Zhang et al., 2015; Bender et al., 2015; Storer et al., 2017; Teutscherova et al., 2018). Most of these existing studies have performed reductionist experimental designs with artificial soil substrates which did not integrate indigenous nitrifiers, denitrifiers and AMF community and as to limit competition and exaggerate the strong effect that AMF could induce on mineral soil N uptake. Here, AMF might have directly altered autotrophic nitrifiers and denitrifiers and thus reduced soil N_2O emissions in oxic and anoxic conditions, respectively (Veresoglou et al., 2012a; Veresoglou et al., 2018). Independently of AMF-induced increases in crop productivity; we here offer to date evidence that AMF mitigate N_2O emissions in both conditions.

The contradictory results in Chapter 3-AMF increased N_2O potential emission rates and denitrification ratio and the results we found here (Chapter 5) could be explained by the fact we sampled soil cores differing from AMF treatments but not from aggregate treatments. Moreover, in Chapter 3, we quantified the response of AMF on ex-situ denitrification rates and related N_2O potential emission rates at the endpoint harvest over a short period of time less than four hours (short-term effect), possibly masking the detrimental effect of AMF on cumulative N_2O emissions while in Chapter 5 we monitored N_2O emissions for 3 days (long-term effect). Likewise, these contrasting results uncover the spatial and temporal heterogeneous nature of soil N_2O production (Butterbach-Bahl et al., 2013). Further research studies need to be carried out to determine temporal effect of AMF (short-term and long-term AMF effects) on denitrification.

Finally, using unsterilized arable soils with intact soil aggregates inoculated or not with a model AMF species, *R. irregularis*, we increased the ecological relevance of our results by

showing that AMF affect N_2O potential emissions, but also potential denitrification activity and denitrification potential ratio compared to the existing studies (Bender et al., 2014; Lazcano et al., 2014; Zhang et al., 2015; Bender et al., 2015; Storer et al., 2017; Teutscherova et al., 2018).

Conclusion

Our main objective was to address how AMF influence denitrification potential and N_2O emissions under a range of realistic experimental conditions with native soil aggregate states and mimicking low diversity native agricultural soils and medium-diversity grassland communities. Overall, these results clearly demonstrate that AMF strongly influence actual denitrification rates, denitrification potential activity and N_2O emission rates under realistic experimental settings which increase the ecological relevance of our study and challenge the existing studies. AMF effects are ecological context-dependent. AMF increased N_2O potential emission rates and denitrification ratio via AM hyphal production and soil aggregation promotion in a single host system relevant for agricultural systems, but plant diversity masked AMF-induce changes in N_2O potential emission rates in a natural grassland plant diversity. Moreover, our study showed that the influence of AMF on denitrification could be inconsistent between denitrification types (ex-situ potential DEA and N_2O emission rates and in-situ denitrification rates and cumulative N_2O fluxes). The results that we found here might be related to the interactive effects of AMF with non-AMF indigenous soil communities and are, thus, of potential importance for the mitigation of climate change and management of global N_2O emissions in field conditions.

Further research studies should be directed to:

- address interacting effects of AMF and soil aggregation on denitrification in a plant community;
- design experiments to deepen understand which dominant N_2O production pathway is particularly affected by AMF;
- determine whether AMF also affect denitrification and related total N_2 emissions;

- assess gene expression and the response of microbial nitrifiers and denitrifier and nondenitrifiers in the presence or absence of AMF to fully understand the mechanisms behind the reduction and increase in N_2O production;

- perform field-based studies in the order to assess the interactive effect of AMF with non-AMF soil organisms on denitrification under a wide range of soil types and environmental conditions in both natural and agricultural systems;

- quantify the response of AMF on N_2O and N_2 emissions in a complete plant AMF soil system;

- Investigate the short-terms and long-term effects of AMF on denitrification, N_2O and N_2 emission rates;

- measure denitrification activity and related N_2O and N_2 emissions from soils using complementary techniques (e.g., DEA, isotopic method or gas flow incubation method) and this would help reduce the uncertainty of N_2O production.

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Contribution to Chapters

I. Okiobe, S.T., Pirhofer-Walzl**,** K**.,** Leifheit, E.F., Veresoglou, S.D., 201X. Understanding the role of arbuscular mycorrhizal fungi in environmental controls of denitrification and soil N_2O emissions: a review.

Own contributions: I conceived the ideas of the paper, performed literature research. wrote the manuscript and designed the conceptual framework and tables. All authors reviewed the manuscript.

II. Okiobe, S.T., Augustin, J., Mansour, I., Veresoglou, S.D., 2019. Disentangling direct and indirect effects of arbuscular mycorrhiza on nitrous oxide activity and denitrification.

Own contributions: I conceived the ideas of the project and wrote the proposal thereafter funded by DAAD (under the supervision of MCR and SDV), ran greenhouse experiment, performed laboratory work and DEA assay (with IM), recorded all data, performed calculations of denitrification rates and statistical analyses. SDV mentored statistical analyses. I wrote the manuscript. All authors reviewed the manuscript.

III. Okiobe, S.T., Rillig, M.C., Magkdi, M., Augustin, J., Veresoglou, S.D., 201X. Plant diversity masks arbuscular mycorrhiza-induced changes in N_2O potential emissions in experimental plant communities.

Own contributions: Design work (together with SDV and MCR), collected materials, greenhouse experiment run, performed laboratory work and DEA assay (with MM) and recorded all data, performed calculations of denitrification rates and statistical analyses. SDV mentored statistical analyses. I wrote the manuscript. All authors reviewed the manuscript.

IV. Okiobe, S.T., Veresoglou, S.D., 201X. Arbuscular mycorrhiza induces no changes in nitrate availability, but mitigates nitrous oxide emissions from a native fertile agricultural soil using a gas flow system.

Own contributions: Design work (together with SDV), collected materials, performed laboratory work and recorded all data, performed calculations of gas fluxes. SDV mentored statistical analyses. I wrote the manuscript. All authors reviewed the manuscript.

Curriculum Vitae

Academic profile

2010-2012: University of Yaounde I, MSc in Plant biotechnology, Grade: very good.
2008-2010: University of Maroua, Professional MSc in Live Sciences, Grade: good.
2007-2008: University of Yaounde I, Maitrise in Plant biology, Grade: quite well.
2006-2007: University of Yaounde I, Bachelor degree in Plant biology, Grade: quite well.

Pratical experience

2013-2014: Agence Universitaire de la Francophonie (AUF), Project building capacity.
2013-2014: African Model Forest Network (RAFM)/B-Adapt project (Canadian and Cameroonian project), Supervisor of microbial biofertilizer production at the Microbial biofertilizer Center, Abong-Mbang, East Region, Cameroon
2010-2016: Modern GHS of Sangmelima, Sud-Cameroon, Secondary and High School Teacher in Earth and Lives Sciences.

Oral presentations

2018-2019: Disentangling direct and indirect effects of mycorrhiza on potential nitrous oxide and denitrification activities. Annual Meeting British Ecological Society, 16 - 19 December, ICC Birmingham, UK
2016-2017: Science Slam"A love story between plant root and mycorrhizal fungi: Eco-technology for the reduction of greenhouse gas emissions from agricultural soils" Wissenschafts Forum Berlin am Gendarmenmarkt Markgrafenstraße 37. 12 May 2017. Berlin, Germany.
2016-2017: "Effects of arbuscular mycorrhizal fungi inoculation and soil aggregation on potential denitrification rates from agricultural soils" at the 4 th Leibniz PhD Student Symposium, 28-29 September 2017, Berlin Germany.
2014-2015: "Organic agriculture in Cameroon: Innovations and perpectives" at the first international symposium of the African Biological Agricultural Federation (ABAF) at the Cremin-Cam room conference, 11-13 October, 2014. Yaounde, Cameroon.
2010-2011: Oral presentation in a digital video conference entitled "Environment and food security" at the Embassy of the Unites States of America, 15-16, September 2010, Yaounde, Cameroon.

Poster presentation

2013-2014. Improvement of arbuscular mycorrhizal fungi inoculum production by nutrient concentration solution and soil texture variation. Poster publication at the 8 th International Conference on Mycorrhiza at Marrakesh, Morocco, October 15-17 th.

Written articles

1- **Okiobe et al. 2015.** Improvement of arbuscular mycorrhizal fungi inoculum production by nutrient concentration solution and soil texture variation. International Journal of Agronomy and Agricultural Research 6: 7-20.
2- **Okiobe et al. 2012**. Effects of arbuscular mycorrhizal fungi on growth performances of two contrasted rice varieties in vertisol, Extreme-north region, Cameroon. A dissertation submitted in partial fulfillment of the award of the secondary and high school teachers' diploma in earth and live sciences. Annales of University of Maroua. Cameroon.

Appendix A - Supplementary Material for Chapter 3.

Table A1. Techniques and instruments used for the characterization of the soil

Measurement	Technique/Instrument
Total organic C and N	DIN ISO 10694, Leco Instruments GmbH.
pH, conductivity	DIN ISO 10390, DIN ISO 11265, TitraMaster85
NO_3^-	DIN EN 12260, CFA-SAN
Total P and K	DIN EN 25663, AAS-iCE 3300, GalleryTM Plus
Soil texture	DIN EN 1484, ISO 8245, TOC-Vcph

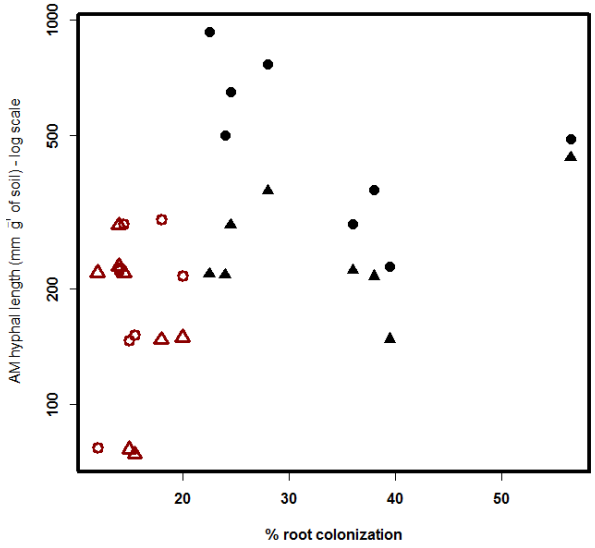

Fig. A1. Relationship between % root colonization (*x*-axis) and AM hyphal length (*y*-axis) in the mesocosms. Red open symbols stand for mesocosms that did not receive additional AM inoculum and filled black symbols for those that received propagules of *R. irregularis*. Cycles describe rhizosphere compartments whereas triangles hyphosphere ones (note that % root colonization is considered identical for the compartments that originate from the same mesocosm).

Appendix 1: Model Selection Statistics

Table S1. AIC statistics of our model selection procedure when we used WSA as a response variable. The lower the AIC value the better is the fit of the model. For model selection we used the mixed effects form of the repeated measures model. The optimal model is highlighted in grey. We highlight in each case the parameters which were included in the models.

Model Name	Df	AIC	AM treatment	Soil Aggregation Treatment	AM:Soil Aggr. Treatment	Hyphae (log)		Compartment
modla	7	134.23	X	X	X			X
modlc	8	132.10	X	X	X	X		X
modld	5	147.32		X		X		
modlg	6	139.06	X	X				X
modlh	6	138.22		X		X		X

Table S2. AIC statistics of our model selection procedure when we used nitrous oxide potential activity as a response variable. The lower the AIC value the better is the fit of the model. For model selection we used the mixed effects form of the repeated measures model. The optimal model is highlighted in grey. We highlight in each case the parameters which were included in the models.

Model Name	Df	AIC	AM treatment	Soil Aggregation Treatment	AM:Soil Aggr. Treatment	Hyphae (log)	WSA	Compartment
modla	7	148.84	X	X	X			X
modlb	8	151.42	X	X	X		X	X
modlc	8	147.43	X	X	X	X		X
modld	5	153.30		X		X		
modle	6	155.43		X		X	X	
modlf	6	169.63	X			X	X	
modlg	6	150.53	X	X				X
modlh	6	149.61		X		X		X

Table S3. AIC statistics of our model selection procedure when we used total denitrification rates as a response variable. The lower the AIC value the better is the fit of the model. For model selection we used the mixed effects form of the repeated measures model. The optimal model is highlighted in grey. We highlight in each case the parameters which were included in the models.

Model Name	Df	AIC	AM treatment	Soil Aggregation Treatment	AM:Soil Aggr. Treatment	Hyphae (log)	WSA	Compartment
modla	7	187.90	X	X	X			X
modlb	8	180.66	X	X	X		X	X
modlc	8	185.54	X	X	X	X		X
modld	5	193.68		X		X		
modle	6	193.61		X		X	X	
modlf	6	191.85	X			X	X	
modlg	6	191.39	X	X				X
modlh	6	191.62		X		X		X

Table S4. AIC statistics of our model selection procedure when we used the square root transformed ratio between nitrous oxide and total N_2 activity as a response variable. The lower the AIC value the better is the fit of the model. For model selection we used the mixed effects form of the repeated measures model. The optimal model is highlighted in grey. We highlight in each case the parameters which were included in the models.

Model Name	Df	AIC	AM treatment	Soil Aggregation Treatment	AM:Soil Aggr. Treatment	Hyphae (log)	WSA	Compartment
modla	7	-18.69	X	X	X			X
modlb	8	-17.76	X	X	X		X	X
modlc	8	-13.74	X	X	X	X		X
modld	5	-27.55		X		X		
modle	6	-22.10		X		X	X	
modlf	6	5.76	X			X	X	
modlg	6	-22.31	X	X				X
modlh	6	-21.20		X		X		X

Appendix 2: Optimal model statistics

We maintain the output of the models from R. This includes several non-significant digits. We used the following abbreviations: AM: arbuscular mycorrhizal treatment, rhizo: plant compartment treatment, aggr: soil aggregation treatment, hyphae: AMF hyphal density, WSA: Water stable soil aggregate percentage.

Optimal Model 1: Response variable WSA

```
>summary(modela<-aov(WSA~Error(subject/rhizo)+log(hyphae)+AM*aggr+rhizo,
data=ourdata))


Error: subject
             Df Sum Sq Mean Sq F value  Pr(>F)
log(hyphae)   1  69.41   69.41  11.804 0.00557 **
AM            1  62.47   62.47  10.624 0.00761 **
aggr          1  73.25   73.25  12.458 0.00472 **
AM:aggr       1  22.66   22.66   3.854 0.07542 .
Residuals    11  64.68    5.88
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1


Error: subject:rhizo
             Df Sum Sq Mean Sq F value   Pr(>F)
log(hyphae)   1   1.44    1.44   0.706 0.414981
```

```
rhizo         1  35.35   35.35  17.288 0.000967 ***
Residuals    14  28.62    2.04
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1
```

```
>summary(modelab<-aov(WSA~Error(subject/rhizo)+log(hyphae)+aggr*AM+rhizo,
data=ourdata))


Error: subject
             Df Sum Sq Mean Sq F value   Pr(>F)
log(hyphae)   1  69.41   69.41  11.804 0.005567 **
aggr          1 135.34  135.34  23.017 0.000556 ***
AM            1   0.38    0.38   0.065 0.803224
aggr:AM       1  22.66   22.66   3.854 0.075422 .
Residuals    11  64.68    5.88
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1


Error: subject:rhizo
             Df Sum Sq Mean Sq F value   Pr(>F)
log(hyphae)   1   1.44    1.44   0.706 0.414981
rhizo         1  35.35   35.35  17.288 0.000967 ***
Residuals    14  28.62    2.04
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1
```

Optimal Model 2: Response variable nitrous oxide potential activity rates “rate” – square root transformed

```
>   summary(modelb<-aov(rate~Error(subject/rhizo)+log(hyphae)+AM*aggr+rhizo,
data=ourdata))


Error: subject
             Df Sum Sq Mean Sq F value   Pr(>F)
log(hyphae)   1  241.0   241.0   43.14 4.04e-05 ***
AM            1  174.1   174.1   31.17 0.000164 ***
aggr          1  334.5   334.5   59.88 8.95e-06 ***
```

```
AM:aggr      1    4.1     4.1    0.73 0.411156
Residuals   11   61.4     5.6
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1


Error: subject:rhizo
            Df Sum Sq Mean Sq F value Pr(>F)
log(hyphae)  1  46.01   46.01   7.646 0.0152 *
rhizo        1   8.74    8.74   1.452 0.2482
Residuals   14  84.25    6.02
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1
> summary(modelbb<-aov(rate~Error(subject/rhizo)+log(hyphae)+aggr*AM+rhizo,
data=ourdata))


Error: subject
            Df Sum Sq Mean Sq F value   Pr(>F)
log(hyphae)  1  241.0   241.0  43.139 4.04e-05 ***
aggr         1  506.3   506.3  90.637 1.21e-06 ***
AM           1    2.3     2.3   0.414    0.533
aggr:AM      1    4.1     4.1   0.730    0.411
Residuals   11   61.4     5.6
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1


Error: subject:rhizo
            Df Sum Sq Mean Sq F value Pr(>F)
log(hyphae)  1  46.01   46.01   7.646 0.0152 *
rhizo        1   8.74    8.74   1.452 0.2482
Residuals   14  84.25    6.02
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1
```

Optimal Model 3: Response variable potential denitrification activity

```
>summary(modelc<-aov(rateAC~Error(subject/rhizo)+WSA+AM*aggr+rhizo,
data=ourdata))


Error: subject
          Df Sum Sq Mean Sq F value  Pr(>F)
WSA        1  39.50   39.50   3.555 0.08605 .
AM         1   7.74    7.74   0.697 0.42167
aggr       1  35.32   35.32   3.178 0.10222
AM:aggr    1 126.21  126.21  11.357 0.00625 **
Residuals 11 122.25   11.11
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1


Error: subject:rhizo
          Df Sum Sq Mean Sq F value Pr(>F)
WSA        1    0.2    0.18   0.007  0.934
rhizo      1   67.0   67.04   2.686  0.124
Residuals 14  349.5   24.96
>        summary(modelcb<-aov(rateAC~Error(subject/rhizo)+WSA+aggr*AM+rhizo,
data=ourdata))


Error: subject
          Df Sum Sq Mean Sq F value  Pr(>F)
WSA        1  39.50   39.50   3.555 0.08605 .
aggr       1  36.23   36.23   3.260 0.09840 .
AM         1   6.83    6.83   0.615 0.44954
aggr:AM    1 126.21  126.21  11.357 0.00625 **
Residuals 11 122.25   11.11
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1


Error: subject:rhizo
          Df Sum Sq Mean Sq F value Pr(>F)
WSA        1    0.2    0.18   0.007  0.934
```

```
rhizo      1   67.0   67.04   2.686  0.124
Residuals 14  349.5   24.96
```

Optimal Model 4: Response variable denitrification ratio

```
>summary(modeld<-aov(ratio~Error(subject/rhizo)+log(hyphae)+aggr,
data=ourdata))


Error: subject
            Df Sum Sq Mean Sq F value   Pr(>F)
log(hyphae)  1 0.8321  0.8321   53.62 5.81e-06 ***
aggr         1 1.6917  1.6917  109.03 1.09e-07 ***
Residuals   13 0.2017  0.0155
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1


Error: subject:rhizo
            Df  Sum Sq  Mean Sq F value Pr(>F)
log(hyphae)  1 0.00292 0.002922   0.284  0.602
Residuals   15 0.15417 0.010278
```

Model 5: Differences in Nitrate concentrations across treatments

```
Error: subject
          Df Sum Sq Mean Sq F value Pr(>F)
Residuals  3 0.8598  0.2866


Error: as.factor(subject):Mesh
          Df Sum Sq Mean Sq F value Pr(>F)
Mesh       1 20.458  20.458   15.77 0.0285 *
Residuals  3  3.891   1.297
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1


Error: Within
          Df Sum Sq Mean Sq F value  Pr(>F)
```

```
AM         1  0.134   0.134   0.196 0.66288
aggr       1  9.059   9.059  13.216 0.00155 **
AM:aggr    1  0.082   0.082   0.119 0.73338
Residuals 21 14.395   0.685
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1
```

Model 6: Sensitivity test for root growth

```
>anova(modla<-lme(as.numeric(rate)~AM*aggr + rhizo + AM:rhizo,
random=~1|subject, data=ourdata))
```

	numDF	denDF	F-value	p-value
(Intercept)	1	14	171.77209	<.0001
AM	1	12	0.48814	0.4981
aggr	1	12	130.20648	<.0001
rhizo	1	14	7.29829	0.0172
AM:aggr	1	12	0.78964	0.3917
AM:rhizo	**1**	**14**	**1.69107**	**0.2145**

```
>anova(modlb<-lme(as.numeric(rateAC)~AM*aggr+WSA + rhizo + AM:rhizo,
random=~1|subject, data=ourdata))
```

	numDF	denDF	F-value	p-value
(Intercept)	1	13	658.8928	<.0001
AM	1	12	0.4381	0.5206
aggr	1	12	0.1969	0.6651
WSA	1	13	2.2930	0.1539
rhizo	1	13	4.8734	0.0459
AM:aggr	1	12	6.1014	0.0295
AM:rhizo	**1**	**13**	**0.0859**	**0.7741**

```
>anova(modlc<-lme(as.numeric(ratio)~log(hyphae) + aggr + rhizo +
rhizo:(log(hyphae)), random=~1|subject, data=ourdata))
```

	numDF	denDF	F-value	p-value
(Intercept)	1	14	435.6090	<.0001
log(hyphae)	1	13	28.8248	0.0001
aggr	1	14	138.3609	<.0001

rhizo	1	13	0.2456	0.6284
log(hyphae):rhizo	**1**	**13**	**1.4083**	**0.2566**

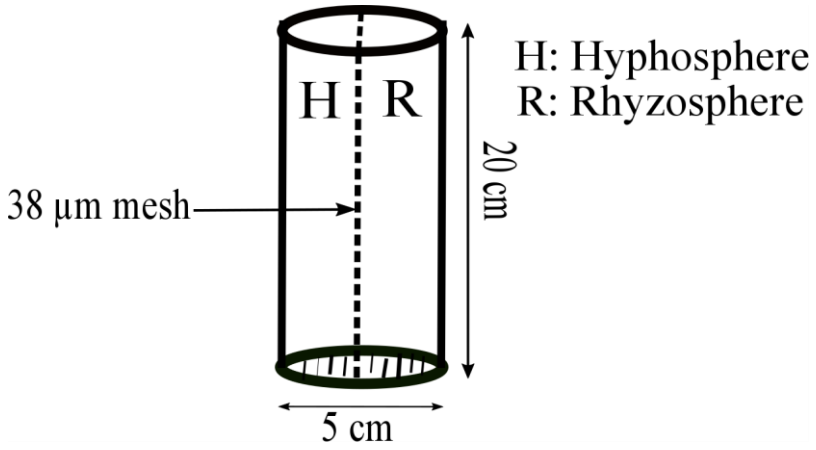

Fig. A1. Experimental mesocosm used in Chapter 3.

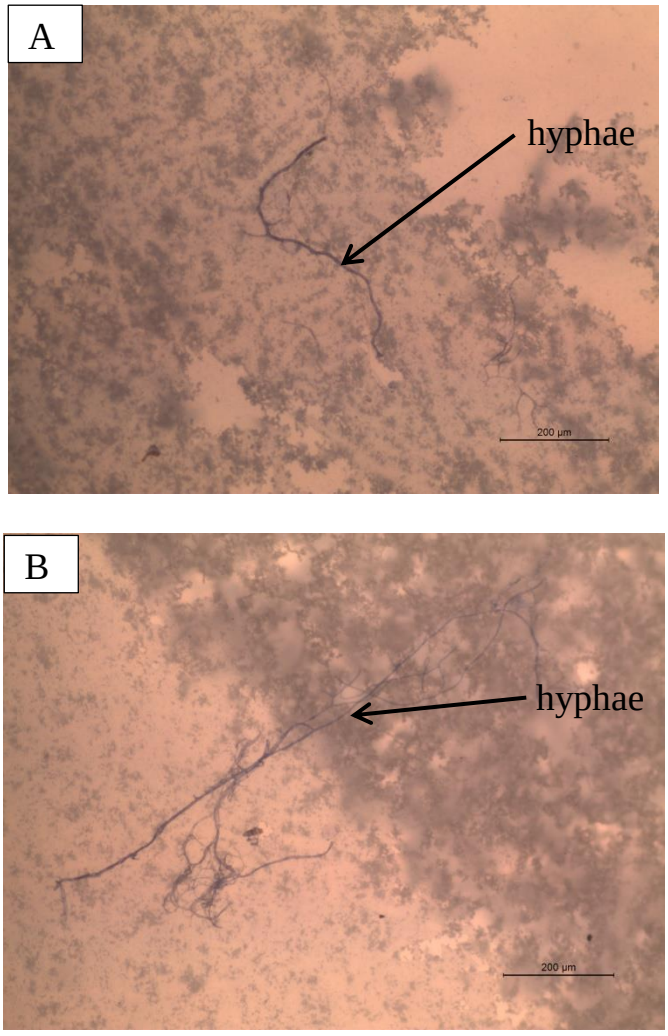

Fig. A2. AM hyphae in soil. A: noninoculated soil and B: inoculated soil with AMF

Appendix B - Supplementary Material for Chapter 4.

Appendix 1. Models – experimental design

Response variable: N_2O potential emissions

```
>summary(model1<-aov(denitrification ~ productivity + myc * shannon, data=N2O))
Df Sum Sq Mean Sq F value Pr(>F)
productivity  1    7.3    7.31   0.451 0.5076
myc           1    0.3    0.32   0.020 0.8893
shannon       1   89.9   89.92   5.554 0.0262 *
myc:shannon   1    0.4    0.45   0.028 0.8689
Residuals    26  420.9   16.19
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1
>summary(model2<-aov(denitrification ~ productivity + myc * evenness, data=N2O))
Df Sum Sq Mean Sq F value  Pr(>F)
productivity  1    7.3    7.31   0.495 0.48775
myc           1    0.3    0.32   0.022 0.88409
evenness      1  126.3  126.32   8.566 0.00703 **
myc:evenness  1    1.5    1.54   0.104 0.74934
Residuals    26  383.4   14.75
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1
```

Response variable: Total potential denitrification

```
>summary(model3<-aov(denitrification ~ productivity + myc * shannon, data=full))
Df Sum Sq Mean Sq F value   Pr(>F)
productivity  1  112.1  112.15   7.686 0.009958 **
myc           1  275.6  275.59  18.889 0.000176 ***
shannon       1    9.5    9.48   0.649 0.427357
myc:shannon   1   45.5   45.54   3.121 0.088584 .
Residuals    27  393.9   14.59
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1
>summary(model4<-aov(denitrification ~ productivity + myc * evenness, data=full))
Df Sum Sq Mean Sq F value   Pr(>F)
productivity  1  112.1  112.15   8.693  0.00652 **
myc           1  275.6  275.59  21.363 8.41e-05 ***
evenness      1   29.9   29.92   2.320  0.13938
```

```
myc:evenness  1   70.7   70.70   5.480  0.02687 *
Residuals    27  348.3   12.90
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1
```

Response variable: Denitrification Ratio

```
>summary(model5<-aov(denitrification ~ productivity + myc * shannon, data=full2))
Df Sum Sq Mean Sq F value Pr(>F)
productivity  1  0.528  0.5276   4.204 0.0505 .
myc           1  0.691  0.6909   5.506 0.0269 *
shannon       1  0.671  0.6706   5.344 0.0290 *
myc:shannon   1  0.001  0.0014   0.011 0.9163
Residuals    26  3.263  0.1255
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1
>summary(model6<-aov(denitrification ~ productivity + myc * evenness, data=full2))
Df Sum Sq Mean Sq F value Pr(>F)
productivity  1  0.528  0.5276   4.309 0.0479 *
myc           1  0.691  0.6909   5.643 0.0252 *
evenness      1  0.737  0.7372   6.022 0.0211 *
myc:evenness  1  0.015  0.0145   0.119 0.7331
Residuals    26  3.183  0.1224
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1
```

Models – explain plant community dynamics

```
>anova(myrda, by="terms", permutations=99999)
Permutation test for rda under reduced model
Terms added sequentially (first to last)
Permutation: free
Number of permutations: 99999

Model: rda(formula = data2[-20, ] ~ total_den + nitrous_oxide + den_ratio + productivity + WSA
+ hyphae + shannon + evenness, data = den, scale = F)
Df  Variance      F  Pr(>F)
total_den      1 0.0043950 3.2823 0.02227 *
nitrous_oxide  1 0.0029531 2.2055 0.08067 .
den_ratio      1 0.0015804 1.1802 0.30322
```

```
productivity   1 0.0043577 3.2545 0.02300 *
WSA            1 0.0004495 0.3357 0.87869
hyphae         1 0.0021491 1.6050 0.17020
shannon        1 0.0117156 8.7495   4e-05 ***
evenness       1 0.0025573 1.9098 0.11414
Residual      22 0.0294580
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1
```

Table 1a. Techniques and instruments used for the characterization of the soil

Measurement	Technique/Instrument
Total organic C and N	DIN ISO 10694, Leco Instruments GmbH.
pH, conductivity	DIN ISO 10390, DIN ISO 11265, TitraMaster85
NO_3^-	DIN EN 12260, CFA-SAN
Total P and K	DIN EN 25663, AAS-iCE 3300, GalleryTM Plus
Soil texture	DIN EN 1484, ISO 8245, TOC-Vcph

Table 1b. Plant species used in this experiment

Grasses (90%)	Forbs (5%)	Legumes (5%)
Agrostis capillaris (Ac);	*Achillea millefolium (Am);* Asteraceae	*Medicago lupulina (Ml);* Fabaceae
Bromus hordeaceus (Bh)	*Leucanthemum ircutianum vulgare (Li);* Asteraceae	*Trifolium pretense (Tp); Fabaceae*
Bromus secalinus (Bs)	*Plantago lanceolate (Pl);* Plantaginaceae	*Lotus corniculatus (Lc);* Fabaceae
Cynosurus cristatus (Cc)	*Prunella vulgaris (Pv);* Lamiaceae	*Medicago reticula (Mr);* Fabaceae
Festuca guestfalica ovina (Fg)	*Sanguisorba minor (Sm);* Rosaceae	
Festuca rubra (Fr)		
Lolium perenne (Lp)		
Poa angustifolia (Pa)		
Poa nemoralis (Pn)		

Fig B1. Mesocosm experiment Chapter 4

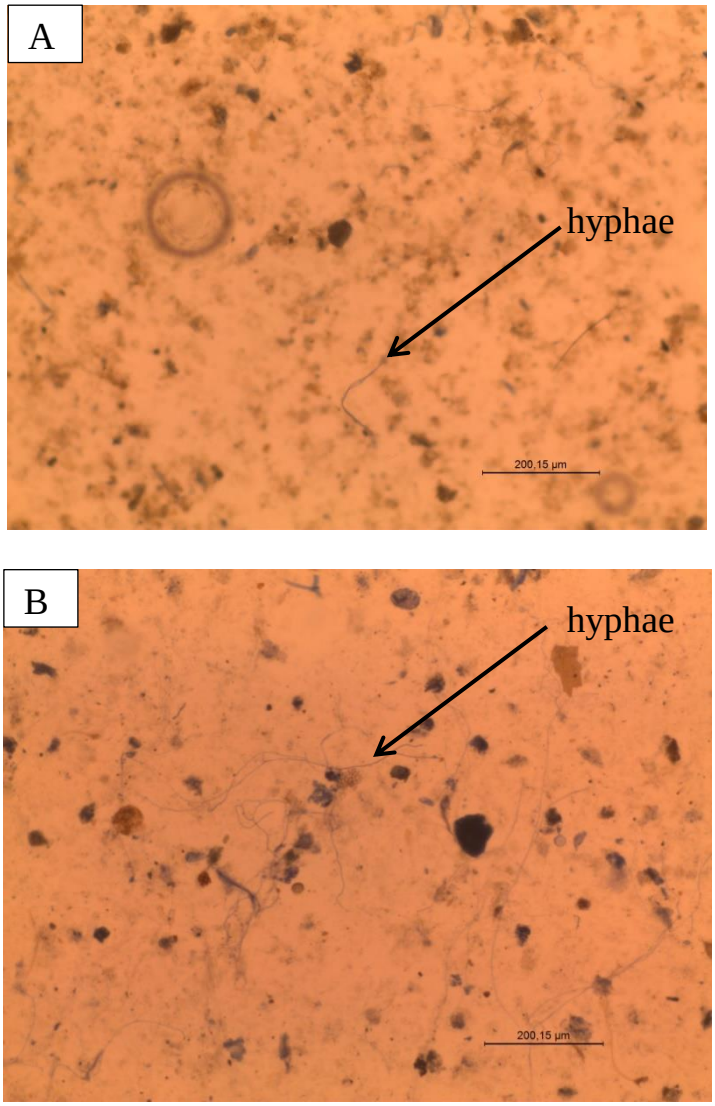

Fig B2. AM hyphae in soil. A: noninoculated soil and B: inoculated soil with AMF

Appendix C - Supplementary Material for Chapter 5.

Appendix A. Model statistics

The response variable is convexity (der). The predictor is either AM hyphal length or nitrate.

Response variable convexity

```
> ourmodel1<-aov(der ~ N2O + Error(subject))
>summary(ourmodel1)

Error: subject
Df  SumSq Mean Sq F value  Pr(>F)
AM         1 0.08597 0.08597   26.33 0.00683 **
Residuals  4 0.01306 0.00327
---
Signif.codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

Error: Within
Df Sum Sq Mean Sq F value Pr(>F)
Residuals  6 0.1094 0.01823
```

Predictor AM hyphal density

```
> ourmodel2<-aov(der ~ hyphae + Error(subject))
>summary(ourmodel2)

Error: subject
          Df  SumSq Mean Sq F value Pr(>F)
hyphae     1 0.07608 0.07608   13.26 0.0219 *
Residuals  4 0.02295 0.00574
---
Signif.codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

Error: Within
          Df Sum Sq Mean Sq F value Pr(>F)
Residuals  6 0.1094 0.01823
```

Predictor nitrate concentration

```
> ourmodel3<-aov(der ~ nitrate + Error(subject))
>summary(ourmodel3)

Error: subject
          Df  SumSq Mean Sq F value Pr(>F)
nitrate    1 0.04884 0.04884   3.892   0.12
Residuals  4 0.05019 0.01255

Error: Within
          Df Sum Sq Mean Sq F value Pr(>F)
Residuals  6 0.1094 0.01823
```

Appendix B. Characteristics of soil used in this experiment

Soil variables	Values
pH-water	5.6
NH_4^+-N (mg. g^{-1} soil)	30
NO_3^--N (mg. g^{-1} soil)	138
Total P (mg. g^{-1} soil)	469
Available P (mg. g^{-1} soil)	379
Available K (mg. g^{-1} soil)	1313
Total organic matter (%)	0.54
Total inorganic carbon (%)	0.02
Total nitrogen (%)	0.06
Electrical conductivity ($\mu S.cm^{-1}$)	86.30
Texture	
Sand (%)	69.8
Silt (%)	20.7
Clay (%)	9.5

Appendix C. Calculation of gas fluxes see Fiedler et al. 2017

$$f = \frac{M \times p \times v \times dc}{R \times T \times A}$$

where f is the flux (N_2 mg m^{-2} h^{-1}, N_2O: ng m^{-2} h^{-1}), M, molar mass in g mol^{-1} (N_2: 28, N_2O: 44), p the air pressure (Pa), v the air flow (L h^{-1}), R the gas constant (8.31 J mol^{-1} K^{-1}), T the temperature inside the chamber (K), A the area of the incubation vessel (m^2) and dc the difference of gas concentrations (N_2 ppm, N_2O: ppb) between inlet and outlet of a vessel.