Thèse de Doctorat

Ecole Doctorale Environnement-Santé

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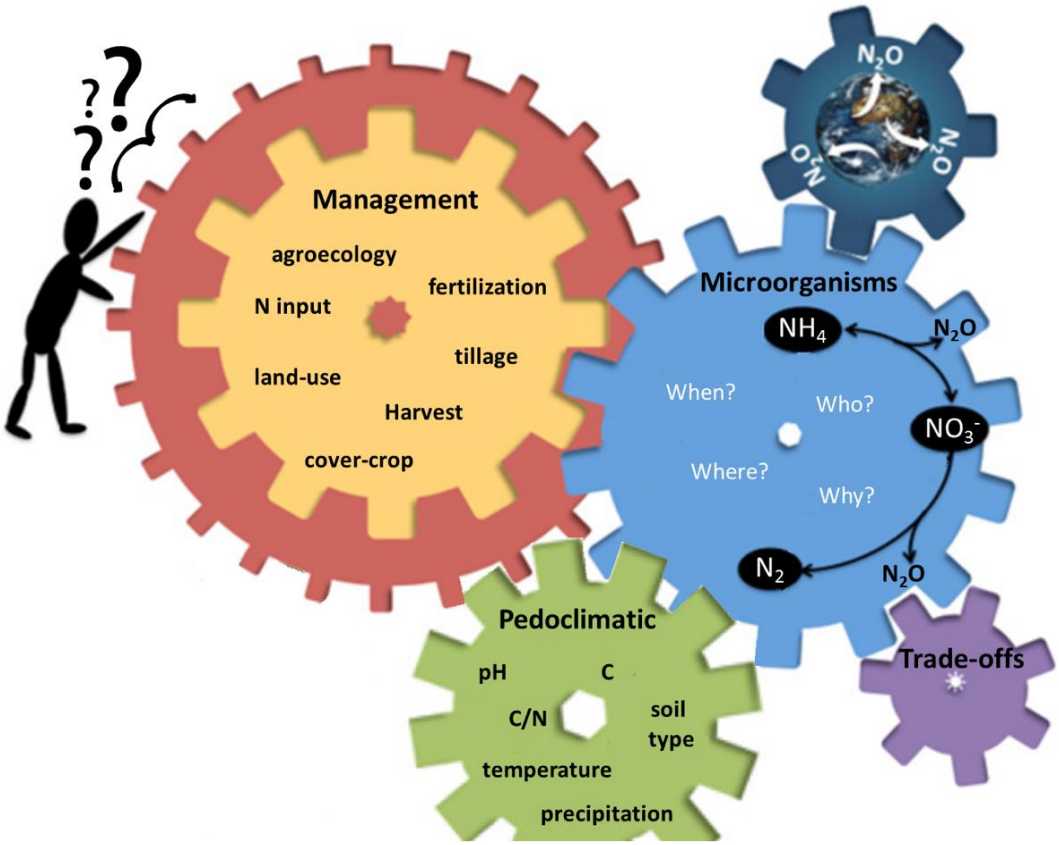

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Soutenue le 16 Décembre 2016 à Dijon

LUIZ A. DOMEIGNOZ HORTA

M[me] Lydie CHAPUIS-LARDY, IRD, Montpellier, France	Rapporteur
M. Xavier LE ROUX, INRA, Lyon, France	Rapporteur
M. Denis ANGERS, AAC/AAFC, Québec, Canada	Examinateur
M. David WENDEHENNE, INRA, Dijon, France	Examinateur
M. Laurent PHILIPPOT, INRA, Dijon, France	Directeur de thèse
M. Aymé SPOR, INRA, Dijon, France	Co-encadrant de thèse

Université de Bourgogne
Ecole Doctorale Environnement Santé
Dijon, France

INRA UMR 1347 Agroécologie
Dijon, France

Abstract

Nitrous oxide (N_2O) is an important greenhouse gas (GHG) and the main ozone depleting substance. Agricultural soils are the main anthropogenic-induced source of this GHG. The concentration of N_2O in the atmosphere is steadily increasing, but we still lack knowledge on the factors controlling its production and consumption in soils. The reduction of N_2O to N_2 by microorganisms harboring the N_2O reductase gene (*nosZ*) is the only known biological process able to consume this GHG. Recent studies revealed a previously unknown clade of N_2O-reducers which was shown to be important to the N_2O sink capacity of soils. This thesis seeks to gain a greater understanding on the ecology of N_2O-reducers in agricultural soils. A combination of laboratory incubation and field experiments were used to gain knowledge on the importance of N_2O-producers and N_2O-reducers to the soil N_2O production. Additionally, the potential of agricultural practices to modify those microbial communities were assessed.

We showed experimentally, in laboratory incubations, that the addition of a non-denitrifying strain *Dyadobacter fermentans*, which possesses the previously unaccounted N_2O reductase NosZII, reduced N_2O production in 1/3 of the tested soils. Remarkably, after addition of the *nosZ*II strain, some soils became a N_2O sink, as negative rates were recorded. This experiment provided unambiguous evidence that the overlooked non-denitrifying *nosZ*II bacteria can contribute to N_2O consumption in soil.

Our evaluation of agricultural field experiments showed limited impact of agricultural practices on the microbial communities except for tillage management, and differences observed between an annual and a perennial cropping system. Increasing tillage management enhanced *nosZ*II diversity. Higher diversity of the *nosZ*II clade was also observed in the annual cropping system than in the perennial cropping system. Overall, the recently identified clade of N_2O-reducers was more sensitive to environmental variables than the previously known clade (*nosZ*I). The community structure of these two groups was explained by common and uncommon soil properties suggesting niche specialization between the two N_2O-reducers.

In an attempt to understand the relationship between the microbial communities and process rates, we assessed the potential denitrification and nitrification rates, and *in situ* N_2O emissions. Potential N_2O production and potential denitrification activity were used to calculate the denitrification end-product ratio. The diversity of *nosZ*II was negatively related to the N_2O:N_2 ratio and explained the highest fraction of its variation (26%), while the

potential N_2O production and potential denitrification activity were mainly explained by the soil properties. To better evaluate the contribution of different factors to the *in situ* emissions, more than 70000 N_2O measurements were subdivided into different ranges, from low to high rates. Interestingly, the low range of *in situ* N_2O emissions was only related to soil pH, while the high ranges were also strongly related to the microbial communities. This result suggests that the "base-line" N_2O emissions might be more regulated by soil edaphic conditions than by microorganisms, the lasts being more important for the high emissions ranges. Among the significant microbial variables, we found that the diversity of *nosZ*II was negatively related to the high ranges of *in situ* N_2O emissions.

In conclusion, our results highlight the relevance of the second clade of N_2O-reducers to the fate of N_2O in soil. Our results also suggest niche differentiation between the two N_2O-reducing clades with *nosZ*II being more responsive to environmental variables. Agricultural practices showed limited impact on the two guilds. Further research is needed to test the niche specialization between the two groups, to disentangle their controlling factors, and to evaluate their potential for N_2O mitigation.

Keywords: microbial ecology, nitrogen, greenhouse gas, agricultural practices, denitrification, nitrous oxide, nosZ

Résumé

Le protoxyde d'azote (N_2O) est un gaz à effet de serre (GES) important et la principale substance attaquant la couche d'ozone. Les sols agricoles sont la principale source anthropique de ce GES. La concentration de N_2O dans l'atmosphère est en constante augmentation, mais nous manquons de connaissances sur les facteurs contrôlant sa production et sa consommation dans les sols. La réduction du N_2O en N_2 par des microorganismes porteurs du gène codant pour la N_2O réductase (*nosZ*) est le seul processus biologique capable de réduire ce GES. Des études récentes ont mis en évidence un clade précédemment inconnu de réducteurs du N_2O qui interfère de manière significative avec la quantité de N_2O produite dans les sols. Cette thèse a cherché à mieux comprendre l'écologie des réducteurs du N_2O dans les sols agricoles.

Une combinaison d'expériences d'incubation en laboratoire mais aussi d'expériences en plein champs a été utilisée pour essayer de mieux comprendre la production de N_2O dans le sol, en analysant l'influence conjointe des producteurs et réducteurs de N_2O. Nous avons aussi évalué l'impact des pratiques agricoles et leurs potentiels à modifier ces communautés microbiennes. Suite aux essais réalisés en laboratoire, nous avons montré que l'ajout d'une souche non-dénitrifiante *Dyadobacter fermentans*,possédant la N_2O réductase NosZII, permettait de réduire la production de N_2O dans 1/3 des sols testés. Certains sols sont même devenus consommateurs de N_2O suite à l'ajout de la souche *nosZ*II. Cette expérience a démontré la contribution des bactéries *nosZ*II non-dénitrifiantes dans la consommation de N_2O dans le sol.

D'autre part, nos analyses en contexte agricole ont montré que les pratiques agricoles testées ont peu d'influence sur les communautés microbiennes considérées, les exceptions étant le travail du sol (labour), et le système de culture (annuel ou pérenne). L'intensifiant du travail du sol induit une augmentation de la diversité de *nosZ*II. Nous observons le même phénomène dans le système de culture annuel comparé au système de culture pérenne. D'autres résultats nous permettent aussi d'affirmer que le clade récemment identifié de réducteurs du N_2O est plus sensible aux variables environnementales que le clade précédemment connu (*nosZ*I). Les variations de propriétés du sol, notamment pH et C:N structurent les communautés microbiennes appartenant à ces 2 clades indiquant une spécialisation de niche pour chacun de ces deux clades de N_2O-réducteurs.

Pour mieux comprendre les relations entre les communautés microbiennes et les processus impliqués, nous avons évalué les activités potentielles de dénitrification et de nitrification, et les émissions de N_2O *in situ*. La production potentielle de N_2O et l'activité potentielle de dénitrification ont été utilisées pour calculer le ratio de production de N_2O (N_2O:N_2). La diversité du clade *nosZ*II est négativement corrélée au ratio N_2O:N_2, et explique à elle seule la plus grande part de variance observée du ratio N_2O:N_2. Les variations de production potentielle de N_2O et d'activité potentielle de dénitrification sont elles expliquées principalement par les variations de propriétés du sol. Afin de mieux évaluer la contribution des différents facteurs édaphiques et microbiologiques aux variations d'émission *in situ* de N_2O, 70000 mesures ont été subdivisées en différentes gammes d'émission de N_2O, d'émissions dites de base à des émissions élevées. Fait intéressant, les variations d'émissions *in situ* de N_2O dites de base sont seulement liées à des variations du pH du sol, alors que les variations d'émissions dites élevées sont également fortement associées aux variations de diversité des communautés microbiennes. Parmi les variables microbiennes importantes, nous avons constaté que la diversité des *nosZ*II est négativement liée aux émissions de N_2O *in situ* dites élevées.

En conclusion, nos résultats mettent en évidence l'importance du clade *nosZ*II pour le cycle du N_2O dans le sol. Ce clade *nosZ*II semble plus sensible aux variables environnementales mais relativement robuste aux pratiques agricoles évaluées. D'autres études de l'écologie de ces réducteurs de N_2O sont nécessaires afin de démêler les facteurs contrôlant leur structure et leur diversité, pour évaluer leur potentiel d'atténuation pour les émissions de N_2O.

Mots-clés: écologie microbienne, azote, gaz à effet de serre, les pratiques agricoles, la dénitrification, l'oxyde nitreux, nosZ

Dedication

“Live as if you were to die tomorrow. Learn as if you were to live forever.”

Mahatma Gandhi

“Feliz aquele que transfere o que sabe e aprende o que ensina.”

“Happy are the ones who transfer what they know and learn what they teach.”

Cora-Coralina

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List of Publications

Chapter I Domeignoz-Horta L.A., Putz M., Spor A., Bru D., Breuil M.C., Hallin S., Philippot L. *Soil Biol*. *Biochem*., 2016. Non-denitrifying nitrous oxide-reducing bacteria - an effective N_2O sink in soil.

Chapter II Domeignoz-Horta L. A., Spor A., Bru D., Breuil M-C., Bizouard F., Léonard J. and Philippot L. *Front*. *Microbiol*., 2015. The diversity of the N_2O reducers matters for the N_2O:N_2 denitrification end-product ratio across an annual and a perennial cropping system.

Chapter III Domeignoz-Horta L.A., Peyrard C., Bru D., Breuil M.C., Bizouard F., Mary B., Léonard J., Philippot L., Spor A. Effects of agricultural practices and soil properties on soil N_2O-reducing bacteria and *in situ* N_2O emissions. Manuscript in preparation to be submitted to Global Change Biology

Abbreviations

N	Nitrogen
N_r	Reactive Nitrogen
NO_3^-	Nitrate
NO_2^-	Nitrite
NO	Nitric Oxide
N_2O	Nitrous Oxide
N_2	Dinitrogen gas
N_2OR	Nitrous Oxide Reductase
P450Nor	Cytochrome P450 Nitric Oxide Reductase
AOB	Ammonia Oxidizer Bacteria
AOA	Ammonia Oxidizer Archaea
nirK	Gene encoding Nitrite reductase
nirS	Gene encoding Nitrite reductase
nosZ	Gene encoding Nitrous Oxide Reductase
*nosZ*I	Gene from Clade I encoding Nitrous Oxide Reductase
*nosZ*II	Gene from Clade II encoding Nitrous Oxide Reductase
IPCC	Intergovernmental Panel on Climate Change
EF	N_2O Emission Factor
pO_2	Oxygen Partial Pressure

1. Introduction

1.1 The Nitrogen Problem: "too much of a good thing"

Free nitrogen, N_2, constitutes 78% of Earth's atmosphere. However, this immense reserve of N is accessible only to N_2-fixing bacteria and archaea, which reduce it into ammonium making it available for other living organisms. Vitousek & Howarth (1991) estimated that about 0.1% of the N_2 pool was biologically fixed in the biosphere, thus limiting primary production in terrestrial and marine environments. Nevertheless, this picture started to change rapidly after Fritz Haber and Carl Bosch discovered in the beginning of the 20th century a process able to convert the almost non-limited reserve of N_2 from the atmosphere to reactive forms of N (N_r). This discovery allowed the industrial synthesis of nitrogen fertilizers (Trautmann et al., 1989). The Haber-Bosch process, named after the discoverers, marked the beginning of a massive human intervention on the N cycle. Today more than half of the world's population consumes food produced with N fixed by the Haber-Bosch process (Smil 2002). Although this process became very important to sustain human life, its use in modern agriculture can cause major environmental problems (Galloway & Cowling, 2002).

At a planetary scale, the addition of nitrogen in ecosystems due to intensive agriculture changed the global nitrogen cycle. Thus, the rate of change in this cycle is recognized to be one of the fastest compared to other biogeochemical cycles (Gruber & Galloway, 2008; Steffen et al., 2015). Anthropogenically fixed nitrogen via the Haber-Bosch process already exceeded terrestrial biological nitrogen fixation before the end of the 20th century (Galloway & Cowling, 2002). During 2008 alone, ammonia supply via the Haber-Bosch reached 9.7 x 10^{12} mol N (Figure 1) (Canfield et al., 2010). Most of this N_r is used for fertilizer production. However, approximately 50% of all applied fertilizer is estimated to be lost to the environment via runoff or gaseous products (Venterea et al., 2012; Galloway & Cowling, 2002).

The N applied as fertilizer can cause various negative effects in the environment as soil acidification (Matson et al., 1999) and increasing the emissions of the greenhouse gas N_2O (Smith et al., 2008; Saggar et al., 2013). In addition, increased nitrogen deposition due to volatilization of N-based fertilizers was shown to decrease plant diversity (review in Bobbink et al., 2010). Finally, nitrate leaching may lead to eutrophication of water bodies and estuaries (Vitousek et al., 1997; Robertson & Vitousek, 2009; Sutton et al., 2011). Moreover, the same

atom of N_r can cause a sequence of effects between terrestrial ecosystems, the atmosphere, fresh water and marine systems. This sequence of effects is designated as the "nitrogen cascade" to illustrate that a unique N_r atom could produce more than one undesired effect to the environment (Galloway et al., 2003).

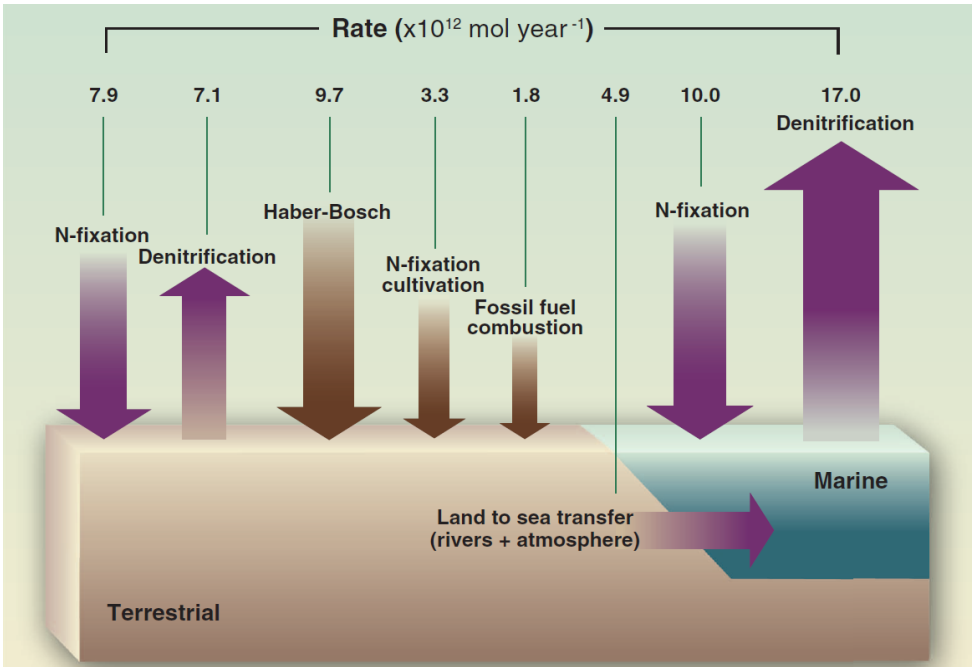

Fig. 1. Rates of nitrogen flux in the nitrogen cycle. Arrow size shows the relative size of the flux and brown arrows represent anthropogenic inputs (Canfield et al., 2010).

An assessment of nitrogen pollution cost in Europe is estimated to range from 70 up to 320 billion euros yearly (Sutton et al., 2011). The highest societal costs are affiliated to air and water quality. These numbers would even increase if contributions of N pollution to climate change (enhanced emissions of the greenhouse gas N_2O) were taken into account. Overall, N_2O emissions associated with agriculture are projected to rise from about 6.4 Tg N_2O-N yr^{-1} in 2010 to 7.6 Tg N_2O-N yr^{-1} by 2030 (Reay et al., 2012).

1.2 Nitrous Oxide: no laughing matter, but a potent greenhouse gas

First described by Joseph Priestley in 1772, nitrous oxide (N_2O) is commonly known as "laughing gas" since the 18th century. This colourless and odourless gas has anesthetic properties. More recently, it was discovered that N_2O binds to vitamin B_{12} and inactivates it (Sullivan et al., 2013). Thus, N_2O consumption can cause vitamin B_{12} deficiency and dangerously harm health (Flippo & Holder et al., 1993). However, societal concern regarding

N_2O is increasing because of its contribution to climate change. This gas has a long life time (110 years) and a global warming potential 298 times that of carbon dioxide on a 100 year time scale per unit of weight (Bates et al., 2008). N_2O is also currently the dominant ozone depleting substance and should remain so for this century (Ravishankara et al., 2009).

Atmospheric nitrous oxide concentration increased by 20% since the beginning of the industrialization (Montzka et al., 2011). A recent estimate suggests that if no mitigation measure is taken, anthropogenic N_2O emissions would almost double by 2050 relative to 2005 values (Davidson et al., 2014). Yearly N_2O emissions accounts for about 10% of global warming, being the third most important GHG after carbon dioxide and methane (Bates et al., 2008; Thomson et al., 2012).

Pre-industrial emissions, which are considered to represent the N_2O emitted by natural earth ecosystems without human interference, are estimated to reach 11 Tg N_2O-N y^{-1} (IPCC 2013). These estimates represent two thirds of total global emissions of this GHG. The remaining third is due to anthropogenic activities, and agriculture is responsible for the largest fraction with 66% of total anthropogenic emissions (Davidson & Kanter, 2014). This is primarily the result of nitrogen fertilizer input into agroecosystems (Smith et al., 2012). Human-induced emissions are however not exclusive to agricultural sites and Skiba et al. (2009) showed that N deposition in non-agricultural ecosystems enhanced N_2O emissions. Equally relevant are the indirect N_2O emissions due to NO_3^- leaching (Reay et al 2009).

Top-down or bottom-up methodologies are used to estimate N_2O emissions. The first are based on changes in atmospheric N_2O concentration over time. Contrary to top-down, bottom-up estimates rely on extrapolation of flux measurements of individual ecosystems to larger regions, continents, and finally to the global scale. The total input of N_r is a predictor of N_2O emissions. Thus, it is currently used to estimate national N_2O inventories using a N_2O emission factor approach (EF) (de Klein et al., 2006).

The Intergovernmental Panel on Climate Change (IPCC) estimated that 1% of applied synthetic fertilizers results in N_2O emissions, *i.e*. every 1000 kg of N input results in 10 kg of N_2O-N emitted directly from the soil. An EF of 1% assumes a linear relationship between N input and N_2O emissions, however increasing evidences suggest that fertilizer induced emissions respond exponentially rather than linearly to N input (McSwiney & Robertson, 2005; Hoben et al., 2011; Kim et al., 2013; Shcherbak et al., 2014). A literature review of N_2O emissions from fertilized fields reported approximately 1 kg N_2O-N ha^{-1} y^{-1} at application rates of 25 up to 150 kg N ha^{-1} y^{-1} and a dramatic increase when N input surpasses 250 kg N ha^{-1} y^{-1} (Bouwman et al., 2002). McSwiney & Robertson (2005) showed an

exponential increase in N_2O emissions at N input higher than 100 kg N ha^{-1} y^{-1}, which corresponds to input rate for the maximum crop harvest. Shcherbak et al. (2014) confirmed these previous findings suggesting an exponential increase in N_2O emissions when N inputs exceed crop needs.

Considering an EF default value of 1% has important consequences for national GHG inventories. N_2O emissions might be underestimated where a high rate of applied N occurs, whereas emissions might be over-estimated in regions under low fertilization. For example, high N input rates of 500 kg N ha^{-1} y^{-1}, which are common in China's North Plain region (Vitousek et al., 2009), will lead to underestimations of N_2O emissions by about 50% with an EF of 1%. The use of a higher EF, as suggested by some authors (Grace et al., 2011; Smith et al., 2012; Griffis et al., 2013), would not solve the problem of under- and overestimating N_2O emissions due to contrasting N inputs in different regions. Shcherbak et al (2014) suggested the use of an N-rate-dependent ΔEF model, could be a more appropriate method to estimate N_2O emissions than adopting a default value. The use of a constant EF may also explain why regional bottom-up estimates of N_2O are not equivalent to top-down estimates (Reay et al., 2012). Taking into account that N inputs varies greatly worldwide (Vitousek et al., 2009), simulation of N_2O emissions in grassland and arable soils showed large variations between different regions (Fig. 2) (Stehfest & Bouwman, 2006).

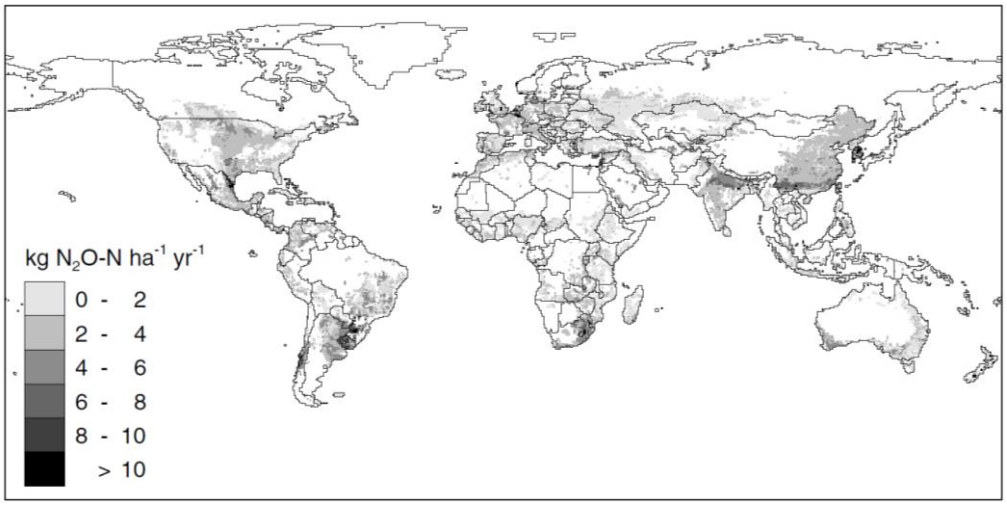

Fig. 2. Simulated annual N_2O emissions from agriculture and grasslands (Stehfest & Bouwman, 2006).

The assumption of a constant EF also disregards biological thresholds that influence N_2O emissions. Although N application rate is considered to be the major factor influencing N_2O emissions, other factors contribute to it. Some known factors influencing N_2O emissions are soil temperature, soil water content, carbon content, and crop types

(Butterbach-Bahl et al., 2013; Shcherbak et al., 2014). Those will be further discussed in this manuscript.

Terrestrial ecosystems are not only a source of N_2O, but can also act as a sink for this GHG. Unfortunately, most estimations of GHG sequestration ignore N_2O sink by the environment (Tian et al., 2016; Chapuis-Lardy et al., 2007). When negative fluxes have been reported in the literature, they were often not considered except for highlighting that little information is available on the capacity of soils to take up N_2O (Fenn et al., 1996; Smith et al., 1998; Butterbach-Bahl et al., 2002; Kiese et al., 2003; Xu et al., 2004). Schlesinger (2013) reviewed previous studies in an attempt to estimate the global potential of soils to act as nitrous oxide sinks and suggested a rather small uptake of the global net flux from soils to the atmosphere (about 5 % of 6.2-9.4 Tg N y^{-1}, *i.e.* ~ 0.3 Tg N yr^{-1}. This estimation might however be biased, considering that negative fluxes were frequently observed but commonly omitted from datasets (Chapuis-Lardy et al., 2007).

Considering that fertilizer applications are estimated to increase up to 60% before 2030 (Smith et al., 2007) and that soil N_2O emissions continue rising (Reay et al., 2012) (Figure 3), it is urgent to better understand the factors underlying soil N_2O production and the potential of soil to reduce this GHG. While the influence of pedo-climatic factors on N_2O production has been extensively studied in the past, the importance of the underlying microbial communities has been acknowledged just recently.

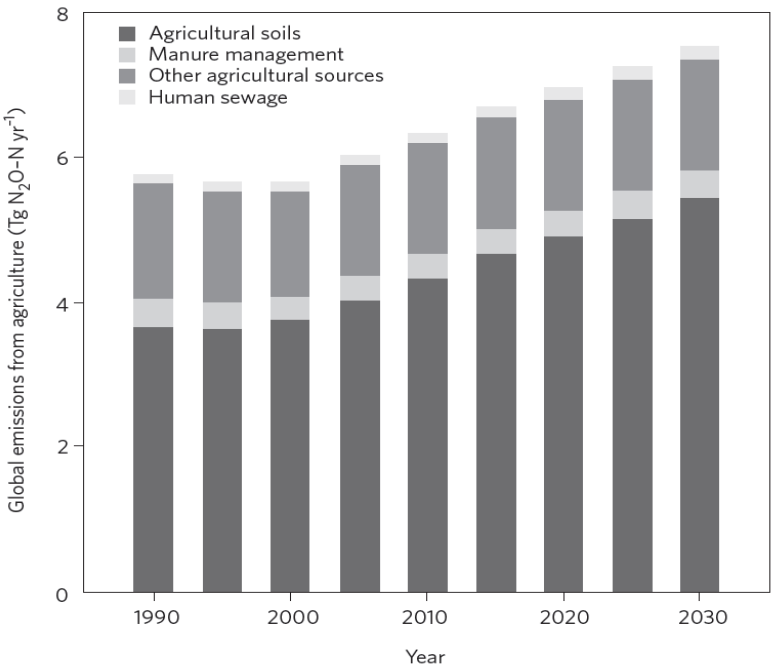

Fig. 3. Global N_2O emissions from agriculture from 1990 and estimated values for 2020 and 2030 (Reay et al., 2012).

1.3 Biological processes responsible for N_2O production and consumption

N_2O production can occur abiotically *via* chemodenitrification (Samarkin et al., 2010). However, it is microbially-mediated processes that dominate N transformations (Figure 4). Within this manuscript, only biological N pathways will be considered.

Multiple interrelated and connected biological pathways, sharing intermediate products, catalyse the production of N_2O (Figure 4 and 5). In soils, N_2O production is primarily attributed to denitrification and nitrification (Hu et al., 2015; Philippot et al., 2007; Snyder et al., 2009).

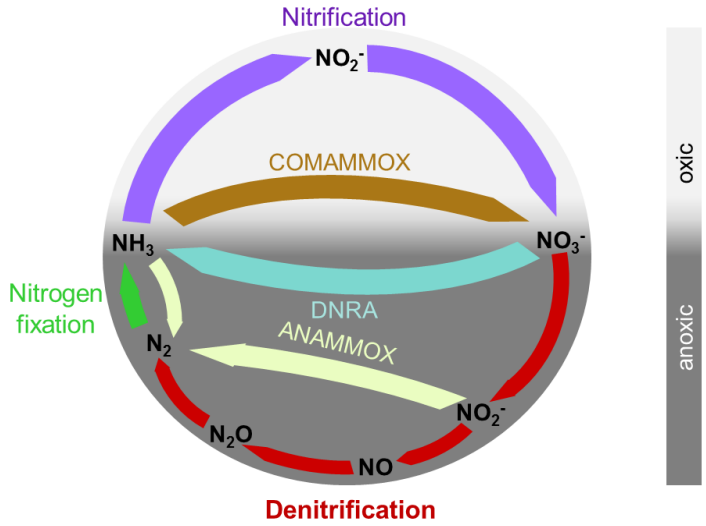

Fig. 4. Simplified schematic representation of major pathways of nitrogen cycling.

1.3.1 Nitrification

Nitrification is an important pathway in agroecosystems as it is converting ammonium (NH_4^+) to nitrate (NO_3^-) (Figures 4 and 5). While NH_4^+ has a propensity to bind to soil particles, its conversion to NO_3^- leads to significant losses of soil nitrogen through leaching (Vitousek et al., 2000). The loss of NO_3^- not only represents a wastage of fertilizer, estimated at billions of euros yearly, but it is also very costly to the ecosystems (Robertson & Vitousek, 2009). The NO_3^- anion is a major problem for water pollution, causing eutrophication of rivers and lakes but also algal blooms in the ocean (Sutton et al., 2011).

Nitrification is the aerobic oxidation of ammonia to nitrate via nitrite ($NH_3 \rightarrow NH_2OH/HNO \rightarrow NO_2^- \rightarrow NO_3^-$). This process was first described as performed by two functionally defined group of microbes (Winogradsky, 1890; Prosser & Nicol 2012). The ammonia oxidizers are responsible for the oxidation of ammonia to nitrite. This step is

performed by chemolithoautotrophs microorganisms, which utilise CO_2 as carbon source, NH_3 as electron donor and O_2 as electron acceptor. Two physiologically distinct groups of microorganisms perform this step- Ammonia Oxidizing Archaea (AOA) and Ammonia Oxidizing Bacteria (AOB) (Prosser & Nicol 2012)- which is catalyzed by the ammonia monooxygenase (AMO) encoded by the *amoA* gene. Ammonia oxidation is usually considered as the rate-limiting step of nitrification (Zumft, 1997; Prosser & Nicol 2012). It is also the controlling step for N_2O production by AOB or AOA. AOB generate N_2O via two processes: *i*) the incomplete hydroxylamine (NH_2OH) oxidation to NO and further to N_2O, and *ii*) through nitrifier denitrification. The mechanisms by which AOA produces N_2O are unclear (Walker et al., 2010). Some authors suggest that N_2O may be produced abiotically by oxidation of compounds such as NH_2OH, NO or NO_2^- (Harper et al., 2015). Further studies are still needed to elucidate the N_2O production mechanisms by AOA.

Differences in pH tolerance, ammonia affinity and mixotrophy between AOA and AOB were suggested as important factors for niche specialisation between these two groups (Erguder et al., 2009; Valentine et al., 2007; Prosser & Nicol 2012). For example, it has been reported that AOB predominate in alkaline soils while AOA are more prevalent in acidic soils (He et al., 2012; Hu et al., 2014; Yao et al., 2011). N supply are also important for AOA and AOB, while AOB was shown to be favoured by high NH_3 concentrations, AOA prefers low NH_3 concentrations (Di et al., 2010). Prosser & Nicol (2012) draw attention to the fact that no single factor should be used to discriminate between these two groups, as both shows an important physiological diversity that enables them to grown and be active in very distinct soils. These authors highlight the need for more studies on culture organisms that represent dominant environmental communities rather than studies relying solely on correlations to better understand their physiological diversity and the environmental factors important for AOA and AOB.

Until recently, the inability to distinguish nitrification by AOA and AOB impeded to assess their relative contribution to soil N_2O emissions. For the first time, Hink et al. (2016) could evaluate the relative N_2O production by AOA and AOB during aerobic ammonia oxidation using 1-octyne as a specific inhibitor of AOB (Taylor et al., 2013). These authors showed that AOA dominated oxidation of mineralisation-derived ammonia and the corresponding N_2O production. However, when NH_4^+ was added to soil microcosms AOB was responsible for its oxidation to NO_3^- and the corresponding N_2O emissions. To our knowledge, it is the only study disentangling the contribution of the AOA and AOB to N_2O emissions.

The second step of nitrification is performed by the nitrite-oxidizing bacteria (NOB), which include several genera as Nitrobacter (α-Proteobacteria), Nitrococcus (γ-Proteobacteria), Nitrospina (δ-Proteobacteria) and Nitrospira (Nitrospira class). These genera have very various metabolic pathways, Nitrobacter members are predominantly chemolithoautotrophic, Nitrospina bacteria use the tricarboxylic pathway to fix CO_2, while others have also heterotrophic metabolism and are considered mixotrophs or facultative autotrophs. These genera oxidize NO_2^- to NO_3^- by the nitrite oxidoreductase (NOR), completing the nitrification pathway.

A recent discovery revealed novel microorganisms belonging to the Nitrospira genus that completely oxidizes ammonia to nitrate via nitrite, a process denominated Comammox (van Kessel et al., 2015; Daims et al., 2015). This discovery changed the old dogma that nitrification was a process performed by two group of functionally defined organisms (ammonia-oxidizing Bacteria/Archaea and nitrite-oxidizing Bacteria) (Kuypers, 2015). First assessments of organisms performing Comammox showed they were widespread in terrestrial ecosystems (van Kessel et al., 2015; Daims et al., 2015), suggesting that they are important for ammonia oxidation in this environment. Until now there is no evidence that Comammox microorganisms can produce N_2O.

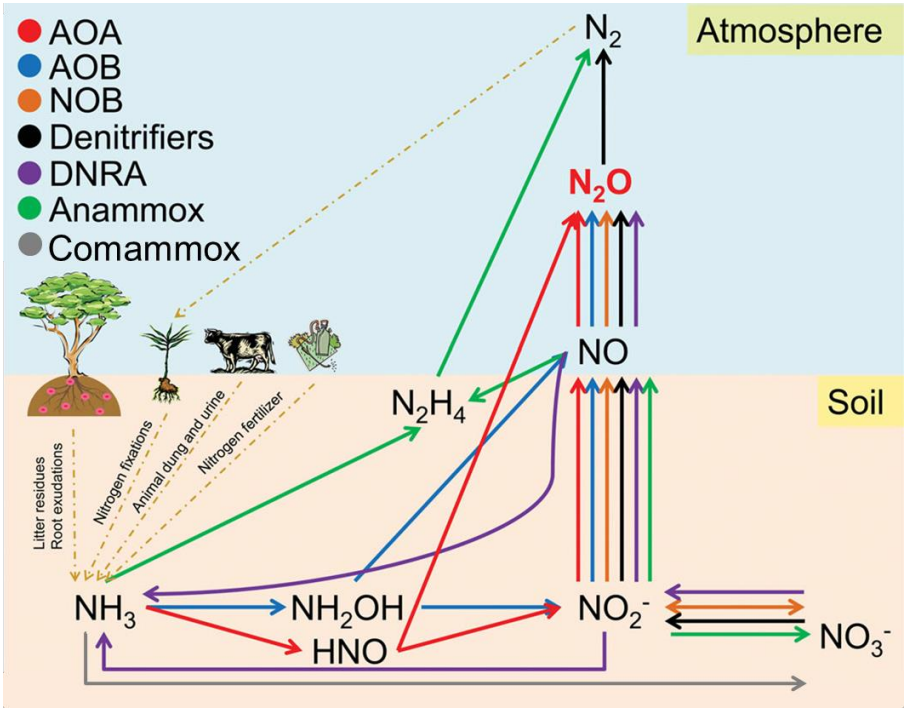

Fig. 5. Simplified schematic representation of major pathways of the nitrogen cycle (Modified from Hu et al., 2015).

Heterotrophic nitrification is the oxidation of inorganic and organic reduced forms of N, to NO_3^-, by a wide range of fungi and heterotrophic bacteria (Killham, 1990). There is little evidence that heterotrophs gain energy and can grow on nitrification (Prosser et al., 2005). Moreover, N_2O production by this process remained for long overlooked. Some studies acknowledge that this process might be relevant to N_2O emissions, and may increase in importance in acidic soils (Stange et al., 2013; Zhang et al., 2015). Further studies are needed to assess the relevance of heterotrophic nitrification for N_2O production. In this manuscript however we restrain our discussion to autotrophic nitrification.

1.3.2 Denitrification

From an ecosystem perspective, denitrification can be considered as an important service for the environment (Hooper et al., 2005) because it removes NO_3^-, which in excess is a pollutant. It is also the only known biological process able to consume N_2O (Figure 5 and 6). However, from an agricultural perspective, denitrification represents a huge loss of fertilizer since it converts bioavailable forms of N to N_2O or N_2.

Denitrification is a facultative heterotrophic respiratory pathway by which soluble nitrogen oxides (NO_3^- and NO_2^-) are reduced to gaseous nitrogen oxides (NO, N_2O and N_2) under low O_2 partial pressure (Figure 6) (Zumft, 1997). Denitrification is a four-step reduction of nitrate to dinitrogen gas coupled to an electron transport chain generating a proton gradient across the membrane for energy conservation (Wasser et al., 2002). Denitrification is a modular pathway (Graf et al., 2014; Philippot 2002; Zumft, 1997). While some organisms can completely reduce NO_3^- to dinitrogen gas, others can lack one or several steps in this cascade of reduction. The first step, the reduction of NO_3^- to NO_2^-, is performed by a periplasmic or by a membrane-associated nitrate reductase which catalytic unit is encoded by the *napA* or *narG* genes, respectively. The next step ($NO_2^- \rightarrow NO$) is considered as the defining step of denitrification because it transforms a soluble nitrogen oxide into a soluble nitrogen gas (Zumft, 1997). This step is catalyzed by either a copper- or a cytochrome cd_1- nitrite reductase, encoded by the *nirK* or *nirS* genes, respectively (Glockner et al., 1993). Both NirK and NirS proteins are localized in the periplasm, but differ regarding their structure and catalytic site. The NirK protein is a multi-copper enzyme with copper ions as ligands in the catalytic center. In contrast, NirS has iron ions as ligands within its catalytic center (Wasser et al., 2002). The next step, carried out by the nitric oxide reductase (Nor), is not exclusive to denitrifiers as NO is highly cytotoxic, and non-denitrifying organisms can also have Nor for

detoxification (Zumft, 2005). Nor is a membrane-associated protein, which has three different variants (*cnorB*, *qnorB* or *norYS*). The final step of denitrification ($N_2O \rightarrow N_2$), catalyzed by the nitrous oxide reductase (N_2OR), has its catalytic subunit encoded by the *nosZ* gene. Recently, a second clade of nitrous oxide reductase has been identified, herein named *nosZ*II (Sanford et al., 2012; Jones et al., 2013).

The capacity to denitrify is present in bacteria, Archaea and eukarya (fungi or benthic foraminifera) (Prendergast-Miller et al., 2011; Risgarrd-Petersen et al., 2006). Denitrification is a common trait in fungi (Maeda et al., 2015). This reductive process is performed via the copper nitrite reductase NirK and a cytochrome P450 nitric oxide reductase. In fungi, NirK is associated with the mitochondrial respiratory chain, coupled to the synthesis of ATP (Shoun et al., 2012). Fungi are able to perform denitrification in the absence of O_2, but also under microaerobic conditions (Laughlin & Stevens; 2002; Philipps et al., 2016). Fungal denitrification is of environmental concern because fungi do not possess N_2O-Reductase, N_2O being consequently the final product (Maeda et al., 2015; Mothapo et al., 2015).

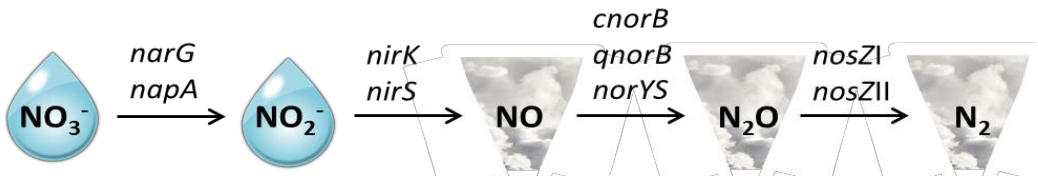

Fig. 6. The denitrification pathway, with the soluble and gaseous nitrous oxides indicated. The genes encoding the enzymes responsible for catalyzing each step are listed above.

1.3.2.1 A large, diverse and previously unrecognized group of N_2O reducers

The *nosZII* gene has been found in Bacteria and Archaea that denitrify but also in those that perform the dissimilatory nitrate reduction to ammonium (DNRA; see section 1.4.4) (Jones et al., 2011). The microorganisms belonging to the *nosZ*II clade are more diverse than those from the *nosZ*I clade. Thus, the clade I consists of members from alpha, beta, gamma-proteobacteria and archaea. The clade II gathers members from diverse phyla such as the previously mentioned groups, but also from others as Bacteroidetes, Firmicutes and Epsilon-proteobacteria (Figure 7). The first quantification of *nosZ*II-community abundance revealed that this clade is at least as abundant as *nosZ*I in various environments (Jones et al., 2013). From the 10 arable soils studied, 9 soils showed higher relative abundance of *nosZ*II

compared to *nosZ*I. Altogether, these results indicate that the abundance and the diversity of N_2O-reducing microbial communities were largely underestimated in previous studies.

The N_2OR encoded by *nosZ*I and *nosZ*II clades have distinctive translocation pathways. While *nosZ*I secretes the N_2OR across the cytoplasmic membrane via the twin-arginine translocation (Tat) pathway, it is the Sec-type pathway that is predominant on *nosZ*II clade except for a hyperthermophilic archaea (*Ferroglobus placidus*) and two thermophilic bacteria from the phylum Chloroflexi (*Thermomicrobium roseum* and *Sphaerobacter thermophilum*) (Fig.7) (Jones et al., 2013). These two translocation mechanisms are different (Natale et al., 2008). The Sec-type is transporting unfolded proteins, while the Tat pathway transports folded proteins. Some authors suggested that another important difference between the two translocation mechanisms is their energetic costs, with the Sec pathway being less energetically costly than the Tat one (Lee et al., 2006). Nevertheless, there is no agreement in the literature as other studies indicated that Sec and Tat transporters requires similar energy levels (Alder & Theg, 2003; Palmer & Berks, 2012).

The genomic loci encoding for the N_2OR is part of the *nos* cluster, which contains genes encoding for accessory proteins necessary for N_2OR maturation and functioning. The *nos* cluster of each *nosZ* clade also presents important differences. Contrary, to *nosZ*I, *nosZ*II lacks *nosR* and *nosX* that are relevant for *nosZ* expression (Cuypers et al., 1992; Sanford et al., 2012; Zumft et al, 2007). Another relevant distinction within the *nos* cluster of *nosZ*II is the presence of a gene (TM) encoding a transmembrane protein, placed just downstream of *nosZ* gene whose function remains unknown (Sanford et al., 2012). Sanford et al., (2012) also identified differences in the histidine residues implicated in the binding of the catalytic copper center (Cu_Z). While clade *nosZ*I exhibited highly conserved residues in the Cu_Z-binding motifs, *nosZ*II showed little residues conservation. The use of different secretory pathways and differences at the Cu_Z-binding motif suggests potentially distinct mechanisms and structural differences that could result in different growth efficiency and distinct capacities to reduce N_2O.

Comparative genomics revealed that co-occurrence patterns of denitrification genes were not randomly distributed across taxa. Specific patterns were found to be absent or dominant within different taxonomic groups. Some taxa had all denitrification genes and were characterized as complete denitrifiers. Others did not possess any *nosZ* gene making them a potential source of N_2O (Graf et al., 2014). For example, most Ascomycota have a *nir* and a *nor* gene, but are missing the gene encoding for the N_2OR. In contrast, Bacteroidetes showed a higher number of genomes with a *nos* gene than with a *nir* gene, making this phylum a

potential candidate for explaining N_2O sink capacity in soils. Remarkably, from the 652 microbial genomes analysed by Graf et al. (2014) 52% of the genomes containing *nosZ*II are lacking *nirK* or *nirS* genes, while only 17% of the genomes with *nosZ*I are lacking these genes. This suggests that *nosZ*II rather than *nosZ*I organisms would be potential candidates for explaining N_2O sink capacities.

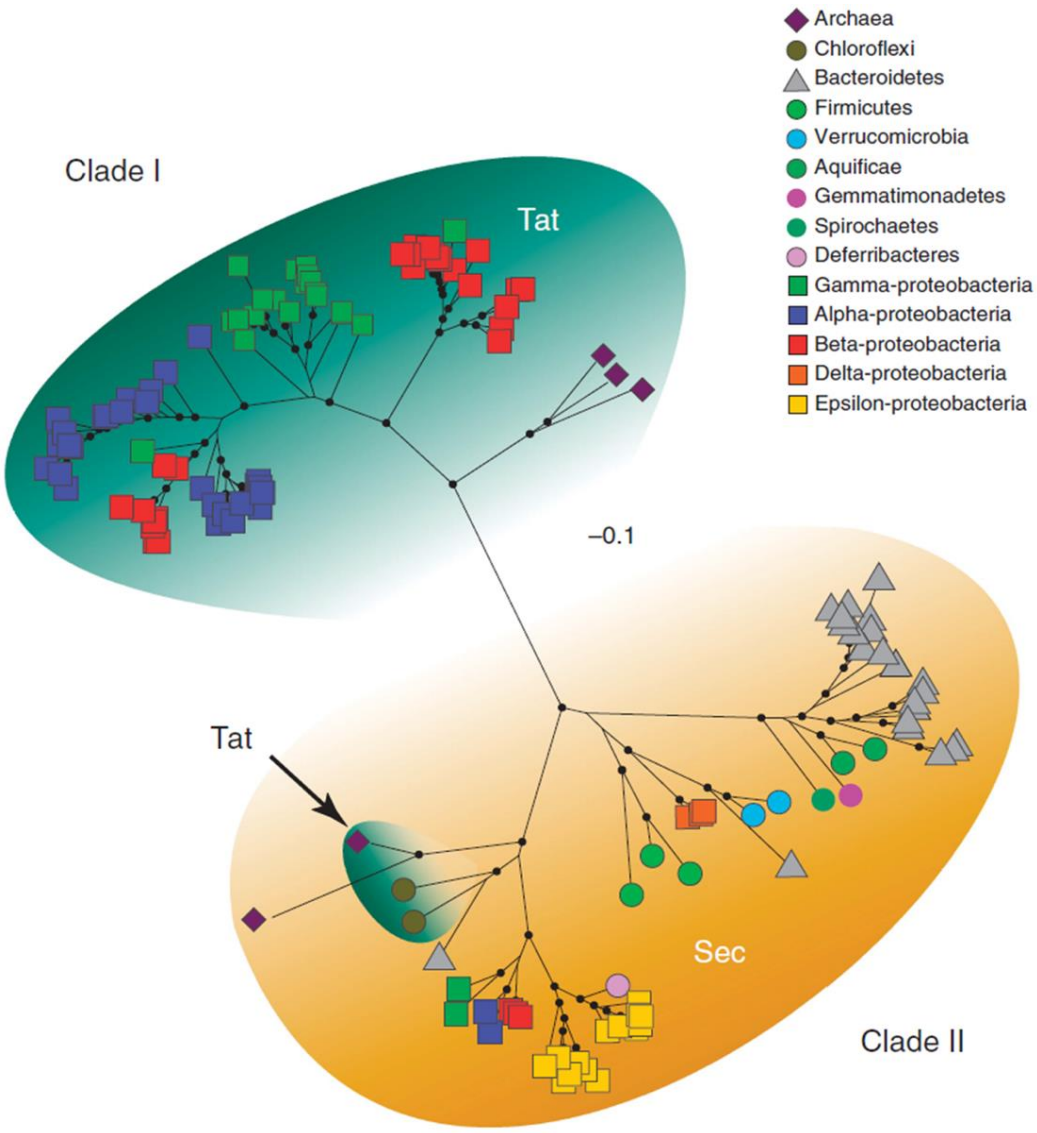

Fig. 7. Maximum likelihood phylogeny of nosZ amino-acid sequences obtained from genomes. The peptide occurrences for the different translocation pathways are indicated. Symbols correspond to the major taxonomic groups. Nodes with > 70% bootstrap support (n=500) are indicated by dots.

1.3.3 Codenitrification

Codenitrification was described as the utilization of NO_2^- and of another N atoms from NH_4^+ or amines to generate a hybrid N-N bond of N_2O or N_2. Concomitantly, non-hybrid N-N bonds are formed via the conventional denitrification pathway. The N_2O formation by the co-metabolic pathway of codenitrification was further observed with NH_2OH, N_3^-, NH_4^+, hydrazine (H_2N-NH_2) and salicyl hydroxamic acid ($C_7H_7NO_3$) (Spott et al., 2011).

Codenitrification has been studied to a great extent in fungi. Tanimoto et al. (1992) showed that *F. oxysporum* produced N_2O resulting from codenitrification and denitrification. Shoun et al., 1992 showed that other fungi species, *F. solani* and *C. tonkinense*, produced N_2 via codenitrification. Codenitrification products (N_2 or N_2O) vary according to the redox state of the nitrogen donor, while N_2 is formed from amines, N_2O is generated from imines and azide (Shoun et al., 2012). The enzyme catalyzing N_2O production by fungi via codenitrification is the cytochrome P450NOR (Su et al., 2004), while in bacteria the responsible enzyme is the cytochrome cd1NIR (Spott et al., 2011).

A review of the literature by Spott et al. (2011) suggests that codenitrification might be an ubiquitous pathway of microbial N gas formation in soil. It also has been suggested that codenitrification could be an important source of N_2O (Kammann et al., 2008; Roobroeck et al., 2010). However, we still lack studies that quantify the isotopic signature of N_2O produced by codenitrification and studies that look at this process within soil samples. Thus, the contribution of codenitrification to soil N_2O production remains largely unknown (Baggs and Philippot 2010). Apart from being a source of N_2O, codenitrification can be relevant from an environmental perspective, because it plays a role in N immobilization by bonding inorganic N forms (NO_2^-) with organic forms during N gas production.

1.3.4 Dissimilatory Nitrate Reduction to Ammonia (DNRA)

Nitrate reduction to nitrite is the first step of denitrification, but also the first step of the Dissimilatory NO_3^- Reduction to Ammonia (DNRA). This is a two-step process involving the NO_3^- reduction to NO_2^-, and its subsequent reduction to NH_4^+ (Figure 5). DNRA is a strictly anaerobic process that predominates in reductant-rich environments, such as marine sediments, sulfur thermal vents, and gastrointestinal animal tracts (Mohan & Cole, 2007). There is evidence that not only bacteria but also fungi can perform DNRA (Rütting et al., 2011; Stief et al., 2014).

Different genes encode the enzymes involved in DNRA depending on whether this process is occurring in the cytoplasm or periplasm. If occurring in the cytoplasm, *nar* and *nir* genes are involved in the two-step reduction, while *nap* or *nrf* genes are involved if occurring in the periplasm. The *nar* and *nap* genes are homologous to the denitrification genes, while the *nir* is a different gene coding for a nitrite siroheme NADH-dependent reductase. Unlike denitrification, which is a facultative anaerobic process, DNRA has no aerobic counterpart. DNRA is the third biological pathway known to produce N_2O as a by-product of the NO_2^- reduction stage (Rütting et al., 2011). The N_2O produced by this process cannot be further reduced within the DNRA, thus it would be advantageous for microorganisms performing DNRA to also perform the last step of denitrification ($N_2O \rightarrow N_2$), resulting in an energetic gain. The Epsilonproteobacteria *Wolinella succinogenes* was shown for example to both perform DNRA and reduce N_2O (Kern & Simon, 2009; Simon et al., 2004).

DNRA N_2O production is likely limited in soils (Inselsbacher et al., 2010; Silver et al., 2005). However, Silver et al. (2001) showed that DNRA rates in upland tropical forest soils were three times higher than denitrification and nitrification rates. DNRA may be relevant from an environmental perspective as it can avoid the loss of N by converting NO_3^- to NH_4^+.

1.4 Environmental factors influencing N_2O fluxes

The main factors affecting denitrification, nitrification and the corresponding N_2O emissions in soils are: oxygen availability and water content, soil N, available carbon (C), and pH.

- Oxygen supply and water content

Oxygen concentration determines if the predominant N pathway in soils is anaerobic or aerobic (*i.e* denitrification and nitrification, respectively). The main factors determining O_2 concentration (O_2 partial pressure; pO_2) are the soil water content and O_2 consumption by plant roots and microorganisms (respiration).

Numerous studies showed higher denitrification activity and N_2O emissions under low pO_2 associated with high soil water content (Ledgard et al., 1999; de Klein & Van Logtestjin 1994; Peyrard et al., 2016a). de Klein & Van Logtestjin (1994) showed a soil water threshold with increasing denitrification rates once such value was reached. The soil water threshold value varies according to soil type and is herein named water-filled pore space

(WFPS), and is normally close to the field capacity or higher. Interestingly, the N_2O:N_2 ratio increases with increasing pO_2 (Firestone et al., 1980) suggesting that O_2 inhibits the N_2OR (Morley et al., 2005).

Some soil properties can also influence pO_2 playing a role on defining if denitrification or nitrification are occurring. Some of these soil properties are: porosity, texture, compaction and drainage (IFA/FAO 2001). Accordingly, Mosquera et al., (2007) showed that severe soil compaction could double N_2O emissions, probably due to enhanced denitrification under low pO_2.

- Soil pH

Historically, soil pH received special attention, advocated as the "master variable" responsible for affecting numerous soil properties and processes. I will therefore summarize the findings regarding pH and these two most important processes involved in N_2O fluxes (*i.e*. nitrification and denitrification), and in regards to the proportion of N_2O produced during denitrification, herein named N_2O/N_2 ratio.

Various studies reported a significant relationship between pH and denitrification rates (Bandibas et al., 1994; Scholefield et al., 1997). Neutral to alkaline pH was reported to favors denitrification (Wijler & Delwiche, 1954; Nagele & Conrad, 1990), but denitrification can still occur in low pH soils (Saggar et al., 2013). In a review article Šimek & Cooper (2002) discuss that the low denitrification activity at low pH, may be due to an indirect effect as low pH can decrease available carbon and nitrogen mineralization.

Nitrification rates are also strongly controlled by soil pH (Jiang et al., 2015; Song et al., 2016; Che et al., 2015; de Gannes et al., 2014), firstly by its effect on NH_3 availability, which decreases under low pH. Thus, low nitrification rates are normally recorded in low pH soils, and consequently low N_2O derived from ammonia oxidation (Baggs et al., 2010). However, Jung et al (2014) recorded the production of high N_2O yields under acidic conditions by a AOA strain. Most of the studies addressing the relationship between pH and nitrification has focused on the relative contribution of ammonia oxidizers to the nitrification rates (Gubry-Rangin et al., 2010; He et al., 2012; Hu et al., 2014; Yao et al., 2011). Further studies are needed to understand N_2O production by nitrification.

Soil pH has also been suggested to control the N_2O:N_2 ratio (Firestone et al., 1980). Firestone et al (1980) showed that in the absence of measurable quantities of NO_3^-, soil acidity had very limited influence on the N_2O:N_2 ratio. However, when NO_3^- was added,

significantly more N_2O was produced during denitrification at pH 4.9 than 6.5. These results were corroborated by Koskinen & Keeney (1982), who showed that at pH 4.6 and 5.4, N_2O was the main product of denitrification, while at pH 6.9 N_2 was the predominant denitrification end-product. Cuhel et al. (2010) recorded an increase in N_2O:N_2 under low pH, which was due to changes in the total denitrification activity, while the production of N_2O remained unchanged. These results show that low pH can increase the proportion of N_2O as end-product of denitrification, which was attributed to an unsuccessful assembly of the N_2OR under low pH (Liu et al., 2014).

- Carbon and Nitrogen Content

Denitrification is primarily performed by heterotrophic microorganisms, and as such it is dependent of available carbon as electron donor (Benckiser et al., 2015). Increasing soil organic carbon enhances denitrification rates and N_2O emissions (Huang et al., 2004; Saggar et al., 2013). As previously discussed, changes in pH influences carbon availability, which will impact denitrification rates (Koskinen & Keeney 1982). The accessibility of available C to microorganisms is especially important in field conditions. Normally, dissolved organic carbon (DOC) is more easily accessible than more stable C sources such as cellulose or lignin in soils. It is normally accepted that increasing carbon supply decreases the N_2O:N_2 (Smith and Tiedje, 1979; Weier et al., 1993), nevertheless, the total amount of N_2O produced by denitrification may be enhanced by C addition. Any process that influences the rate of C mineralization in soils (e.g., temperature, incorporation of crop residues, drying-wetting cycles, tillage, liming, organic or inorganic fertilizer input, root exudates) can have a large impact on denitrification rates and corresponding N_2O emissions. Some of these factors will be further discussed in this manuscript.

The availability of N, especially of NH_4^+ and NO_3^- is important to determine the rates of nitrification and denitrification, respectively. Soil concentrations of NH_4^+ and NO_3^- depend from mineralization rates, plant N uptake, microbial immobilization, leaching and diffusion. The concentration of NO_3^- is one of the important factors that influence the N_2O:N_2 ratio in denitrification, with higher NO_3^- concentrations generally increasing the N_2O:N_2 (Firestone et al., 1980; Saggar et al., 2013). This might be due to the fact that denitrifiers might obtain more energy by reducing NO_3^- than N_2O (Trudinger et al., 1980).

Moreover, not only the concentration of available carbon or nitrogen are relevant, the C/N ratio is also important. Huang et al (2004) showed that N_2O emissions were influence

by the type of plant residues. This study showed a negative correlation between the C/N ratio of the residue incorporated and the cumulative N_2O emissions.

1.5 Agricultural practices influence on N_2O emissions and N_2O-reducers

Agricultural practices can not only directly affect the composition of microorganisms and their activities by altering N and C availability, water content (irrigation), but also indirectly by changing soil properties (Figure 10). While there is a very large body of literature reporting the effect of numerous agricultural practices on microbial communities involved in N_2O emissions and the corresponding fluxes, this section will mostly focus on those related to my experimental work.

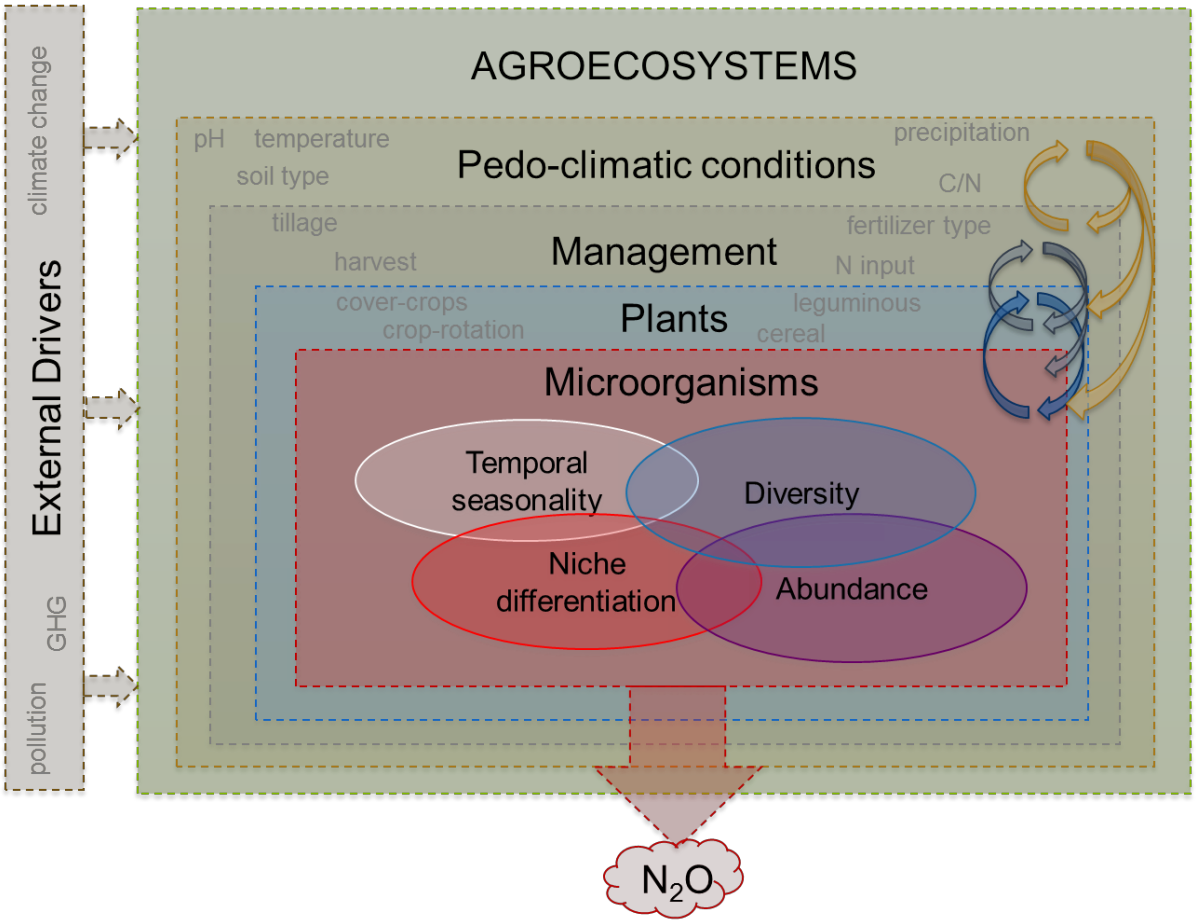

Fig. 10. Simplified conceptual framework of processes influencing the microorganism involved on N_2O fluxes within agroecosystems. The arrows on the right side show the interaction between the different compartments, which influence the microorganism and consequently the N_2O fluxes. Within each compartment, some examples of important factors shown in gray.

1. 5.1. N_2O emissions

- Fertilization

The influence of fertilization has been the most extensively studied agricultural practice on denitrification and nitrification in the last decades (Braker & Conrad, 2011). Not surprisingly, fertilization increases available N forms, and therefore enhances N turnover, leading to high denitrification and N_2O:N_2 ratio, and high nitrification rates, and consequently increased N_2O emissions (Thangarajan et al., 2013; Skiba and Smith 2000).

Fertilizers can be divided into two groups: organic and inorganic, which may lead to different effects on the processes rates and N_2O emissions. For example, organic fertilizer stimulates more than inorganic fertilizers denitrification rates and corresponding N_2O emissions, as it also represents an input of available carbon (Barton et al., 2001; Rochette et al., 2000).

Fertilization can also have an impact on denitrification and nitrification indirectly by changing soil pH. For example, ammonium sulfate ($(NH_4)_2SO_4$) is can for decrease soil pH and consequently lowering potential denitrification rates (Enwall et al., 2005). Moreover, due to the feedstuffs provided to animals, some types of organic fertilizers such as animal manure or sewage sludge are a source of heavy metal contamination in soils. Heavy metal contamination in soil was shown to affect negatively the denitrification activity (Philippot et al., 2007).

Fertilizer type can also affect nitrification. Despite the fact that ammonia oxidizers are traditionally considered to be obligate autotrophs, there is increasing evidence that organic source of N stimulates AOA, while inorganic N stimulates AOB (Zhou et al., 2015). Hink et al (2016) suggest that AOA may produce lower N_2O yields than AOB. Considering that AOA could be stimulated by organic fertilizers, while AOB is enhanced by inorganic, these authors suggest that organic fertilizers could reduce N_2O emissions by nitrification by enhancing AOA. More studies are needed better understand the effect of different N sources on N_2O emissions by nitrification.

- Crops

Plant species can influence denitrification and nitrification processes by modifying the soil properties next to the rots, the so-called rhizosphere. By taking up NH_4^+ and NO_3^-, plants

change the substrate availability for the respective processes. The rhizodeposition of carbon compounds is a source of electron donors to denitrifiers. Plants can also influence the availability of oxygen in the rhizosphere by reducing it through root respiration or increasing it through transpiration and increased water uptake. During litter decomposition and plant senescence, the plant is also a source of N compounds and carbon to microorganisms.

Comparison of denitrification rates between planted soil and bulk soil showed higher rates for planted soils, with exponentially increasing rates with soil NO_3^- concentration (Mahmood et al., 1997). Additionally, carbon exudation by the roots (Philippot and Hallin, 2011; Henry et al., 2008), and plant-influence on air-filled porosity (Bakken 1988; Prade and Trolldenier 1988) are recognized as important factors stimulating denitrification. A review by Rochette & Janzen (2005) proposed that N_2O emissions induced by leguminous plants were due to N release by the roots and decomposition of crops residues, but not associated with the process of biological nitrogen fixation *per se*. Shcherbak et al., (2014) showed in a global meta-analysis that fertilized leguminous plants were the only crop type which significantly changed the soil N_2O emissions factor. They discussed that such results are very likely because fertilization exceeded legume crop needs' faster than other crops. These results highlighted the importance of having fertilization rates that do not exceed the crop needs to avoid increasing N_2O emissions. Graf et al., (2016) showed no significant effect of barley or sunflower in the potential denitrification activity, potential N_2O production or N_2O:N_2 ratio in two different soils.

Regarding nitrification, plants can both stimulate or inhibit it. Stimulation is mostly due to enhanced N availability due to root exudates. However, certain plants exudate chemical nitrification inhibitors in their rhizosphere (Subbarao et al., 2006; 2009). These compounds block the ammonia mono-oxygenase and the hydroxylamine oxidoreductase of ammonia oxidizers, and their release is controlled by the concentration of ammonium in the rhizosphere. It has been suggested that biological nitrification inhibition by some plants is a strategy to compete with both microorganism and other plants for nitrogen (Subbarao et al., 2012). Subbarao et al. (2009) showed cultivated for three years consecutively of plants inhibiting nitrification lead to reduced abundance of ammonia-oxidizing microorganisms, lower nitrification rates and N_2O emissions. These results demonstrated that plants play an important role in controlling processes within the N-cycle and N_2O emissions.

Crop residue decomposition also represents a source of N and C and can enhance N_2O production after harvest (Aulakh et al., 1991). One attempt to avoid the loss of reactive N

after harvest, is planting cover crops (Basche et al., 2014), even if the incorporation of those residues into the soil may enhance the N_2O emissions after harvest (Peyrard et al., 2016b).

- Tillage

Soil tillage, which consist of "mechanical or soil-stirring actions exerted on soil to modify soil conditions for the purpose of nurturing crops" (El Titi., 2003). Thus, tillage *per se* consists of soil reversion, which causes changes on soil properties such as aggregate size, moisture, porosity and redistribution of plant residues on a deep soil horizon.

Reduced tillage or no-tillage may lead to increase carbon sequestration in soil and have been therefore proposed as a strategy for climate change mitigation (Six et al., 2004; Lal et al., 2004; Smith et al., 2008). However, Six et al., (2004) showed that the increase in N_2O emissions in NT systems was responsible for positive net GHGs fluxes. There is no consensus on tillage management impact on N_2O emissions. While in some soils reduced tillage enhanced N_2O emissions (Rochette et al., 2008), in other soils it decreased (Robertson et al., 2000) or had no influence (Marland et al., 2001). Tillage influence on N_2O emission depends also on the duration of its application, climate conditions (Six et al., 2004), as well as soil type (Rochette et al., 2008).

Finally, beyond the individual effect of single management practices, the important role of interactions between practices makes the influence of a single management practice on the N_2O emissions even more uncertain.

1.5.2 N_2O-reducers

Agricultural practices were shown to affect not only the total microbial community (Hartmann et al., 2015; Geisseler et al., 2016) but also several functional groups involved in N_2O production (Hallin et al., 2009; Hartmann et al., 2015; Melero et al., 2011; Morales et al., 2010; Le Roux et al., 2008; Attard et al., 2010; Wang et al., 2015). In this section, I will restrict the discussion to the management practices evaluated in the agricultural sites studied in this thesis.

Several studies showed that different fertilization regimes modified the *nosZ*I community composition (Enwall et al., 2005; Hallin et al., 2009; Yang et al., 2015; Dambreville et al., 2006). The analysis of the impact of six different fertilization regimes for 50 years (unfertilized, unfertilized bare fallow, $Ca(NO_3)_2$, $(NH_4)_2SO_4$, manure and sewage

sludge) showed that the *nosZ*I community differed the most in the $(NH_4)_2SO_4$ and sewage sludge compared to the other treatments. Since, the long-term application of $(NH_4)_2SO_4$ and sewage sludge resulted in a significant decreased soil pH, the effects of these treatments was, at least partially, attributed to the indirect effect of soil acidification. In contrast, Clark et al. (2012) found no differences in the *nosZ*I community composition between 0 N input plots, inorganic fertilized plots with NH_4NO_3 and farmyard manure amended plots in another long term fertilization experiment. Interestingly, in this 160 year-old experimental site, the distinct fertilization regimes did not impact strongly soil properties except for an increase in organic carbon in the manure amended plots (2.83 % against 0.9 %, 1.13 %, 1.20 % for manure amended plots at 0 kg N h^{-1}, 144 kg N h^{-1} and 288 kg N h^{-1}, respectively) (Clark et al. 2012). Dambreville et al. (2006) also observed differences in the *nosZ*I community structure between plots fertilized with ammonium nitrate and pig manure.

Fertilization can also influence denitrifier abundance (Hallin et al., 2009; Kong et al., 2010, Yang et al., 2015). Hallin et al. (2009) showed that the abundance of the *nosZ*I community was significantly lower in the plots treated with sewage sludge than in the ones with manure. Yang et al (2016) evaluated the influence of different N input levels (0 kg N h^{-1}; medium with 130 or 118 kg N h^{-1}; and high with 260 or 249 kg N h^{-1}) on *nosZI* abundances in three Canadian soils and also reported higher relative abundances under medium fertilization compared to the control, but not significant differences at the higher fertilization level. These results suggest fertilizer type rather than fertilization rates may be more important drivers for the *nosZ*I.

Another practice impacting N_2O-reducers is tillage (Baudoin et al., 2009; Morales et al., 2010; Tatti et al., 2015). Some studies showed that no-tillage can increase the abundance of *nosZ*I (Baudoin et al., 2009; Morales et al., 2010), while other observed higher abundance under full tillage (Tatti et al., 2015). One consequence of tillage is the incorporation of crop residues into the soil, while in no-tillage management crop residues remain in the soil top layer. Miller et al (2008) investigated the impact of addition of different crop residues (red clover and barley straw) in soil microcosms, but found no significant differences in *nosZI* community abundance.

There are very few studies looking at the effect of cropping systems on denitrifier community and more specifically on N_2O-reducers. Thompson et al. (2016) investigated the effect of annual and perennial cropping systems on the *nosZ*I community. A higher abundance of the *nosZ*I community was found in the perennial field than in the annual one, a difference that became inexistent after the perennial field was ploughed a year later. The community

composition of *nosZ*I was also different between the two cropping systems. The perennial system showed higher diversity, which decreased after ploughing. Due to several differences between the two cropping systems, such as crop types (annual cropping planted with corn (*Zea mays*) monoculture, while the legume *Medicago sativa* and the perennial grass *Phleum pratense* were present at perennial site) and tillage management, these authors could not attribute these modifications in *nosZ*I diversity and structure to a specific factor, but rather to the distinct cropping systems.

To our knowledge, no study evaluated the impact of agricultural practices on the clade II of N_2O reducers. Jones et al. (2014) reported that distinct soil properties were important to explain the abundance and diversity of *nosZ*I and *nosZ*II suggesting niche differentiation between both N_2O-reducer clades. Accordingly, agricultural practices may have differential impacts on these two groups, and therefore could foster one or the other. Thus, Harter et al., (2016) showed a different response of *nosZ*I and *nosZ*II clade to biochar amendment in soil microcosms, with resulted in an increase in *nosZ*I clade relative abundance while *nosZ*II decreased. Along with these changes, shifts in the community structure were also observed. For example, the relative abundance of the *nosZ*II Bacteroidetes *Pedobacter saltans* increased by 24 % after addition of biochar. Interestingly, *P. saltans* DSM12145 is a strain only reducing N_2O as it does not possess any denitrification gene except *nosZ*. As such, increasing the relative abundance of *P saltans* strains carrying exclusively the N_2OR could have a potential to decrease soil N_2O emissions. These results suggest that management may, directly or indirectly by changing soil properties, impact the two N_2O-reducing clades, with potential consequences for the N_2O emissions.

1.6 Relevance of community abundance, composition and diversity for understanding N_2O fluxes

Microorganisms have a central role on biogeochemical process. Nevertheless, the importance of the relative abundance and composition of functional groups to predict processes rates remains widely debated (Rölling et al., 2007; Graham et al., 2014; Nemergut et al., 2014).

Numerous individual studies showed a positive correlation between abundance of N cycling communities and their respective potential activity (Petersen et al., 2012; Philippot et al., 2009; Hallin et al., 2009; Dong et al., 2009). Petersen et al. (2012) showed with a path model approach that soil properties were important to explain the abundance of functional genes, which in turn related to the potential nitrification and denitrification rates (Figure 8).

On the other hand, several studies reported no correlation between gene abundance and potential rates (Attard et al., 2011; Dandie et al., 2008; Miller et al., 2008; Baudoin et al., 2009). A common aspect between these studies is the small range in variation for the potential denitrification rates and genes abundance between treatments. Accordingly, Petersen et al. (2012) suggested that gene abundance may not be a good predictor for relatively small changes in potential activities, but a better predictor when large differences were observed.

One of the reasons that might explain the absence of relationship between gene abundance and process rate is post-transcriptional gene regulations. The presence of a gene in a population is not a guarantee that this gene has been transcribed, the protein translated, or that the protein is active. Thus, more research on the factors controlling transcription, translation, and enzyme activity to better understand the relationship between a gene pool and the corresponding process rate is needed as suggested by Rocca et al. (2015). Furthermore, our understanding of how many different functional groups might be participating in the production or consumption of a molecule might be still not complete. Two good examples are the recently identified N_2O reducers clade (Sanford et al., 2012; Jones et al., 2013), and the discovery of archaea ammonia oxidizers (Konneke et al., 2005).

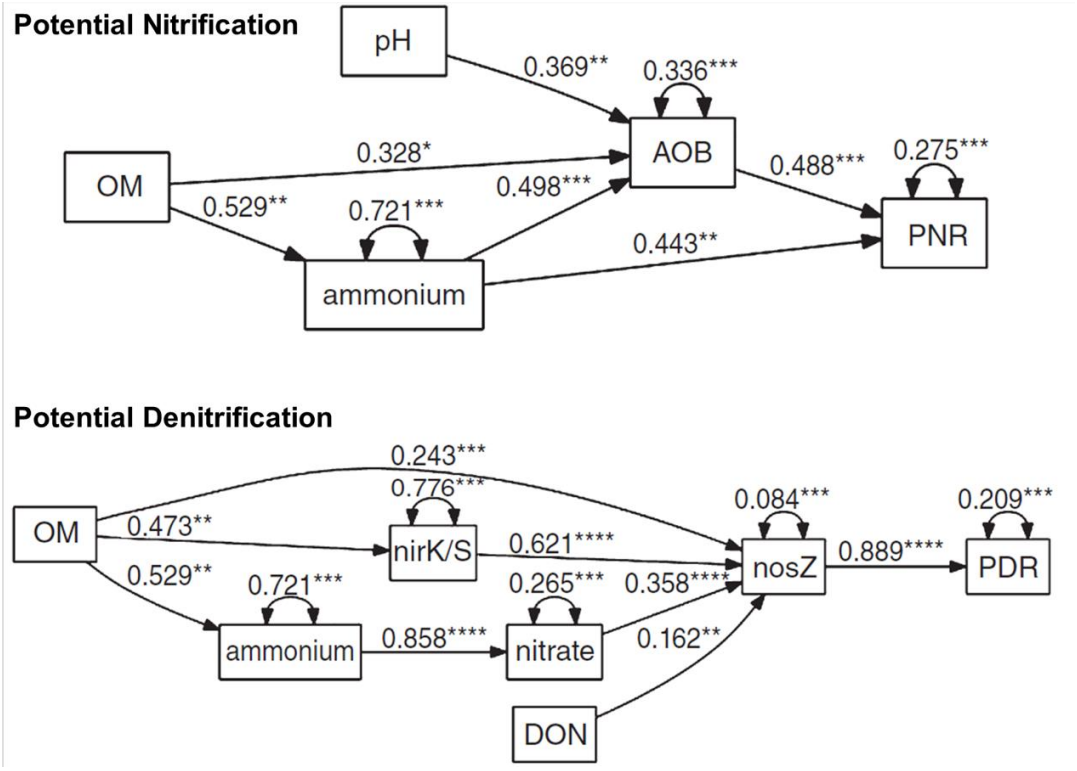

Fig. 8. Path diagram representing the final model to describe the relative importance of different variables explaining the rates of potential nitrification activity (PNA), and potential denitrification activity (PDR).

Besides the abundance, the relevance of microbial community composition for process rates has also been investigated with large discrepancies between studies (Le Roux et al., 2008; Enwall et al., 2005; Attard et al., 2011; Dandie et al., 2008; Baudoin et al., 2009; Graham et al., 2016). For example, Le Roux (2008) showed that a shift in the ammonia-oxidizers community structure was related to modifications of the nitrification rates. Other studies demonstrated no changes in process rates despite different community compositions (Enwall et al., 2005; Attard et al., 2011; Dandie et al., 2008). Attard et al. 2011 suggested that the soil properties rather than the denitrifiers abundance or diversity were important to determine PDA. On the other hand, Jones et al. (2014) showed that the soil N_2O sink capacity was mostly explained by the abundance and phylogenetic diversity of *nosZ*II community, which mediated the influence of edaphic factors (Figure 9; Jones et al., 2014).

To assess the importance of microorganisms for predicting ecosystem processes, Graham et al. (2016) evaluated the value of environmental variables and microbial community structure both independently and in combination for explaining N cycling processes. Environmental variables were the strongest predictors but on average, models with both environmental and microbial predictors explained more variation in processes than environmental models alone (R^2 of 0.65 against 0.56). They also showed that only 29% of their dataset were significantly improved by adding information on microbial community structure. It is important to consider that process based models *implicitly* consider microorganisms by accounting for variation in factors that influence microbial community composition such as pH (Lauber et al., 2009), moisture (Nemergut et al., 2010), and substrate availability (Legg et al., 2012). Thus, it is not completely surprising that a relatively small increase in explanatory power is achieved when adding microbial predictors to increase explanatory power of models that contain environmental variables important to drive those microorganisms.

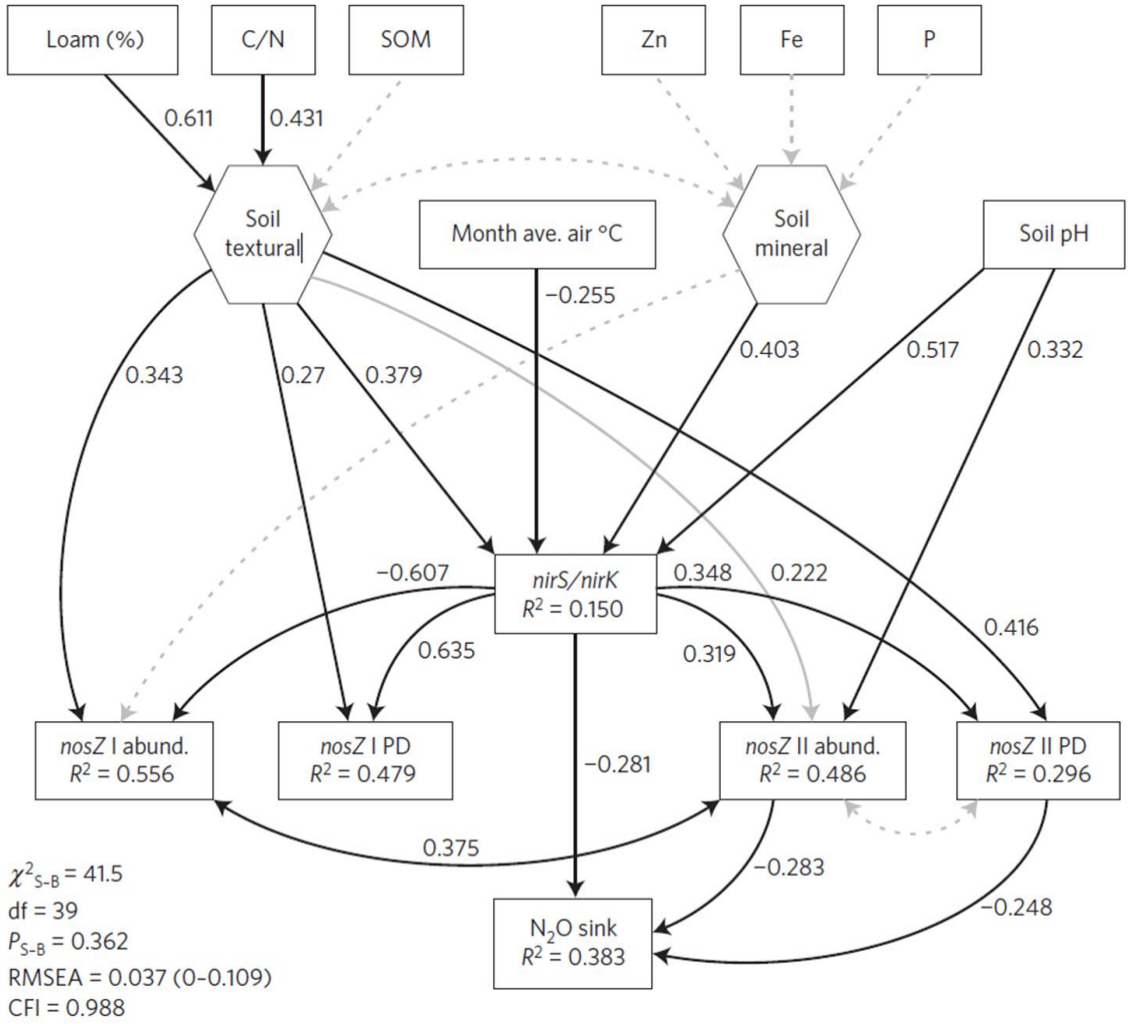

Fig. 9. Structural equation model showing the relative importance of soil abiotic properties and denitrifiers community characteristics for the soil N_2O sink capacity (Jones et al., 2014).

2 Aim and Objective

The major purpose of this thesis was to investigate the ecology of N_2O-producers and N_2O-reducers in agricultural soils, and their relation to the soil N_2O production. My thesis started just after the discovery of the *nosZ*II clade. Thus, we aimed to increase the understanding of this clade's relevance for soil N_2O reduction. For this purpose, we performed experiments at different scales. First, a laboratory incubation experiment was set up to test if *nosZ*II N_2O-reducers can take up N_2O produced by indigenous soil communities and therefore reducing its emissions. Secondly, in one experimental site, we investigated the influence of different agricultural practices on the N_2O-producers and N_2O-reducers as well as their influence on the soil N_2O production. Finally, we applied the same approach to multiple sites with distinct pedo-climatic conditions and broader category of agricultural practices, to investigate their effects on microorganisms involved in N_2O production and reduction, in relation with *in situ* N_2O emissions.

I. **Chapter I: Laboratory incubation** - Non-denitrifying nitrous oxide-reducing bacteria - an effective N_2O sink in soil.

II. **Chapter II: One site approach** - The diversity of the N_2O reducers matters for the N_2O:N_2 denitrification end-product ratio across an annual and a perennial cropping system.

III. **Chapter III: Multi-site approach** - Effects of agricultural practices and soil properties on soil N_2O-reducing bacteria and *in situ* N_2O emissions.

3 Chapter I

Non-denitrifying nitrous oxide-reducing bacteria - an effective N_2O sink in soil

Luiz A. Domeignoz-Horta

Martina Putz

Aymé Spor

David Bru

Marie-Christine Breuil

Sara Hallin

Laurent Philippot

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Short Communication

Non-denitrifying nitrous oxide-reducing bacteria - An effective N_2O sink in soil

L.A. Domeignoz-Horta [a], M. Putz [b], A. Spor [a], D. Bru [a], M.C. Breuil [a], S. Hallin [b], L. Philippot [a, *]

[a] Agroécologie, AgroSup Dijon, INRA, Univ. Bourgogne Franche-Comté, F-21000, Dijon, France
[b] Swedish University of Agricultural Sciences, Department of Forest Mycology and Plant Pathology, Uppsala, Sweden

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ABSTRACT

Nitrous oxide (N_2O) is an important greenhouse gas and fundamental questions about the capacity of soil microbial communities to act not only as sources, but also as sinks for N_2O remains unanswered. We evaluated the capacity of non-denitrifying N_2O-reducers to mitigate the production of this greenhouse gas in soil. We showed experimentally that the addition of the non-denitrifying strain *Dyadobacter fermentans*, which possesses the previously unaccounted N_2O reductase NosZII, to 11 different soils significantly reduced N_2O production of up to 189% in more than 1/3 of the soils. The magnitude of this effect was significantly influenced by the soil pH and C/N ratio. Overall, our results provide unambiguous evidence that the overlooked non-denitrifying NosZII-type bacteria can contribute to N_2O consumption in soil.

Nitrous oxide (N_2O) is both the major ozone depleting substance and a potent greenhouse gas having a global warming potential 298 times that of CO_2 (Ravishankara et al., 2009; Thomson et al., 2012). It is mainly produced by the microbial processes denitrification and nitrification (Hu et al., 2015), with arable soils being the largest source (Montzka et al., 2011; Shcherbak et al., 2014). Until recently, denitrification was the only known biological process reducing N_2O through the N_2O reductase encoded by the *nosZ* gene (Chapuis-Lardy et al., 2007). However, a new lineage of the N_2O-reductase (*nosZ* clade II), which is abundant and widespread in soils, has been identified (Jones et al., 2013; Orellana et al., 2014; Sanford et al., 2012). Intergenomic comparisons revealed that about 51% of the organisms belonging to *nosZ* clade II lack a nitrite reductase and are therefore unable to denitrify (Graf et al., 2014). Their importance as N_2O sinks in soil have been suggested in studies showing that their diversity was positively related to the soil N_2O sink capacity (Jones et al., 2014) and a strong predictor of the N_2O/N_2 denitrification end-product ratio (Domeignoz-Horta et al., 2015).

Here, we experimentally test the proposed role of non-denitrifying *nosZ* clade II microorganisms for soil N_2O reduction capacity. For this purpose, we manipulated soil microbial communities by adding different amounts of *Dyadobacter fermentans*, a *nosZ*II bacterial strain lacking any known denitrification genes, in 11 different soils and monitored the N_2O production and denitrification rates by the indigenous soil communities. Soils with contrasting properties were sampled from 0 to 15 cm depth in long-term agricultural experimental sites in Ireland, Sweden and England (Supplementary Table S1). Samples were sieved (4 mm) and distributed across 130 microcosms (each with 60 g dry weight soil) with 3–4 replicate microcosms per soil and for each inoculation treatment. Soil DNA was extracted with the ISO11063 protocol (Petric et al., 2011) and the abundance of *nirK*/*nirS* and *nosZ*I/*nosZ*II genes was quantified by real time PCR and used as a proxy for potentially N_2O-producing and N_2O-reducing indigenous bacteria, respectively (Jones et al., 2013; Philippot et al., 2011; Fig. S1). *Dyadobacter fermentans* strain NS114^T (=DSM18053) was grown aerobically in R2A media as recommended by the DSMZ microbial collection. When the optical density reached approximately 0.7, cells were washed by centrifugation, and inoculated in the microcosms at two concentrations; 10^6 (denoted low inoculation, LI) and 10^8 cells g^{-1} dry soil (denoted high inoculation, HI), in addition to a

* Corresponding author.
E-mail address: laurent.philippot@dijon.inra.fr (L. Philippot).

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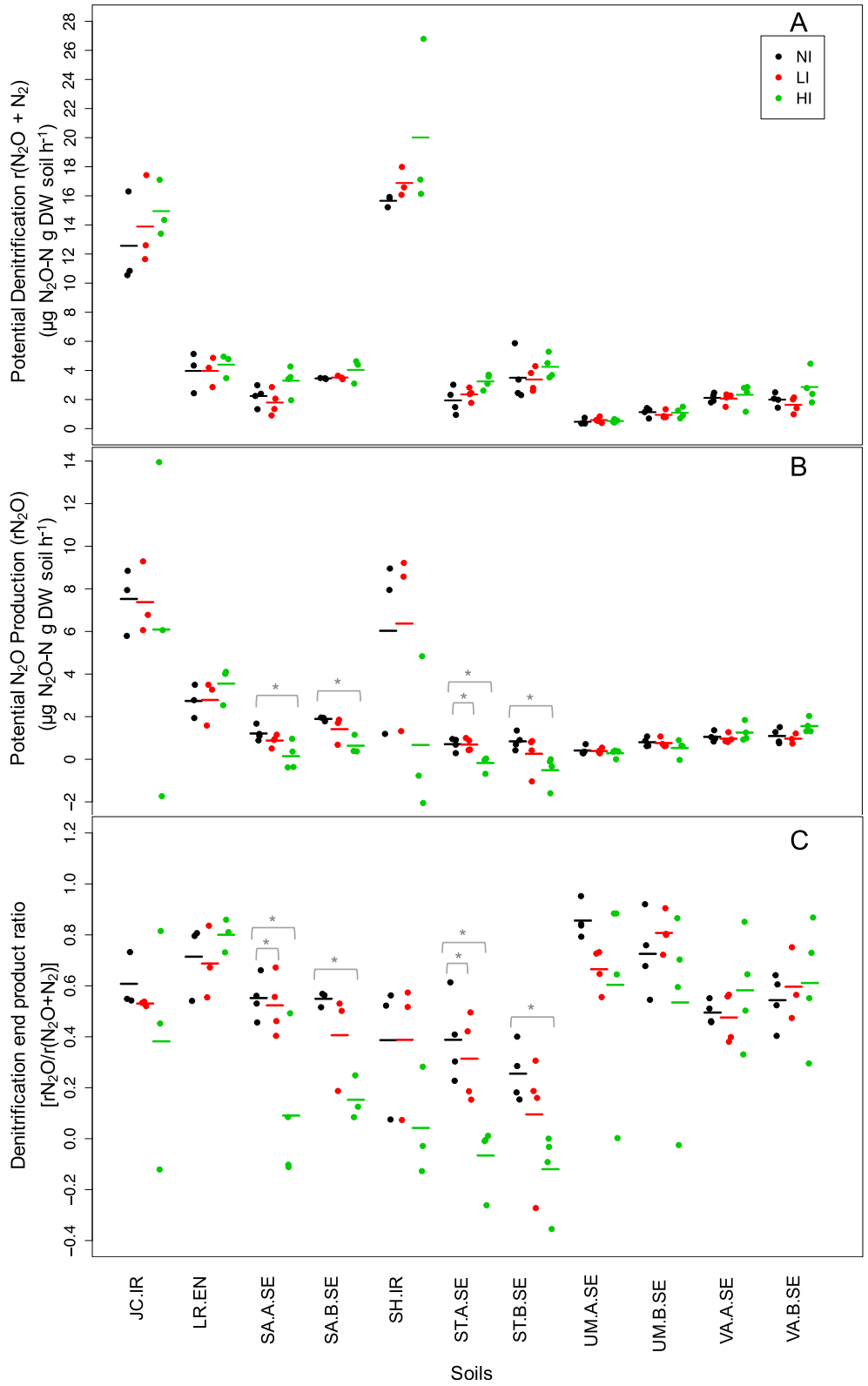

Fig. 1. Impact of *Diadobacter fermentans* addition on potential denitrification rate (A), potential N_2O production rate (B) and gaseous end-product ratio (C) in the soils. Individual points represent the replicates for non-inoculated microcosms (NI; black), low inoculation (LI; red) and high inoculation (HI; green), whereas horizontal lines represent the average per treatment for each soil. Significant differences are indicated (*$P < 0.05$, **$P < 0.001$).

Table 1
Percentage of variance explained by biotic and abiotic variables for the activities in the non-inoculated soils (NI) and for the ratios and differences between the N_2O production rates at the highest inoculation level (HI) and the NI soil. Significant variables are indicated (*$P < 0.05$, **$P < 0.01$) in addition to the directions of the correlations in case of positive (+) or negative (−) correlations.

Activity	pH	Clay (%)	Loam (%)	C/N	16S rRNA[a]	*nirK/nirS*	*nosZ*I (%)	*nosZ*II (%)	*nosZ*I/*nosZ*II	*nosZ*I/(*nirK*+*nirS*)	*nosZ*II/(*nirK*+*nirS*)	Residuals
$NI^{(N_2O)}$	1.2	9.3* (+)	1.1	24.1** (−)	2.8	4.6	6.8* (+)	6.7	0.2	3.8	0.4	38.9
$NI^{(N_2O+N_2)}$	2.1	6.0** (+)	0.3	47.1** (−)	2.3	7.5** (+)	7.2** (+)	7.1** (+)	0.4	2.5	0.1	17.4
$NI^{(N_2O/N_2O+N_2)}$	60.1** (−)	2.5	7.1* (+)	1.9	0.2	0.3	0.0	0.0	0.3	0.1	0.0	27.4
$HI^{(N_2O)}/NI^{(N_2O)}$	50.4** (−)	9.8** (+)	0.0	0.2	5.7** (+)	1.5	3.6* (−)	4.7* (−)	0.4	1.1	0.3	22.3
$NI^{(N_2O)} - HI^{(N_2O)}$	10.8** (+)	0.1	2.5	19.4** (+)	3.8	17.3* (+)	6.1* (+)	1.5	0.0	1.7	0.0	36.8

[a] Expressed in number of copies ng DNA^{-1}.

non-inoculated control with equivalent amount of sterile KNO_3 solution (denoted no inoculation, NI). Soil microcosms were brought to 80% of the maximum water holding capacity (WHC) with a 3 mM KNO_3 solution and incubated for 3 h; a procedure known to induce denitrification by the indigenous soil community (Ekpete and Cornfield, 1964). To determine maximum WHC, duplicate samples of each soil were weighed after allowing soil samples to saturate with water in a cylinder and then again after placing the cylinder on an absorbent membrane so that excess water is drawn away by gravity for 5 h and finally after drying at 105 °C for 12 h. After 3 h, potential denitrification and potential N_2O production rates were measured by incubating soil samples in airtight flasks under anoxic conditions with and without acetylene, respectively as previously described (Jones et al., 2014). Gas samples were taken from the headspace every 30 min for 150 min and N_2O concentrations were measured using a gas chromatograph equipped with an EC-detector (Trace GC Ultra, Thermo Scientific). To confirm that *D. fermentans* is incapable of denitrification, its ability to utilize nitrogen oxides as electron acceptors was tested. Fifty mL of R2A media supplemented with 2, 4, 8 and 16 mM of NO_3^-, NO_2^- or N_2O as the only electron acceptor and 2 mM acetate was used to monitor the growth.

The non-inoculated soils showed potential denitrification rates ranging from 0.1 to 8 μg N_2O-N g^{-1} dry weight soil h^{-1} with strong differences between soil (Fig. 1A; $P < 0.001$). Similar ranges are commonly reported in the literature (e.g. Attard et al., 2011; Keil et al., 2015; Lagomarsino et al., 2016). The potential N_2O production rates corresponded to 10–95% of the denitrification rates. It has long been recognized that the proportion of N_2O emitted by denitrification is highly variable and, for example, soil pH or oxygen availability are known factors influencing the denitrification end products (Butterbach-Bahl et al., 2013; Firestone et al., 1980). Accordingly, pH was the strongest driver of the potential N_2O production to denitrification rate ratio $[rN_2O/r(N_2O+N_2)]$ ($P < 0.001$). Nevertheless, a negative correlation was observed between the relative abundance of the indigenous *nosZ*II abundance and the potential N_2O production ($r = -0.35$, $P < 0.05$) and $[rN_2O/r(N_2O+N_2)]$ ($r = -0.64$, $P < 0.0001$). Altogether this is in line with previous studies suggesting that the abundance of microbial populations can add predictive power to N-cycling processes (Graham et al., 2016; Jones et al., 2014; Petersen et al., 2012), but it can be obscured by various factors occurring at other levels of regulation (Rocca et al., 2014; Röling, 2007).

D. fermentans NS114^T is a Bacteroidetes and its genome shows the presence of the recently identified *nosZ*II-type N_2O reductase encoding gene but lacks both types of nitrite reductase genes (Lang et al., 2009). This suggests that this strain is not a denitrifier, being only capable of reducing N_2O, but unable to use other forms of nitrogen oxides as electron acceptors. Accordingly, the strain did not grow in the R2A media using nitrate or nitrite as sole electron acceptors (Supplementary Fig. S2). By contrast, growth was observed with N_2O, which was concomitant with a decrease in N_2O concentration (Supplementary Figs. S2 and S3). This indicates that *D. fermentans* NS114^T can respire N_2O and grow, as previously reported for the non-denitrifying *nosZ*II-type *Anaeromyxobacter dehalogenans* strain 2CP-C and *Dechloromonas aromatica* strain RCB (Sanford et al., 2012; Yoon et al., 2016).

Addition of *D. fermentans* cells to the soil microcosms at the highest, but not the lower concentration, significantly decreased N_2O production in 1/3 of the soils ($P < 0.05$), while the potential denitrification rates were not significantly affected (Fig. 1A–C). In average, the reduction in N_2O production was 51%, and up to 189% in some soil microcosms since negative rates were recorded. The significant reduction of N_2O production in several soils after inoculation of *D. fermentans* demonstrates that non-denitrifying bacteria with *nosZ* are capable to eliminate the N_2O emitted by other microorganisms in soil.

The reduction of N_2O production after adding *D. fermentans* varied between soils. Even if we cannot rule it out, such variability in the effect of the *D. fermentans* on N_2O production was unlikely due to differences in the survival of the inoculated strain between soils because of the short incubation period of 3 h. A model selection analysis was performed to analyze the contribution of putative biotic or abiotic variables to such differences (Table 1). We found that the N_2O mitigation potential of the added *nosZ*II strain, calculated both as ratio and as difference in N_2O production between the inoculated and control microcosms, was significantly related to biotic and abiotic factors that also explained differences in the $[rN_2O/r(N_2O+N_2)]$ ratio in the non-inoculated soil. Most of these correlations provide limited insights in the underlying mechanisms. However, the positive relationships between soil pH or the C/N ratio and the amount of N_2O reduced by *D. fermentans* suggests that the capacity of the strain to mitigate N_2O emissions was hampered in soil with lower pH or C/N ratio. This is in accordance with findings from Jones et al. (2014) who reported a strong influence of both soil pH and C/N ratio on the *nosZ*II community.

While Graf et al. (2014) showed that a large fraction of organisms belonging to *nosZ* clade II do not harbor denitrification genes, the present study provide direct evidence that these non-denitrifying N_2O-reducing bacteria can contribute to lowering the net N_2O production in soil. Thus, agricultural practices fostering *nosZ* clade II microbial communities could be an alternative strategy to reduce N_2O emissions and research should seek to increase the understanding of the ecology of these organisms and how they are favored. Overall, our result reinforces the potential of soil microorganisms to enhance ecosystem functioning and emphasize the need for research on the management of soil biodiversity for ecological intensification of agroecosystems, as suggested by others (Bender et al., 2016; Gaba et al., 2014).

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.soilbio.2016.09.010.

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Supplementary Material

Table S1. Sampling sites, soil texture and chemical properties.

Soil	Country	Land Use	Fertilisation level	pH	Clay[1,2,3]	Silt[1,23]	Sand[1,2]	Total-C[2]	Total-N[2]	C:N	SOM[2]	CEC[3,4]
JC.IR	Ireland	Arable land	NA	5.9^{e} ± 0.2	21.1^{e} ± 0.3	38.3^{b} ± 1.1	40.6^{abc} ± 1.4	26.8^{b} ± 3.4	2.6^{b} ± 0.2	10.4^{ef} ± 0.3	46.4^{b} ± 5.9	8.6^{de} ± 1.4
SH.IR	Ireland	Pasture	Low	6.6^{cd} ± 0.3	36.7^{bc} ± 0.6	41.7^{b} ± 1.7	21.7^{de} ± 2.2	64.0^{a} ± 8.4	6.6^{a} ± 0.7	9.7^{g} ± 0.2	110.7^{a} ± 14.6	25.1^{a} ± 0.8
LR.EN	England	Pasture	Low	5.5^{f} ± 0.2	32.7^{cd} ± 4.6	29.6^{c} ± 1.2	37.8^{bc} ± 5.8	45.6^{a} ± 4.9	4.8^{a} ± 0.5	9.6^{g} ± 0.0	78.8^{a} ± 8.5	17.7^{b} ± 5.5
UM.A.SE	Sweden	Arable land	Low	5.9^{e} ± 0.2	8.1^{f} ± 1.0	62.7^{a} ± 5.7	29.1^{cde} ± 6.6	22.5^{bc} ± 4.8	1.5^{cd} ± 0.3	15.0^{a} ± 0.4	39.0^{bc} ± 8.3	3.7^{f} ± 0.7
UM.B.SE	Sweden	Arable land	High	5.9^{e} ± 0.2	7.6^{f} ± 1.2	58.4^{a} ± 3.0	33.9^{cd} ± 3.8	24.8^{b} ± 4.5	1.6^{c} ± 0.3	15.1^{a} ± 0.4	42.9^{b} ± 7.8	4.1^{ef} ± 0.4
SA.A.SE	Sweden	Arable land	Low	6.6^{c} ± 0.4	46.2^{a} ± 1.3	45.3^{b} ± 0.5	8.4^{f} ± 1.1	15.8^{d} ± 1.4	1.3^{cd} ± 0.1	11.8^{b} ± 0.1	27.3^{d} ± 2.5	13.5^{bc} ± 1.8
SA.B.SE	Sweden	Arable land	High	6.7^{bc} ± 0.1	47.8^{a} ± 1.6	44.9^{b} ± 1.4	7.2^{f} ± 0.2	17.1^{cd} ± 1.4	1.5^{cd} ± 0.1	11.6^{bc} ± 0.1	29.5^{cd} ± 2.4	14.5^{bc} ± 0.2
ST.A.SE	Sweden	Arable land	Low	7.1^{ab} ± 0.2	26.2^{de} ± 5.4	24.0^{c} ± 4.6	49.7^{ab} ± 9.5	10.5^{e} ± 0.8	1.0^{e} ± 0.1	10.8^{de} ± 0.1	18.1^{e} ± 1.5	11.2^{cd} ± 1.7
ST.B.SE	Sweden	Arable land	High	7.2^{a} ± 0.1	24.3^{e} ± 2.8	23.5^{c} ± 2.3	52.1^{a} ± 5.0	12.4^{de} ± 2.0	1.1^{de} ± 0.2	11.1^{cd} ± 0.1	21.5^{de} ± 3.5	11.7^{cd} ± 1.1
VA.A.SE	Sweden	Arable land	Low	6.4^{cd} ± 0.2	41.7^{ab} ± 2.0	39.4^{b} ± 0.9	18.7^{ef} ± 2.0	12.5^{de} ± 0.8	1.2^{cde} ± 0.1	10.1^{fg} ± 0.2	21.6^{de} ± 1.4	12.6^{cd} ± 0.7
VA.B.SE	Sweden	Arable land	High	6.3^{de} ± 0.1	42.3^{ab} ± 2.0	40.0^{b} ± 0.8	17.6^{ef} ± 2.5	13.1^{de} ± 0.6	1.3^{cde} ± 0.1	10.1^{fg} ± 0.1	22.7^{de} ± 1.0	12.0^{cd} ± 1.1

Soil properties were measured by the INRA Laboratory of Soil Analysis (Arras, France) using standard procedures. Means and 95% confidence interval of soil physicochemical properties are presented and significant differences between soils are indicated with different letters(ANOVA followed by Tukey's HSD test, $P < 0.05$); NA:not available, [1]Size fractions based on µm *Atterberg scale;[2]g kg^{-1}; [3]Cation exchange capacity, [5]cmol kg^{-1}.*

Supplementary Material

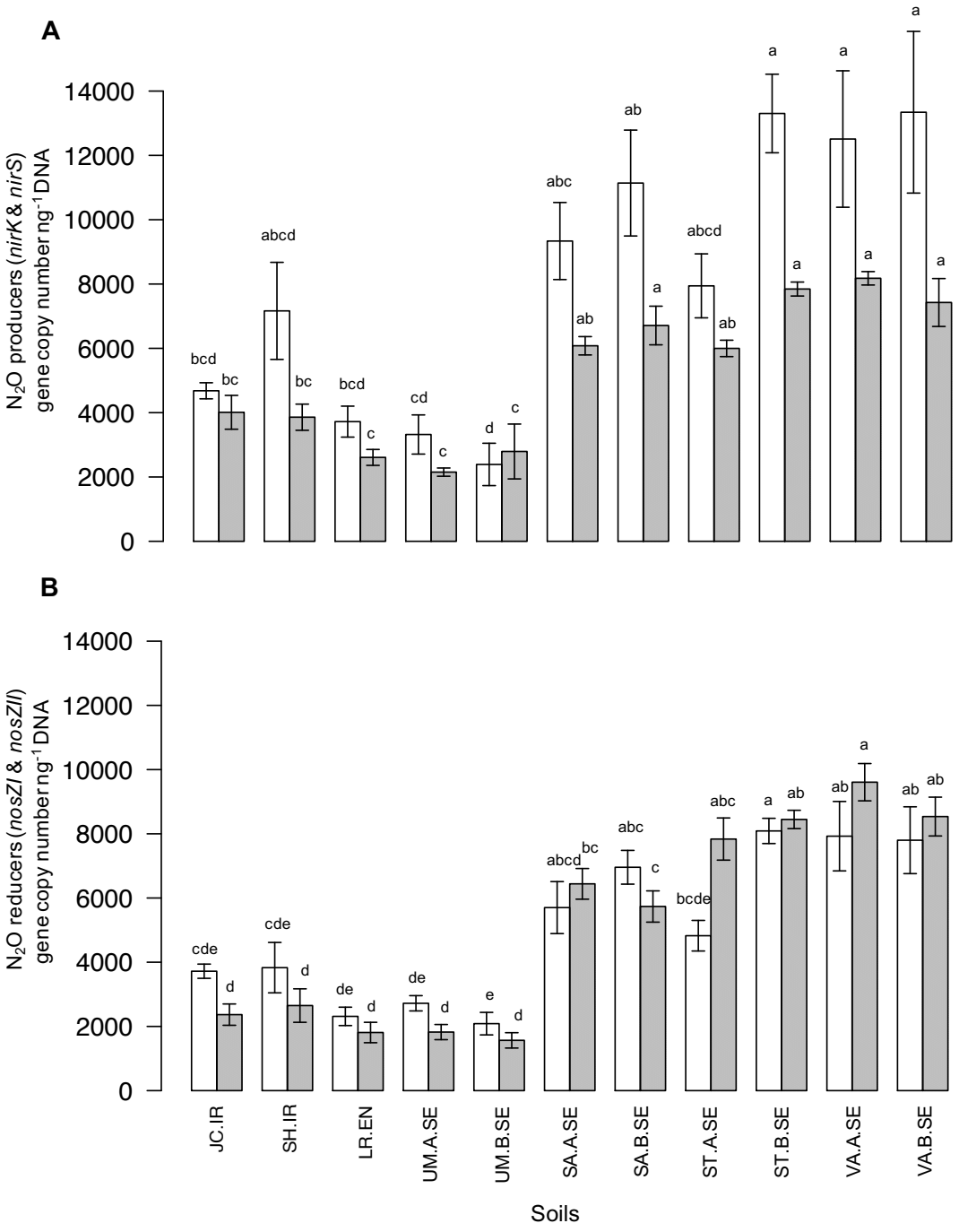

Fig. S1. Abundance of indigenous microbial communities in the soils. A) N_2O-producers (*nirK;* white, *nirS*; gray) B) N_2O-reducers (*nosZ*I; white, *nosZ*II; gray). For each gene, significant differences between soils are indicated with different letters (ANOVA followed by Tukey's HSD test, $P<0.05$; mean ± SE, n=4).

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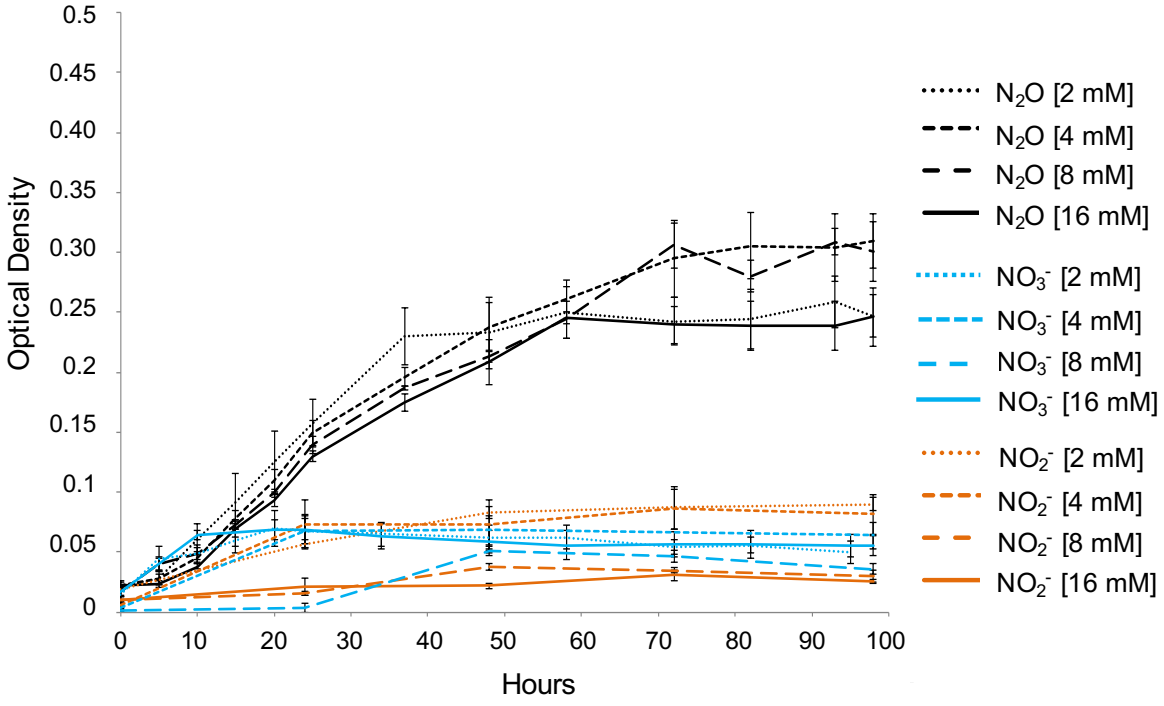

Fig. S2. Growth curves of *Diadobacter fermentans* with different nitrogen oxides as only electron acceptors at different concentrations in the liquid phase. Error bars indicate SE (n=4).

Supplementary Material

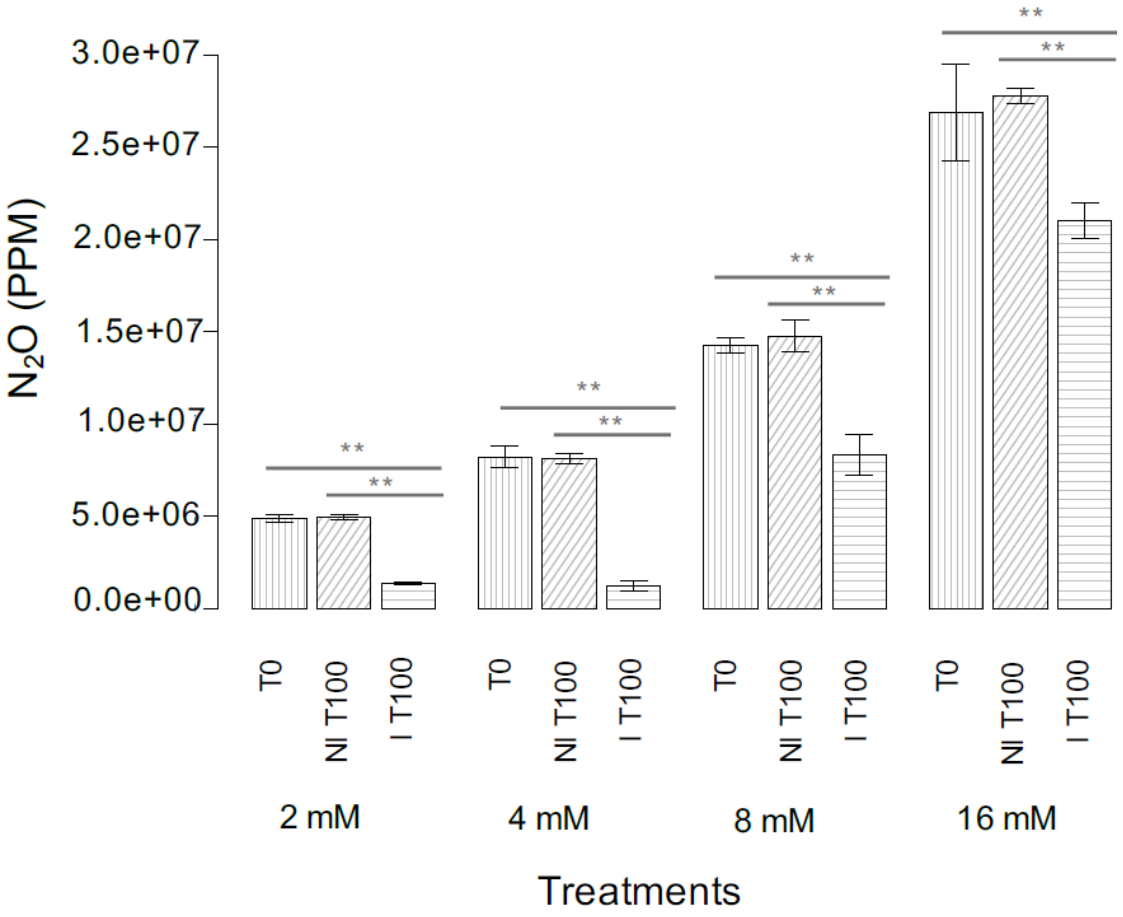

Fig. S3. N_2O reduction by *Diadobacter fermentans* in pure culture grown at 2, 4, 8 and 16 mM of N_2O in the liquid phase. The N_2O concentration in the headspace is shown at the start (T0) and after 100 hours of incubation with (IT100) or without (NI T100) the strain. Significant differences are indicated (**P*<0.05, ***P*<0.001; mean ± SE, n=4).

4 Chapter II

The diversity of the N_2O reducers matters for the N_2O:N_2 denitrification end-product ratio across an annual and a perennial cropping system

Luiz A. Domeignoz-Horta

Aymé Spor

David Bru

Marie-Christine Breuil

Florian Bizouard

Jöel Léonard

Laurent Philippot

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The diversity of the N_2O reducers matters for the N_2O:N_2 denitrification end-product ratio across an annual and a perennial cropping system

*Luiz A. Domeignoz-Horta[1], Aymé Spor[1], David Bru[1], Marie-Christine Breuil[1], Florian Bizouard[1], Joël Léonard[2] and Laurent Philippot[1]**

[1] INRA, UMR 1347 Agroécologie, Dijon, France, [2] INRA, UR 1158 AgroImpact, Laon, France

Agriculture is the main source of terrestrial emissions of N_2O, a potent greenhouse gas and the main cause of ozone layer depletion. The reduction of N_2O into N_2 by microorganisms carrying the nitrous oxide reductase gene (*nosZ*) is the only biological process known to eliminate this greenhouse gas. Recent studies showed that a previously unknown clade of N_2O-reducers was related to the capacity of the soil to act as an N_2O sink, opening the way for new strategies to mitigate emissions. Here, we investigated whether the agricultural practices could differently influence the two N_2O reducer clades with consequences for denitrification end-products. The abundance of N_2O-reducers and producers was quantified by real-time PCR, and the diversity of both *nosZ* clades was determined by 454 pyrosequencing. Potential N_2O production and potential denitrification activity were used to calculate the denitrification gaseous end-product ratio. Overall, the results showed limited differences between management practices but there were significant differences between cropping systems in both the abundance and structure of the *nosZII* community, as well as in the [$rN_2O/r(N_2O+N_2)$] ratio. More limited differences were observed in the *nosZI* community, suggesting that the newly identified *nosZII* clade is more sensitive than *nosZI* to environmental changes. Potential denitrification activity and potential N_2O production were explained mainly by the soil properties while the diversity of the *nosZII* clade on its own explained 26% of the denitrification end-product ratio, which highlights the importance of understanding the ecology of this newly identified clade of N_2O reducers for mitigation strategies.

Keywords: *nosZ*, greenhouse gas, agroecology, diversity, nitrous oxide, agricultural practices, nitrogen cycling

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Tim Daniell,
The James Hutton Institute, UK

Reviewed by:
Gwen-Aelle Grelet,
Landcare Research - Manaaki Whenua, New Zealand
Adrian Langarica-Fuentes,
The James Hutton Institute, UK

****Correspondence:***
Laurent Philippot,
INRA, UMR 1347 Agroécologie,
17 Rue Sully, F-21065 Dijon, France
Laurent.Philippot@dijon.inra.fr

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Introduction

Nitrous oxide (N_2O) is one of the six gases subject to restriction by the Kyoto Protocol, which aims at reducing anthropogenic greenhouse gas (GHG) emissions. N_2O is both directly and indirectly of importance for the Earth's climate. Firstly, it is a potent greenhouse gas with a long life time of 110 years and a global warming potential 298 times that of carbon dioxide on a 100-year time scale and per unit of weight. Thus, N_2O is the third most important GHG contributing to about 10%

of annual global warming (Bates et al., 2008; Thomson et al., 2012). Secondly, after the success of the Montreal Protocol for phasing out chlorofluorocarbons (CFCs), N_2O is today the dominant ozone-depleting substance (Ravishankara et al., 2009). The atmospheric concentration of N_2O has been rising over the past 100 years resulting in a concentration 19% higher than pre-industrial levels (Montzka et al., 2011) with an estimated increase of N_2O emissions of up to 60% by 2050 (relative to 1900 values) (Bouwman et al., 2013).

N_2O emissions are, to a great extent, the result of microbial processes such as nitrification and denitrification. However, denitrification is also the only known sink for N_2O. Denitrification is a microbial respiratory pathway through which soluble forms of nitrogen, i.e., nitrate (NO_3^-) and nitrite (NO_2^-), are sequentially transformed into NO, N_2O and N_2 gases via four enzymatic systems (Tiedje et al., 1982; Zumft, 1997). The reduction of soluble NO_2^- into NO and N_2O is catalyzed by copper- or cd_1- nitrite reductases and nitric oxide reductases, respectively (Zumft, 2005). Nitrous oxide reductase, whose catalytic subunit is encoded by the *nosZ* gene, is the last enzyme of the pathway. It converts the GHG N_2O into inert N_2, which accounts for 78% of the atmospheric gases, and is, therefore, the key enzyme involved in the N_2O sink. It is now recognized that denitrification is a modular process (Graf et al., 2014). Thus, while some microorganisms harbor all denitrification enzymes and can potentially perform the complete pathway, others either lack the nitrous oxide reductase gene and produce only N_2O as the denitrification end product (Philippot et al., 2011), or are only able to reduce N_2O (Sanford et al., 2002).

Recent studies have identified a previously undescribed *nosZ* clade, herein after named *nosZII*, which is diverse and widespread in the environment (Sanford et al., 2012; Jones et al., 2013; Orellana et al., 2014). Genome analyses showed that an important fraction of the microorganisms that possess this *nosZII* gene also harbor a highly truncated version of the denitrification pathway without any nitrite reductase or N_2O-producing nitric oxide reductase, and, therefore, can only consume N_2O (Graf et al., 2014). The abundance and phylogenetic diversity of *nosZII* microorganisms was found to mediate the soil N_2O sink capacity in European soils (Jones et al., 2014), showing the importance of understanding the ecology of this microbial guild for mitigating N_2O emissions.

Agriculture accounts for about 60% of all N_2O emissions from global anthropogenic sources (Syakila and Kroeze, 2011). A compilation of more than 1215 measurements of N_2O emissions from agricultural and natural soils showed that agricultural practices, such as the N application rate, type of crop and type of fertilizer, affected the emissions (Stehfest and Bouwman, 2006). More recently, Shcherbak et al. (2014) reported that the response of soil N_2O emissions to nitrogen fertilizer was nonlinear for synthetic fertilizers and most crop types. The effect of agricultural practices has often been described in terms of changes in soil substrates or environmental conditions, which can also affect soil microbial communities in various ways. For example, soil amendment with peat stimulated the relative abundance of the Alphaproteobacteria, but reduced the relative abundance of Firmicutes (Wessen et al., 2010). Organic farming increased richness, decreased evenness and shifted the structure of the soil microbial community when compared with conventionally managed soils amended with mineral fertilizers (Hartmann et al., 2014) Shifts in abundance and structure of the denitrifier community have also been reported in response to the fertilization regime (Hallin et al., 2009; Clark et al., 2012; Tatti et al., 2014) or in response to land use intensity (Meyer et al., 2013). However, very little is known about how soil management could affect microorganisms belonging to the newly described *nosZII* clade while agricultural practices that foster these microorganisms are of interest for mitigating N_2O emissions.

This study was therefore set out to determine how the *nosZI* and *nosZII* N_2O-reducing communities responded to various agricultural practices in two different arable farming systems. It also assessed the relationships between diversity, composition, and abundance of the N_2O-reducing microbial communities and N-gas production (N_2O and N_2) by denitrification. The study was based on two randomized block experiments localized at the same site, one with an annual rotation with 5 different management practices (ORE) and one with a perennial crop system with 4 different management practices (BE).

Results

Potential N_2O Production, Potential Denitrification Activity (N_2O+N_2) and Denitrification End-product Ratio [$rN_2O/r(N_2O+N_2)$]

To assess the activity of the N_2O reducing microbial communities, the potential N_2O production and potential denitrification activity (PDA) were quantified and used to calculate the denitrification end-product ratio [$rN_2O/r(N_2O+N_2)$]. The potential activity of denitrifying microorganisms varied in all cropping systems, ranging from 0.03 ($CI_{95\%} = [0- 0.09]$) to 0.85 ($CI_{95\%} = [0.79 - 0.91]$) and 0.17 ($CI_{95\%} = [0.07 - 0.27]$) to 1.51 ($CI_{95\%} = [1.41 - 1.61]$) $\mu g\ N_2O\text{-}N\ g^{-1}$ soil DW h^{-1} for potential N_2O and PDA, respectively (**Figures 1A,B**). The [$rN_2O/r(N_2O+N_2)$] ratio ranged between 0.18 ($CI_{95\%} = [0.08–0.28]$) and 1 ($CI_{95\%} = [0.9–1.1]$) (**Figure 1C**) and was significantly higher ($P < 0.001$) for BE than for ORE cropping system with an average of 0.65 ($CI_{95\%} = 0.81–0.48$) and 0.29 ($CI_{95\%} = 0.24–0.34$), respectively. There were significant differences in [$rN_2O/r(N_2O+N_2)$] between the early (ME) and late harvest (ML) practices for plots planted with *Miscanthus giganteus* ($P < 0.05$) (**Figure 1B**) with the denitrification end product being mainly N_2O in the early harvested plots with a [$rN_2O/r(N_2O+N_2)$] close to 1 (**Figure 1C**). For switchgrass, there was the same tendency to have a higher [$rN_2O/r(N_2O+N_2)$] with the early harvest practice, although this was not significant.

Abundance of Total Bacteria, N_2O-producers and N_2O-reducers

The genes encoding catalytic enzymes involved in N_2O production (*nirK* and *nirS*) and N_2O reduction (*nosZI* and

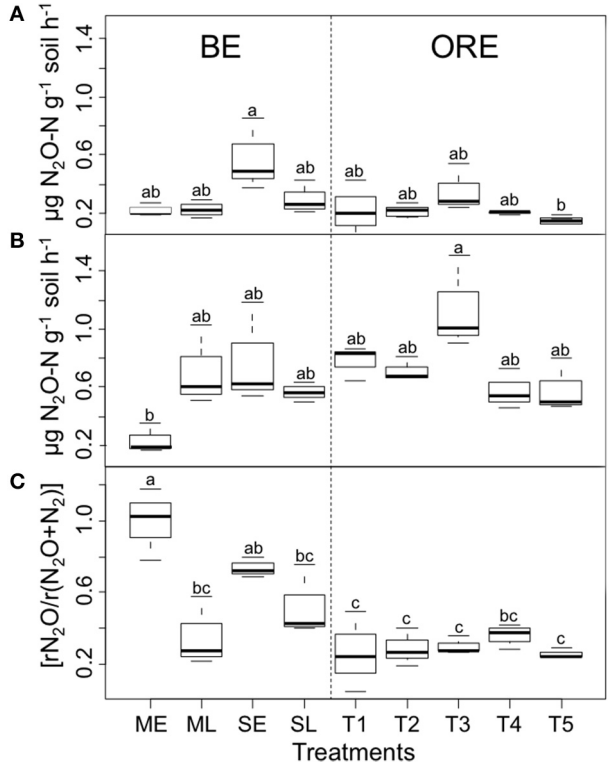

FIGURE 1 | Potential N_2O emissions (A), potential denitrification activity (B) and denitrification gaseous end-product ratio [$rN_2O/r(N_2O+N_2)$] (C). Means ± sem per treatments within each experimental block are given. BE and ORE represent the respective cropping systems. Significant differences between treatments are indicated with different letters (anova followed by Tukey HSD test, $P < 0.05$).

nosZII) were quantified by Real-Time quantitative PCR (qPCR) and used as proxies for the abundances of the corresponding functional communities. The 16S rRNA gene copy number, which was used to estimate the abundance of the total bacteria community, varied from 1.3×10^9 to 5.1×10^9 copy numbers g-1 oil DW without any significant difference between treatments (data not shown). The relative abundances of *nirS* and *nirK* communities were similar, ranging from 2.5 to 7.6% of the total bacterial community (Figure S1). The *nosZI* community was significantly ($P < 0.05$) more abundant than the *nosZII* community, ranging from 4.9 to 8.5% and 0.28 to 2.9% of the total bacteria, respectively (Figure S1). No significant differences in N_2O-producers and N_2O-reducers abundances were found between treatments (Figure S1). However, *nosZII* abundance was higher in BE than in ORE ($P < 0.01$).

N_2O-reducer Diversity

To assess whether the agricultural practices could drive the composition and structure of the N_2O-reducer community, the diversity of both *nosZI* and *nosZII* clades was characterized by 454 pyrosequencing. 123,130 *nosZI* and 121,500 *nosZII* sequences were obtained from samples after quality filtering. Similarity-based clustering of sequences gave an average of 162 ($CI_{95\%}$ = 133–191) and 312 ($CI_{95\%}$ = 279–345) OTUs for *nosZI* and *nosZII* respectively in BE, and 158 ($CI_{95\%}$ = 136–180) and 355 ($CI_{95\%}$ = 330–380) OTUs for *nosZI* and *nosZII* respectively in ORE. In both BE and ORE, the *nosZII* community had a significantly higher richness than *nosZI* ($P < 0.0001$). Within BE and ORE, the agricultural practices had no significant effect on the α-diversity of the N_2O-reducing communities (Table S1). An analysis of *nosZI* and *nosZII* phylogenies showed that the most abundant sequences in ORE and BE were similar (Figures S2, S3), with *nosZII* sequences affiliated mainly to *nos*Z from Bacteroidetes while *nosZI* sequences were affiliated to *nos*Z from Alphaproteobacteria and Betaproteobacteria. Further examination of the β-diversity by non-metric multidimensional scaling (NMDS) and analysis of similarity (ANOSIM) showed (**Figures 2A,B**) no clustering of samples according to the agricultural practices. However, differences in both *nosZ* communities between annual rotation and perennial cropping systems were significant, but stronger for *nosZII* communities, ($R = 0.43$, $P < 0.0001$ and $R = 0.77$, $P < 0.0001$ for *nosZI* and *nosZII* respectively). Fitting the environmental variables onto the ordination plot showed that pH and calcium were significant explanatory variables ($P < 0.05$) for the community structure of both guilds. The *nosZI* community structure was also related to the water content while the abundance of nitrite reductase genes (*nirK*) and sand content were related to the *nosZII* community structure (**Figure 2B**). The [$rN_2O/r(N_2O+N_2)$] surface was fitted onto the ordination plot and showed a strong relationship between the *nosZII* community structure, its diversity and the denitrification end products with a lower proportion of N_2O produced when the *nosZII* diversity increased (**Figure 2B**).

Denitrification Activity and End Product Ratio as a Function of Soil Properties, Abundance and Diversity of the N_2O-reducing Community

We used variance partitioning technique to quantify the relative contribution of the different groups of variables to the variation in N_2 production by denitrification across samples (**Figures 3A–D**). The physical and chemical characteristics of the soil, the abundance of N_2O producers and reducers, and the diversity of N_2O reducers were used as explanatory variables. After model selection using multiple linear regressions (Table S2), the physical and chemical properties of the soil were found to be the variables that contributed most to the potential N_2O production and PDA, explaining up to 29 and 45% of the variance, respectively (**Figures 3B,C**). In contrast, the [$rN_2O/r(N_2O+N_2)$] was mostly explained by the diversity of the N_2O-reducers (26%). Interactions between physical and chemical properties of the soil and the diversity of the N_2O-reducing communities accounted for 26 and 17% of potential N_2O production and [$rN_2O/r(N_2O+N_2)$], respectively (**Figures 3B,D**). The importance of *nosZII* diversity for the end product ratio of denitrification was also suggested by the strong negative correlation between the ($rN_2O/r(N_2O+N_2)$) and the *nosZII* diversity (r = -0.70, $P < 0.0001$) (Figure S4). The abundance of the communities studied made only a marginal contribution, explaining 2% of the variance in [$rN_2O/r(N_2O+N_2)$].

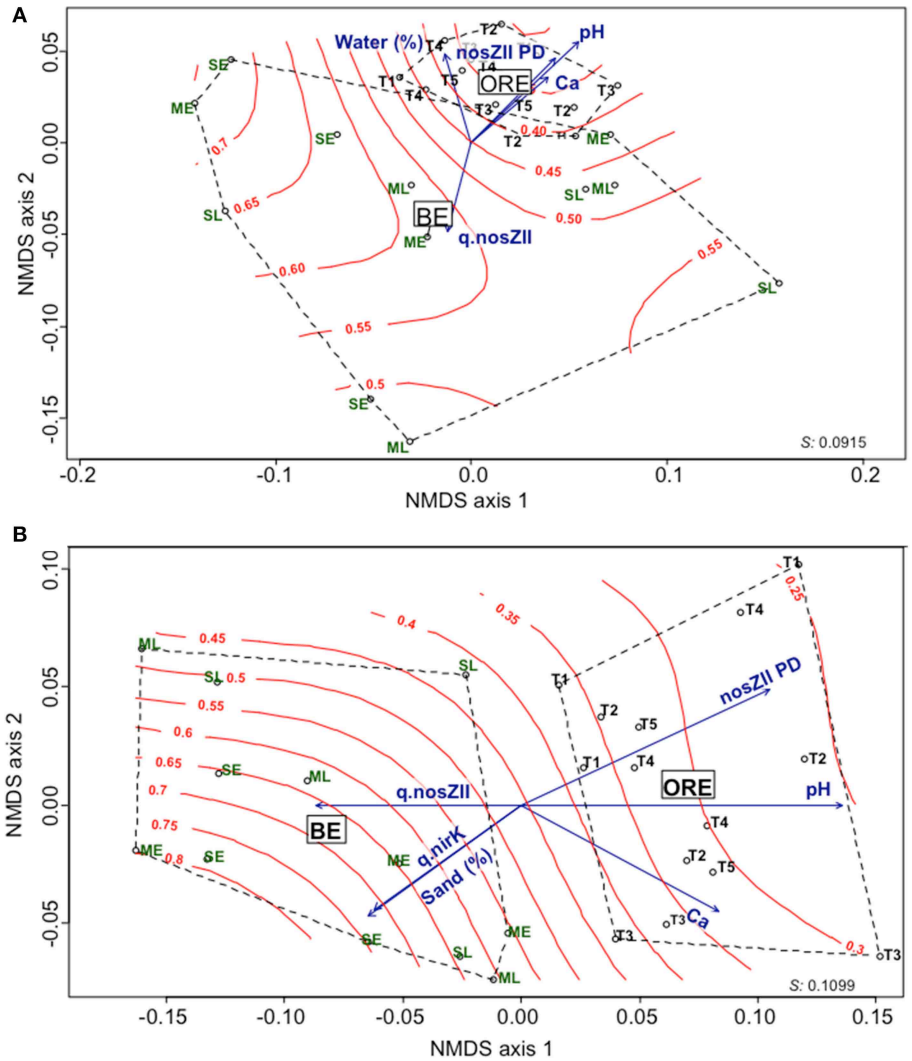

FIGURE 2 | NMDS ordinations of *nosZ* weighted unifrac distance matrices. (A) Variation in *nosZI* community structure. **(B)** Variation in *nosZII* community structure. Red curves represent the final denitrification product [$rN_2O/r(N_2O/N_2)$]. Significant explanatory variables are represented as blue vectors ($P < 0.05$), Ca (calcium g kg^{-1} dw soil), q.nosZII and q.nirK correspond to the quantification by qPCR of nitrous oxide reductase community (clade II) and copper nitrite reductase community, respectively (copy number g^{-1} dw soil); sand and water content are expressed in percentage. The lengths of the arrows are proportional to the strength of the correlation. Stress values are indicated at the bottom right of each panel.

Discussion

The recent identification of a previously unknown clade of N_2O reducers (Sanford et al., 2012; Jones et al., 2013), whose abundance and phylogenetic diversity are critical for the soil N_2O sink capacity (Jones et al., 2014), raised the question of whether it would be possible to mitigate N_2O GHG emissions by selecting agricultural practices that favor these microorganisms. A large body of literature shows that land management can drive microbial communities (Clark et al., 2012; Lauber et al., 2013; Hartmann et al., 2014). Here, our intent was not to compare the impact of individual agricultural practices between the two cropping systems but rather to assess how practices for a particular system, such as tillage, residue management, quantity and nature of N inputs in ORE and biomass crop species and harvest date in BE, affect N_2O-reducing communities and N_2 production. We did not observe any significant effect of the practices for either BE or ORE on either the diversity or the abundance of the N_2O-reducing communities (Table S1 and Figure S1). In contrast, previous results showed differences in the denitrifier community under different fertilization regimes (Hallin et al., 2009) or tillage systems (Melero et al., 2011). Significant effects of the cropping systems on the total bacterial communities were also reported (Hartmann et al., 2014). One common feature of these studies is that they were based on long term experiments that had been running for up to 50 years. This discrepancy with our results could be due to the fact that the ORE and BE systems were established only in 2010 and 2006, respectively, which has not allowed a strong differentiation

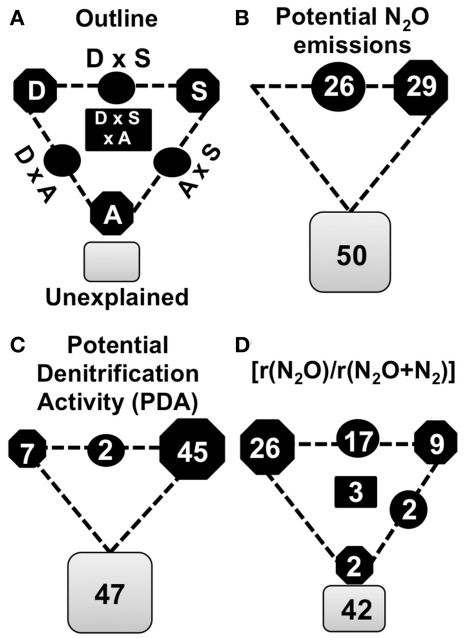

FIGURE 3 | Variation partitioning of denitrification activities. (A) Variance in denitrification activities was partitionned into *nosZ* diversity **(D)**, soil physicochemical properties (S), denitrifiers abundance **(A)** and by combinations of predictors. Geometric areas are proportional to the respective percentages of explained variation. The edges of the triangle depict the variation explained by each factor alone. Percentages of variation explained by interactions of two or all factors are indicated on the sides and in the middle of the triangles, respectively. **(B)** Variance partitioning of potential N_2O emissions. **(C)** Variance partitioning of potential denitrification activity (PDA). **(D)** Variance partitioning of final denitrification product [$r(N_2O/r(N_2O+N_2)$]. The variables used for each variation partitioning are indicated in the Table S2.

in soil properties between practices within a cropping system (**Table 1**). On the other hand, the comparison of ORE and BE did show differences in the N_2O reducing communities with a higher *nosZII* abundance for ORE than for BE ($P < 0.01$). *NosZII* community richness estimated as OTU numbers was also significantly higher in ORE than in BE (Table S1). Furthermore, NMDS also showed significant clustering according to perennial and annual rotation cropping systems for *nosZII* and to a lesser extent for *nosZI* (**Figures 2A,B**). Since it is not possible to distinguish the effect of practices from soil legacy or age effects between ORE and BE, it cannot be concluded that the observed changes in the *nosZII* community were due to differences in agricultural practices. However, the stronger response of the *nosZII* community structure and abundance indicates that this recently identified clade is more sensitive to environmental changes than the *nosZI* clade and is therefore more likely to be driven by the land use type.

The agricultural practices studied also had little impact on PDA and N_2O production. However, larger differences were observed when calculating the denitrification gaseous end-product ratio ($rN_2O/r(N_2O+N_2)$) with, in particular, early harvest of *M. giganteus* giving a significantly higher proportion of N_2O than late harvest. Previous studies at BE showed that late harvest of *M. giganteus* gave a significant net input and accumulation of organic matter due to senescence and leaf fall between early and late harvest (Amougou et al., 2011, 2012; Cadoux et al., 2014). This type of mulch is known to improve soil moisture by reducing soil surface evaporation, resulting in a lower partial pressure of oxygen (pO_2). This is consistent with our results since previous studies have shown that production of N_2O relative to N_2 during denitrification in soils is strongly influenced by carbon availability and pO_2 (Firestone et al., 1980; Murray and Knowles, 2004; Walker et al., 2008; Giles et al., 2012; Saggar et al., 2013) with, for example decreasing proportion of N_2O produced during denitrification with decreasing O_2 concentrations (Firestone et al., 1980). However, the harvesting date had no significant effect on the denitrification gaseous end-product ratio for switchgrass. This might be due to the fact there is no leaf deposition between late and early harvest for switchgrass. Similarly, to the N_2O reducer community structure, significant differences in the N_2O ratio were observed between the BE and ORE cropping systems with a lower end-product ratio in ORE ($P < 0.001$). In agreement with previous results (Jones et al., 2014), a strong negative correlation was found between the ($rN_2O/r(N_2O+N_2)$) ratio and *nosZII* diversity (r = -0.70, $P < 0.0001$) (Figure S4), highlighting the importance of *nosZII* N_2O reducers in the N_2O-reducing capacity of a soil.

We found that the abundance of *nosZII* N_2O reducers was significantly correlated with several soil properties such as pH, Ca concentration, soil moisture and total N. This was not the case for *nosZI* which was correlated solely with the C:N ratio, confirming that the *nosZII* clade is more sensitive to changes in environmental conditions than the *nosZI* clade. The analysis of the community structure showed that two N_2O-reducer clades shared a certain number of explanatory variables but there were also distinct explanatory variables for each clade. The structure of both communities was driven by pH and calcium, while water content was related to clade I and sand to clade II (**Figures 3A,B**). To our knowledge, the difference in the response of the two N_2O reducer clades to soil properties has only been assessed in one previous study (Jones et al., 2014). Using structural equation modeling, Jones et al. (2014) showed that soil texture was a more important driver of the abundance of the *nosZI* community whereas soil pH affected the abundance of the *nosZII* community only. Overall, our results, which showed that different factors influenced the *nosZI* and *nosZII* clades, confirm niche partitioning between the two N_2O reducing communities. They also indicate that agricultural practices could affect *nosZI* and *nosZII* communities in different ways with consequences for N_2O reduction.

Variation partitioning analysis was applied to disentangle the contribution of soil properties, the abundance of N_2O producers and reducers, and the diversity of N_2O reducing microbial communities to the production of N_2O and N_2 as the denitrification end products. Potential N_2O production (29%) and PDA (45%) were mostly explained by soil properties. Water content, C/N ratio, Cation Exchange Capacity (CEC) and pH were the major soil properties explaining N_2O production, while sand content, total nitrogen and the C/N ratio explained PDA. Accordingly, nitrate and carbon availability, pO_2 and related variables such as water content and soil texture are well known proximal factors regulating N_2O and PDA rates (Baggs, 2011; Giles et al., 2012). pH is also known to be a

TABLE 1 | Description of treatments and soil physicochemical properties.

Treatment	Cropping system	Crops	Management practices[1]	N input[2]	% Water	pH	% Clay[3]	% Loam[3]	% Sand[3]	Total C[4]	Total N[4]	OM[4]	Ca[4]	CEC[5,6]
ML	BE	Miscanthus (*M. giganteus*)	Late harvest	120	$21.7^{c} \pm 0.9$	$7.2^{ab} \pm 0.5$	20.2 ± 3.6	74.0 ± 3.3	5.6 ± 0.4	11.5 ± 1.0	1.0 ± 6.10^{-2}	19.9 ± 1.9	1.3 ± 0.6	11.1 ± 2.9
ME	BE	Miscanthus (*M. giganteus*)	Early harvest	120	$23.0^{bc} \pm 0.1$	$7.4^{ab} \pm 0.6$	20.9 ± 1.7	73.5 ± 2.4	6.0 ± 1.3	10.9 ± 0.6	1.0 ± 4.10^{-2}	18.9 ± 1.0	2.3 ± 2.6	11.6 ± 1.0
SL	BE	Switchgrass (*P. virgatum*)	Late harvest	120	$23.3^{ab} \pm 1.1$	$7.4^{ab} \pm 0.5$	21.9 ± 2.4	72.3 ± 1.3	5.7 ± 1.1	11.8 ± 0.8	1.1 ± 6.10^{-2}	20.5 ± 1.3	1.3 ± 0.6	12.3 ± 1.1
SE	BE	Switchgrass (*P. virgatum*)	Late harvest	120	$24.7^{abc} \pm 0.6$	$6.8^{b} \pm 0.1$	20.1 ± 3.3	73.8 ± 2.8	6.0 ± 0.5	12.0 ± 0.4	1.1 ± 5.10^{-2}	20.7 ± 0.8	1.0 ± 0.0	10.8 ± 1.8
T1	ORE	Peas, rapeseed, wheat, barley, corn, wheat	Conventional tillage, straw addition, high N input, non-legume intercrop	100	$25.1^{ab} \pm 0.9$	$7.9^{a} \pm 0.2$	21.1 ± 4.0	74.1 ± 3.4	4.5 ± 0.5	10.7 ± 0.8	1.0 ± 4.10^{-2}	18.5 ± 1.4	3.0 ± 2.9	12.3 ± 1.7
T2	ORE	Peas, rapeseed, wheat, barley, corn, wheat	Reduced tillage, straw addition,high N input, non-legume intercrop	100	$24.8^{ab} \pm 0.5$	$7.9^{a} \pm 0.3$	17.7 ± 0.5	77.0 ± 0.06	4.9 ± 0.4	10.8 ± 0.2	1.0 ± 1.10^{-2}	18.6 ± 0.5	3.7 ± 2.8	10.5 ± 0.5
T3	ORE	Peas, rapeseed, wheat, barley, corn, wheat	Reduced tillage, straw removal, high N input, non-legume intercrop	100	$24.4^{ab} \pm 1.0$	$7.9^{a} \pm 0.2$	20.5 ± 2.8	74.4 ± 3.2	4.7 ± 0.7	11.4 ± 0.7	1.1 ± 6.10^{-2}	19.7 ± 1.3	4.0 ± 5.8	12.1 ± 1.5
T4	ORE	Peas, rapeseed, wheat, barley, corn, wheat	Conventional tillage, straw addition, low N input, non-legume intercrop	40	$25.2^{a} \pm 1.1$	$8.0^{a} \pm 0.2$	19.8 ± 3.3	75.1 ± 3.3	4.8 ± 0.2	10.7 ± 0.4	1.0 ± 5.10^{-2}	18.6 ± 0.8	3.3 ± 2.8	11.7 ± 1.9
T5	ORE	Peas, rapeseed, wheat, barley, corn, wheat	Conventional tillage, straw addition, low N input, legume intercrop	40	$25.0^{ab} \pm 0.4$	$8.1^{a} \pm 0.1$	17.8 ± 1.6	77.1 ± 1.2	4.8 ± 0.5	10.7 ± 0.3	1.0 ± 5.10^{-2}	18.4 ± 0.6	3.4 ± 1.7	10.4 ± 1.0

Means and 95% confidence interval of soil physicochemical properties are given per treatment within each experimental block. Significant differences between treatments are indicated with different letters: "a", "b", and "c" (anova followed by Tukey HSD test, $P < 0.05$).

[1] "Late" and "early" harvest correspond to February and October, respectively, "conventional tillage" is applied to a 20 cm depth while "reduced tillage" to 6 cm, "intercrop" is a non-leguminous crop for all treatments at ORE except for T5; [2] N input (Kg N ha^{-1} y^{-1}); [3] Size fractions based on μm Atterberg scale; Clay = 2 μm, Loam = 2-50 μm, and Sand = 50-2000 μm; [4] g kg^{-1}; [5] cation exchange capacity; [6] cmol kg^{-1}.

master regulator of biological processes in soils (Enwall et al., 2005; Giles et al., 2012; Petersen et al., 2012; Saggar et al., 2013; Liu et al., 2014) including the reduction of N_2O into N_2 by nitrous oxide reductase (Firestone et al., 1980). Unlike other studies (Hallin et al., 2009; Petersen et al., 2012; Jones et al., 2014), the abundance of the N_2O producing and reducing communities did not explain variations in PDA nor in potential N_2O production (**Figures 3B,C**). According to Petersen et al. (2012) the abundance of denitrifiers was correlated with PDA only when large variations in fluxes were observed, which was not the case in this study. In contrast to potential N_2O production and PDA, the variance in the proportion of N_2O emitted by denitrification [$rN_2O/r(N_2O+N_2)$] was mostly explained by the diversity of the *nosZII* clade (26%), and the interaction between this diversity and soil properties (17%) (**Figure 3D**). This is in agreement with previous research showing a positive correlation between the ability of the soil to consume N_2O and the phylogenetic diversity of the *nosZII* clade (Jones et al., 2014). It also illustrates the importance of the *nosZII* community in determining the nature of the denitrification gaseous end-products through their capacity to act as a N_2O sink.

Overall, our results showed that the newly described *nosZII* clade is the strongest predictor of the [$rN_2O/r(N_2O+N_2)$] ratio while soil properties are the main drivers of potential denitrification and N_2O production. They also showed that the two clades of N_2O reducers were not affected by the same soil properties, suggesting niche partitioning. The *nosZII* clade was more sensitive to environmental changes than the *nosZI* clade, which may make it easier to foster this group using agricultural practices as a new strategy for mitigating N_2O emissions. Further studies are required to determine the effect of different agricultural practices on the abundance and diversity of the *nosZII* clade, in sites with different pedoclimatic conditions to provide more information on the ecology of this recently described functional guild. Moreover, due to the increasing evidence that fungi can produce N_2O and N_2 by denitrification and co-denitrification (Laughlin and Stevens, 2002; Wei et al., 2014; Maeda et al., 2015), respectively, the response of these microorganisms to agricultural practices should also be considered to circumvent any tradeoff.

Materials and Methods

Experimental Design and Sampling

Soil samples were collected in October 2013 from two randomized field experiments ORE (49°52′25.615″N, 3°1′53.914″E) and BE (49°52′19.29″N, 3°0′47.267″E) located at the same site near Estrées-Mons, France, which has both annual rotation and perennial crop systems, as well as various agricultural practices. A description of the practices can be found in **Table 1**. Replicated plots with the same practices were randomly distributed within each block experiment. Briefly, the ORE experiment, which consisted in 5 treatments (T1–T5), was set up in 2010 to study the effect of soil tillage, crop residue management, fertilization rate and substitution of mineral N input by fixation by legumes on biogeochemical cycles and soil biodiversity (www.soere-acbb.com). The BE experiment was set up in 2006 to compare the productivity and environmental impacts of various energy crop systems including perennial crops such as *Miscanthus giganteus* and *Panicum virgatum* and differences in management practices such as early or late harvest (ML, ME, SL, and SE). Three replicate samples were collected for each combination of cropping system and management practices, each being a composite sample of five subsamples (soil cores of 2.5 cm by 20 cm) from each plot. Samples were frozen (−20°C) until further analysis. The physical and chemical soil characteristics were measured for all samples (INRA Laboratory of Soil Analysis, Arras, France) (**Table 1**).

Potential Denitrification Activity (PDA) and Potential N_2O production

Potential denitrification activity (N_2O + N_2) and potential nitrous oxide production (N_2O) were measured using the acetylene inhibition technique (Yoshinari et al., 1977). For each sample 10 g of fresh weight soil was wetted with 20 ml of distilled water and was amended with a final concentration of 3 mM KNO_3, 1.5 mM succinate, 1 mM glucose, and 3 mM acetate. To determine the potential denitrification activity, acetylene was added to reach 0.1 atm partial pressure followed by 30 min incubation at 25°C and agitation (175 rpm). Gas samples were taken every 30 min for 150 min (Pell et al., 1996). The N_2O concentrations were determined using a gas chromatograph (Trace GC Ultra, Thermo Scientific) equipped with an EC-detector.

Nucleic Acid Extraction and Abundance of Bacterial Communities

DNA extraction for all samples was performed in accordance with ISO 11063 (Petric et al., 2011). 0.25 g of soil was homogenized with a 1 ml homogenization buffer for 30 s at 1600 rpm in a mini-bead beater cell disruptor (Mikro-Dismembrator S; B Braun Biotech International), followed by centrifugation at 14000 × g for 1 min to eliminate soil and cell debris. For protein precipitation, supernatant was incubated on ice for 10 min with 1/10 volume of 3M sodium acetate and centrifuged (14000 × g, 5 min, 4°C). The DNA was precipitated by adding one volume of cold isopropanol (−20°C) over 24 h. The mix was then centrifuged for 30 min at 14,000 g (4°C), the resulting pellet was washed with 70% ethanol, and the DNA was resuspended with 100 μL of TE buffer (pH 8). The DNA was purified in two steps: first using polyvinylpolypyrrolidone (PVPP) microbiospin columns (Bio-Rad, CA, USA), and then a Sepharose 4G column (Sigma-Aldrich, United Kingdom). The DNA quality was checked by electrophoresis on agarose gel and quantified by spectrofluorometer using the Quant-iT PicoGreen® dsDNA Assay Kit (Invitrogen, Cergy-Pontoise, France) following the manufacturer's instructions.

The abundance of denitrifiers was assessed by real-time quantitative PCR (qPCR) by targeting N_2O-producers, *nirK* and *nirS* (Henry et al., 2004; Kandeler et al., 2006) and N_2O-reducers *nosZI* (Henry et al., 2006; Jones et al., 2013) and *nosZII* (Table S3). Abundance of total bacteria was assed using 16S rRNA primers (Muyzer et al., 1993) as previously described (López-Gutiérrez et al., 2004). qPCR Reactions were carried out in a StepOnePlus

Real time PCR System (Life Technologies, Carlsbad, CA, USA). The abundance was based on the increasing fluorescence intensity of the SYBR Green dye during amplification. The qPCR assay was carried out in a 15 µl reaction volume containing 1 ng of DNA, 7.5 µl of SYBRgreen PCR Master Mix (Absolute qPCR SYBR GreenRox, Thermo, Courtaboeuf, France), 1 µM of each primer, 250 ng of T4 gene 32 (QBiogene, Illkrich, France). Before assessing the abundance of the bacterial communities, an inhibition test was performed by mixing DNA extracts with a known amount of control plasmid DNA and no inhibition was detected. Three independent quantitative qPCR assays were performed for each gene. Controls and no-template controls giving null or negligible values were run for each quantitative qPCR assay. The qPCR efficiencies for the various genes ranged between 70 and 96%.

Phylogenetic Diversity of N_2O-reducers

A diversity analysis of *nosZI* and *nosZII* was performed by 454 pyrosequencing as previously described in Jones et al. (2014). Briefly, the DNA was prepared using a two-step PCR procedure (Berry et al., 2012). In the first step, 20 PCR cycles were performed with primers *nosZI* and *nosZII* (Table S4) in a 25 µl reaction volume containing 5 µl 5 × Taq Buffer (GoTaq, Promega, Madison, U.S.A.), 2 µM of each primer, 250 ng of T4 gene 32 (QBiogene, Illkrich, France), 0.125 µl of DNA Polymerase (GoTaq, Promega, Madison, U.S.A.), 200 µM(each) deoxyribonucleoside triphosphate, and 1 ng of template DNA. In the second step, 4 µL of the PCR products of the first reaction were amplified in a 50 µl reaction volume containing 10 µl 5 × Taq Buffer (GoTaq, Promega, Madison, U.S.A.), 200 µM (each) deoxyribonucleoside triphosphate, 1 µM of each primer, 0.25 µl of DNA Polymerase (GoTaq G2, Promega, Madison, U.S.A.). In the second PCR 15 or 18 cycles PCR were performed using the forward primers preceded by 10 bp-long barcodes, the sequencing key and the forward sequencing adapter; the reverse primers being only preceded by the sequencing key and the reverse sequencing adapter as described in Jones et al. (2014) (Table S4).Because there were only very small amounts of products for *nosZII* after the first PCR, the second PCR for this gene was extended to 18 cycles. The product of 3 independent second PCR was then gel extracted and purified using the QIAEX II kit (Qiagen; France). Pyrosequencing was performed by Genoscreen (Lille, France) on a Roche's 454 FLX Genome Sequencer according to manufacturer's instructions.

Sequence Processing

The QIIME pipeline (Caporaso et al., 2010a) was used for quality trimming of raw 454 pyrosequencing data (QIIME version 1.8.0). The minimum and maximum sequence lengths were 230 and 410 bp respectively. Sequences with an average score below 25 using a sliding window of 50 bp were discarded. After quality checking, 123,130 sequences were found for *nosZI* and 121,500 sequences for *nosZII*. Sequences were then processed using the "pick_otus.py" script within QIIME, and the "usearch" option (Edgar, 2010) with reference-based and *de novo* chimera checking, and clustering of sequences at 97% similarity. Raw sequences were deposited at the NCBI under the accession number SRP058080. The process of raw sequence submission was greatly simplified by using the *make.sra* command of Mothur software (Schloss et al., 2009).

nosZ phylogeny

Reference sequences for *nosZ* were downloaded from all 4135 draft and completed microbial genome nucleotide sequences available in the National Center for Biology Information (NCBI) (Jones et al., 2014). These reference sequences were used as templates for aligning 454 reads with PYNAST (Caporaso et al., 2010b). Phylogenetic trees for *nosZI* and *nosZII* were constructed with fastree (Price et al., 2010) and ITOL was used to visualize and manipulate of the trees (Letunic and Bork, 2007).

Statistical Analysis

Statistical analysis and graphics were produced using the R statistical software, R version 3.0.3, (R Core Team, 2013) and the *agricolae* (Mendiburu, 2014) and *vegan* (Oksanen et al., 2015) packages. The effect of agricultural practices and cropping systems was determined by analysis of variance and *post hoc* Tukey HSD test. Collinearity between explaining variables within each group (soil properties, microbial community abundances, and denitrifiers diversity) was checked, and one of each pair of collinear variables was kept for subsequent analyses. Non-metric MultiDimensional Scaling (NMDS) of the Unifrac distance matrices (unweighted and weighted) was used to describe community structure. Ordinations with the lowest stress values were used. The soil properties, community abundances and diversity were plotted onto the ordination map as vectors. Permutation tests ($n = 10000$) were used to test the significance of vector fits and only significant ones were depicted ($P < 0.05$). Vector and surface fitting of variables within ordinations were performed using the *envfit* and *ordisurf* functions in the vegan package respectively. ANalysis Of SIMilarity (ANOSIM) was used to test for significant differences in community structure between cropping systems (permutations = 1999, $P < 0.05$).

Significant explanatory variables of [$rN_2O/r(N_2O+N_2)$], potential N_2O and PDA were chosen by linear regression and model selection (backward) and by minimizing the Akaike Information Criterion (AIC). The statistical significance was assessed by 1000 permutations of the reduced model. The resulting significant explanatory variables (Table S2) were used to access their contribution to explaining the variation of potential N_2O, PDA and [$rN_2O/r(N_2O+N_2)$], using the function *varpart* (Peres-Neto et al., 2006).

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Supplementary Material

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2015.00971

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Supplementary Material

The diversity of the N2O reducers matters for the relative proportion of N_2O emitted by denitrification across cropping systems.

L. A. Domeignoz-Horta, A. Spor, D. Bru, M.C. Breuil, F. Bizouard, J. Léonard, L. Philippot*

***Correspondence:** Corresponding Author: Laurent.Philippot@dijon.inra.fr

Figure S1 Abundances of N_2O-producers (*nirK* and *nirS*) and N_2O –reducers (*nosZI* and *nosZII*). Means ± sem per treatments within each experimental block are given. Significant differences between treatments are indicated with different letters (anova followed by Tukey HSD test, P<0.05).

Figure S2 Phylogenetic placement of *nosZI* pyrosequencing reads within a reference phylogeny. The phylogeny was inferred using maximum likelihood analysis of full-length *nosZ* amino acid sequences obtained from microbial genomes. The concentric barplots plotted around the phylogenies represent the two block experiments BE and ORE, in red and green respectively. The bars sizes correspond to the relative abundance of each OTU with respect to total read counts. Colors of branches denote taxonomic affiliation of source organisms for reference *nosZ* sequences.

Figure S3 Phylogenetic placement of *nosZII* pyrosequencing reads within a reference phylogeny. The phylogeny was inferred using maximum likelihood analysis of full-length *nosZ* amino acid sequences obtained from microbial genomes. The concentric barplots plotted around the phylogenies represent the two block experiments BE and ORE, in red and green respectively. The bars sizes correspond to the relative abundance of each OTU with respect to total read counts. Colors of branches denote taxonomic affiliation of source organisms for reference *nosZ* sequences.

Figure S4 Correlation of *nosZII* phylogenetic diversity with the final denitrification product [$rN_2O/r(N_2O+N_2)$]. Pearson correlation was calculated between Faith's PD index of *nosZII* clade and [$rN_2O/r(N_2O+N_2)$], (r = -0.70, P<0.0001).

Table S1 Description of treatments and soil physicochemical properties. Means and 95% confidence interval of soil physicochemical properties are given per treatment within each experimental block. Significant differences between treatments are indicated with different letters (anova followed by Tukey HSD test, P<0.05).

Table S2 Diversity of the two *nosZ* clades. Means and 95% confidence interval of diversity indices for both *nosZ* clades are given per treatment within each experimental block. Significant differences between treatments are indicated with different letters (anova followed by Tukey HSD test, P<0.05).

Table S3 Selected explaining variables used for the variation partitioning analysis for each denitrification activity variable. Diversity of *nosZ* clades is represented by Faith's PD index, water and sand are expressed in %, TN represents total nitrogen (g/kg), and CEC the cation exchange capacity (cmol+/kg). Abundance (nbc/ng DNA) of N_2O producers and reducers is expressed by q.nirK, q.nirS and q.nosZI, q.nosZII, respectively.

1.1. Supplementary Figures

Figure S1

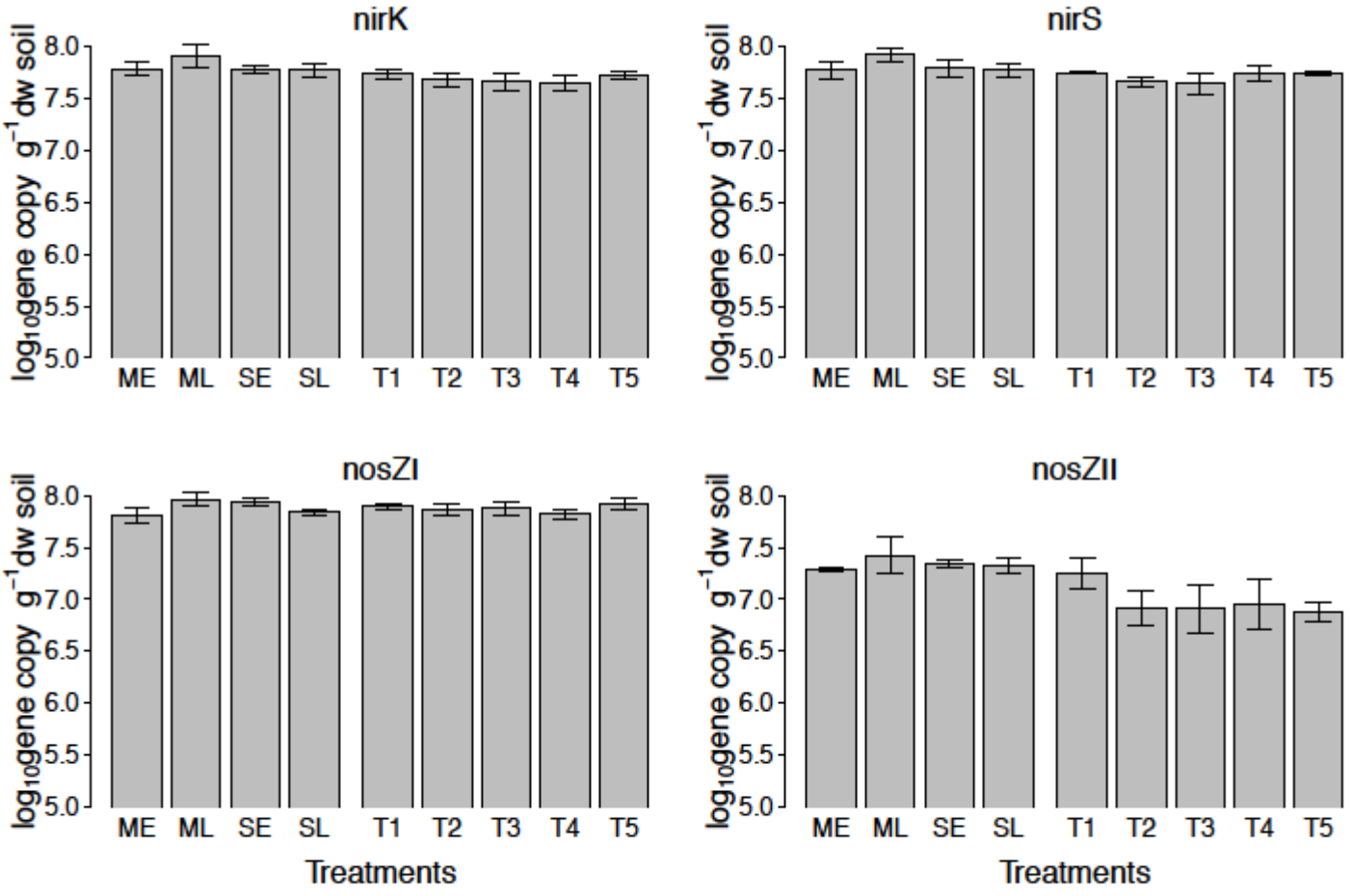
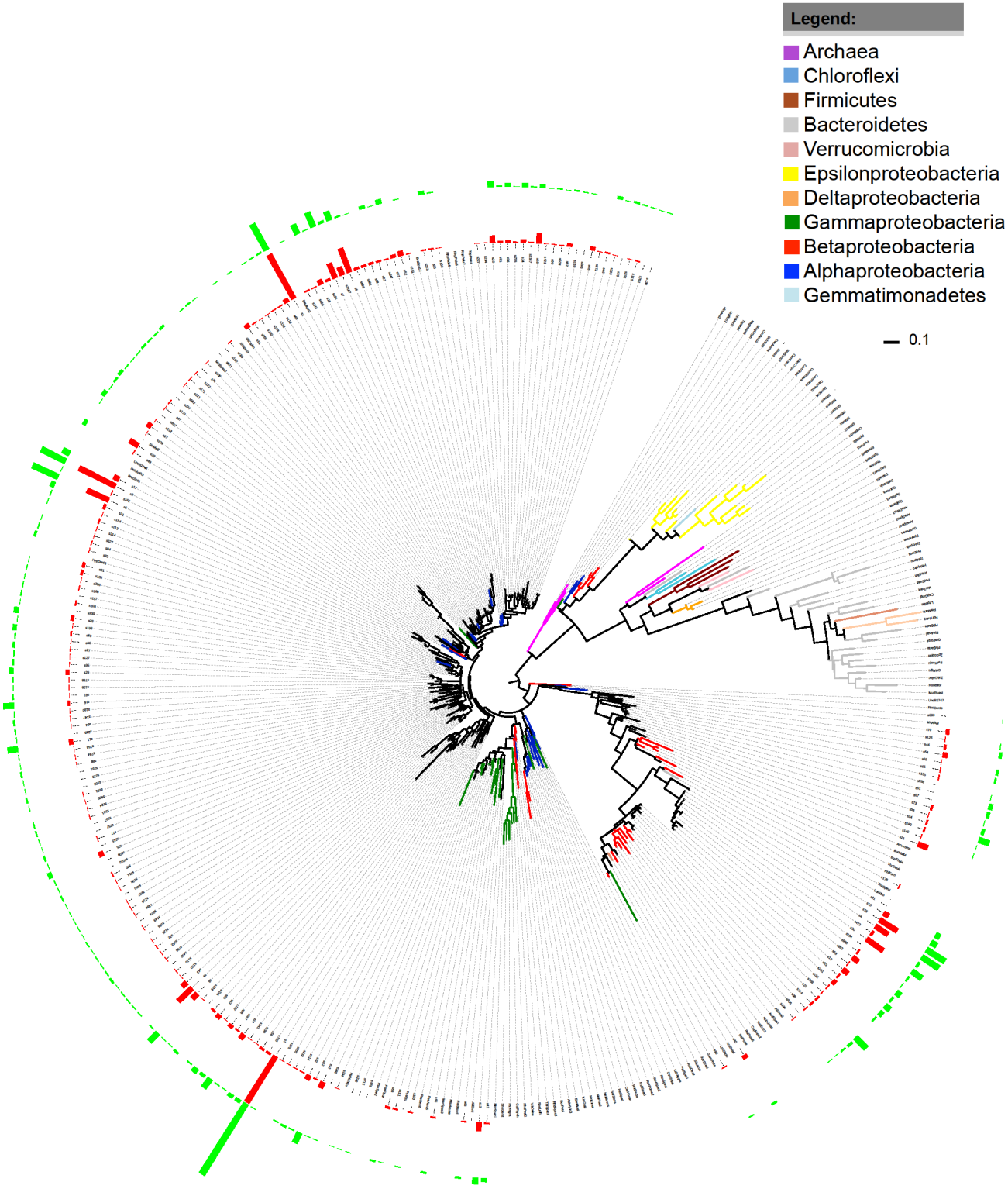


Figure S2.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Firmicutes
Bacteroidetes
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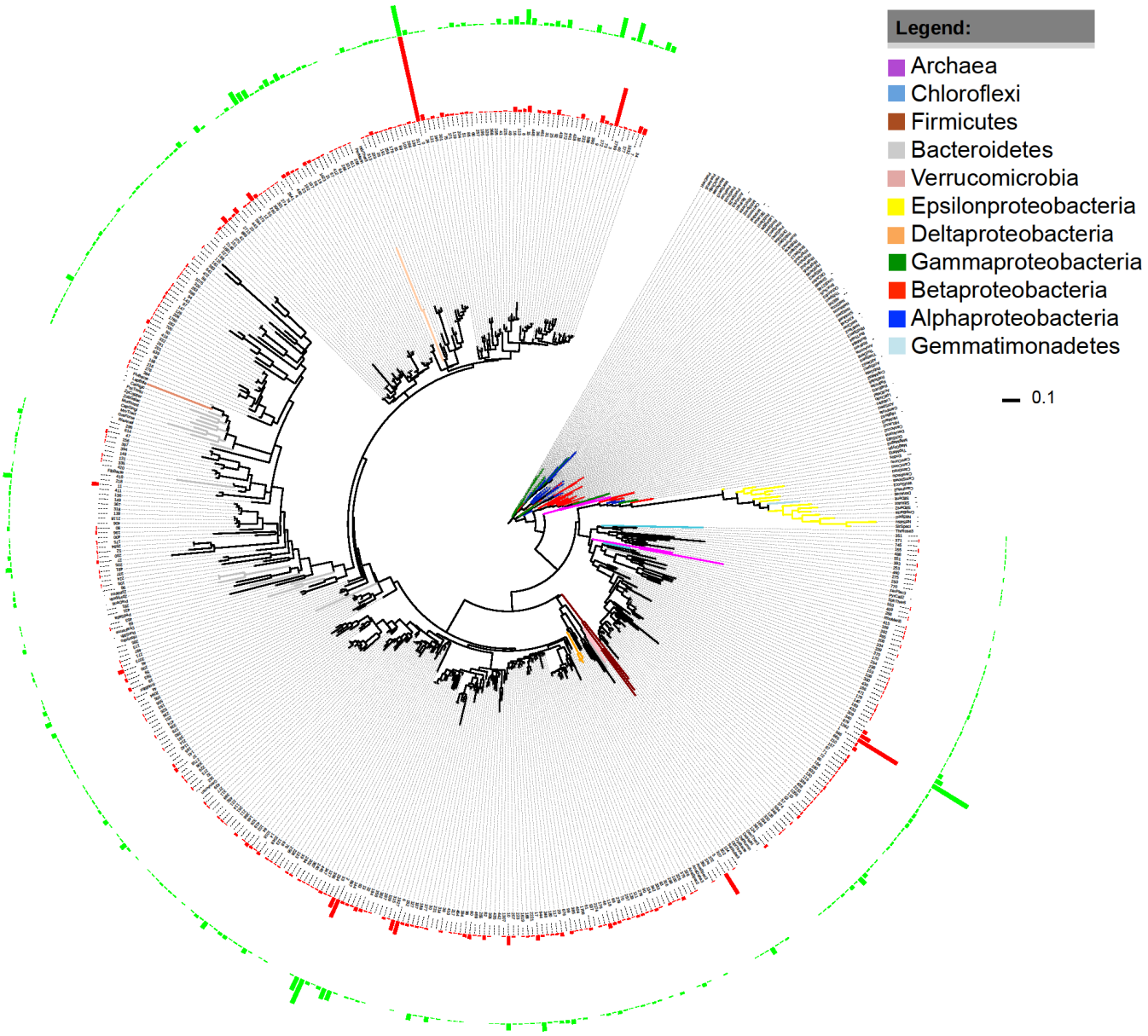
Betaproteobacteria
Alphaproteobacteria
Gemmatimonadetes
0.1

Figure S3


Legend:
Archaea
Chloroflexi
Firmicutes
Bacteroidetes
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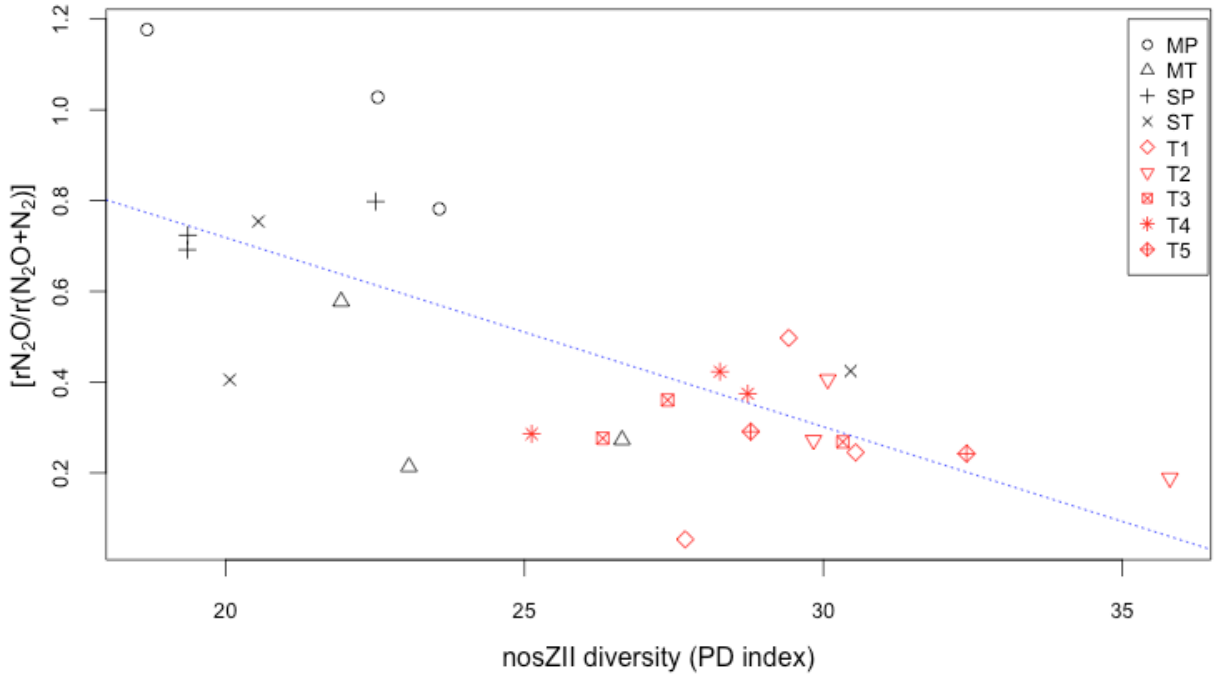
Epsilonproteobacteria
Deltaproteobacteria
Gammaproteobacteria
Betaproteobacteria
Alphaproteobacteria
Gemmatimonadetes
0.1

Figure S4


MP
MT
SP
ST
T1
T2
T3
T4
T5
[rN2O/r(N2O+N2)]
0.2
0.4
0.6
0.8
1.0
1.2
20
25
30
35
nosZII diversity (PD index)

1.2. Supplementary Tables

Table S1

Treatment	Description	% Water	pH	% Clay§	% Loam§	% Sand§	Total C*	Total N*	OM*	Ca*	cec
ML	Miscanthus (*M. giganteus*) late harvest	21.7^{c} ± 0.9	7.2^{ab} ± 0.5	20.2 ± 3.6	74 ± 3.3	5.6 ± 0.4	11.5 ± 1.0	1 ± 6.10^{-2}	19.9 ± 1.9	1.3 ± 0.6	11 ± 2
ME	Miscanthus (*M. giganteus*) early harvest	23^{bc} ± 0.1	7.4^{ab} ± 0.6	20.9 ± 1.7	73.5 ± 2.4	6.0 ± 1.3	10.9 ± 0.6	1 ± 4.10^{-2}	18.9 ± 1	2.3 ± 2.6	11 ±
SL	Switchgrass (*P. virgatum*) late harvest	23.3^{ab} ± 1.1	7.4^{ab} ± 0.5	21.9 ± 2.4	72.3 ± 1.3	5.7 ± 1.1	11.8 ± 0.8	1.1 ± 6.10^{-2}	20.5 ± 1.3	1.3 ± 0.6	12 ± 1
SE	Switchgrass (*P. virgatum*) late harvest	24.7^{abc} ± 0.6	6.8^{b} ± 0.1	20.1 ± 3.3	73.8 ± 2.8	6.0 ± 0.5	12 ± 0.4	1.1 ± 5.10^{-2}	20.7 ± 0.8	1 0	10 ± 1
T1	Conventional tillage, straw addition, N input, non-legume intercrop	25.1^{ab} ± 0.9	7.9^{a} ± 0.2	21.1 ± 4.0	74.1 ± 3.4	4.5 ± 0.5	10.7 ± 0.8	1 ± 4.10^{-2}	18.5 ± 1.4	3.0 ± 2.9	12 ±1
T2	Reduced tillage, straw addition, N input, non-legume intercrop	24.8^{ab} ± 0.5	7.9^{a} ± 0.3	17.7 ± 0.5	77.0 ± 0.06	4.9 ± 0.4	10.8 ± 0.2	1 ± 1.10^{-2}	18.6 ± 0.5	3.7 ± 2.8	10 ± 0
T3	Reduced tillage, straw removal, N input, non-legume intercrop	24.4^{ab} ± 1.0	7.9^{a} ± 0.2	20.5 ± 2.8	74.4 ± 3.2	4.7 ± 0.7	11.4 ± 0.7	1.1 ± 6.10^{-2}	19.7 ± 1.3	4 ± 5.8	12 ± 1
T4	Conventional tillage, straw addition, Low N input, non-legume intercrop	25.2^{a} ± 1.1	8^{a} ± 0.2	19.8 ± 3.3	75.1 ± 3.3	4.8 ± 0.2	10.7 ± 0.4	1 ± 5.10^{-2}	18.6 ± 0.8	3.3 ± 2.8	11 ± 1
T5	Conventional tillage, straw addition, Low N input, legume intercrop	25^{ab} ± 0.4	8.1^{a} ± 0.1	17.8 ± 1.6	77.1 ± 1.2	4.8 ± 0.5	10.7 ± 0.3	1 ± 5.10^{-2}	18.4 ± 0.6	3.4 ± 1.7	10 ± 1

Table S2

Treatment	*nosZI*					*nosZII*				
	OTUs	PD	chao1	Simpson reciprocal	Shannon	OTUs	PD	chao1	Simpson reciprocal	Shannon
ML	167 ± 21.3	9 ± 0.5	178 ± 19.7	24 ± 14.8	5 ± 0.5	322^{bc} ± 23.7	23^{abc} ± 2.7	464^{ab} ± 57.8	40^{c} ± 21.9	6^{c} ± 4.10^{-1}
ME	181 ± 44	11 ± 2.4	227 ± 94.7	30 ± 7.8	6 ± 4.10^{-1}	318^{bc} ± 37.6	21^{bc} ± 2.9	472^{ab} ± 94.4	33^{c} ± 11.3	6^{c} ± 3.10^{-1}
SL	163 ± 9	9 ± 0.4	196 ± 23.8	24 ± 6.6	5 ± 1.10^{-1}	315^{bc} ± 49.2	23^{abc} ± 6.6	461^{ab} ±159.5	52^{bc} ± 21.4	7^{bc} ± 4.10^{-1}
SE	138 ± 19	8 ± 1.2	165 ± 25.1	21 ± 13.0	5 ± 4.10^{-1}	294^{c} ± 31.1	20^{c} ± 2.05	457^{b} ± 67.7	34^{c} ± 5.2	6^{c} ± 2.10^{-1}
T1	161 ± 30	9 ± 0.6	191 ± 38.1	14 ± 5.0	5 ± 4.10^{-1}	373^{ab} ± 26.2	29^{ab} ± 1.6	558^{ab} ± 56.8	92^{ab} ± 10.4	7^{ab} ± 6.10^{-2}
T2	161 ± 4	9 ± 0.4	196 ± 23.9	18 ± 1.6	5 ± 7.10^{-2}	382^{ab} ± 29.0	31^{a} ± 3.8	547^{ab} ± 38.1	98^{a} ± 16.1	7^{ab} ± 1.10^{-1}
T3	176 ± 12.7	10 ± 1.1	203 ± 18.3	16 ± 1.4	5 ± 9.10^{-2}	368^{abc} ± 24.8	28^{abc} ± 2.3	526^{ab} ± 58.6	84^{ab} ± 18.6	7^{ab} ± 1.10^{-1}
T4	175 ± 35.1	10 ± 1.9	223 ± 58.5	18 ± 10.1	5 ± 5.10^{-1}	369^{abc} ± 18.6	27^{abc} ± 2.2	526^{ab} ± 21.2	93^{ab} ± 14.40	7^{ab} ± 1.10^{-1}
T5	179 ± 34.3	10 ± 1.1	237 ± 74.9	16 ± 0.3	5 ± 2.10^{-1}	404^{a} ± 14.1	30^{a} ± 2.1	621^{a} ± 7.1	110^{a} ± 20.9	7^{a} ± 8.10^{-2}

Table S3.

	Potential N_2O	PDA	$[rN_2O/r(N_2O+N_2)]$
Diversity nosZ clades	*nosZI*, *nosZII*	*nosZI*, *nosZII*	*nosZII*
Soil Physicochemical properties	water, pH, C/N, cec	sand, TN, C/N	sand, C/N
Abundance (qPCR)	q.nosZII	-	q.nirK, q.nosZI

5 Chapter III

Effects of agricultural practices and soil properties on soil N_2O-reducing bacteria and in situ N_2O emissions

Luiz A. Domeignoz-Horta

Céline Peyrard

David Bru

Marie-Christine Breuil

Florian Bizouard

Eric Justes

Bruno Mary

Jöel Léonard

Laurent Philippot

Aymé Spor

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TITLE: Effects of agricultural practices and soil properties on soil N_2O-reducing bacteria and *in situ* N_2O emissions

Domeignoz-Horta[1] L.A., Peyrard C.[2], Bru D.[1], Breuil M.C.[1], Bizouard F.[1], E. Justes[3], Mary B.[2], Léonard J.[2], Philippot L[1*], Spor A.[1]

[1]Agroécologie, AgroSup Dijon, INRA, Univ. Bourgogne Franche-Comté, F-21000 Dijon, France

[2] AgroImpact, INRA, UR1158, F-02000 Laon, France

[3]AGIR, INRA-ENSAT, UMR 1248, F-31000 Toulouse, France

*Corresponding author

laurent.philippot@dijon.inra.fr

Tel: 33 (0) 3 80 69 33 46

Fax: 33 (0) 3 80 69 32 24

Keywords: agroecosystems, nitrogen cycling, land-use, tillage, denitrification, nitrification, microbial diversity, greenhouse gas

Abstract

Agriculture is the main source of terrestrial N_2O emissions, a potent greenhouse gas and the main cause of ozone depletion. The reduction of N_2O into N_2 by microorganisms carrying the nitrous oxide reductase gene (*nosZ*) is the only biological process known to eliminate this greenhouse gas. Recent studies showed that a previously unknown clade of N_2O-reducers (*nosZII*) was related to the capacity of the soil to act as a N_2O sink. However little is known about how this group responds to different agricultural practices. Here, we investigated how N_2O-reducers were affected by agricultural practices and evaluated the consequences for N_2O emissions. The abundance of N_2O-reducers and N_2O producers both ammonia oxidizers and denitrifiers was quantified by real-time PCR, and the diversity of both *nosZ* clades was determined by 454 pyrosequencing. Denitrification and nitrification potential activities and *in situ* N_2O emissions were assessed. Overall, our results showed that the *nosZ*II clade of N_2O-

reducers was more sensitive to environmental factors and agricultural practices than the *nosZ*I clade. Tillage increased the diversity of this clade while it did not affect the diversity of the *nosZ*I clade. To better access the contribution of different factors to the *in situ* N_2O emissions we subdivided these into ranges from low to high rates. Interestingly, the low rate was only related to soil pH, while the high rates were also strongly related to the microbial communities. Particularly, the *nosZ*II clade abundance and diversity across agricultural systems were negatively correlated with the *in situ* N_2O emissions.

Introduction

Terrestrial ecosystems can not only release but also capture greenhouse gases (GHG). Most estimations of greenhouse gas sinks are accounting either for carbon sequestration to capture CO_2 or CH_4 but seldom for N_2O (Six et al., 2004; Chapuis-Lardy et al., 2007; Tian et al., 2016). Nevertheless, N_2O is an important potent GHG with a global warming potential (GWP) over 100 years of about 298 and 3 times that of CO_2 and CH_4, respectively. This gas is also the dominant ozone depleting substance after the suppression of CFCs by the Kyoto Protocol (Ravishankara et al., 2009). Anthropogenic nitrogen (N) input via industrial N_2 fixation to produce fertilizers, are responsible for doubling the natural rate of terrestrial nitrogen fixation (Canfield et al., 2010). As a result, N_2O concentrations are estimated to increase up to 60% by 2050 compared to the beginning of last century (Bouwman et al., 2013).

Most of N_2O is emitted from soils, with agricultural soils being the main source of anthropogenic N_2O emissions and are estimated to contribute to 59% of total global emissions by 2030 (Hu et al., 2015). The emissions of this GHG are, to a great extent, the result of microbially driven processes such as denitrification and nitrification (Snyder et al., 2009; Hu et al., 2015). Denitrification is also the only known sink for this GHG. This is a respiratory process during which soluble nitrogen oxides (NO_3- and NO_2-) are reduced into gaseous forms (NO, N_2O and N_2). The reduction of soluble NO_2^- to gaseous forms is catalyzed by the nitrite reductase encoded by the *nirK* or *nirS* genes. The other denitrification step important for N_2O-emissions is the reduction of N_2O to N_2, which is catalyzed by the N_2O reductase encoded by the *nosZ* gene. Denitrification is described as a modular process (Graf et al., 2014). Thus, some organisms are able to perform the complete pathway, while others either lack the N_2O reductase and therefore produce N_2O as final denitrification product (Philippot et al., 2011) or can only reduce N_2O without producing it (Sanford et al., 2012), and are

therefore a potential sink for this GHG. Recently a new N_2O reducing clade has been identified (Sanford et al., 2012; Jones et al., 2013), herein named *nosZII*, which is diverse and abundant in soils (Jones et al., 2014; Orellana et al., 2014; Domeignoz-Horta et al., 2015). An analysis of the sequenced genomes of *nosZ*II strains revealed that about half of them harbor the nitrous oxide reductase but none of the nitrite reductases, which highlight their potential importance in mitigating N_2O emissions (Graf et al., 2014). This assumption was confirmed by Jones et al. (2014), who showed that the abundance and diversity of the *nosZ*II community were the main drivers determining the soil N_2O sink capacity. Such results stressed the importance of understanding the response of this clade to environmental factors, and of identifying agricultural practices which could foster this clade as a possible N_2O mitigation strategy.

Several studies have investigated the influence of agriculture practices on microorganisms involved in N_2O production (Hallin et al., 2009; Kong et al., 2010; Bissett et al., 2014; Hartmann et al., 2015). Fertilization, which is a practice known for increasing N_2O emissions (Smith, 2007; Shcherbak et al., 2014), was also shown to affect the denitrifier community (Hallin et al., 2009; Clark et al., 2012). Other practices of importance in the climate change debate such as no-tillage (Six et al., 2004; Smith, 2007; Powlson et al., 2014) can influence both the N_2O emissions and the corresponding microbial communities (Six et al., 2004; Melero et al., 2011; Tatti et al., 2015). However, due to its recent identification, there is little information available concerning the effects of farming systems on the *nosZ*II community.

Here we assessed how the microbial communities responsible for N_2O sources both nitrifiers and denitrifiers) and sinks (N_2O-reducers) responded to different agricultural practices at different sites across France. We also investigated the relationships between these microbial communities, their corresponding potential activities and *in situ* N_2O fluxes. We hypothesized that soil properties and agricultural practices would differentially affect the *nosZ*I and *nosZ*II communities, and that the *nosZ*II community rather than the *nosZ*I community would be negatively related to *in situ* N_2O fluxes.

Material and Methods

Experimental Design and Sampling

Soil samples were collected in spring 2014 from 4 experimental sites: SOERE ACBB Grandes Cultures (49.522° N, 3.153° E), Biomass & Energy (B&E) (49.521° N, 3.047° E), Essay A (48.327° N, 2.381° E), and Mic-Mac (43.527° N, 1.506° E). The first two sites are located at Estrées-Mons, the third at Boigneville and the fourth at Auzeville, all being in France. The sites undergo various agricultural practices (Tab. 1). All experimental sites consist of three blocks, each comprising different treatments in randomized plots. Briefly, the MONS-SOERE experiment, which consisted in 6 treatments (T1–T6), was set up in 2010 to study the effect of soil tillage, crop residue management, fertilization rate and N-fixation by legumes on biogeochemical cycles and soil biodiversity (www.soere-acbb.com). The Mons-B&E experiment was set up in 2006 to compare the productivity and environmental impacts of various energy crop systems including perennial crops such as *Miscanthus giganteus* (M) and *Panicum virgatum* (Switchgrass, S) with differences in management practices such as early (E) or late harvest (L) and with N-fertilization (N) or without fertilization (Switchgrass only). The Auzeville experiment, which consisted in 6 treatments, was set up in 2010 to study the impact of reduced fertilization (Low Inputs and Very Low Inputs), intercropping (AS) and of the use of cover crops (C) (Tab. 1). The site in Boigneville was set up in 1970 to study the effect of tillage management with three different intensities: Full Inversion Tillage (FIT), Shallow Tillage (ST) and No-Tillage (NT) (Tab. 1).

Three replicate samples were collected for each treatment, each being a composite sample of five subsamples (soil cores of 2.5 cm by 20 cm) from each plot. Samples were frozen (−20°C) until further analysis. The physical and chemical soil characteristics were measured for all samples (INRA Laboratory of Soil Analysis, Arras, France) (Tab. 1).

Potential Denitrification Activity and Potential Nitrification Activity and *in situ* Fluxes

Potential denitrification activity (PDA) was measured using the acetylene inhibition technique as described by Yoshinari et al. (1977). For each sample,10 g of soil (fresh weight) was added in a flask with 20 ml of distilled water, 3 mM KNO_3, 1.5 mM succinate, 1 mM glucose, and 3 mM acetate (final concentrations). The flasks were then sealed, the atmosphere replaced by helium and acetylene, a specific inhibitor of the N_2O reductase was added to reach 0.1 atm

partial pressure. Flasks were incubated at 25°C and agitated at 175 rpm. Gas samples were taken every 30 min for 150 min (Pell et al., 1996). The N_2O concentrations were determined using a gas chromatograph (Trace GC Ultra, Thermo Scientific) equipped with an EC-detector. Potential nitrification activity (PNA) was performed according to ISO 15685. Briefly, 1.4 mM sulfate ammonium was added to 10g of fresh weight soil supplemented with 500 mM of sodium chlorate to block the oxidation of nitrite. Ammonium oxidation rates were determined in each sample by measuring the accumulated nitrite every 2 hours during 6 hours via a colorimetric assay (Kandeler, 1995). In addition to the potential activities, more than 70.000 measurements of *in situ* chambers were used to assess the N_2O emissions from the different treatments. These measurements were subdivided in percentiles corresponding to basal emissions (25%), median emissions (50%) and high emissions (75%, 90%, 95% and 99%).

DNA Extraction and Abundance of Microbial Communities

DNA extraction was performed from 0.25 g of soil from each replicate sample in accordance with the ISO 11063 (Petric et al., 2011). The DNA quality was checked by electrophoresis on agarose gel and quantified by spectrofluorometer using the Quant-iT PicoGreen® dsDNA Assay Kit (Invitrogen, Cergy-Pontoise, France) following the manufacturer's instructions.

Abundances of the total bacterial community and of microbial communities potentially involved in N_2O production or N_2O reduction were determined by real-time quantitative PCR (qPCR). The nitrification gene *amoA* and the denitrification genes *nirK* and *nirS* were used as molecular markers to quantify the bacterial (AOB) and thaumarchaeal (AOA) ammonia-oxidizing and the denitrifying communities, respectively (Bru et al., 2011). The *nosZ*I and *nosZ*II genes were used to target the N_2O-reducers (Jones et al., 2013). Abundance of total bacteria was assessed using 16S rDNA primers as previously described (Lopez-Gutierrez et al., 2004). QPCR Reactions were carried out in a Step One Plus (Life Technologies, Carlsbad, CA, USA) with 15µl reaction volume containing 1 ng of DNA, 7.5µl of SYBRgreen PCR Master Mix (Absolute QPCR SYBR GreenRox, Thermo, Courtaboeuf, France), 1 µM of each primer, 250 ng of T4 gene 32 (QBiogene, Illkrich, France). Three independent quantitative qPCR assays were performed for each gene. No-template controls giving null or negligible values were run for each quantitative qPCR assay. Inhibition in qPCR assay was tested by mixing soil DNA extracts with either control plasmid DNA (pGEM-T Easy Vector, Promega, France) or water. The measured cycle threshold (Ct) values obtained for the different DNA

extracts and the controls with water were not significantly different indicating that no inhibition occurred. The qPCR efficiencies for the various genes ranged between 70 and 94%.

Diversity of the N_2O-reducers

The diversity of *nosZ*I and *nosZ*II communities was analyzed by 454 pyrosequencing. Briefly, the amplicons were prepared using a two-step PCR procedure (Berry et al., 2011), In the first step 20 PCR cycles were used to amplify *nosZ*I and *nosZ*II primers (Jones et al., 2014). The second step used 4 µl from the first PCR and 15 PCR cycles were performed with barcoded primers. The second PCR for *nosZ*II was extended to 18 cycles because a small amount of products was produced during the first PCR. PCR products were gel purified and pooled using the QIAEX II kit (Qiagen; France). Pyrosequencing was performed by Genoscreen (Lille, France) on a Roche's 454 GS FLX+ Genome Sequencer according to manufacturer's instructions.

Sequence Processing

The QIIME pipeline (Caporaso et al., 2010) was used for quality trimming of raw 454 pyrosequencing data (QIIME version 1.8.0). The minimum sequence lengths were 230 and 410 bp for *nosZ*I and *nosZ*II, respectively. Sequences with an average score below 25 using a sliding window of 50 bp were discarded. Sequences were then processed using the 'pick_otus.py' script within QIIME, and the 'usearch' option (Edgar, 2010) with reference-based and *de novo* chimera checking, and clustering of sequences at 97% similarity. Raw sequences were deposited at the NCBI under the accession number (not finalized). The process of raw sequence submission was greatly simplified by using the *make.sra* command of Mothur software (Schloss et al., 2009).

Statistical Analysis

Statistical analysis were performed using the R statistical software (R Core Team, 2013) and the *agricolae* (Mendiburu, 2014) and *vegan* (Oksanen et al., 2015) packages. The effect of agricultural practices was determined by analysis of variance and post hoc Tukey HSD test. Non-metric MultiDimensional Scaling (NMDS) of the Unifrac distance matrices (unweighted

and weighted) was used to describe communities' structure. Ordinations with the lowest stress values were used. Permutation tests (n=10000) were used to test for the significance of soil properties, community abundances and diversity explanatory variables of communities' structure and only significant ones were depicted (P<0.05). ANalysis Of SIMilarity (ANOSIM) was used to test for significant differences in communities' structure between experimental sites (permutations=1999, P<0.05).

Results

Soil Chemical and Textural Properties

The four sites showed significant differences in soil chemical and textural properties (Tab. 1). None of the measured soil properties were however affected by the agricultural practices within a site except for the pH, which was significantly lower in the VLI cropping systems at Auzeville (Tab. 1).

Potential Denitrification Activity ($N_2O + N_2$), Potential Nitrification Activity and *in situ* N_2O emissions.

Differences between the four experimental sites were observed for both PDA and PNA. The highest PDA were observed for the two sites located in Mons with rates of 1.2 and 1.1 µg N_2O-N g^{-1} DW soil h^{-1} for SOERE et B&E respectively. Average PDA rates at Auzeville and Boigneville ranged between 0.3 and 0.5 µg N_2O-N g^{-1} DW soil h^{-1} soil (Fig. S1). The SOERE and B&E also showed higher PNA with average rates of 0.3 and 0.2 µg NO_2-N g^{-1} DW soil h^{-1}, respectively (Fig. S2). A much lower PNA rate of 0.01 µg NO_2-N g^{-1} DW soil h^{-1} was observed at Auzeville. No significant difference between treatments within a site was observed for both potential activities.

In contrast, *in situ* N_2O fluxes, which were estimated by more than 70 000 measurements, were impacted by a few agricultural practices. For instance, time of harvest influenced emissions at Mons-B&E, with early harvest showing lower fluxes than late harvest for both bioenergy crops. The use of cover crops also decreased N_2O emissions at Auzeville. At Boigneville, tillage management had no significant impact despite ST showing higher emissions than NT and FIT for all percentiles but 25% (Fig 1). Fertilization had no influence on *in situ* N_2O emissions at Auzeville (Fig. 1).

Abundance of Total Bacteria, Archaea, N_2O-producers and N_2O-reducers

In all soil samples, AOA were more abundant than AOB, with AOA/AOB ratios ranging from 7 up to over 40 in the AS treatment at Auzeville. Similarly to the total bacterial and archaeal communities, only significant differences between sites but not between treatments within a site were observed for both AOA and AOB, with the lowest abundances observed in Auzeville (Fig. S3). Neither the abundances of denitrifiers N_2O producers (Fig. S4) nor of N_2O reducers (Fig. S5) were affected by agricultural practices. However, contrasted trends were observed for the two clades of N_2O reducers, with the abundance of the *nosZ*I community decreasing with tillage intensity while the *nosZ*II community remained stable (Fig S5).

Table 1. **Soil chemical and textural properties.** SOM represent the soil organic matter.

Site	Treatment	Management[1]	pH	% Clay[2]	% Loam[2]	% Sand[2]	SOM[3]	Total-C[3]	Total-N[3]	C:N	CEC[4,5]	% Water
Auzeville	LI	Low N input	8.1^{a} ± 0.2	30.2^{abc} ± 1.6	36.7^{e} ± 1.1	30.7^{a} ± 6.1	16.4^{bcd} ± 2.7	9.5^{bcd} ± 1.6	1.0^{abcd} ± 0.2	9.2 ± 0.5	18.4^{abc} ± 3.5	17.2^{bc} ± 0.4
	LI.C	Low N input & CC	7.4^{abcd} ± 1.0	30.6^{abc} ± 3.7	38.1^{e} ± 1.0	29.2^{a} ± 7.8	16.7^{cd} ± 3.2	9.7^{abcd} ± 1.9	1.0^{cd} ± 0.2	9.4 ± 0.1	16.8^{abcd} ± 4.5	18.2^{abc} ± 1.2
	VLI	Very low N input	6.3^{cd} ± 0.4	29.4^{abcd} ± 1.6	35.6^{e} ± 2.9	34.9^{a} ± 2.0	14.3^{d} ± 1.4	8.3^{d} ± 0.8	0.9^{d} ± 0.1	9.1 ± 0.4	14.6^{abcde} ± 2.5	15.2^{c} ± 1.9
	VLI.C	Very low N input & CC	6.5^{cd} ± 0.3	29.0^{abcd} ± 2.6	35.5^{e} ± 1.6	35.3^{a} ± 1.3	15.5^{cd} ± 1.9	9.0^{cd} ± 1.1	1.0^{bcd} ± 0.1	9.3 ± 0.3	14.5^{abcde} ± 1.2	15.6^{c} ± 1.1
	AS	Intercropping	8.1^{a} ± 0.3	32.8^{a} ± 2.4	35.9^{e} ± 3.0	30.5^{a} ± 6.3	17.1^{abcd} ± 2.3	9.8^{abcd} ± 1.3	1.0^{abcd} ± 0.2	9.5 ± 0.3	20.5^{a} ± 2.7	16.7^{bc} ± 3.2
	AS.C	Intercropping & CC	7.7^{ab} ± 0.7	32.5^{ab} ± 2.4	35.6^{e} ± 3.4	29.8^{a} ± 9.2	17.1^{abcd} ± 1.5	9.9^{abcd} ± 0.9	1.1^{abcd} ± 0.1	9.3 ± 0.1	19.6^{ab} ± 3.8	16.6^{bc} ± 3.5
Mons - B&E	MLN	Miscanthus late harvest & N input	7.2^{abcd} ± 0.6	20.2^{e} ± 3.6	74.1^{abc} ± 3.4	5.6^{b} ± 0.4	19.9^{ab} ± 1.9	11.5^{abc} ± 1.1	1.1^{abcd} ± 0.1	10.6 ± 0.4	11.2^{de} ± 3.0	20.6^{abc} ± 1.0
	MEN	Miscanthus early harvest & N input	7.4^{abc} ± 0.6	20.9^{e} ± 1.8	72.8^{abcd} ± 2.2	6.0^{b} ± 1.3	18.9^{abc} ± 1.1	10.9^{abc} ± 0.7	1.1^{abcd} ± 0.0	10.2 ± 0.2	11.6^{de} ± 1.1	19.5^{abc} ± 0.7
	SLN	Switchgrass late harvest & N input	7.4^{abc} ± 0.6	21.9^{de} ± 2.5	72.3^{abcd} ± 1.4	5.7^{b} ± 1.1	20.5^{a} ± 1.3	11.8^{ab} ± 0.8	1.2^{abc} ± 0.1	10.3 ± 0.2	12.3^{cde} ± 1.1	22.6^{a} ± 2.2
	SEN	Switchgrass early harvest & N input	6.8^{bcd} ± 0.1	20.1^{e} ± 3.4	73.8^{abc} ± 2.9	6.0^{b} ± 0.5	20.7^{a} ± 0.9	12.0^{ab} ± 0.5	1.1^{abcd} ± 0.1	10.8 ± 0.2	10.8^{e} ± 1.8	18.3^{abc} ± 1.5
	SL	Switchgrass late harvest & no N input	7.1^{abcd} ± 0.5	20.9^{e} ± 1.8	73.6^{abcd} ± 1.8	5.5^{b} ± 0.0	21.4^{a} ± 1.2	12.4^{a} ± 0.7	1.1^{abcd} ± 0.1	10.9 ± 0.0	13.0^{abcde} ± 1.8	20.1^{abc} ± 1.2
	SE	Switchgrass early harvest & no N input	7.3^{abc} ± 0.5	20.4^{e} ± 5.2	74.1^{abc} ± 4.9	5.4^{b} ± 0.8	19.6^{ab} ± 0.8	11.4^{abc} ± 0.5	1.1^{abcd} ± 0.1	10.8 ± 0.2	12.7^{cde} ± 2.8	17.6^{abc} ± 0.6
Mons - SOERE	T1	High N input, full Inversion tillage, straw return & nonlegume as CC	7.9^{a} ± 0.3	21.1^{e} ± 4.1	74.1^{a} ± 3.4	4.5^{b} ± 0.6	18.5^{abc} ± 1.4	10.7^{abcd} ± 0.8	1.1^{abcd} ± 0.0	10.1 ± 0.4	12.5^{cde} ± 2.0	20.2^{abc} ± 0.8
	T2	High N input, shallow tillage, straw return & non legume as CC	7.9^{a} ± 0.3	17.7^{e} ± 0.5	77.0^{a} ± 0.1	4.9^{b} ± 0.5	18.6^{abc} ± 0.5	10.8^{abcd} ± 0.3	1.1^{abcd} ± 0.0	10.3 ± 0.3	10.5^{e} ± 0.5	21.8^{ab} ± 0.8
	T3	High N input, shallow tillage, straw export & non legume as CC	7.9^{a} ± 0.3	20.5^{e} ± 2.8	74.4^{abc} ± 3.2	4.7^{b} ± 0.7	19.7^{ab} ± 1.4	11.4^{abc} ± 0.8	1.1^{abcd} ± 0.1	10.4 ± 0.4	12.1^{cde} ± 1.5	18.8^{abc} ± 0.5
	T4	Low N input, full inversion tillage, straw return & non legume as CC	8.0^{a} ± 0.2	19.8^{e} ± 3.3	75.1^{ab} ± 3.4	4.8^{b} ± 0.2	18.6^{abc} ± 0.9	10.7^{abcd} ± 0.5	1.0^{abcd} ± 0.1	10.4 ± 0.2	11.7^{de} ± 2.0	20.9^{abc} ± 3.4
	T5	Low N input, full inversion tillage, straw return & legume as CC	8.1^{a} ± 0.1	17.8^{e} ± 1.7	77.1^{a} ± 1.3	4.8^{b} ± 0.6	18.4^{abc} ± 0.7	10.7^{abcd} ± 0.4	1.0^{abcd} ± 0.1	10.3 ± 0.5	10.4^{e} ± 1.0	21.8^{abc} ± 2.5
	T6	Switchgrass & no N added	8.2^{a} ± 0.1	17.5^{e} ± 3.0	76.5^{a} ± 3.4	5.7^{b} ± 0.5	17.9^{abcd} ± 1.4	10.4^{abcd} ± 0.9	1.0^{abcd} ± 0.1	10.6 ± 1.4	11.7^{de} ± 1.6	20.1^{abc} ± 2.4
Boingville	FIT	Full inversion tillage	6.6^{cd} ± 0.4	25.1^{bcde} ± 2.7	66.4^{d} ± 1.6	8.4^{b} ± 1.1	20.4^{ab} ± 3.6	11.8^{abc} ± 2.5	1.1^{ab} ± 0.2	10.6 ± 0.7	15.6^{abcde} ± 2.6	20.1^{abc} ± 0.4
	ST	Shallow tillage	6.2^{d} ± 0.2	25.0^{bcde} ± 1.7	67.2^{cd} ± 1.9	7.8^{b} ± 0.4	19.8^{ab} ± 1.1	11.4^{ab} ± 0.6	1.0^{abc} ± 0.0	10.8 ± 0.3	13.4^{abcde} ± 1.2	17.1^{bc} ± 1.9
	NT	No tillage	6.2^{d} ± 0.2	24.1^{cde} ± 0.5	68.1^{bcd} ± 1.8	7.8^{b} ± 1.5	23.1^{ab} ± 1.2	13.4^{abc} ± 0.7	1.1^{a} ± 0.0	11.2 ± 0.4	12.8^{bcde} ± 0.5	19.7^{abc} ± 3.5

Means and 95% confidence interval of soil physicochemical properties are given per treatment within each experimental block. Significant differences between treatments are indicated with different letters: "a", "b", and "c" (anova followed by Tukey HSD test, $P < 0.05$). [1]CC represent cover crop, "*Late" and "early" harvest correspond to February and October, respectively*; [2]*Size fractions based on* µm *Atterberg scale; Clay = 2µm, Loam = 2-50µm, and Sand = 50-2000µm;* [3]*g kg^{-1};* [4]*Cation exchange capacity,* [5]*cmol kg^{-1}.*

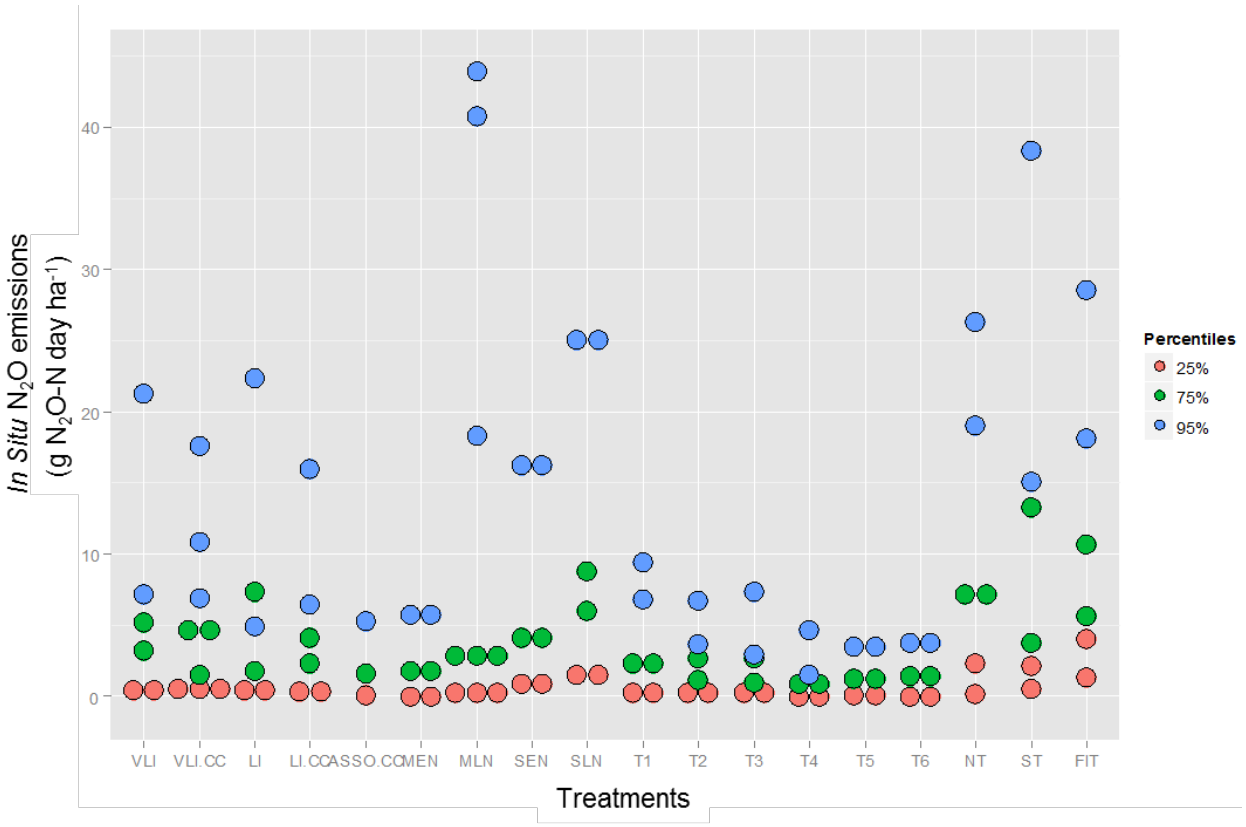

Fig. 1. ***In situ*** **N_2O emissions.** Representation of low emissions range (25%) and high emissions range (75 and 95%).

N_2O-reducers Diversity

To assess the influence of agricultural practices on the composition and structure of N_2O-reducers, the diversity of *nosZ*I and *nosZ*II communities was determined by 454 pyrosequencing. Clustering based on similarity of sequences (97%) gave an average of 113 ($CI_{95\%}$= 106 - 120), 103 ($CI_{95\%}$= 94 - 112), 94 ($CI_{95\%}$= 89 – 99), and 119 ($CI_{95\%}$= 107 – 131) OTUs for *nosZI* at Auzeville, Mons-B&E, Mons-SOERE and Boigneville, respectively (Tab. 2). The *nosZ*II *clade* was more diverse with 224 ($CI_{95\%}$= 206 - 242), 226 ($CI_{95\%}$= 209 - 243), 241 ($CI_{95\%}$= 231 - 251), and 202 ($CI_{95\%}$= 174 - 228) OTUs for Auzeville, Mons-B&E, Mons-SOERE and Boigneville, respectively. Among the studied agricultural practices, only tillage had a significant effect on the *nosZ*II community only. Thus, tillage intensity significantly increased the diversity of *nosZ*II, with a PD of 25.8 in the FIT treatment compared to 19.4 and 20.4 in ST and NT treatments (Tab. S1). Both OTUs richness and chao1 were also significantly higher in FIT than in NT for the *nosZ*II community (Tab. S1). We tested for clustering of samples by sites using an ANOSIM. It showed an *R* coefficient equal to 0.30 and

0.67 for *nosZ*I and *nosZ*II, respectively (P<0.001), which indicates that the clustering of samples by site is significant for both clades but higher for the *nosZ*II clade (Fig. 2).

Soil Properties, Abundance and Diversity of Microbial Communities in Relation to the Potential Activities and *in situ* N_2O emissions

Soil properties were correlated both to PDA and PNA. pH was positively correlated to both potential activities while a negative relationship was found for clay (Tab. S3). Soil organic matter (SOM) was also positively related to PDA, but not PNA. Ammonia oxidizing archaea (AOA) but not their bacterial counterpart was significantly related to PNA. Similarly, the abundances of *nirK*-, *nirS*-, *nosZ*I- and *nosZ*II-communities were positively related to PDA (Tab. S3).

The pH was negatively related to the the entire range of *in situ* N_2O emissions. Interestingly, it was the only variable related to the lowest emissions' range (25%). The abundance of the AOA and to a lower extent of *nirK*- and *nosZ*II-communities were also negatively related to the *in situ* emissions. Thus, AOA was related to all ranges of *in situ* N_2O emissions except for the lowest range, while *nirK* related to 75% and 90% percentiles and *nosZ*II only to the 75% percentile. The diversity of the *nosZ*II-community estimated using the Simpson reciprocal index, but not that of the *nosZ*I-community, was negatively related to i*n situ* N_2O emissions for all percentiles higher than 50% (Tab. S4).

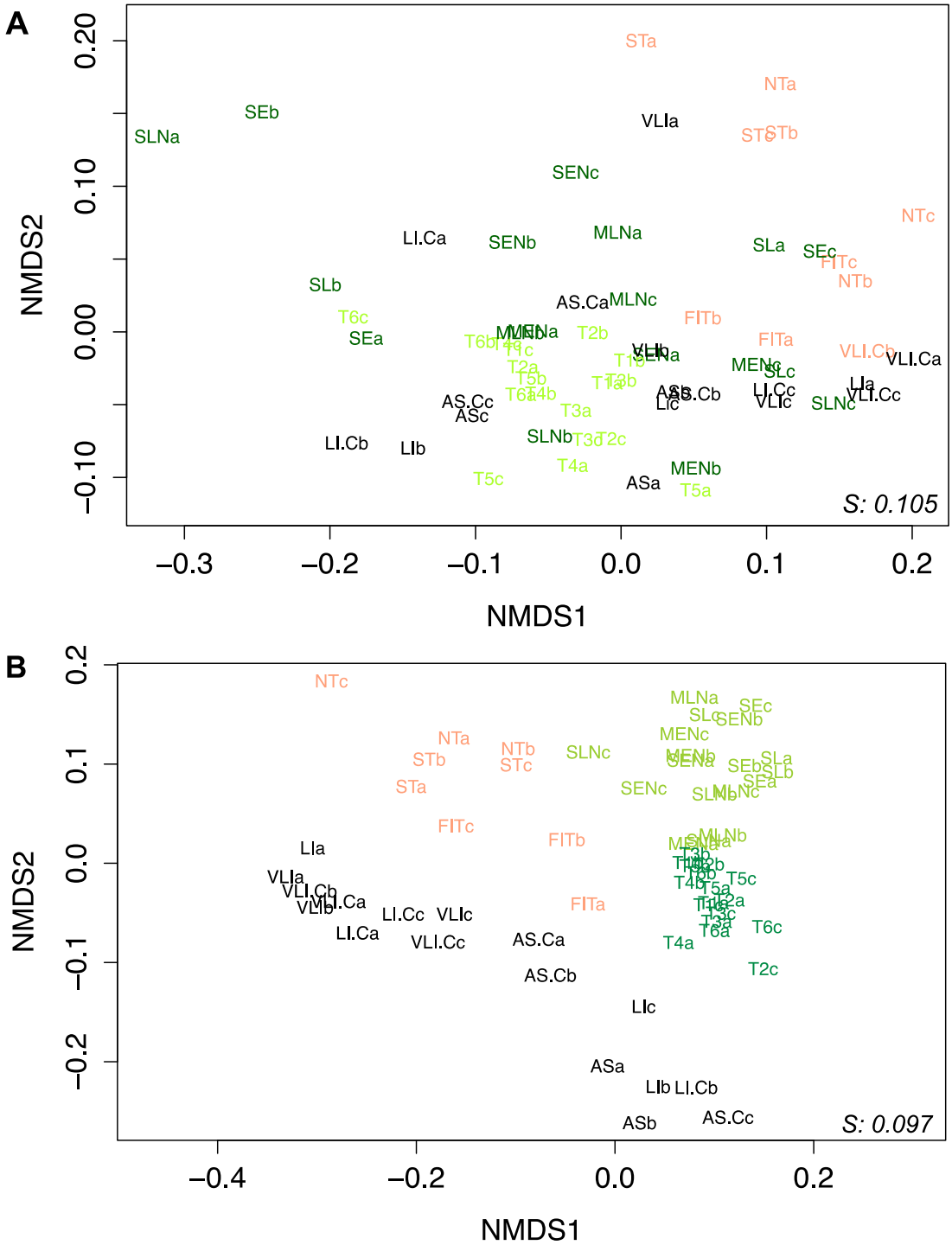

Fig. 2. **NMDS ordinations of *nosZ* weighted unifrac distance matrices. (A)** Variation in *nosZ*I community structure. **(B)** Variation in *nosZ*II community structure. Samples for each sites are represented in different colors: Auzeville (black), Boigneville (salmon), Mons - B&E (light green) and Mons - SOERE (dark green). Stress values are indicated at the bottom right of each panel.

Discussion

Agricultural Practices Influence on Potential Activities and *in situ* N_2O Emissions

Comparison of agricultural practices showed an effect of the harvesting date on *in situ* N_2O emissions at Boigneville with higher N_2O emissions at late than at early harvest for both perennial crops (*i.e*. Miscanthus and Switchgrass) (Fig. 1). Contrarily to late harvest, early harvest in autumn prevents so-called "biomass losses" due to leaf senescence in winter. The leaf litter in late harvest treatment represents a significant carbon input (Amougou et al., 2011, 2012) and creates a mulch layer, which increases soil moisture by reducing soil evaporation. Such conditions are known to be favorable to N_2O emissions by denitrification. However, we did not find significant differences in PDA between harvesting dates. Such discrepancy is likely explained by the fact that PDA reflects the denitrification enzymatic pool at the moment of sampling while *in situ* N_2O emissions were monitored over 3 years. Differences between early and late harvest treatments could also affect the N_2O:N_2 denitrification end product ratio rather than PDA as previously observed (Domeignoz-Horta et al. 2015).

No effect of the tillage regime on N_2O fluxes, PDA and PNA was observed in our study (Fig. 1). There is no consensus regarding the tillage effect on N_2O emissions. While some studies showed that no-till or reduced tillage promotes N_2O emissions (Baggs et al.2003, Smith et al., 2008), others reported on the contrary that tillage increased N_2O emissions (Robertson et al., 2000; Chatskikh et al, 2007) or even that it had no influence (Marland et al., 2001). The effect of tillage can also depend on no-till duration and climate conditions (Six et al., 2004; van Kessel et al., 2013) and soil type (Rochette et al., 2008).

Responses of N_2O-producing and N_2O-reducing Microbial Communities to Agricultural Practices

None of the studied agricultural practices resulted in significant shifts in the abundances of N_2O producers (*nirS*- and *nirK*- denitrifiers and AOA and AOB) or N_2O reducers (*nosZ*I or *nosZ*II). There are large discrepancies between the results reported in different studies investigating the responses of N-cycling communities to agricultural practices. Thus, abundances of ammonia-oxidizers or of denitrifiers were shown to be differently affected by the fertilization regime (Hallin et al., 2009; Cui et al. 2015; Sun et al. 2015). All types of mineral and organic fertilizers affected the *nirK*-community while the *nirS*-community

showed a negative response only to the ammonium sulfate and sewage sludge treatments (Hallin et al., 2009). Similarly, among the four fertilization treatment used, Cui et al. (2015) observed that *nirS*-, *nirK*- and *nosZ*-communities only increased in the two treatments fertilized with pig manure organic alone or in combination with mineral fertilizers. Other practices such as direct seeding, mulch-based cropping and weed management were shown to affect the abundance of denitrifier communities (Baudouin et al. 2009; Gulden et al., 2015). The lack of significant differences in the abundance of N-cycling communities in our study could be due to very small shifts in soil properties by the studied practices at our experimental sites (Tab. 1). However, we did found that the diversity of the *nosZ*II clade was significantly increased by tillage intensity at the Boigneville site (Tab. 2). A similar tendency of increasing *nosZII* diversity with increasing tillage was observed at Mons – SOERE between the treatments T1 and T2. Tillage *per se* consist of soil inversion, which causes changes on the redistribution of plant residues in deeper soil horizons, and may change or not soil properties as aggregate size and porosity (Chan et al., 2003; Strudley et al., 2008; Govaerts et al., 2009). These changes may impact soil water flow and aeration, which can also influence the microorganisms. Despite there not being consensus in the literature whether no till may lead to increase carbon sequestration in soil (Six et al., 2004; Angers et al., 2008), it has been proposed as a strategy for climate change mitigation (Lal et al., 2004; Smith et al., 2007; Smith et al., 2008). While our study was not designed to identify which changes in soil properties due to tillage were driving the studied community, our results suggest that full tillage rather than NT or ST fosters the *nosZ*II community, which can potentially act as a soil N_2O sink.

Soil Properties Influence on N_2O Producing and Reducing Microbial Communities and their Activities

Comparison of all plots across the four different experimental sites showed that pH was significantly related to PDA and PNA (Tab 3) and *in situ* N_2O emissions (Tabs 3 and 4). Accordingly, a large body of literature highlighted the importance of soil pH for nitrification, denitrification and N_2O fluxes (Šimek and Cooper, 2002; Hallin et al., 2009). A significant negative relationship was also observed between clay and both potential activities, while SOM was only positively related to PDA. Decreases in N-cycling activities as soil clay content increases has been previously reported and attributed to the fact that clay can protect

organic N from breakdown and also fix mineralized-N in the form of ammonium (Fortuna et al., 2016).

Our results confirm previous work suggesting that the recently discovered clade of N_2O reducers is more sensitive to environmental factors than *nosZ*I (Jones et al., 2014; Domeignoz-Horta et al., 2015) (Fig. 2A-B). Thus, the analysis of the structure of the N_2O reducing community shows a stronger cluster by site for *nosZ*II than for *nosZ*I. Common explanatory variables for *nosZ*I and *nosZ*II community structure were pH, AOA, C/N ratio, while sand, total N, cec (cation exchange capacity) only correlated to the *nosZ*II community structure (Tab. S2). These results confirm the existence of niche differentiation between these two clades as previously suggested (Domeignoz-Horta et al., 2015; Graf et al., 2016).

Relationships between N_2O-producing and reducing microbial communities, their corresponding potential activities and *in situ* N_2O fluxes

Abundances of N_2O-producers and N_2O-reducers as well as the diversity of the *nosZ*I-community were positively related to the PDA, which is in accordance with previous works (Hallin et al., 2009; Petersen et al., 2012; Jones et al., 2014) (Tab. 3). Due to their recent identification, only a few studies have investigated the ecology of the *nosZ*II-community and even less in relation to their activities (Orellana et al., 2014; Graf et al., 2016). In an earlier work, Jones et al. (2014) showed that both the diversity of the abundance of the *nosZ*II-community were important drivers of the soil N_2O sink capacity. More recently, we found that the diversity of *nosZ*II community was the strongest predictor of the N_2O:N_2 ratio measured by potential activity assays (Domeignoz-Horta et al., 2015) while it did not explain variation in PDA. This might be due to the fact that about 51% of the bacterial genomes having *nosZII* lack either *nirK* and *nirS* and are therefore contributing only to N_2O reduction but not to PDA (Graf et al., 2014). We also found that potential nitrification activity was significantly related to the abundance of AOA but not to AOB. The relative contribution of bacteria and archaea to the first step of nitrification is still unclear despite an increasing body of literature (Lu et al., 2015; Stergren et al., 2015; Wang et al., 2015). It has been proposed that both groups of ammonia oxidizers have different ecological niches and that no single factor can discriminate these two groups (Prosser and Nicol, 2012). For example, AOB are described as being more sensitive to low pH than AOA while AOA for having a higher affinity for ammonium concentrations than AOB (Di et al., 2010). Similarly to our findings,

several studies showed a predominant role of AOA in soil nitrification (Schauss et al., 2009; Gubry-Rangin et al., 2010; Verhame et al., 2011;Zhang et al., 2012).

When analyzing the relationships between the studied microbial communities and measurements of about 70.000 *in situ* N_2O emissions across all sites, we found that the diversity of the *nosZ*II-community was negatively related to the N_2O emissions for all percentiles higher than 50%. A negative relationship between the *nosZ*II-community and *in situ* emissions was also observed for the 75% percentile only. Morales et al. (2010) previously reported a positive correlation between N_2O emissions and the *nirS* gene abundance minus the *nosZI* gene abundance. However, to our knowledge, our study is the first one showing a link between *in situ* N_2O fluxes and the newly identified *nosZ*II-community. This strengthen the hypothesis that the *nosZII*-community can act as a N_2O sink as suggested both by soil surveys (Jones et al. 2014; Domeignoz-Horta 2015) and by comparative genomics indicating that *nosZII* bacteria are often genetically capable to reduce N_2O but not to produce it (Graf et al., 2014). Accordingly, Domeignoz-Horta et al. (2016) demonstrated in a manipulation experiment a direct correlation between the abundance of N_2O reducers and N_2O production. Thus, addition of a *Dyadobacter fermetans*, a *nosZ*II strain, significantly reduced N_2O production by denitrification of up to 189% in more than 1/3 of the studied soils. Interestingly, we also found that *in situ* N_2O emissions were negatively related to the abundance of AOA (Tab. S4). This could be due to lower emissions of N_2O by AOA than by AOB during ammonia oxidation in soil (Hink et al., 2016). This hypothesis is supported by our results showing a correlation between PNA and AOA but not AOB. We also found that the lowest range of N_2O emissions (25%) was only related to soil pH, and not to the communities. This suggests that these "baseline" emissions are more dependent on soil properties than on microorganisms.

Conclusions

The recent discovery of the overlooked *nosZ*II clade of N_2O-reducers, which are related to the soil N_2O sink capacity (Jones et al., 2014), opened up new possibilities to mitigate the emissions of this greenhouse gas by, for example, selecting agricultural practices fostering this clade. Collectively, our results highlight the higher sensitivity of the *nosZ*II- than the *nosZ*I-community to environmental factors. However, despite significant variations in the *nosZ*II community across the studied sites, only a few of the assessed agricultural practices resulted in shifts in either the abundance or the diversity of this community. Thus, tillage

regime had the stronger impact on the *nosZ*II community with increasing diversity as tillage intensity increases. Nevertheless, comparison of all plots across the different sites indicates for the first time that a higher abundance or diversity of the *nosZ*II community was concomitant with lower *in situ* fluxes, which confirms the suggested importance of this guild for decreasing N_2O emissions.

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Supplemental Material

Tab. S1. Diversity of the two *nosZ* clades. Means and 95% confidence interval of diversity indices for both *nosZ* clades are given per treatment within each site. Significant differences between treatments are indicated with different letters (anova followed by Tukey HSD test, $P<0.05$).

		***nosZ*I**				***nosZ*II**			
Site	Treatment	OTUs	Chao1	PD	Simpson reciprocal	OTUs	Chao1	PD	Simpson reciprocal
Auzeville	LI	114 ± 10	135.3 ± 7.9	8.7 ± 0.7	24.4 ± 9.3	242 ± 68	339.2 ± 103.6	21.9 ± 2.2	30.5 ± 31.5
	LI.C	113 ± 4	142.1 ± 17.7	8.3 ± 0.4	20.0 ± 12.8	230 ± 5	328.0 ± 34.1	21.2 ± 2.9	23.7 ± 6.9
	VLI	115 ± 34	134.6 ± 39.5	8.7 ± 2.8	25.2 ± 14.2	185 ± 39	252.0 ± 47.7	18.0 ± 5.2	24.3 ± 26.7
	VLI.C	112 ± 11	137.9 ± 15.8	8.9 ± 1.4	29.4 ± 3.2	197 ± 40	263.0 ± 58.5	21.0 ± 4.2	26.0 ± 23.9
	AS	106 ± 22	125.6 ± 32.4	8.2 ± 1.0	14.4 ± 7.4	256 ± 14	351.3 ± 20.4	23.9 ± 3.8	26.9 ± 3.1
	AS.C	122 ± 28	147.8 ± 36.1	9.0 ± 1.8	22.1 ± 7.2	238 ± 17	299.3 ± 35.7	22.6 ± 2.9	52.4 ± 20.9
Mons B&E	MLN	94 ± 20	108.6 ± 17.9	7.3 ± 1.2	19.1 ± 7.5	224 ± 44	319.8 ± 72.2	22.4 ± 4.8	16.6 ± 6.4
	MEN	92 ± 6	108.6 ± 15.5	7.3 ± 0.1	19.7 ± 3.8	233 ± 34	344.6 ± 18.2	22.9 ± 3.0	19.5 ± 12.5
	SLN	100 ± 11	131.1 ± 49.2	8.2 ± 1.2	11.0 ± 4.2	216 ± 2	328.6 ± 7.1	22.2 ± 2.0	18.6 ± 7.7
	SEN	106 ± 34	127.4 ± 46.6	8.4 ± 2.2	27.6 ± 14.2	197 ± 14	308.1 ± 58.2	20.5 ± 0.7	11.8 ± 4.2
	SL	123 ± 33	153.0 ± 46.7	9.1 ± 2.3	26.1 ± 15.7	248 ± 83	393.4 ± 104.5	24.4 ± 7.3	19.9 ± 14.3
	SE	105 ± 16	122.4 ± 27.4	8.2 ± 1.0	15.5 ± 7.2	238 ± 38	368.7 ± 25.6	23.1 ± 3.2	16.0 ± 9.8
Mons SOERE	T1	97 ± 7	124.4 ± 20.3	7.7 ± 0.2	16.9 ± 4.7	249 ± 8	387.4 ± 26.1	22.4 ± 0.5	29.1 ± 7.3
	T2	88 ± 14	100.3 ± 15.5	7.2 ± 0.7	15.8 ± 3.0	261 ± 17	354.2 ± 45.0	22.7 ± 1.2	41.0 ± 15.2
	T3	89 ± 13	103.3 ± 16.3	7.4 ± 0.8	19.6 ± 8.5	235 ± 18	374.2 ± 80.1	22.3 ± 1.5	26.6 ± 8.8
	T4	91 ± 10	119.8 ± 16.3	7.5 ± 0.8	17.2 ± 7.24	227 ± 22	319.1 ± 46.6	20.2 ± 0.7	25.2 ± 2.3
	T5	97 ± 23	135.4 ± 11.6	7.7 ± 1.2	16.78 ± 2.03	242 ± 28	370.3 ± 82.1	21.4 ± 2.8	32.9 ± 14.5
	T6	105 ± 16	126.9 ± 12.2	8.1 ± 1.0	21.5 ± 7.4	230 ± 47	334.4 ± 84.9	20.5 ± 2.4	35.7 ± 19.8
Boigneville	FIT	126 ± 15	153.9 ± 17.5	9.4 ± 1.0	24.6 ± 2.2	246^{a} ± 37	335.6^{a} ± 68.3	25.8^{a} ± 1.5	45.4 ± 19.2
	ST	112 ± 16	128.9 ± 20.9	10.1 ± 1.5	26.1 ± 11.6	188^{ab} ± 22	236.7^{ab} ± 32.8	20.4^{b} ± 2.0	36.9 ± 8.2
	NT	120 ± 36	144.2 ± 42.0	9.6 ± 0.6	27.4 ± 8.8	173^{b} ± 26	223.1^{b} ± 24.1	19.4^{b} ± 2.0	24.0 ± 17.5

Tab S2. Significant explanatory variables for *nosZ*I and *nosZ*II community structure.

Community Structure	Soil Physic-chemical	Abundance	Diversity
*nosZ*I	pH, C/N	AOA, *nosZ*I, *nosZ*II, *nosZ*I/*nosZ*II	*nosZ*I (PD)
*nosZ*II	pH, C/N, Total N, cec, sand	AOA, AOB, *nosZ*II	*nosZ*I (PD)

Tab S3. Correlation coefficients between potential denitrification and nitrification activities, and soil properties, functional guilds abundances and N_2O-reducers diversity.

Potential Activities	pH	Clay	Sand	Loam	SOM	q.AOA	q.AOB	q.*nirK*	q.*nirS*	q.*nosZ*I	q.*nosZ*II	*nosZ*I PD	*nosZ*II PD	*nosZ*I SR	*nosZ*II SR
Nitrification (PN)	0.81***	-0.41**	ns	ns	ns	0.46***	ns	-	-	-	-	-	-	-	-
Denitrification (PDA)	0.40**	-0.54***	ns	ns	0.46**	-	-	0.30***	0.35*	0.34*	0.35*	0.34*	ns	-0.33*	ns

Pearson correlation coefficients. Significant correlations are indicated by *P<0.05, ** P<0.01; *** P<0.001; *** P<0.0001.

Tab. S4. Correlation coefficients between *in situ* N_2O emissions and soil properties, abundance and diversity of microorganisms.

N_2O "in situ" fractions"	pH	Clay	Sand	Loam	SOM	q.AOA	q.AOB	q.*nirK*	q.*nirS*	q.*nosZ*I	q.*nosZ*II	*nosZ*I PD	*nosZ*II PD	*nosZ*I SR	*nosZ*II SR
25%	-0.38*	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
50%	-0.58**	NS	NS	NS	NS	-0.41*	NS	NS	NS	NS	NS	NS	NS	NS	NS
75%	-0.66**	NS	NS	NS	NS	-0.52**	NS	-0.35*	NS	NS	-0.37*	NS	NS	NS	-0.43*
90%	-0.70**	NS	NS	NS	NS	-0.55**	NS	-0.36*	NS	NS	NS	NS	NS	NS	-0.49**
95%	-0.71**	NS	NS	NS	NS	-0.48**	NS	NS	NS	NS	NS	NS	NS	NS	-0.50**
99%	-0.63*	NS	NS	NS	NS	-0.41*	NS	NS	NS	NS	NS	NS	NS	NS	-0.45**

Spearman correlation coefficients. Significance of *P<0.05, **P <0.01.

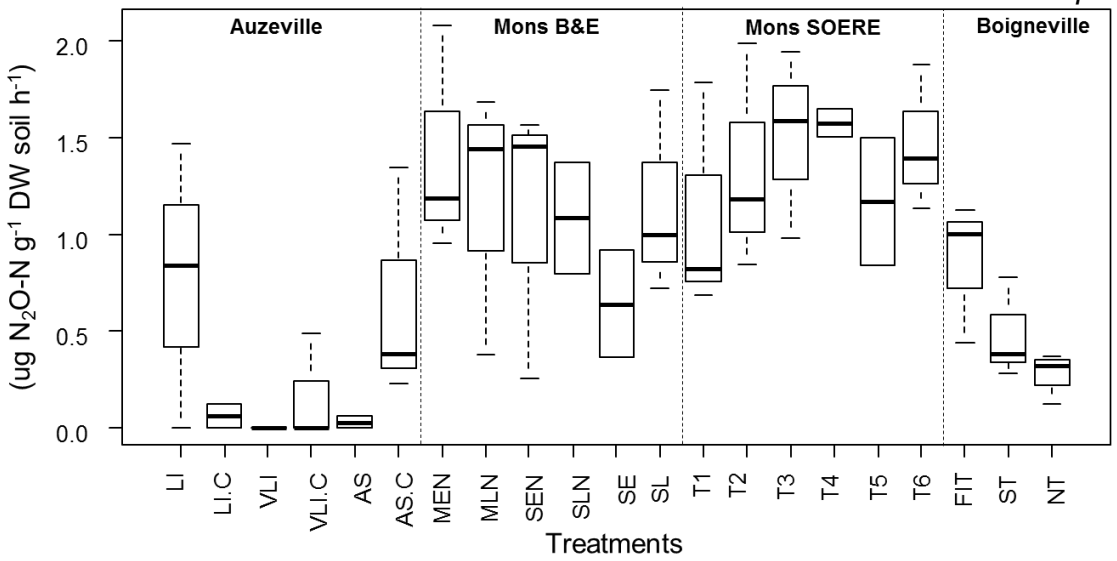

Fig S1. Potential Denitrification Activity (N_2O+N_2; PDA). Significant differences between treatments within a site are indicated with different letters (anova followed by Tukey HSD test, $P<0.05$)

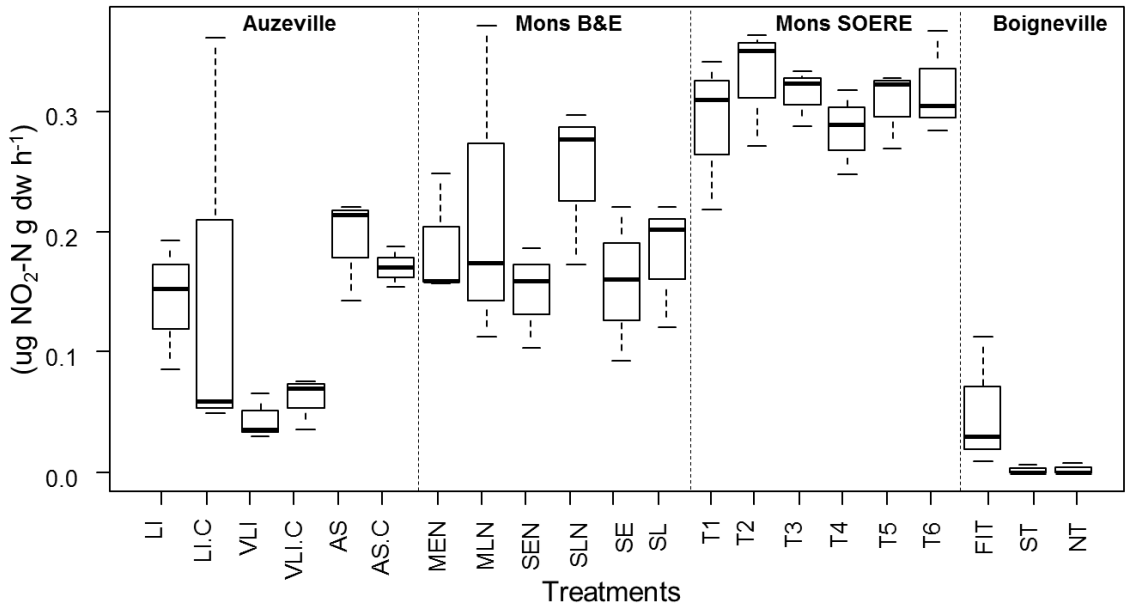

Fig S2. Potential Nitrification Activity (PNA). Significant differences between treatments within a site are indicated with different letters (anova followed by Tukey HSD test, $P<0.05$).

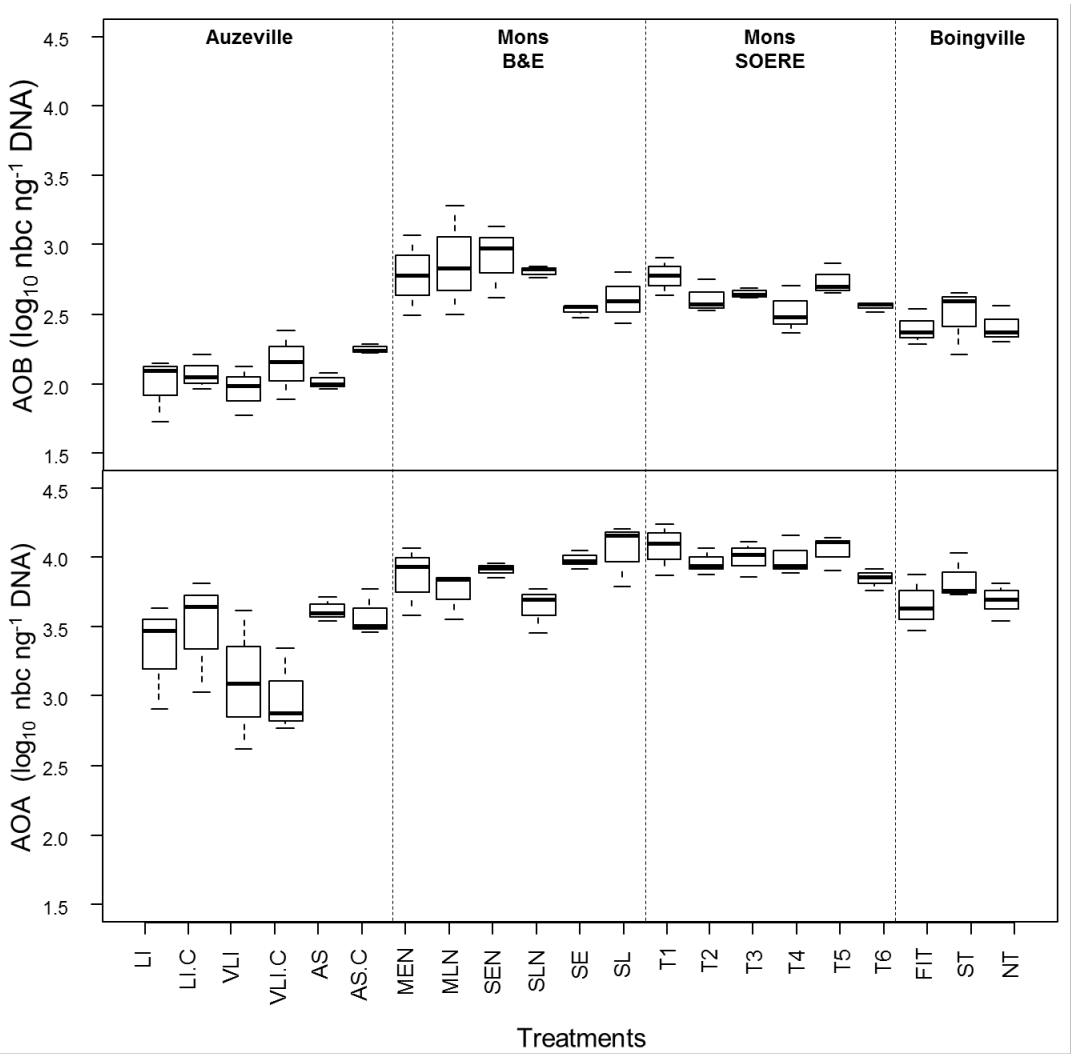

Fig S3. Abundance of Ammonia-oxidizers bacteria (AOB) and Archaea (AOA). Significant differences between treatments within a site are indicated with different letters (anova followed by Tukey HSD test, $P<0.05$)

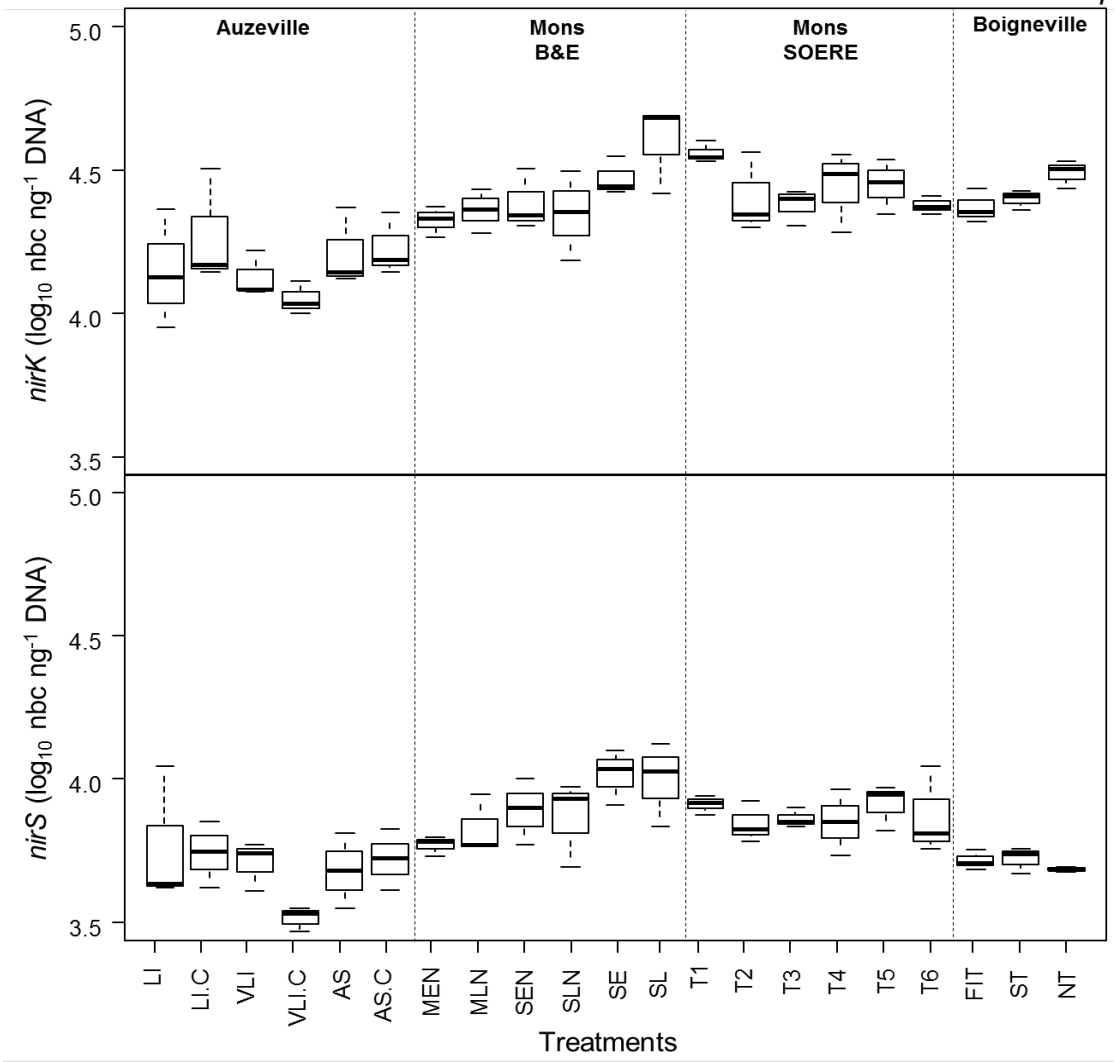

Fig S4. Abundance of *nirK* and *nirS*. Significant differences between treatments within a site are indicated with different letters (anova followed by Tukey HSD test, $P<0.05$)

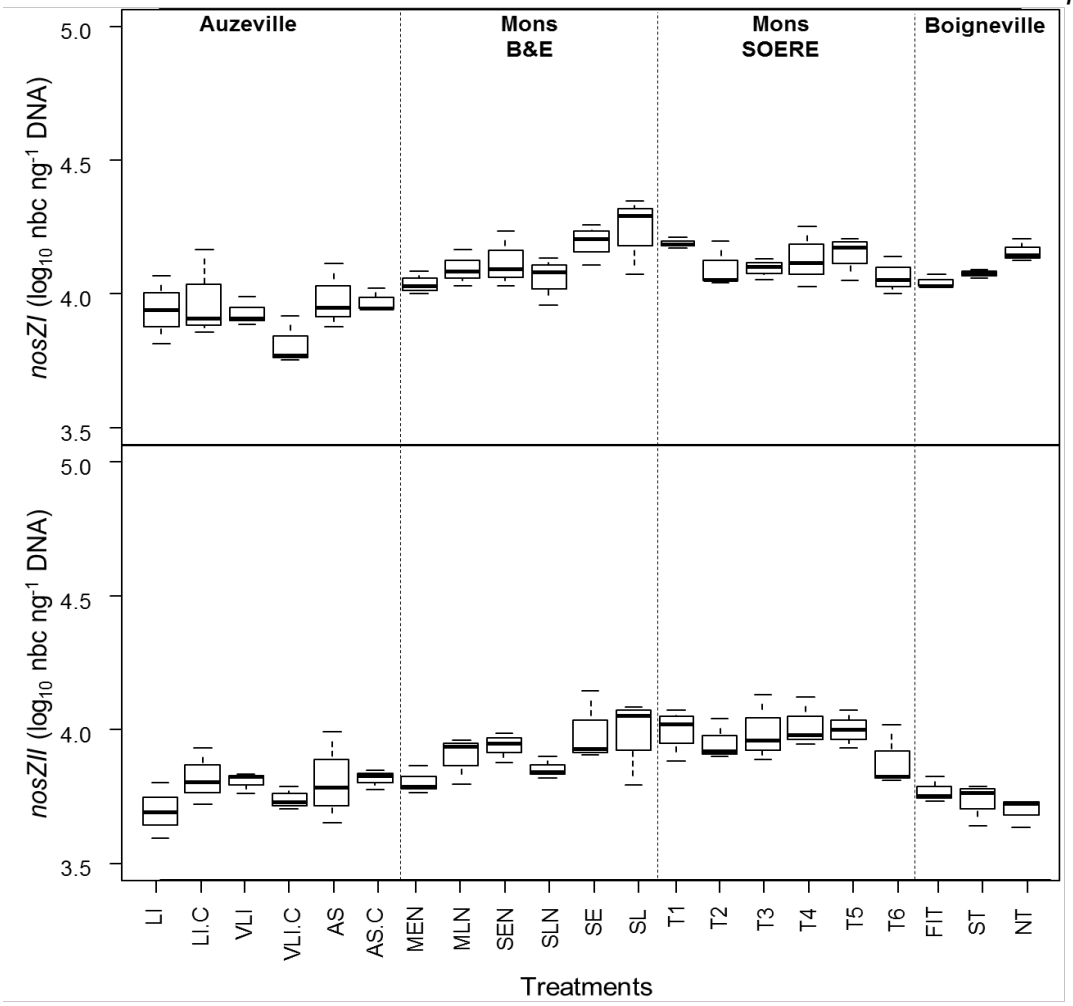

Fig S5. Abundance of *nosZ*I and *nosZ*II. Significant differences between treatments within a site are indicated with different letters (anova followed by Tukey HSD test, $P<0.05$)

6 General Discussion

I started this thesis shortly after the discovery of a second clade of N_2O reducers, which has been reported to be abundant and highly diverse in agricultural soils (Sanford et al., 2012; Jones et al., 2013; Orellana et al., 2014). In this context, we aimed to evaluate the relevance of this guild for soil N_2O emissions at different scales from a laboratory incubation experiment up to multiple experimental sites under different management practices.

6.1 *nosZ*II N_2O-reducers matters for soil N_2O reduction

In **Chapter I** we evaluated the influence of altered proportions of *nosZ*II N_2O reducing bacteria on the end-product of denitrification. A *Dyadobacter fermentans* strain was selected as a model organism because sequencing of the complete genome indicated that it lacked all the denitrification genes except *nosZ*, encoding a clade II-type N_2O reductase. Our results showed that increasing abundance of *Dyadobacter fermentans* resulted in a decrease of the N_2O production in 1/3 of the tested soils. Previously, Philippot et al. (2011) had manipulated the proportion of N_2O-producers by inoculating *Agrobacterium tumefaciens*, a strain producing N_2O but not capable to reduce it, into three Swedish soils. They showed an increase in N_2O production with higher *A. tumefaciens* inoculation levels. However, in one of the three tested soils, the N_2O/N_2 ratio was unaffected even after addition of the highest number of *A. tumefaciens* cells. This suggested that the indigenous denitrifier community was capable to uptake the N_2O produced by *A. tumefaciens*. Altogether these two studies investigating respectively the importance of denitrifiers lacking *nosZ* and of N_2O reducers having only *nosZ*, came to the same conclusion that the relative abundance of N_2O-reducers can be of importance for explaining soil N_2O production.

In our study, the N_2O reducing capacity of a single non-denitrifying *nosZ*II strain has been evaluated. An intergenomic comparison of denitrifiers by Graf et al. (2014) showed that about 51 % of the genomes from *nosZ*II strains do not possess any nitrite reductase gene, while for the *nosZ*I clade this proportion is about 17%. Additionally, both PCR-based approaches and metagenomic analyses indicated that the *nosZ*II clade is either as abundant as the *nosZ*I clade in soils or even more abundant in some ecosystems (Jones et al., 2013; Orellana et al., 2014). Such results highlight the role of the *nosZ*II guild as a potential N_2O

sink. The reduction of N_2O by a few other *nosZ*II strains was evaluated in recent studies. Similarly to *D. fermentans*, *Anaeromyxobacter dehalogenans* was also able to grow using N_2O as the sole electron acceptor and achieved a 1.5-fold higher cell yield compared to the complete denitrifier *Pseudomonas stutzeri* DCP-Ps1 (Sanford et al., 2012). This suggests that its N_2O-reductase may operate with higher efficiency than the respiratory machinery of a complete denitrifier. Yoon et al. (2016) also compared the growth yield and N_2O consumption kinetics between two *nosZ*I and three *nosZ*II strains. Two gammaproteobacteria, *Pseudomonas stutzeri* and *Shewanella loihica*, were used as model organisms for the *nosZ* clade I, while *Anaeromyxobacter denitrificans*, a deltaproteobacteria, and two betaproteobacteria, *Dechloromonas aromatica* and *Dechloromonas denitrificans*, were selected for the *nosZ* clade II (Yoon et al., 2016). A diversity in responses within both N_2O-reducers clades regarding Michaelis-Menten parameters *Ks* and V*max* under growth with N_2O was observed. *nosZ*I strains presented a higher V*max* than *nosZ*II (ranging between 0.44 and 4.16 against 0.017 to 0.461 μmol min^{-1} mg $biomass^{-1}$, for *nosZ*I and *nosZ*II, respectively). Regarding *Ks*, *nosZ*I strains showed also a higher range with 7.0 to 35.5 μM (N_2O), while *nosZ*II strains recorded from 0.32 to 1.34 μM (N_2O). The authors suggested that the *nosZ*I clade could be considered as an r-strategist and *nosZ*II k-strategists. This would imply that the last shows advantage if growing on nutrient-limited soil environment, while the first would have a competitive advantage when substrate is plentiful. However, only a few strains have yet been studied and they do not cover the existing diversity within each *nosZ* clade. Thus, the *nosZ*I clade has a relatively low diversity and is composed by alpha-, beta-, and gamma-, proteobacteria. In contrast, the *nosZ*II clade is much more diverse including bacteria from the previous phyla but also from more distinct phyla such as Bacteroidetes, Firmicutes, Verrucomicrobia, Aquificae, and others. Therefore, the number of strains studied is too limited to draw general conclusions about the ecophysiology of clade I and clade II N_2O reducers and more studies are needed in order to make a robust comparison of the physiology of these two functional groups.

We reported reductions in N_2O production by the addition of *D. fermentans* up to 189 % since negative rates were recorded, showing that some soils could be potential sinks for N_2O. In the literature, most studies have focused on N_2O production rather than on N_2O consumption. In a comprehensive review, Chapuis-Lardy et al. (2007) showed that even though numerous studies acknowledged negative N_2O fluxes, these measurements were mostly considered as experimental noise. Here we showed that a soil with increased

proportion of N_2O-reducers may act as a N_2O sink. However, in several soils, we did not find any effect of *D. fermentans* addition. This could be due to multiple factors: *i*) no survival of the strain in these soils, *ii*) a significant effect of pH and C/N on the capability of the strain to reduce N_2O in the different soils. Accordingly, Liu et al. (2014) suggested that low soil pH diminishes N_2O reduction due to an unsuccessful assembly of the N_2O reductase. pH has been shown by various studies as a major variable controlling the proportion of N_2O produced by denitrification (Šimek & Cooper 2002) and N_2O emissions (Benckiser et al 2015; Snyder et al., 2009). The strain we used might be sensitive to pH. We would suggest using physiologically distinct strains to identify other controlling factors and quantify the strain-specific pH range for performing N_2O-reduction. The other soil property that was related to the capacity of *D. fermentans* to reduce N_2O was the C/N ratio. There is consent among authors that concentration and supply rate of bioavailable organic carbon is limiting growth for heterotrophic bacteria (Coleman et al., 2004). Therefore, it is not surprising that differences in the C/N ratio can impact the capacity of *D. fermentans* to reduce N_2O. It is also worth noticing that the C/N ratio was one of the soil properties driving the *nosZ*II community in Jones et al. 2014.

Our study was the first to show that a *nosZ*II strain inoculated in soils can reduce N_2O produced by the indigenous microbial communities. Previous studies investigated the capacity of plant-symbiotic bacteria having the *nosZ*I gene to reduce N_2O emissions. Hénault and Revellin (2011) inoculated soybean plants with three *Bradyrhizobium japonicum* (USDA 110, MSDJ G49 and a mutant UA 110 with deleted *nosZ*) and observed N_2O consumption in the treatments with the functional *nosZ*. Interestingly, the inoculums with *B. japonicum* were able to reduce N_2O under both anaerobic and aerobic conditions, suggesting that this could be an efficient solution to reduce N_2O in the field with low dependence on the O_2 partial pressure. These results were confirmed at a field scale by Itakura et al. (2013), who showed that post-harvest N_2O emissions due to soybean nodules degradation could be mitigated by the inoculation of *B. japonicum* with enhanced N_2O-reductase activity. The potential of free living denitrifying *nosZ*I strains from the genus *Azospirillum* sp. (gamma-proteobacteria) and *Herbaspirillum* sp. (beta-proteobacteria) to reduce N_2O production was also shown, with most of the strains reducing N_2O emissions in a soil growing a Fabaceae (*Trifolium pratense*), but not in a soil with a Poaceae (*Phleum pretense*) (Gao et al., 2016). These authors also evaluated if the inoculation of the strain could promote plant growth at the same time of reducing N_2O. They concluded that most of the strains enhanced the Fabaceae growth and to a

less extent the Poaceae. Interestingly, some strains had a positive effect on one plant but not on the other suggesting specific strain-plant interactions. Coupling the reduction of N_2O emissions with a positive effect on plant productivity could be an incentive for farmers to inoculate strains having such properties in the fields. Another promising strategy is to add N_2O-reducing strains with the fertilizer as it is often reported that the peak in emissions is observed after fertilization (Butterbach-Bahl., 2013; Akiyama et al., 2010). This has been tested successfully by Nishiwaza et al. (2014), who added eight different denitrifying strains (*Azoarcus* sp. KH32C, KS11B, and KS30A; *Niastella* sp. KS31B; *Dechloromonas* sp. KS31F; *Dyella* sp. YH11B and YS9C; *Burkholderia* sp. TSO47-3) to pellet poultry manure and observed that N_2O emissions were significantly lowered (up to 60%). Gao et al. (2016) and Nishiwaza et al. (2014) used canonical denitrifying strains and the drawback is that it could also enhance fertilizer losses while the addition of N_2O-reducers only could be more beneficial.

The incorporation of modified non-extraneous soil microorganisms with enhanced enzymatic functions or the addition of exogenous microorganisms must be done with caution. Whitney & Gabler (2008) suggested that rapid evolution can be frequent among invasive organisms. Given the fast generation time of microorganisms and their propensity to horizontal gene transfer, such evolutionary aspects should not be overlooked when adding microorganisms to the soil. Moreover, Mallon et al. (2015) argued that the invasion may cause a shift in the indigenous microbial diversity, which can impact ecosystem function. Possible deleterious consequences of manipulating microbial communities and therefore biotic interactions must not be neglected and deserve special attention in future research (Gaba et al., 2015).

6.2 Assessing management influences on the microbial communities and their activities

There is a large body of literature addressing the impact of various agricultural practices on microorganisms (Cheneby et al., 2004; Hallin et al., 2009; Melero et al., 2011; Clark et al., 2012; Fierer et al., 2013; Lauber et al., 2013; Hartmann et al., 2015). Due to differences in pedo-climatic conditions we cannot compare practices between sites, thus our intent was rather to assess how practices within a particular site affect the microbial communities responsible for N_2O production, N_2O reduction and their activities.

Overall, we found in **Chapter II** and **III** a limited impact of the agricultural practices on the studied microorganisms and the site effect was stronger. The exception was tillage in Boigneville. Increasing tillage intensity raised significantly the diversity of *nosZ*II and although not significantly, it seemed to enhance the abundance of this clade while it did not change *nosZ*I diversity or abundance. Comparing our results for the *nosZ*II clade to the literature is impossible because, to our knowledge, no other study is available. We will therefore restrain our comparisons to the *nosZ*I clade. Melero et al. (2011) studied the abundance of *nosZ*I genes under different tillage management in a vertisol and observed that the effect of tillage varied with the sampling time. Thus, in the 0-30 cm soil depth during wheat growth, the *nosZ*I clade abundance was higher in the No-Tillage (NT) treatment than in the Full Inversion Tillage (FIT) one while the opposite was found after crop harvesting. These authors also assessed the 30-50 cm soil profile, which showed no significant differences during the growing season, but higher abundance of *nosZ*I under FIT after harvest. Our sampling design did not allow us to draw conclusions regarding temporal variability because we had only 1 or 2 sampling time points but another study reported that the sampling time had no significant effect of the abundance of the *nosZ*I community, which was mostly driven by soil pH (Bru et al. 2011). Tatti et al. (2015) also studied the abundance of clade I under FIT and NT during two winters and observed higher abundance of this clade under FIT than NT. Differences were however significant only in one of the winters. The authors related this difference between winters to differences in snow depth which protects the soil against cold temperatures. On the contrary, *nosZ* clade I community structure responded to tillage regimes at all sampling dates and during the two winters. Regarding *in situ* N_2O emissions, Potential Denitrification Activity (PDA) and Potential Nitrification Activity (PNA), no significant differences were observed between the different tillage management. Large discrepancies were reported in the literature about the effect of tillage on N_2O emissions. Thus, some studies indicated tillage promotes N_2O emissions (Smith et al., 2008) and in others that it reduces it (Robertson et al., 2000), or had no influence at all (Marland et al., 2001). Tillage practices can modify the quality and quantity of soil organic carbon by changing soil structure, soil moisture, and the distribution of residues in the soil profile (Hussain et al., 1999). No Tillage can have positive effects for soil structure including soil aggregation (Six et al., 1999), therefore modifying properties that can impact N_2O emissions like decreasing soil porosity (Pastorelli et al., 2013), and increasing bulk density (Logsdon & Karlen, 2004). These changes may lead to a soil with lower pO_2, enhancing anoxic conditions that can favor

denitrification. Moreover, tillage management may impact differently microbial communities in different soils. Rochette (2008) suggested that NT more than doubled N_2O emissions in heavy clay soil compared to NT. Venterea et al. (2005) showed that NT effect on N_2O emissions was also dependent on fertilizer type with increased N_2O emissions by urea application in NT, while anhydrous ammonia lowered the emissions. Overall, these studies highlight the importance of considering management practices in a more global context and not restraining the analyses to only one of the multiple practices for understanding the N_2O fluxes.

An agricultural practice which had no effect on the microbial communities but impacted the *in situ* N_2O emissions and the N_2O:N_2 ratio was the harvest date in the B&E experimental site. We observed a trend of higher *in situ* N_2O emissions at late harvest compared to early harvest for both perennial crops (*i.e*. Miscanthus and Switchgrass). We hypothesized that this is due to enhanced conditions favoring denitrification and thus effecting N_2O production. Contrarily to late harvest, early harvest in Autumn prevents leaves senescence during winter which results in litter deposition. Additionally, Amougou et al. (2011) observed that N concentrations in the plant litter is lower in the late compared to the early harvest, which was shown to decrease decomposition rates (Trinsoutrot et al., 2000) enhancing litter accumulation. The higher deposition and accumulation of litter in the late harvest represents a significant carbon input (Amougou et al., 2011, 2012), which creates a mulch layer that reduces soil evaporation. Consequently, these conditions can favor denitrification and enhance N_2O production. Peyrard et al. (2016a) investigated N_2O emissions in Miscanthus early and late harvest at the same experimental site and observed that harvest date had the stronger effect on N_2O emissions compared to fertilizer type or year. Late harvest resulted in emissions which were in average five times higher emissions than in the early harvest treatment. The harvest date also impacted the N_2O:N_2 ratio. In particular, early harvest of *M*. *giganteus* showed a significantly higher proportion of N_2O produced during denitrification than late harvest. Previous studies reported that production of N_2O relative to N_2 during denitrification in soils is influenced by carbon availability and pO_2 (Firestone et al., 1980; Murray and Knowles, 2004; Giles et al., 2012;). The proportion of N_2O produced during denitrification in soils was shown to increase with increasing pO_2 (Firestone et al., 1980), which is in accordance with our results as the N_2O:N_2 is higher in the early harvest treatment, without the mulch layer and consequently lower moisture content (Peyrard et al. 2016a). The B&E experimental site was set up to compare the productivity and environmental

impacts of Miscanthus and Switchgrass, two energy crops. Our results showed that different harvest dates had consequences for the N_2O fluxes, which highlights the importance of the agricultural practices for avoiding high N_2O emissions. Accordingly, Cadoux et al. (2014) evaluated the productivity versus global warming potential of six different bioenergy crops and observed the highest benefit with Miscanthus early harvest, which resulted in the lowest amount of GHG emissions per hectare. Drewer et al. (2012) showed that bioenergy crops only emit less GHGs than annual crops if they receive no fertilizer or very low fertilization rates. To justify the implementation of bioenergy crops, it is important to demonstrate that their production represents a net reduction in GHG emissions. Our results suggest that timing of harvest may play an important role for determining the best cost/benefit scenario for such crops.

In **Chapter II,** we also observed differences between the annual cropping system (ORE) compared to a perennial cropping system (B&E). Thus, the *nosZ*II community was more diverse in the ORE compared to B&E, while *nosZ*I diversity was not impacted. Similarly, the abundance of *nosZ*II was higher in the B&E experiment than in the ORE, but we did not observe any difference in *nosZ*I abundance. One of the differences between these two block-experiments is that in the annual cropping system the soil was tilled (full or shallow tillage) while in the perennial block-experiment the soil was not. Because of several other differences between these two block-experiments (crop types, fertilization rate and the presence of legume as a cover crop in some treatments in ORE), we can not attribute the observed differences to the presence or absence of tillage or to any other individual practices. Regarding N_2O production we did not observe significant differences in the potential N_2O production or PDA. However, we showed a higher proportion of N_2O produced during denitrification (N_2O:N_2) in the perennial site compared to the annual one. Measurements of daily N_2O emissions by Thompson et al. (2016) showed significantly lower emissions in the perennial fields compared to the annual ones (1.81 ng N_2O-N m^{-2} s^{-1} and 10.01 ng N_2O-N m^{-2} s^{-1} for the perennial and annual fields, respectively). A higher *nosZ*I gene abundance was also reported in the perennial system compared to the annual system. However, it should be noted here that this study suffers from an inadequate experimental design to test for differences between annual and perennial cropping systems since the two treatments were not replicated. Thus, the observed differences on N_2O fluxes or *nosZ*I community could be due to other differences between the two sites that were not taken into account.

Surprisingly, the other agricultural practices investigated in my work did not affect the microbial communities and their activities. Other studies, such as Hallin et al. (2009), showed that different fertilization regimes impacted *nosZ*I abundance and community structure. In this study, soils samples were collected from a 50 years-old experimental site under 6 different treatments: bare fallow unfertilized, unfertilized, calcium nitrate ($Ca(NO_3)_2$), ammonium sulfate ($(NH_4)_2SO_4$), solid cattle manure and sewage sludge. The inorganic fertilized plots received a rate of 80 kg N h^{-1}, while organic fertilizers were applied biannually in an amount of 800 kg ash-free organic matter h^{-1}. Comparison of the two inorganic and of two organic fertilizers revealed that $(NH_4)_2SO_4$ and sewage sludge reduced the abundance of *nosZ*I compared to $Ca(NO_3)_2$ and solid cattle manure, respectively. In our study, within the ORE and the Auzeville sites, the fertilization rates varied but not the fertilizer type. At the ORE, a solution with equal parts of urea and ammonium nitrate (NH_4NO_3) was applied at a rate of 40 and 100 Kg N h^{-1} y^{-1} for the low (T4 and T5) and high (T1, T2 and T3) fertilization treatments, respectively. The plots in Auzeville received NH_4NO_3 at rates of 45 or 90 Kg N h^{-1} y^{-1} for the Very Low Input (VLI) and Low Input (LI) treatments, respectively. We could also hypothesize that it is due to a relatively recent establishment of these sites. However, Clark et al. (2012) evaluated the effect of different fertilization rates (0, 144 and 288 kg N h^{-1} y^{-1}) in a 160 years-old experimental site and did not showed any significant differences on *nosZ*I abundance, nor on N_2O reducers community structure. These results suggested that the type of N may be more important than the rate of fertilization for N_2O-reducers. The different fertilization rates did not influence the N_2O emissions in our study. This could be explained by the fact that the block-experiments selected in our study can be considered as low-input systems regarding N management as they have reduced or split applications that are below the French average of 151 Kg N ha^{-1} y^{-1} (Agreste, 2014). As such, fertilization probably meets the plants requirements without exceed them, and therefore do not lead to fertilizer-driven N_2O emissions as shown by Peyrard et al. (2016b).

To our knowledge, no other study evaluated *nosZ*II clade response to agricultural practices. However Graf et al. (2016) studied the abundance of both N_2O reducing clades in relation to denitrification activity and N_2O production in the rhizosphere of barley (*Hordeum vulgare* "Triple"), sunflower (*Helianthus annuus* IREGI-type) and bulk soil. The denitrification activity and N_2O production were determined by soil type. Interestingly, *nosZ*I was predominant in the rhizosphere, while *nosZ*II in the bulk soil. These results indicated niche differentiation between the two clades. Harter et al. (2016) assessed the impact of the

addition of biochar in soil microcosms on both N_2O-reducers clade. The authors showed that *nosZ*I and *nosZ*II responded differently two weeks after biochar amendment. The abundance of *nosZ*I gene abundance increased while *nosZ*II abundance decreased. Additionally, within these guilds while some members increased their relative abundance, others decreased: two species belonging to the *nosZ*I clade (*Pseudomonas stutzeri* and *Ensifer meliloti*) showed higher abundance in biochar microcosms while another one (*Azoarcus* sp KH32C) was more abundant in the control ones. For *nosZ*II, the authors found one species with higher abundance after biochar amendment (*Pedobacter saltans*), and four that were more abundant in the control (*Flavobacteriaceae bacterium 3519-10*, *Belliella baltica*, *Dechloromonas aromatic*, and *Niastella koreensis*). *P. saltans* showed the strongest shift in relative abundance with 26.7% of the denitrifier community in the control microcosms and 50.9% of the denitrifier community in the biochar microcosms. Sequencing of the complete genome of *Pedobacter saltans* strain DSM 12145 revealed that it carries only the *nosZ* gene and no other denitrification genes. As such an increase in the relative abundance of strains having the same truncated pathway could have important consequences for the soil N_2O sink capacity.

Overall our results showed limited impact of practices on the microbial communities. Geisseler et al. (2016) highlighted the importance of long term field experiments as unique opportunities to measure incrementally small, but cumulatively large impact of management. The only site in which we observed differences between treatments was the Boigneville site, which was established in the 1970s. It is more than 45 years-old and it is the older site used in our study. A few years might be needed before significant changes in soil properties lead to a measurable shift in the microbial communities. Some studies actually reported a lag in the response of microorganisms after changes in management (Habekost et al., 2008; Kulmatiski et al., 2008; Eisenhauer et al., 2010). This could explain why no changes were detectable in the ORE experiment, which has also different tillage management strategies (shallow or full tillage), but which was established only in 2010. Another reason for not detecting changes in this recently established site, might be due to our sampling method. We sampled the 0 to 20 cm soil horizon in a treatment on which only the top 6 cm suffered tillage (shallow tillage in ORE). By sampling the whole horizon, differences between the first centimeters and the deeper horizon could have been masked. A more appropriate approach to assess shallow tillage effects would have been to sample at two depths, for example from 0 to 5 cm and from 15 to 20 cm.

6.3 Relevance of soil N_2O-reducers diversity and abundance for soil N_2O reduction.

There is an intense debate in the literature whether or not we need to take into account for microbial communities to predict ecosystem function (Petersen et al., 2012; Graham et al., 2014; Nemergut et al., 2014; Graham et al., 2016).

In general, our results highlighted the relevance of the *nosZ*II clade for explaining the N_2O fate in soil. In **Chapter II** the diversity of the *nosZ*II clade was negatively correlated to the N_2O:N_2 ratio, and explained alone 26% of its variation. This is in agreement with Jones et al. (2014) who found that *nosZ*II diversity was important to determine the capacity of the soil to act as a sink of N_2O. These authors designed an experiment to investigate the soil N_2O sink capacity across 47 European soils by adding a N_2O producing strain (*A. tumefaciens*) at different concentrations and evaluating how the different soils consumed the N_2O produced by this strain. These authors proposed a N_2O sink index, which is a relative measure of the capacity of soils to reduce the additional N_2O produced by the exogenous strains compared to an "average" theoretical soil. It gives a positive value if the soil is a source of N_2O, and a negative one if it is a sink for this GHG. Their analysis showed that soil properties and climatic conditions influences on the N_2O sink capacity of soils were mediated by the microbial communities and in particular by the *nosZ*II community.

A positive relationship between the abundance of a functional community and the corresponding process rate was observed in a few studies (Dong et al., 2009; Hallin et al., 2009; Philippot et al., 2009; 2010; Petersen et al., 2012), while other investigations did not find such relationship (Attard et al., 2011; Dandie et al., 2008; Baudoin et al., 2009). In **Chapter II**, we found that the abundance of denitrification genes had a marginal importance to explain the denitrification gaseous products explaining only 2% of the N_2O:N_2 ratio variation and did not correlate to the PDA or potential N_2O production. In **Chapter III,** we expanded the approach from **Chapter II** to multiple sites from different locations. We therefore increased the range of variations in gene abundances and in potential activities. We found that all targeted denitrification genes, *nirK, nirS, nosZI and nosZII*, were positively correlated to the PDA. Petersen et al (2012) suggested that gene copy number may be a better predictor of potential rates when the rates showed a larger range of variation. We observed a relatively small increase in PDA range (of ~ ¼), but a higher heterogeneity between samples.

This could explain why in **Chapter III** we observed a relationship between genes abundance and PDA that we did not in the first dataset.

Rölling (2007) applied a biochemistry concept of regulation analysis into microbial ecology. Such analysis aimed at quantifying how biogeochemical fluxes are regulated by the microorganisms performing the process, and is an attempt to uncouple which changes in fluxes are due to changes in abundance or to changes in cell activity. Thus, the author suggested that fluxes are generally, but not always, regulated by modification in cellular activity and not by cell number. However, the author highlighted that most of the studied cases addressed differences in the depth profiles of shallow sediments. These systems are prone to bioturbation and physical mixing, homogenizing the profiles and could hide the relationship between abundance and processes rates (Rölling, 2007). The author hypothesized that under different environmental conditions or other biogeochemical processes the role of the abundance could have been more important than in the studied conditions. Another study tested the global relationship between gene abundance and the corresponding processes rates by performing a meta-analysis of published literature (Rocca et al., 2015). They identified 59 studies reporting the abundance of genes and the rates of the corresponding processes. Fifteen genes were included in their analysis. From those only 6 genes (archaeal *amoA*, bacterial *amoA*, *nirK*, *nirS*, *nosZ, and nirfH*) had more than five studies, the other ones having three (*cnorB* and *narG*), two (*pmoA* and *napA*) or only one study (*mcrA*, *nidA*, *nrfA*, *PHAgn* and *tfdA*). From the genes with more than 5 studies, all genes showed a positive relationship between gene abundance and the corresponding process rate except *nirfH*. Our results suggest that gene abundance could be a good proxy for processes rates especially when a high range in variation is observed as suggested by Petersen et al. (2012). The small number of studies compiled by Rocca et al (2015) in their meta-analysis is an indication that we still need more studies to evaluate the relationship between gene abundance and the corresponding process rates.

In an attempt to identify the biotic and abiotic factors important for low and high N_2O emissions recorded in the field, long term *in situ* N_2O measurements were subdivided in percentiles corresponding to basal emissions (25%), median emissions (50%) and high emissions (75%, 90%, 95% and 99%). pH is a well-known factor regulating N_2O emissions (Baggs, 2010; Giles et al., 2012; Prosser and Nicol 2012; Sîmek et al., 2010) and it is not surprising that it was correlated negatively to the N_2O emissions. Interestingly, pH was the only variable that relate to the lowest N_2O fraction (25%) while the diversity and the

abundance of *nosZ*II, and the abundance of *nirK* were negatively related to the higher fractions of N_2O. Similarly, Morales et al (2010) reported a positive relationship between *nirS* gene abundance minus *nosZI* gene abundance and the N_2O emissions. To our knowledge, this study is the first to study the relationships between the newly identified *nosZ*II community and *in situ* N_2O emissions, which lead to the identification of a negative correlation between *nosZ*II abundance and diversity and *in situ* N_2O emissions. Interestingly, we also observed that *in situ* N_2O emissions were negatively related to the abundance of AOA. Two hypotheses might explain this relationship. One is based on the findings that AOA may produces less N_2O emissions than AOB during ammonia oxidation in soil (Hink et al., 2016), and on the niche specialization between these two groups as suggested by some studies (Prosser & Nicol et al., 2012; Wang et al., 2015). The second hypothesis could be that since pH is positively related to AOA abundance, and negatively related to the *in situ* N_2O emissions, the observed negative relationship between AOA and *in situ* N_2O emissions might be spurious and driven by the pH.

7 Conclusion and Perspectives

Soil microorganisms are major contributors to global biogeochemical cycles, including the N cycle. It is an exciting moment to study the N cycle as recent discoveries revealed that we neglected important functional groups such as the new clade of N_2O reducers and bacteria that can completely oxidize ammonia to nitrate (Comammox). These discoveries changed our perception of the N cycling, and should potentially increase our understanding of N cycling processes.

Our results are a contribution to the understanding of the ecology and of the relevance of this novel N_2O-reducers clade for GHG emissions. Our main findings were:

- Addition of a non-denitrifying *nosZ*II strain to 11 different soils significantly reduced N_2O production in more than 1/3 of the soils. Some soils even became potential sinks of N_2O. Therefore our results provide unambiguous evidence that the overlooked non-denitrifying NosZII-type bacteria can contribute to N_2O consumption in soil.
- Among the studied agricultural practices, the only practice which impacted the N_2O-reducing community was tillage. Increasing tillage intensity enhanced the diversity of the *nosZ*II clade in the 40 years-old study site, while clade I was not affected. Differences in the diversity of the clade II of *nosZ* were also recorded under different cropping systems, with a higher diversity observed under an annual cropping system compared to a perennial cropping system. However, in overall the studied agricultural practices showed little or no effects on the studied microbial communities, likely due to the lack of major changes in soil properties between the different treatments.
- The proportion of N_2O emitted as end-product of denitrification and the *in situ* N_2O emissions was mostly affected by one agricultural practice, which was the timing of harvest for the energy crops. This was attributed to the presence of a mulch layer at late harvest, which creates propitious conditions to denitrification.
- The diversity of the *nosZ*II clade was negatively related to the proportion of N_2O produced as end-product of denitrification and was the stronger predictor of its variation (26%). Similarly, subdivision of the 70000 *in situ* N_2O measurements into

different emission ranges revealed that the highest emissions were negatively related to the diversity of the *nosZ*II clade.

Altogether, our results are strengthening the hypothesis that the *nosZ*II-community might be of importance for mitigating N_2O emissions. However, little is known about the *nosZ*II community and further research is needed for understanding their ecophysiology. Thus, the N_2O-reducing capacity of more strains and the environmental factors controlling it should be evaluated both in pure culture and in soils. Biochemistry approaches are important for characterizing the N_2O-reductase in both clades. To better understand the relevance of N_2O-reducers to the fate of N_2O in soil we need manipulative studies in soil microcosms to confirm field observations. Even though informative, correlative studies are not sufficient to identify with certainty the factors driving N_2O emission in soils.

The hypothesis of niche differentiation between *nosZ*I and *nosZ*II must also be tested experimentally. We identified here some soil properties that could be used to test such hypothesis: C/N and pH. Additionally to the niche differentiation between the two clades, how the community composition within each clade is influenced by the environmental factors must be evaluated. Thus, it is important to account for the proportion of microorganisms possessing only the N_2O-reductase within each group, as we showed that increasing this proportion has the potential to decrease the soil N_2O production. This last finding also highlighted the relevance to consider modularity in the denitrification pathway to understand the gaseous end-product of this process as suggested by Graf et al. (2014). Further studies must complement this approach by contrasting canonical denitrifiers with only N_2O-reducers from *nosZ*I and *nosZ*II clades.

The only study site in which different treatments resulted in modifications in the N_2O-reducing community was the site established since 1970. This results draw attention to the importance of long term field trials for providing the unique opportunity to access incrementally small but cumulative large impacts of management. More efforts are needed to evaluate other agricultural practices, preferentially in long-term field trials. We would also encourage that future studies subdivide the soil profile by depths, as management may not have the same impact on all soil depths.

Regarding the relationships between microbial abundance and diversity, and their respective processes, our results highlighted the relevance of diversity rather than abundance to the fate of N_2O in soil. However, in my thesis I did not explicitly test how the phylogenetic

diversity of both N_2O-reducing guilds relates to functional diversity. Future studies are needed in this direction. Some authors highlighted that we still lack the needed empirical evidence to determine the microbial processes that have a strong link between physiology and phylogeny. Our results suggested that the two N_2O reducing clades might be a promising model-system to deepen our understanding of the importance of phylogenetic diversity to ensure and maintain an important soil function, the soil N_2O reduction.

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