

Ecology of N2O reducing bacteria in arable soils Luiz A. Domeignoz Horta

▶ To cite this version:

Luiz A. Domeignoz Horta. Ecology of N2O reducing bacteria in arable soils. Bacteriology. Université de Bourgogne, 2016. English. NNT: 2016DIJOS031. tel-01494970

HAL Id: tel-01494970 https://theses.hal.science/tel-01494970

Submitted on 24 Mar 2017 $\,$

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers. L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.



Thèse de Doctorat Ecole Doctorale Environnement-Santé

Écologie des bactéries N₂O réductrices dans les sols agricoles

LUIZ A. DOMEIGNOZ HORTA



Écologie des bactéries N₂O réductrices dans les sols agricoles

Thèse de Doctorat Soutenue le 16 Décembre 2016 à Dijon

LUIZ A. DOMEIGNOZ HORTA

M ^{me} Lydie CHAPUIS-LARDY, IRD, Montpellier, Franc	e 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





et de la Maîtrise de l'Energie





Abstract

Nitrous oxide (N₂O) 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 N₂O in the atmosphere is steadily increasing, but we still lack knowledge on the factors controlling its production and consumption in soils. The reduction of N₂O to N₂ by microorganisms harboring the N₂O reductase gene (*nosZ*) is the only known biological process able to consume this GHG. Recent studies revealed a previously unknown clade of N₂O-reducers which was shown to be important to the N₂O sink capacity of soils. This thesis seeks to gain a greater understanding on the ecology of N₂O-reducers in agricultural soils. A combination of laboratory incubation and field experiments were used to gain knowledge on the importance of N₂O-producers and N₂O-reducers to the soil N₂O 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 nondenitrifying strain *Dyadobacter fermentans*, which possesses the previously unaccounted N₂O reductase NosZII, reduced N₂O production in 1/3 of the tested soils. Remarkably, after addition of the *nosZ*II strain, some soils became a N₂O sink, as negative rates were recorded. This experiment provided unambiguous evidence that the overlooked non-denitrifying *nosZ*II bacteria can contribute to N₂O 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 N₂O-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 N₂O-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 N₂O 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 N₂O measurements were subdivided into different ranges, from low to high rates. Interestingly, the low range of *in situ* N₂O 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" N₂O 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 *nos*ZII was negatively related to the high ranges of *in situ* N₂O 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 (N₂O) 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 N₂O 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 N₂O en N₂ par des microorganismes porteurs du gène codant pour la N₂O 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 N₂O qui interfère de manière significative avec la quantité de N₂O produite dans les sols. Cette thèse a cherché à mieux comprendre l'écologie des réducteurs du N₂O 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 N₂O dans le sol, en analysant l'influence conjointe des producteurs et réducteurs de N₂O. 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 N₂O réductase NosZII, permettait de réduire la production de N₂O dans 1/3 des sols testés. Certains sols sont même devenus consommateurs de N₂O suite à l'ajout de la souche *nos*ZII. Cette expérience a démontré la contribution des bactéries *nos*ZII non-dénitrifiantes dans la consommation de N₂O 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 N₂O 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 N₂O-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 N₂O in situ. La production potentielle de N₂O et l'activité potentielle de dénitrification ont été utilisées pour calculer le ratio de production de N₂O (N₂O:N₂). La diversité du clade nosZII est négativement corrélée au ratio N₂O:N₂, et explique à elle seule la plus grande part de variance observée du ratio N₂O:N₂. Les variations de production potentielle de N₂O 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 N₂O, 70000 mesures ont été subdivisées en différentes gammes d'émission de N₂O, d'émissions dites de base à des émissions élevées. Fait intéressant, les variations d'émissions *in situ* de N₂O 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 nosZII est négativement liée aux émissions de N2O in situ dites élevées.

En conclusion, nos résultats mettent en évidence l'importance du clade *nos*ZII pour le cycle du N_2O dans le sol. Ce clade *nos*ZII 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

List of Contents

	List of publications	1
	Abbreviations	2
1.	Introduction	3
	1.1 The Nitrogen Problem: "too much of a good thing"	3
	1.2 Nitrous Oxide: no laughing matter, but a potent greenhouse gas	4
	1.3 Biological processes responsible for N ₂ O production and consumption	8
	1.3.1 Nitrification 1.3.2 Denitrification	8 9
	1.3.2.1 A large, diverse and previously unrecognized group of N_2O reducers	.12
	1.3.3 Codenitrification 1.3.4 Dissimilatory Nitrate Reduction to Ammonia (DNRA)	.15 15
	1.4 Environmental factors influencing N ₂ O fluxes	16
	1.5 Agricultural practices influence on N ₂ O emissions and N ₂ O-reducers	19
	1.5.1. N ₂ O emissions	20
	1.5.2 N ₂ O-reducers	22
	1.6 Relevance of community abundance, composition and diversity for understanding N_2C fluxes) 24
2.	Aim and Objective	.28
3.	Chapter I – Non-denitrifying nitrous oxide-reducing bacteria – an effective N ₂ O sink in soil	29
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	38
5.	Chapter III Effects of agricultural practices and soil properties on soil N ₂ O reducing	
	bacteria and in situ N ₂ O emissions	.57
6.	bacteria and in situ N ₂ O emissions	57 .86
6.	General Discussion 6.1 nosZII N ₂ O-reducers matters for soil N ₂ O reduction	.57 .86 .86
6.	General Discussion 6.1 nosZll N2O-reducers matters for soil N2O reduction 6.2 Assessing management influences on the microbial communities and their activities	.57 .86 .86 .89
6.	 General Discussion 6.1 <i>nosZ</i>II N₂O-reducers matters for soil N₂O reduction. 6.2 Assessing management influences on the microbial communities and their activities 6.3 Relevance of soil N₂O-reducers diversity and abundance for soil N₂O reduction. 	57 86 .86 .89 .95
6. 7.	General Discussion 6.1 nosZII N ₂ O-reducers matters for soil N ₂ O reduction	57 86 .86 .89 .95 .98
6. 7. 8.	General Discussion 6.1 nosZll N2O-reducers matters for soil N2O reduction 6.2 Assessing management influences on the microbial communities and their activities 6.3 Relevance of soil N2O-reducers diversity and abundance for soil N2O reduction Conclusion and Perspectives References	57 86 86 89 95 98

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 N₂O 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 N₂O reducers matters for the N₂O:N₂ 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 N₂O-reducing bacteria and *in situ* N₂O emissions. Manuscript in preparation to be submitted to Global Change Biology

Abbreviations

- N Nitrogen
- N_r Reactive Nitrogen
- NO₃ Nitrate
- NO₂ Nitrite
- NO Nitric Oxide
- N₂O Nitrous Oxide
- N₂ Dinitrogen gas
- N₂OR 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
- nosZI Gene from Clade I encoding Nitrous Oxide Reductase
- nosZII Gene from Clade II encoding Nitrous Oxide Reductase
- IPCC Intergovernmental Panel on Climate Change
- EF N₂O Emission Factor
- pO₂ Oxygen Partial Pressure

1. Introduction

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

Free nitrogen, N₂, constitutes 78% of Earth's atmosphere. However, this immense reserve of N is accessible only to N₂-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 N₂ 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 N₂ 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 N₂O emissions would almost double by 2050 relative to 2005 values (Davidson et al., 2014). Yearly N₂O 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 N₂O emitted by natural earth ecosystems without human interference, are estimated to reach 11 Tg N₂O-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 N₂O emissions. Equally relevant are the indirect N₂O emissions due to NO₃⁻ leaching (Reay et al 2009).

Top-down or bottom-up methodologies are used to estimate N₂O emissions. The first are based on changes in atmospheric N₂O 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 N₂O emissions. Thus, it is currently used to estimate national N₂O inventories using a N₂O 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 N₂O emissions, *i.e.* every 1000 kg of N input results in 10 kg of N₂O-N emitted directly from the soil. An EF of 1% assumes a linear relationship between N input and N₂O 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 N₂O emissions from fertilized fields reported approximately 1 kg N₂O-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. N₂O 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 N₂O 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 N₂O emissions due to contrasting N inputs in different regions. Shcherbak et al (2014) suggested the use of an N-rate-dependent Δ EF model, could be a more appropriate method to estimate N₂O emissions than adopting a default value. The use of a constant EF may also explain why regional bottom-up estimates of N₂O 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 N₂O emissions in grassland and arable soils showed large variations between different regions (Fig. 2) (Stehfest & Bouwman, 2006).



Fig. 2. Simulated annual N₂O 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 N₂O, but can also act as a sink for this GHG. Unfortunately, most estimations of GHG sequestration ignore N₂O 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 N₂O (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 N₂O emissions continue rising (Reay et al., 2012) (Figure 3), it is urgent to better understand the factors underlying soil N₂O production and the potential of soil to reduce this GHG. While the influence of pedo-climatic factors on N₂O 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).

Introduction

1.3 Biological processes responsible for N₂O production and consumption

N₂O 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 (NH₃ \rightarrow NH₂OH/HNO \rightarrow NO₂⁻ \rightarrow NO₃⁻). 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 N₂O production by AOB or AOA. AOB generate N₂O via two processes: *i*) the incomplete hydroxylamine (NH₂OH) oxidation to NO and further to N₂O, and *ii*) through nitrifier denitrification. The mechanisms by which AOA produces N₂O are unclear (Walker et al., 2010). Some authors suggest that N₂O may be produced abiotically by oxidation of compounds such as NH₂OH, NO or NO₂⁻ (Harper et al., 2015). Further studies are still needed to elucidate the N₂O 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 NH₃ concentrations, AOA prefers low NH₃ 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 N₂O emissions. For the first time, Hink et al. (2016) could evaluate the relative N₂O 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 N₂O production. However, when NH_4^+ was added to soil microcosms AOB was responsible for its oxidation to NO_3^- and the corresponding N₂O emissions. To our knowledge, it is the only study disentangling the contribution of the AOA and AOB to N₂O 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 CO₂, while others have also heterotrophic metabolism and are considered mixotrophs or facultative autotrophs. These genera oxidize NO₂⁻ to NO₃⁻ 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, N₂O production by this process remained for long overlooked. Some studies acknowledge that this process might be relevant to N₂O 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 N₂O 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 (NO₃⁻ and NO₂⁻) are reduced to gaseous nitrogen oxides (NO, N₂O and N₂) under low O₂ 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 NO₃⁻ 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 (NO₂ \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 O₂, but also under microaerobic conditions (Laughlin & Stevens; 2002; Philipps et al., 2016). Fungal denitrification is of environmental concern because fungi do not possess N₂O-Reductase, N₂O 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 N₂O 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 N₂O-reducing microbial communities were largely underestimated in previous studies.

The N₂OR encoded by *nosZ*I and *nosZ*II clades have distinctive translocation pathways. While *nosZ*I secretes the N₂OR across the cytoplasmic membrane via the twinarginine 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 N₂OR is part of the *nos* cluster, which contains genes encoding for accessory proteins necessary for N₂OR 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 that could result in different growth efficiency and distinct capacities to reduce N₂O.

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 N₂O (Graf et al., 2014). For example, most Ascomycota have a *nir* and a *nor* gene, but are missing the gene encoding for the N₂OR. 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.

Introduction

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 N₂O or N₂. Concomitantly, non-hybrid N-N bonds are formed via the conventional denitrification pathway. The N₂O formation by the cometabolic pathway of codenitrification was further observed with NH_2OH , N_3^- , NH_4^+ , hydrazine (H₂N-NH₂) and salicyl hydroxamic acid (C₇H₇NO₃) (Spott et al., 2011).

Codenitrification has been studied to a great extent in fungi. Tanimoto et al. (1992) showed that *F. oxysporum* produced N₂O resulting from codenitrification and denitrification. Shoun et al., 1992 showed that other fungi species, *F. solani* and *C. tonkinense*, produced N₂ via codenitrification. Codenitrification products (N₂ or N₂O) vary according to the redox state of the nitrogen donor, while N₂ is formed from amines, N₂O is generated from imines and azide (Shoun et al., 2012). The enzyme catalyzing N₂O 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 N₂O (Kammann et al., 2008; Roobroeck et al., 2010). However, we still lack studies that quantify the isotopic signature of N₂O produced by codenitrification and studies that look at this process within soil samples. Thus, the contribution of codenitrification to soil N₂O production remains largely unknown (Baggs and Philippot 2010). Apart from being a source of N₂O, codenitrification can be relevant from an environmental perspective, because it plays a role in N immobilization by bonding inorganic N forms (NO₂⁻) 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 N₂O as a by-product of the NO₂⁻ reduction stage (Rütting et al., 2011). The N₂O 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 (N₂O \rightarrow N₂), resulting in an energetic gain. The Epsilonproteobacteria *Wolinella succinogenes* was shown for example to both perform DNRA and reduce N₂O (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 N₂O fluxes

The main factors affecting denitrification, nitrification and the corresponding N₂O 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 N₂O emissions under low pO₂ 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 pO₂ (Firestone et al., 1980) suggesting that O₂ inhibits the N₂OR (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 N₂O emissions, probably due to enhanced denitrification under low pO₂.

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 N₂O fluxes (*i.e.* nitrification and denitrification), and in regards to the proportion of N₂O produced during denitrification, herein named N₂O/N₂ 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 NH₃ availability, which decreases under low pH. Thus, low nitrification rates are normally recorded in low pH soils, and consequently low N₂O derived from ammonia oxidation (Baggs et al., 2010). However, Jung et al (2014) recorded the production of high N₂O 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 N₂O 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 N₂O 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, N₂O was the main product of denitrification, while at pH 6.9 N₂ was the predominant denitrification end-product. Cuhel et al. (2010) recorded an increase in N₂O:N₂ under low pH, which was due to changes in the total denitrification activity, while the production of N₂O remained unchanged. These results show that low pH can increase the proportion of N₂O as end-product of denitrification, which was attributed to an unsuccessful assembly of the N₂OR 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 N₂O 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 N₂O:N₂ (Smith and Tiedje, 1979; Weier et al., 1993), nevertheless, the total amount of N₂O 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 N₂O 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 N₂O 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 N₂O emissions and N₂O-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.

Introduction

1. 5.1. N₂O 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 N₂O emissions. For example, organic fertilizer stimulates more than inorganic fertilizers denitrification rates and corresponding N₂O 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 N₂O 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 N₂O emissions by nitrification by enhancing AOA. More studies are needed better understand the effect of different N sources on N₂O 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

Introduction

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 N₂O 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 N₂O 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 N₂O emissions. Graf et al., (2016) showed no significant effect of barley or sunflower in the potential denitrification activity, potential N₂O production or N₂O:N₂ 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 N₂O emissions. These results demonstrated that plants play an important role in controlling processes within the N-cycle and N₂O emissions.

Crop residue decomposition also represents a source of N and C and can enhance N₂O 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 N₂O emissions in NT systems was responsible for positive net GHGs fluxes. There is no consensus on tillage management impact on N₂O emissions. While in some soils reduced tillage enhanced N₂O 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 N₂O 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 N₂O-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 *nosZI* 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 NH₄NO₃ 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 *nosZI* 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 *nosZI*.

Another practice impacting N₂O-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 *nosZI* (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 N₂O-reducers. Thompson et al. (2016) investigated the effect of annual and perennial cropping systems on the *nosZI* community. A higher abundance of the *nosZI* 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 N₂O 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 N₂O-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 N₂O as it does not possess any denitrification gene except *nosZ*. As such, increasing the relative abundance of *P saltans* strains carrying exclusively the N₂OR could have a potential to decrease soil N₂O emissions. These results suggest that management may, directly or indirectly by changing soil properties, impact the two N₂O-reducing clades, with potential consequences for the N₂O emissions.

1.6 Relevance of community abundance, composition and diversity for understanding N₂O 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 N₂O 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 N₂O-producers and N₂Oreducers in agricultural soils, and their relation to the soil N₂O production. My thesis started just after the discovery of the *nos*ZII clade. Thus, we aimed to increase the understanding of this clade's relevance for soil N₂O reduction. For this purpose, we performed experiments at different scales. First, a laboratory incubation experiment was set up to test if *nos*ZII N₂Oreducers can take up N₂O 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 N₂O-producers and N₂O-reducers as well as their influence on the soil N₂O 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 N₂O production and reduction, in relation with *in situ* N₂O emissions.

- I. Chapter I: Laboratory incubation Non-denitrifying nitrous oxide-reducing bacteria an effective N₂O sink in soil.
- II. Chapter II: One site approach The diversity of the N₂O reducers matters for the N₂O:N₂ 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 N₂O-reducing bacteria and *in situ* N₂O 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

Soil Biology & Biochemistry 103 (2016) 376-3379.

Soil Biology & Biochemistry 103 (2016) 376-379

Contents lists available at ScienceDirect

Soil Biology & Biochemistry

journal homepage: www.elsevier.com/locate/soilbio

Short Communication

Non-denitrifying nitrous oxide-reducing bacteria - An effective $N_2 O$ 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

ARTICLE INFO

Article history: Received 23 May 2016 Received in revised form 3 September 2016 Accepted 18 September 2016

Keywords: Greenhouse gas nosZ Mitigation Microbial community Nitrogen Denitrification

ABSTRACT

Nitrous oxide (N₂O) 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 N₂O remains unanswered. We evaluated the capacity of non-denitrifying N₂O-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 N₂O reductase NosZII, to 11 different soils significantly reduced N₂O 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 N₂O consumption in soil.

© 2016 Elsevier Ltd. All rights reserved.

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 CO₂ (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 N₂O through the N₂O reductase encoded by the nosZ gene (Chapuis-Lardy et al., 2007). However, a new lineage of the N₂O-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 N₂O sinks in soil have been suggested in studies showing that their diversity was positively related to the soil N₂O sink capacity (Jones et al., 2014) and a strong predictor of the N₂O/N₂ denitrification end-product ratio (Domeignoz-Horta et al., 2015).

Here, we experimentally test the proposed role of nondenitrifying nosZ clade II microorganisms for soil N₂O reduction capacity. For this purpose, we manipulated soil microbial communities by adding different amounts of Dyadobacter fermentans, a nosZII bacterial strain lacking any known denitrification genes, in 11 different soils and monitored the N2O production and denitrification rates by the indigenous soil communities. Soils with contrasting properties were sampled from 0 to 15 cm depth in longterm 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 nosZI/nosZII genes was quantified by real time PCR and used as a proxy for potentially N₂O-producing and N₂O-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; 10⁶ (denoted low inoculation, LI) and 10^8 cells g⁻¹ dry soil (denoted high inoculation, HI), in addition to a









Fig. 1. Impact of *Diadobacter fermentans* addition on potential denitrification rate (A), potential N₂O 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 ₂ O
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	рН	Clay (%)	Loam (%)	C/N	16S rRNA ^a	nirK/nirS	nosZI (%)	nosZII (%)	nosZl/nosZlI	nosZl/(nirK+nirS)	nosZII/(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
$NI^{\left(N_{2}O+N_{2}\right)}$	2.1	6.0** (+)	0.3	47.1** (-)	2.3	7.5** (+)	7.2** (+)	7.1** (+)	0.4	2.5	0.1	17.4
$NI^{(N_{2}O/N_{2}O+N_{2})}$	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 KNO3 solution (denoted no inoculation, NI). Soil microcosms were brought to 80% of the maximum water holding capacity (WHC) with a 3 mM KNO₃ 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 N₂O 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 N₂O 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 μ g N₂O-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 N₂O production rates corresponded to 10-95% of the denitrification rates. It has long been recognized that the proportion of N₂O 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 N2O production to denitrification rate ratio [rN₂O/r(N₂O+N₂)] (P < 0.001). Nevertheless, a negative correlation was observed between the relative abundance of the indigenous nosZII abundance and the potential N₂O production (r = -0.35, P < 0.05) and [rN₂O/ $r(N_2O+N_2)$] (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 N₂O 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 N₂O, 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 N₂O, which was concomitant with a decrease in N₂O concentration (Supplementary Figs. S2 and S3). This indicates that *D. fermentans* NS114^T can respire N₂O 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 N₂O 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 N₂O production was 51%, and up to 189% in some soil microcosms since negative rates were recorded. The significant reduction of N₂O production in several soils after inoculation of *D. fermentans* demonstrates that non-denitrifying bacteria with *nosZ* are capable to eliminate the N₂O emitted by other microorganisms in soil.

The reduction of N₂O 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 N₂O 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 N₂O mitigation potential of the added nosZII strain, calculated both as ratio and as difference in N₂O 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 N₂O reduced by D. fermentans suggests that the capacity of the strain to mitigate N₂O 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 nosZII 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 nondenitrifying N₂O-reducing bacteria can contribute to lowering the net N₂O production in soil. Thus, agricultural practices fostering *nosZ* clade II microbial communities could be an alternative strategy to reduce N₂O 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).

Acknowledgments

This work was supported by the European Union (Marie Curie ITN NORA, FP7- 316472). The authors thank Maren Emmerich and

Örjan Berglund for helping with sample collection. In memory of the NORA partners W.F.M. Röling and Simon de Vries.

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.

References

- Attard, E., Recous, S., Chabbi, A., De Berranger, C., Guillaumaud, N., Labreuche, J., Philippot, L., Schmid, B., Le Roux, X., 2011. Soil environmental conditions rather than denitrifier abundance and diversity drive potential denitrification after changes in land uses. Global Change Biology 17, 1975–1989.
- Bender, S.F., Wagg, C., van der Heijden, M.G.A., 2016. An underground revolution: biodiversity and soil ecological engineering for agricultural sustainability. Trends in Ecology & Evolution S0169–5347 (16), 00061–00066.
 Butterbach-Bahl, K., Baggs, E.M., Dannenmann, M., Kiese, R., Zechmeister-
- Butterbach-Bahl, K., Baggs, E.M., Dannenmann, M., Kiese, R., Zechmeister-Boltenstern, S., 2013. Nitrous oxide emissions from soils: how well do we understand the processes and their controls? Philosophical Transactions Royal Society London B Biologial Sciences 368, 20130122.
- Chapuis-Lardy, L., Wrage, N., Metay, A., Chotte, J.-L., Bernoux, M., 2007. Soils, a sink for N₂O? A review. Global Change Biology 13, 1–17.
- Domeignoz-Horta, L.A., Spor, A., Bru, D., Breuil, M.-C., Bizouard, F., Leonard, J., Philippot, L., 2015. The diversity of the N₂O reducers matters for the N₂O: N₂ denitrification end-product ratio across an annual and a perennial cropping system. Frontiers in Microbiology 6, 971.
- Ekpete, D.M., Cornfield, A.H., 1964. Losses, through denitrification, from soil of applied inorganic nitrogen even at low moisture contents. Nature 201, 322–323.
- Firestone, M.K., Firestone, R.B., Tiedje, J.M., 1980. Nitrous-oxide from soil denitrification - factors controlling its biological production. Science 208, 749–751.
- Gaba, S., Fried, G., Kazakou, E., Chauvel, B., Navas, M.-L., 2014. Agroecological weed control using a functional approach: a review of cropping systems diversity. Agronomy for Sustainable Development 34, 103–119.
- Graf, D.R., Jones, C.M., Hallin, S., 2014. Intergenomic comparisons highlight modularity of the denitrification pathway and underpin the importance of community structure for N₂O emissions. PLoS One 9, e114118.
- Graham, E.B., Knelman, J.E., Schindlbacher, A., Siciliano, S., Breulmann, M., Yannarell, A., Bemans, J.M., Abell, G., Philippot, L., Prosser, J., Foulquier, A., Yuste, J.C., Glanville, H.C., Jones, D.L., Angel, F., Salminen, J., Newton, R.J., Buergmann, H., Ingram, L.J., Hamer, U., Siljanen, H.M.P., Peltoniemi, K., Potthast, K., Baneras, L., Hartmann, M., Banerjee, S., Yu, R.-Q., Nogaro, G., Richter, A., Koranda, M., Castle, S.C., Goberna, M., Song, B., Chatterjee, A., Nunes, O.C., Lopes, A.R., Cao, Y., Kaisermann, A., Hallin, S., Strickland, M.S., Garcia-Pausas, J., Barba, J., Kang, H., Isobe, K., Papaspyrou, S., Pastorelli, R., Lagomarsino, A., Lindstrom, E.S., Basiliko, N., Nemergut, D.R., 2016. Microbes as engines of ecosystem function: when does community structure enhance predictions of ecosystem processes? Frontiers in Microbiology 7, 214.
- Hu, H.-W., Chen, D., He, J.-Z., 2015. Microbial regulation of terrestrial nitrous oxide formation: understanding the biological pathways for prediction of emission rates. FEMS Microbiology Reviews 39 (5), 729–749.
- Jones, C.M., Graf, D.R.H., Bru, D., Philippot, L., Hallin, S., 2013. The unaccounted yet abundant nitrous oxide-reducing microbial community: a potential nitrous oxide sink. ISME Journal 7, 417–426.
- Jones, C.M., Spor, A., Brennan, F.P., Breuil, M.-C., Bru, D., Lemanceau, P., Griffiths, B., Hallin, S., Philippot, L., 2014. Recently identified microbial guild mediates soil N₂O sink capacity. Nature Climate Change 4, 801–805.

Keil, D., Niklaus, P.A., von Riedmatten, L.R., Boeddinghaus, R.S., Dormann, C.F.,

Scherer-Lorenzen, M., Kandeler, E., Marhan, S., 2015. Effects of warming and drought on potential N₂O emissions and denitrifying bacteria abundance in grasslands with different land-use. FEMS Microbiology Ecology 91, 7.

- Lagomarsino, A., Agnelli, A.E., Pastorelli, R., Pallara, G., Rasse, D.P., Silvennoinen, H., 2016. Past water management affected GHG production and microbial community pattern in Italian rice paddy soils. Soil Biology and Biochemistry 93, 17–27.
- Lang, E., Lapidus, A., Chertkov, O., Brettin, T., Detter, J.C., Han, C., Copeland, A., Del Rio, T.G., Nolan, M., Chen, F., Lucas, S., Tice, H., Cheng, J.F., Land, M., Hauser, L., Chang, Y.J., Jeffries, C.D., Kopitz, M., Bruce, D., Goodwin, L., Pitluck, S., Ovchinnikova, G., Pati, A., Ivanova, N., Mavrommatis, K., Chen, A., Palaniappan, K., Chain, P., Bristow, J., Eisen, J.A., Markowitz, V., Hugenholtz, P., Goeker, M., Rohde, M., Kyrpides, N.C., Klenk, H.P., 2009. Complete genome sequence of Dyadobacter fermentans type strain (NS114(T)). Standards in Genomic Sciences 1, 133–140.
- Montzka, S.A., Dlugokencky, E.J., Butler, J.H., 2011. Non-CO2 greenhouse gases and climate change. Nature 476, 43–50.
- Orellana, L.H., Rodriguez-R, L.M., Higgins, S., Chee-Sanford, J.C., Sanford, R.A., Ritalahti, K.M., Löffler, F.E., Konstantinidis, K.T., 2014. Detecting nitrous oxide reductase (nosZ) genes in soil metagenomes: method development and implications for the nitrogen cycle. MBio 5 3, e01193-e01214.
- Petersen, D.G., Blazewicz, S.J., Firestone, M., Herman, D.J., Turetsky, M., Waldrop, M., 2012. Abundance of microbial genes associated with nitrogen cycling as indices of biogeochemical process rates across a vegetation gradient in Alaska. Environmental Microbiology 14, 993–1008.
- Petric, I., Philippot, L., Abbate, C., Bispo, A., Chesnot, T., Hallin, S., Laval, K., Lebeau, T., Lemanceau, P., Leyval, C., Lindstrom, K., Pandard, P., Romero, E., Sarr, A., Schloter, M., Simonet, P., Smalla, K., Wilke, B.M., Martin-Laurent, F., 2011. Interlaboratory evaluation of the ISO standard 11063 "Soil quality - method to directly extract DNA from soil samples". Journal of Microbiological Methods 84, 454–460.
- Philippot, L., Andert, J., Jones, C.M., Bru, D., Hallin, S., 2011. Importance of denitrifiers lacking the genes encoding the nitrous oxide reductase for N₂O emissions from soil. Global Change Biology 17, 1497–1504.
- Ravishankara, A.R., Daniel, J.S., Portmann, R.W., 2009. Nitrous oxide (N₂O): the dominant ozone-depleting substance emitted in the 21st century. Science 326, 123–125.
- Rocca, J.D., Hall, E.K., Lennon, J.T., Evans, S.E., Waldrop, M.P., Cotner, J.B., Nemergut, D.R., Graham, E.B., Wallenstein, M.D., 2014. Relationships between protein-encoding gene abundance and corresponding process are commonly assumed yet rarely observed. ISME Journal 9 (8), 1693–1699.
- Röling, W.F.M., 2007. Do microbial numbers count? Quantifying the regulation of biogeochemical fluxes by population size and cellular activity. FEMS Microbiology Ecology 62, 202–210.
- Sanford, R.A., Wagner, D.D., Wu, Q., Chee-Sanford, J.C., Thomas, S.H., Cruz-Garcia, C., Rodriguez, G., Massol-Deya, A., Krishnani, K.K., Ritalahti, K.M., Nissen, S., Konstantinidis, K.T., Loeffler, F.E., 2012. Unexpected nondenitrifier nitrous oxide reductase gene diversity and abundance in soils. Proceedings of the National Academy of Sciences of the United States of America 109, 19709–19714.
- Shcherbak, I., Millar, N., Robertson, G.P., 2014. Global metaanalysis of the nonlinear response of soil nitrous oxide (N₂O) emissions to fertilizer nitrogen. Proceedings of the National Academy of Sciences of the United States of America 111, 9199–9204.
- Thomson, A.J., Giannopoulos, G., Pretty, J., Baggs, E.M., Richardson, D.J., 2012. Biological sources and sinks of nitrous oxide and strategies to mitigate emissions. Philosophical Transactions of the Royal Society B-Biological Sciences 367, 1157–1168.
- Yoon, S., Nissen, S., Park, D., Sanford, R.A., Löffler, F.E., 2016. Nitrous oxide reduction kinetics distinguish bacteria harboring clade I NosZ from those harboring clade II NosZ. Applied and Environmental Microbiology 82, 3793–3800.

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

Soil	Country	Land Use	Fertilisation level	Hq	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	$40.6^{ m abc}$ ± 1.4	26.8 ^b ± 3.4	2.6^{b} ± 0.2	$\begin{array}{c} 10.4^{\mathrm{ef}} \\ \pm 0.3 \end{array}$	$46.4^{\rm b}$ ± 5.9	8.6 ^{de} ±1.4
SH.IR	Ireland	Pasture	Low	$6.6^{\rm cd} \pm 0.3$	$36.7^{\rm bc}$ ± 0.6	$41.7^{\rm b}$ ± 1.7	21.7 ^{de} ± 2.2	$64.0^{a} \pm 8.4$	$6.6^{a} \pm 0.7$	$9.7^{g} \pm 0.2$	110.7^{a} ± 14.6	$25.1^{a} \pm 0.8$
LR.EN	England	Pasture	Low	$5.5^{\rm f}$ ± 0.2	32.7 ^{cd} ±4.6	29.6 [°] ± 1.2	$37.8^{\mathrm{bc}} \pm 5.8$	$\begin{array}{l} 45.6^{\mathrm{a}} \\ \pm 4.9 \end{array}$	4.8^{a} ± 0.5	$9.6^{g} \pm 0.0$	78.8 ^a ± 8.5	$17.7^{\rm b}$ ± 5.5
UM.A.SE	Sweden	Arable land	Low	5.9 ^e ± 0.2	8.1^{f} ± 1.0	62.7 ^a ± 5.7	$29.1^{cde} \pm 6.6$	22.5 ^{bc} ± 4.8	$1.5^{\rm cd}$ ± 0.3	$15.0^{a} \pm 0.4$	39.0 ^{bc} ± 8.3	$3.7^{\rm f}$ ± 0.7
UM.B.SE	Sweden	Arable land	High	5.9 ^e ± 0.2	$7.6^{\rm f}$ ± 1.2	58.4 ^ª ± 3.0	$33.9^{cd} \pm 3.8$	24.8 ^b ± 4.5	$\frac{1.6^{\circ}}{\pm 0.3}$	$15.1^{a} \pm 0.4$	42.9 ^b ± 7.8	$4.1^{\mathrm{ef}} \pm 0.4$
SA.A.SE	Sweden	Arable land	Low	$\begin{array}{c} 6.6^{\rm c} \\ \pm \ 0.4 \end{array}$	46.2^{a} ± 1.3	$45.3^{\rm b}$ ± 0.5	8.4 ^f ± 1.1	15.8 ^d ± 1.4	$1.3^{cd} \pm 0.1$	$\frac{11.8^{\text{b}}}{\pm 0.1}$	27.3 ^d ± 2.5	13.5 ^{bc} ± 1.8
SA.B.SE	Sweden	Arable land	High	$6.7^{\rm bc}$ ± 0.1	47.8^{a} ± 1.6	$\begin{array}{l} 44.9^{\mathrm{b}} \\ \pm 1.4 \end{array}$	$7.2^{\rm f}$ ± 0.2	17.1 ^{cd} ± 1.4	1.5 ^{cd} ± 0.1	$11.6^{\rm bc} \pm 0.1$	29.5 ^{cd} ± 2.4	$\begin{array}{l} 14.5^{\mathrm{bc}}\\ \pm 0.2\end{array}$
ST.A.SE	Sweden	Arable land	Low	$7.1^{ab} \pm 0.2$	26.2 ^{de} ± 5.4	24.0 ^c ± 4.6	49.7^{ab} ± 9.5	$\begin{array}{l} 10.5^{\rm e} \\ \pm 0.8 \end{array}$	1.0^{e} ± 0.1	$10.8^{ m de} \pm 0.1$	18.1 ^e ± 1.5	$\frac{11.2^{\rm cd}}{\pm 1.7}$
ST.B.SE	Sweden	Arable land	High	$7.2^{a} \pm 0.1$	24.3 ^e ± 2.8	23.5° ± 2.3	52.1^{a} ± 5.0	12.4 ^{de} ± 2.0	$1.1^{ m de} \pm 0.2$	11.1 ^{cd} ± 0.1	21.5 ^{de} 主3.5	$\frac{11.7^{cd}}{\pm 1.1}$
VA.A.SE	Sweden	Arable land	Low	$6.4^{\rm cd} \pm 0.2$	41.7^{ab} ± 2.0	$39.4^{\rm b}$ ± 0.9	18.7 ^{ef} ± 2.0	12.5 ^{de} ± 0.8	$1.2^{cde} \pm 0.1$	$10.1^{\mathrm{fg}} \pm 0.2$	21.6 ^{de} ± 1.4	$\frac{12.6^{cd}}{\pm 0.7}$
VA.B.SE	Sweden	Arable land	High	$6.3^{\rm de} \pm 0.1$	42.3^{ab} ± 2.0	40.0^{b} ± 0.8	$17.6^{\rm ef} \pm 2.5$	13.1 ^{de} ± 0.6	$1.3^{cde} \pm 0.1$	$\begin{array}{l} 10.1^{\mathrm{fg}} \\ \pm 0.1 \end{array}$	22.7^{de} ± 1.0	12.0 ^{cd} ± 1.1
Soil propertie physicochem	es were measu ical properties	red by the INRA La are presented and	iboratory of Soil significant diffe	Analysis (rences bet	Arras, Franc ween soils are	e) using stand ? indicated wit	lard procedure. th different lett	s. Means and 5 ers(ANOVA foi	15% confidence lowed by Tuke	e interval of sy's HSD tes	soil st, P < 0.05)	

NA: not available, ¹Size fractions based on μm Atterberg scale; ²g kg⁻¹; ³Cation exchange capacity, ³ cmol kg⁻¹.



Fig. S1. Abundance of indigenous microbial communities in the soils. A) N₂O-producers (*nirK*; white, *nirS*; gray) B) N₂O-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).



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).



Fig. S3. N₂O reduction by *Diadobacter fermentans* in pure culture grown at 2, 4, 8 and 16 mM of N₂O in the liquid phase. The N₂O 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

Frontiers in Microbiololy 6:971 (2015) doi: 10.3389/fmicb.2015.00971.





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-Horta¹, Aymé Spor¹, David Bru¹, Marie-Christine Breuil¹, Florian Bizouard¹, Joël Léonard² and Laurent Philippot^{1*}

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

OPEN ACCESS

Edited by:

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

Specialty section:

This article was submitted to Terrestrial Microbiology, a section of the journal Frontiers in Microbiology

Received: 13 July 2015 Accepted: 01 September 2015 Published: 24 September 2015

Citation:

Domeignoz-Horta LA, Spor A, Bru D, Breuil M, Bizouard F, Léonard J and Philippot L (2015) The diversity of the N₂O reducers matters for the N₂O:N₂ denitrification end-product ratio across an annual and a perennial cropping system. Front. Microbiol. 6:971. doi: 10.3389/fmicb.2015.00971

Agriculture is the main source of terrestrial emissions of N₂O, a potent greenhouse gas and the main cause of ozone layer depletion. The reduction of N₂O into N₂ 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 N₂O-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 N₂O reducer clades with consequences for denitrification end-products. The abundance of N₂O-reducers and producers was quantified by real-time PCR, and the diversity of both nosZ clades was determined by 454 pyrosequencing. Potential N₂O 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 nosZll community, as well as in the $[rN_2O/r(N_2O+N_2)]$ ratio. More limited differences were observed in the nosZl community, suggesting that the newly identified nosZII clade is more sensitive than nosZI to environmental changes. Potential denitrification activity and potential N₂O 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 N₂O reducers for mitigation strategies.

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

Introduction

Nitrous oxide (N₂O) is one of the six gases subject to restriction by the Kyoto Protocol, which aims at reducing anthropogenic greenhouse gas (GHG) emissions. N₂O 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, N₂O 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), N₂O is today the dominant ozone-depleting substance (Ravishankara et al., 2009). The atmospheric concentration of N₂O has been rising over the past 100 years resulting in a concentration 19% higher than preindustrial levels (Montzka et al., 2011) with an estimated increase of N₂O emissions of up to 60% by 2050 (relative to 1900 values) (Bouwman et al., 2013).

N2O 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 N₂O. 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, N2O and N2 gases via four enzymatic systems (Tiedje et al., 1982; Zumft, 1997). The reduction of soluble NO₂⁻ into NO and N₂O 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 N₂O into inert N₂, which accounts for 78% of the atmospheric gases, and is, therefore, the key enzyme involved in the N₂O 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 N2O as the denitrification end product (Philippot et al., 2011), or are only able to reduce N₂O (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 N₂O-producing nitric oxide reductase, and, therefore, can only consume N₂O (Graf et al., 2014). The abundance and phylogenetic diversity of *nosZII* microorganisms was found to mediate the soil N₂O sink capacity in European soils (Jones et al., 2014), showing the importance of understanding the ecology of this microbial guild for mitigating N₂O 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* N₂O-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 N₂O-reducing microbial communities and N-gas production (N₂O and N₂) 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 N₂O Production, Potential Denitrification Activity (N₂O+N₂) and Denitrification End-product Ratio $[rN_2O/r(N_2O+N_2)]$

To assess the activity of the N2O reducing microbial communities, the potential N2O 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 ($CI_{95\%} = [1.41 - 1.61]$) μ g N₂O-N g⁻¹ soil DW h⁻¹ for potential N₂O 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 N2O 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, N₂O-producers and N₂O-reducers

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



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 N₂O-producers and N₂O-reducers abundances were found between treatments (Figure S1). However, *nosZII* abundance was higher in BE than in ORE (P < 0.01).

N₂O-reducer Diversity

To assess whether the agricultural practices could drive the composition and structure of the N₂O-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 (CI95% = 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 a-diversity of the N2Oreducing 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 nosZ from Bacteroidetes while nosZI sequences were affiliated to nosZ 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 N₂O 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 N₂O-reducing Community

We used variance partitioning technique to quantify the relative contribution of the different groups of variables to the variation in N₂ production by denitrification across samples (Figures 3A-D). The physical and chemical characteristics of the soil, the abundance of N2O producers and reducers, and the diversity of N2O 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 N₂O production and PDA, explaining up to 29 and 45% of the variance, respectively (Figures 3B,C). In contrast, the [rN₂O/r(N₂O+N₂)] was mostly explained by the diversity of the N2O-reducers (26%). Interactions between physical and chemical properties of the soil and the diversity of the N2O-reducing communities accounted for 26 and 17% of potential N₂O 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 $[rN_2O/r(N_2O+N_2)].$



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 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 N₂O-reducing communities and N₂ 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 N₂O-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



the triangles, respectively. **(B)** Variance partitioning of potential N₂O 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 N2O 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 (pO₂). This is consistent with our results since previous studies have shown that production of N₂O relative to N₂ during denitrification in soils is strongly influenced by carbon availability and pO2 (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 N₂O produced during denitrification with decreasing O₂ concentrations (Firestone et al., 1980). However, the harvesting date had no significant effect on the denitrification gaseous endproduct 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 N2O reducer community structure, significant differences in the N2O 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* N₂O reducers in the N₂O-reducing capacity of a soil.

We found that the abundance of nosZII N2O 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 N2O-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 N₂O 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 N₂O reducing communities. They also indicate that agricultural practices could affect nosZI and nosZII communities in different ways with consequences for N₂O 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

Frontiers in Microbiology | www.frontiersin.org

Treatmer	nt Croppinę system	g Crops	Management practices ¹	N input ²	% Water	Hq	% Clay ³	% Loam ³	% Sand ³	Total C ⁴	Total N ⁴	OM ⁴	Ca ⁴	CEC ^{5,6}
ML	BE	Miscanthus (<i>M.</i> giganteus)	Late harvest	120	21.7 ^c ± 0.9	7.2 ^{ab} ± 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 (<i>M.</i> giganteus)	Early harvest	120	23.0 ^{bc} ± 0.1	7.4 ^{ab} ± 0.6	20.9 ± 1.7	73.5 ± 2.4	6.0 ± 1.3	10.9 ± 0.6	1.0 ± 4.10 ⁻²	18.9 ± 1.0	2.3 ± 2.6	11.6 土 1.0
SL	BE	Switchgrass (P. virgatum)	Late harvest	120	23.3 ^{ab}	7.4 ^{ab} 土 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} ± 0.6	6.8 ^b 土 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
F	ORE	Peas, rapeseed, wheat, barley, corn, wheat	Conventional tillage, straw addition, high N input, non-legume intercrop	100	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	1.0 ± 4.10^{-2}	18.5 ± 1.4	3.0 ± 2.9	12.3 ± 1.7
12	ORE	Peas, rapeseed, wheat, barley, corn, wheat	Reduced tillage, straw addition,high N input, non-legume intercrop	100	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	1.0 ± 1.10 ⁻²	18.6 ± 0.5	3.7 ± 2.8	10.5 ± 0.5
ЦЗ	ORE	Peas, rapeseed, wheat, barley, corn, wheat	Reduced tillage, straw removal, high N input, non-legume intercrop	100	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	4.0 ± 5.8	12.1 ± 1.5
Т4	ORE	Peas, rapeseed, wheat, barley, corn, wheat	Conventional tillage, straw addition, low N input, non-legume intercrop	40	25.2 ^a ± 1.1	8.0 ^a ± 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 土 1.9
T5	ORE	Peas, rapeseed, wheat, barley, corn, wheat	Conventional tillage, straw addition, low N input, legume intercrop	40	25.0 ^{ab} ± 0.4	8.1 ^a ± 0.1	17.8 ± 1.6	77.1 ± 1.2	4.8 ± 0.5	10.7 ± 0.3	1.0 ± 5.10 ^{−2}	18.4 ± 0.6	3.4 ± 1.7	10.4 ± 1.0
Means and	d 95% confide	ence interval of soil phy	ysicochemical properties are give	an per treatmo	ent within each e	experimental bl	ock. Significan	t differences t	etween treatr	ments are indi	cated with differ	rent letters: "a	", "b", and "c	c" (anova

ŝ

o.

0

m | followed by Tukey HSD test, P < 0.05).

¹ "Late" and "early" havest 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; ²N input (Kg N ha⁻¹ y⁻¹); ³Size fractions based on µm Atterberg scale; Clay = 2 µm, Loam = 2-50 µm, and Sand = 50-2000 µm; ⁴g kg⁻¹; ⁵ cation exchange capacity; ⁶ cmol kg⁻¹.

0

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 N₂O into N₂ 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 N₂O producing and reducing communities did not explain variations in PDA nor in potential N₂O 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 N₂O production and PDA, the variance in the proportion of N₂O emitted by denitrification [rN2O/r(N2O+N2)] 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 N2O 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 endproducts through their capacity to act as a N₂O 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 N2O production. They also showed that the two clades of N2O 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 N₂O 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 N2O and N2 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 ORE randomized field experiments (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 N₂O 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 KNO₃, 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 N₂O 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 \times 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 \times g, 5 min, 4°C). The DNA was precipitated by adding one volume of cold isopropanol $(-20^{\circ}C)$ 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 QuantiT 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 N₂O-producers, *nirK* and *nirS* (Henry et al., 2004; Kandeler et al., 2006) and N₂O-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 μ l reaction volume containing 1 ng of DNA, 7.5 μ l of SYBRgreen PCR Master Mix (Absolute qPCR SYBR GreenRox, Thermo, Courtaboeuf, France), 1 μ M 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 N₂O-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 μ l reaction volume containing 5 μ l 5 \times Tag Buffer (GoTaq, Promega, Madison, U.S.A.), $2\,\mu M$ 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 μ l reaction volume containing 10 μ l 5 \times 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 N₂O 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 N₂O, PDA and $[rN_2O/r(N_2O+N_2)]$, using the function *varpart* (Peres-Neto et al., 2006).

Acknowledgments

This research was supported by the European Union (Marie Curie ITN NORA, FP7- 316472) and the French Agency for the Environment and Energy (ADEME) thought the project EFEMAIR-N₂O. We are grateful to Céline Peyrard and Bruno Mary for helpful discussions and suggestions.

Supplementary Material

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

References

- Amougou, N., Bertrand, I., Cadoux, S., and Recous, S. (2012). Miscanthus x giganteus leaf senescence, decomposition and C and N inputs to soil. *Glob. Change Biol. Bioen.* 4, 698–707. doi: 10.1111/j.1757-1707.2012.01192.x
- Amougou, N., Bertrand, I., Machet, J.-M., and Recous, S. (2011). Quality and decomposition in soil of rhizome, root and senescent leaf from Miscanthus x giganteus, as affected by harvest date and N fertilization. *Plant Soil* 338, 83–97. doi: 10.1007/s11104-010-0443-x
- Baggs, E. M. (2011). Soil microbial sources of nitrous oxide: recent advances in knowledge, emerging challenges and future direction. *Curr. Opin. Environ. Sustain.* 3, 321–327. doi: 10.1016/j.cosust.2011.08.011
- Bates, B., Kundzewicz, Z., Wu, S., and Palutikof, J. (2008). "Climate change and water," in *Technical Paper of the Intergovernmental Panel on Climate Change* (Geneva: IPCC).
- Berry, D., Ben Mahfoudh, K., Wagner, M., and Loy, A. (2012). Barcoded primers used in multiplex amplicon pyrosequencing bias amplification. *Appl. Environ. Microbiol.* 78, 612–612. doi: 10.1128/AEM.07448-11
- Bouwman, A. F., Beusen, A. H. W., Griffioen, J., Van Groenigen, J. W., Hefting, M. M., Oenema, O., et al. (2013). Global trends and uncertainties in terrestrial denitrification and N₂O emissions. *Phil. Trans. R Soc. B.* 368, 20130112. doi: 10.1098/rstb.2013.0112
- Cadoux, S., Ferchaud, F., Demay, C., Boizard, H., Machet, J.-M., Fourdinier, E., et al. (2014). Implications of productivity and nutrient requirements on greenhouse gas balance of annual and perennial bioenergy crops. *Glob. Change Biol. Bioeng.* 6, 425–438. doi: 10.1111/gcbb.12065
- Caporaso, J. G., Bittinger, K., Bushman, F. D., DeSantis, T. Z., Andersen, G. L., and Knight, R. (2010b). PyNAST: a flexible tool for aligning sequences to a template alignment. *Bioinformatics* 26, 266–267. doi: 10.1093/bioinformatics/btp636
- Caporaso, J. G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F. D., Costello, E. K., et al. (2010a). QIIME allows analysis of high-throughput community sequencing data. *Nat. Methods* 7, 335–336. doi: 10.1038/nmeth.f.303
- Clark, I. M., Buchkina, N., Jhurreea, D., Goulding, K. W., and Hirsch, P. R. (2012). Impacts of nitrogen application rates on the activity and diversity of denitrifying bacteria in the Broadbalk Wheat Experiment. *Phil. Trans. R. Soc. B Biol. Sci.* 367, 1235–1244. doi: 10.1098/rstb.2011.0314
- Edgar, R. C. (2010). Search and clustering orders of magnitude faster than BLAST. *Bioinformatics* 26, 2460–2461. doi: 10.1093/bioinformatics/btq461
- Enwall, K., Philippot, L., and Hallin, S. (2005). Activity and composition of the denitrifying bacterial community respond differently to long-term fertilization. *Appl. Environ. Microbiol.* 71, 8335–8343. doi: 10.1128/AEM.71.12.8335-8343.2005
- Firestone, M. K., Firestone, R. B., and Tiedje, J. M. (1980). Nitrous-oxide from soil denitrification - factors controlling its biological production. *Science* 208, 749–751. doi: 10.1126/science.208.4445.749
- Giles, M., Morley, N., Baggs, E. M., and Daniell, T. J. (2012). Soil nitrate reducing processes drivers, mechanisms for spatial variation, and significance for nitrous oxide production. *Front. Microbiol.* 3:407. doi: 10.3389/fmicb.2012.00407
- Graf, D. R., Jones, C. M., and Hallin, S. (2014). Intergenomic comparisons highlight modularity of the denitrification pathway and underpin the importance of community structure for N2O Emissions. *PLoS ONE* 9:e114118. doi: 10.1371/journal.pone.0114118
- Hallin, S., Jones, C. M., Schloter, M., and Philippot, L. (2009). Relationship between N-cycling communities and ecosystem functioning in a 50year-old fertilization experiment. *ISME J.* 3, 597–605. doi: 10.1038/ismej. 2008.128
- Hartmann, M., Frey, B., Mayer, J., Mäeder, P., and Widmer, F. (2014). Distinct soil microbial diversity under long-term organic and conventional farming. *ISME* J. 9, 1177–1194. doi: 10.1038/ismej.2014.210
- Henry, S., Baudoin, E., López-Gutiérrez, J. C., Martin-Laurent, F., Brauman, A., and Philippot, L. (2004). Quantification of denitrifying bacteria in soils by nirK gene targeted real-time PCR. J. Microbiol. Methods 59, 327–335. doi: 10.1016/j.mimet.2004.07.002
- Henry, S., Bru, D., Stres, B., Hallet, S., and Philippot, L. (2006). Quantitative detection of the nosZ gene, encoding nitrous oxide reductase, and comparison of the abundances of 16S rRNA, narG, nirK, and nosZ genes in soils. *Appl. Environ. Microbiol.* 72, 5181–5189. doi: 10.1128/AEM.00231-06

- Jones, C. M., Graf, D. R., Bru, D., Philippot, L., and Hallin, S. (2013). The unaccounted yet abundant nitrous oxide-reducing microbial community: a potential nitrous oxide sink. *ISME J.* 7, 417–426. doi: 10.1038/ismej.2012.125
- Jones, C. M., Spor, A., Brennan, F. P., Breuil, M.-C., Bru, D., Lemanceau, P., et al. (2014). Recently identified microbial guild mediates soil N2O sink capacity. *Nat. Clim. Change* 4, 801–805. doi: 10.1038/nclimate2301
- Kandeler, E., Deiglmayr, K., Tscherko, D., Bru, D., and Philippot, L. (2006). Abundance of narG, nirS, nirK, and nosZ genes of denitrifying bacteria during primary successions of a glacier foreland. *Appl. Environ. Microbiol.* 72, 5957–5962. doi: 10.1128/AEM.00439-06
- Lauber, C. L., Ramirez, K. S., Aanderud, Z., Lennon, J., and Fierer, N. (2013). Temporal variability in soil microbial communities across land-use types. *ISME J.* 7, 1641–1650. doi: 10.1038/ismej.2013.50
- Laughlin, R. J., and Stevens, R. J. (2002). Evidence for fungal dominance of denitrification and codenitrification in a grassland soil. Soil Sci. Soc. Am. J. 66, 1540–1548. doi: 10.2136/sssaj2002.1540
- Letunic, I., and Bork, P. (2007). Interactive Tree Of Life (iTOL): an online tool for phylogenetic tree display and annotation. *Phylogenetics* 23, 127–128. doi: 10.1093/bioinformatics/btl529
- Liu, B., Frostegard, A., and Bakken, L. R. (2014). Impaired Reduction of N_2O to N_2 in acid soils is due to a posttranscriptional interference with the expression of nosZ. *MBio* 5:e01383-14. doi: 10.1128/mBio.01383-14
- López-Gutiérrez, J. C., Henry, S., Hallet, S., Martin-Laurent, F., Catroux, G., and Philippot, L. (2004). Quantification of a novel group of nitrate-reducing bacteria in the environment by real-time PCR. J. Microbiol. Methods 57, 399–407. doi: 10.1016/j.mimet.2004.02.009
- Maeda, K., Spor, A., Edel-Hermann, V., Heraud, C., Breuil, M. C., Bizouard, F., et al. (2015). N2O production, a widespread trait in fungi. *Sci. Rep.* 5:9697. doi: 10.1038/srep09697
- Melero, S., Perez-de-Mora, A., Manuel Murillo, J., Buegger, F., Kleinedam, K., Kublik, S., et al. (2011). Denitrification in a vertisol under long-term tillage and no-tillage management in dryland agricultural systems: key genes and potential rates. *Appl. Soil Ecol.* 47, 221–225. doi: 10.1016/j.apsoil.2010.12.003
- Mendiburu, F. (2014). agricolae: Statistical Procedures for Agricultural Research. R package version 1.2-1. Available online at: http://CRAN.R-project.org/ package=agricolae
- Meyer, A., Focks, A., Radl, V., Keil, D., Welzl, G., Schöening, I., et al. (2013). Different land use intensities in grassland ecosystems drive ecology of microbial communities involved in nitrogen turnover in soil. *PLoS ONE* 8:e73536. doi: 10.1371/journal.pone.0073536
- Montzka, S. A., Dlugokencky, E. J., and Butler, J. H. (2011). Non-CO2 greenhouse gases and climate change. *Nature* 476, 43–50. doi: 10.1038/nature10322
- Murray, R. E., and Knowles, R. (2004). Trace amounts of O-2 affect NO and N2O production during denitrifying enzyme activity (DEA) assays. Soil Biol. Biochem. 36, 513–517. doi: 10.1016/j.soilbio.2003.10.027
- Muyzer, G., Dewaal, E. C., and Uitterlinden, A. G. (1993). Profiling of complex microbial-populations by denaturing gradient gel-electrophoresis analysis of polymerase chain reaction-amplified genes-coding for 16S ribosomal-RNA. *Appl. Environ. Microbiol.* 59, 695–700.
- Oksanen, J., Blanchet, F. G., Kindt, R., Legendre, P., Minchin, R. P., O'Hara, R. B., et al. (2015). *vegan: Community Ecology Package*. R package version 2.2-1. Available online at: http://CRAN.R-project.org/package=vegan
- Orellana, L. H., Rodriguez,-R. L. M., Higgins, S., Chee-Sanford, J. C., Sanford, R. A., Ritalahti, K. M., et al. (2014). Detecting Nitrous Oxide Reductase (nosZ) genes in soil metagenomes: method development and implications for the Nitrogen Cycle. *MBio* 5, e01193–e01114. doi: 10.1128/mBio.01193-14
- Pell, M., Stenberg, B., Stenstrom, J., and Torstensson, L. (1996). Potential denitrification activity assay in soil - With or without chloramphenicol? *Soil Biol. Biochem.* 28, 393–398. doi: 10.1016/0038-0717(95)00149-2
- Peres-Neto, P. R., Legendre, P., Dray, S., and Borcard, D. (2006). Variation partitioning of species data matrices: estimation and comparison of fractions. *Ecology* 87, 2614–2625. doi: 10.1890/0012-9658(2006)87[2614:VPOSDM]2.0.CO;2
- Petersen, D. G., Blazewicz, S. J., Firestone, M., Herman, D. J., Turetsky, M., and Waldrop, M. (2012). Abundance of microbial genes associated with nitrogen cycling as indices of biogeochemical process rates acrolauss a vegetation gradient in Alaska. *Environ. Microbiol.* 14, 993–1008. doi: 10.1111/j.1462-2920.2011.02679.x

- Petric, I., Philippot, L., Abbate, C., Bispo, A., Chesnot, T., Hallin, S., et al. (2011). Inter-laboratory evaluation of the ISO standard 11063 "Soil quality - Method to directly extract DNA from soil samples." J. Microbiol. Methods 84, 454–460. doi: 10.1016/j.mimet.2011.01.016
- Philippot, L., Andert, J., Jones, C. M., Bru, D., and Hallin, S. (2011). Importance of denitrifiers lacking the genes encoding the nitrous oxide reductase for N2O emissions from soil. *Glob.Change. Biol.* 17, 1497–1504. doi: 10.1111/j.1365-2486.2010.02334.x
- Price, M. N., Dehal, P. S., and Arkin, A. P. (2010). FastTree 2-approximately maximum-likelihood trees for large alignments. *PLoS ONE* 5:e9490. doi: 10.1371/journal.pone,0.0009490
- Ravishankara, A. R., Daniel, J. S., and Portmann, R. W. (2009). Nitrous Oxide (N2O): the dominant ozone-depleting substance emitted in the 21st Century. *Science* 326, 123–125. doi: 10.1126/science.1176985
- R Core Team. (2013). R: A Language and Environment for Statistical Computing. Vienna: R Foundation for Statistical Computing.
- Saggar, S., Jha, N., Deslippe, J., Bolan, N. S., Luo, J., Giltrap, D. L., et al. (2013). Denitrification and N2O:N-2 production in temperate grasslands: processes, measurements, modelling and mitigating negative impacts. *Sci. Total Environ*. 465, 173–195. doi: 10.1016/j.scitotenv.2012.11.050
- Sanford, R. A., Cole, J. R., and Tiedje, J. M. (2002). Characterization and description of Anaeromyxobacter dehalogenans gen. nov., sp nov., an arylhalorespiring facultative anaerobic myxobacterium. *Appl. Environ. Microbiol.* 68, 893–900. doi: 10.1128/AEM.68.2.893-900.2002
- Sanford, R. A., Wagner, D. D., Wu, Q., Chee-Sanford, J. C., Thomas, S. H., Cruz-Garcia, C., et al. (2012). Unexpected nondenitrifier nitrous oxide reductase gene diversity and abundance in soils. *Proc. Natl. Acad. Sci. U.S.A.*109, 19709–19714. doi: 10.1073/pnas.1211238109
- Schloss, P. D., Westcott, S. L., Ryabin, T., Hall, J. R., Hartmann, M., Hollister, E. B., et al. (2009). Introducing mothur: open-source, platformindependent, community-supported software for describing and comparing microbial communities. *Appl. Environ. Microbiol.* 75, 7537–7541. doi: 10.1128/AEM.01541-09
- Shcherbak, I., Millar, N., and Robertson, G. P. (2014). Global metaanalysis of the nonlinear response of soil nitrous oxide (N2O) emissions to fertilizer nitrogen. *Proc. Natl. Acad. Sci. U.S.A.*111, 9199–9204. doi: 10.1073/pnas.1322434111
- Stehfest, E., and Bouwman, L. (2006). N2O and NO emission from agricultural fields and soils under natural vegetation: summarizing available measurement data and modeling of global annual emissions. *Nutri. Cycl. Agroecosyst.* 74, 207–228. doi: 10.1007/s10705-006-9000-7
- Syakila, A., and Kroeze, C. (2011). The global nitrous oxide budget revisited. Greenh. Gas Measur. Managem. 1, 17–26. doi: 10.3763/ghgmm.2010.0007

- Tatti, E., Goyer, C., Chantigny, M., Wertz, S., Zebarth, B. J., Burton, D. L., et al. (2014). Influences of over winter conditions on denitrification and nitrous oxide-producing microorganism abundance and structure in an agricultural soil amended with different nitrogen sources. *Agric. Ecosyst. Environm.* 183, 47–59. doi: 10.1016/j.agee.2013.10.021
- Thomson, A. J., Giannopoulos, G., Pretty, J., Baggs, E. M., and Richardson, D. J. (2012). Biological sources and sinks of nitrous oxide and strategies to mitigate emissions. *Phil. Trans. R. Soc. B.* 367, 1157–1168. doi: 10.1098/rstb.2011.0415
- Tiedje, J. M., Sexstone, A. J., Myrold, D. D., and Robinson, J. A. (1982). Denitrification - ecological niches, competition and survival. Antonie Van Leeuwenhoek J. Micrro. 48, 569–583. doi: 10.1007/BF00399542
- Walker, J. K., Egger, K. N., and Henry, G. H. (2008). Long-term experimental warming alters nitrogen-cycling communities but site factors remain the primary drivers of community structure in high arctic tundra soils. *ISME J.* 2, 982–995. doi: 10.1038/ismej.2008.52
- Wei, W., Isobe, K., Shiratori, Y., Nishizawa, T., Ohte, N., Otsuka, S., et al. (2014). N2O emission from cropland field soil through fungal denitrification after surface applications of organic fertilizer. *Soil Biol. Biochem.* 69, 157–167. doi: 10.1016/j.soilbio.2013.10.044
- Wessen, E., Hallin, S., and Philippot, L. (2010). Differential responses of bacterial and archaeal groups at high taxonomical ranks to soil management. *Soil Biol. Biochem.* 42, 1759–1765. doi: 10.1016/j.soilbio.2010.06.013
- Yoshinari, T., Hynes, R., and Knowles, R. (1977). Acetylene inhibition of nitrousoxide reduction and measurement of denitrification and nitrogen fixation in soil. Soil Biol.Biochem. 9, 177–183. doi: 10.1016/0038-0717(77)90072-4
- Zumft, W. G. (1997). Cell biology and molecular basis of denitrification. Microbiol. Mol. Biol. Rev. 61, 533–616.
- Zumft, W. G. (2005). Nitric oxide reductases of prokaryotes with emphasis on the respiratory, heme-copper oxidase type. J. Inorg. Biochem. 99, 194–215. doi: 10.1016/j.jinorgbio.2004.09.024

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.

Copyright © 2015 Domeignoz-Horta, Spor, Bru, Breuil, Bizouard, Léonard and Philippot. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) or licensor are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

The diversity of the N2O reducers matters for the relative proportion of N₂O 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 N₂O-producers (*nirK* and *nirS*) and N₂O –reducers (*nosZI* and *nosZII*). Means \pm 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 N₂O producers and reducers is expressed by q.nirK, q.nirS and q.nosZI, q.nosZII, respectively.

1.1. Supplementary Figures



Figure S1

Figure S2.



Figure S3



Figure S4





1.2. Supplementary Tables

Table S1

Γ reatment	Description	% Water	μd	% Clay ^s	% Loam⁵	$\% \text{ Sand}^{\$}$	Total C*	Total N*	OM*	Ca*	cec
	Miscanthus (M. giganteus) late	21.7^{c}	7.2 ^{ab}	20.2	74	5.6	11.5	1	19.9	1.3	11
ML	harvest	± 0.9	± 0.5	± 3.6	± 3.3	± 0.4	± 1.0	$\pm 6.10^{-2}$	± 1.9	± 0.6	±2
	Miscanthus (M. giganteus) early	$23^{\rm bc}$	7.4 ^{ab}	20.9	73.5	6.0	10.9	1	18.9	2.3	11
ME	harvest	± 0.1	± 0.6	± 1.7	± 2.4	± 1.3	± 0.6	$\pm 4.10^{-2}$	± 1	± 2.6	H
SI	Switcheross (D viewertum) loto how not	23.3 ^{ab}	7.4 ^{ab}	21.9	72.3	5.7	11.8	1.1	20.5	1.3	12
31	Switchiglass (r. Virgaium) late lial vest	± 1.1	± 0.5	± 2.4	± 1.3	± 1.1	± 0.8	$\pm 6.10^{-2}$	± 1.3	± 0.6	$^{+}$
GE	Curitation (D. minaction (D. Martine	$24.7^{\rm abc}$	6.8^{b}	20.1	73.8	6.0	12	1.1	20.7	-	10
20	Switchiglass (r. virgaium) late halvest	± 0.6	± 0.1	± 3.3	± 2.8	± 0.5	± 0.4	$\pm 5.10^{-2}$	± 0.8	0	\pm
T.	Conventional tillage, straw addition,	25.1 ^{ab}	7.9 ^a	21.1	74.1	4.5	10.7	1	18.5	3.0	12
11	N input, non-legume intercrop	± 0.9	± 0.2	± 4.0	± 3.4	± 0.5	± 0.8	$\pm 4.10^{-2}$	± 1.4	± 2.9	1
¢.	Reduced tillage, straw addition,	24.8^{ab}	7.9 ^a	17.7	77.0	4.9	10.8	1	18.6	3.7	10
17	N input, non-legume intercrop	± 0.5	± 0.3	± 0.5	± 0.06	± 0.4	± 0.2	$\pm 1.10^{-2}$	± 0.5	± 2.8	0 #
T_2	Reduced tillage, straw removal,	24.4 ^{ab}	7.9 ^a	20.5	74.4	4.7	11.4	1.1	19.7	4	12
CI	N input, non-legume intercrop	± 1.0	± 0.2	± 2.8	± 3.2	± 0.7	± 0.7	$\pm 6.10^{-2}$	± 1.3	± 5.8	+
Т	Conventional tillage, straw addition,	25.2^{a}	8^{a}	19.8	75.1	4.8	10.7	-	18.6	3.3	11
†	Low N input, non-legume intercrop	± 1.1	± 0.2	± 3.3	± 3.3	± 0.2	± 0.4	$\pm 5.10^{-2}$	± 0.8	± 2.8	+
Ţ	Conventional tillage, straw addition,	25^{ab}	8.1^{a}	17.8	77.1	4.8	10.7	1	18.4	3.4	10
C I	Low N input, legume intercrop	± 0.4	± 0.1	± 1.6	± 1.2	± 0.5	± 0.3	$\pm 5.10^{-2}$	± 0.6	± 1.7	± 1

54

Table S2

	Shannon	$6^{\rm c}$	$\pm 4.10^{-1}$	$6^{\rm c}$	$\pm 3.10^{-1}$	$\gamma^{ m bc}$	$\pm 4.10^{-1}$	θ^c	$\pm 2.10^{-1}$	$\gamma^{ m ab}$	$\pm 6.10^{-2}$	$\gamma^{ m ab}$	$\pm 1.10^{-1}$	$\gamma^{ m ab}$	$\pm 1.10^{-1}$	$\gamma^{ m ab}$	$\pm 1.10^{-1}$	7^{a}	$\pm 8 10^{-2}$
I	Simpson reciprocal	$40^{\rm c}$	± 21.9	33°	± 11.3	52^{bc}	± 21.4	34°	± 5.2	92^{ab}	± 10.4	98^{a}	± 16.1	84^{ab}	± 18.6	93^{ab}	± 14.40	110^{a}	± 20.9
nosZL	chao1	464^{ab}	± 57.8	472^{ab}	± 94.4	461^{ab}	± 159.5	457 ^b	± 67.7	558^{ab}	± 56.8	547 ^{ab}	± 38.1	526^{ab}	± 58.6	526^{ab}	± 21.2	621 ^a	± 7,1
	PD	$23^{\rm abc}$	± 2.7	21^{bc}	± 2.9	$23^{\rm abc}$	± 6.6	20°	± 2.05	29^{ab}	± 1.6	31^{a}	± 3.8	28^{abc}	± 2.3	$27^{\rm abc}$	± 2.2	30^{a}	± 2.1
	OTUs	322^{bc}	± 23.7	318^{bc}	± 37.6	315^{bc}	± 49.2	294°	± 31.1	373^{ab}	± 26.2	382^{ab}	± 29.0	$368^{\rm abc}$	± 24.8	$369^{\rm abc}$	± 18.6	404^{a}	± 14 1
	Shannon	5	± 0.5	9	$\pm 4.10^{-1}$	5	$\pm 1.10^{-1}$	5	$\pm 4.10^{-1}$	5	$\pm 4.10^{-1}$	5	$\pm 7.10^{-2}$	5	$\pm 9.10^{-2}$	5	$\pm 5.10^{-1}$	5	$\pm 2.10^{-1}$
ıosZI	Simpson reciprocal	24	± 14.8	30	± 7.8	24	± 6.6	21	± 13.0	14	± 5.0	18	± 1.6	16	± 1.4	18	± 10.1	16	± 0.3
1	chao1	178	± 19.7	227	± 94.7	196	± 23.8	165	± 25.1	191	± 38.1	196	± 23.9	203	± 18.3	223	± 58.5	237	± 74.9
	PD	6	± 0.5	11	± 2.4	6	± 0.4	8	± 1.2	6	± 0.6	6	± 0.4	10	± 1.1	10	± 1.9	10	+ - -
	OTUs	167	± 21.3	181	± 44	163	± 9	138	± 19	161	± 30	161	± 4	176	± 12.7	175	± 35.1	179	± 34.3
	Treatment	1 V L	ML		ME	UT CI	N		0E	Ę	11	Ē	17	٢٦	CI	Ē	1 4	τ	CI



Table S3.

_	Potential N ₂ O	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

Draft: Unpublished manuscript

TITLE: Effects of agricultural practices and soil properties on soil N₂O-reducing bacteria and *in situ* N₂O emissions

Domeignoz-Horta¹ L.A., Peyrard C.², Bru D.¹, Breuil M.C.¹, Bizouard F.¹, E. Justes³, Mary B.², Léonard J.², Philippot L^{1*}, Spor A.¹

¹Agroécologie, AgroSup Dijon, INRA, Univ. Bourgogne Franche-Comté, F-21000 Dijon, France
 ² AgroImpact, INRA, UR1158, F-02000 Laon, France
 ³AGIR, 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 N₂O emissions, a potent greenhouse gas and the main cause of ozone depletion. The reduction of N₂O into N₂ 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 N₂O-reducers (*nosZII*) was related to the capacity of the soil to act as a N₂O sink. However little is known about how this group responds to different agricultural practices. Here, we investigated how N₂O-reducers were affected by agricultural practices and evaluated the consequences for N₂O emissions. The abundance of N₂O-reducers and N₂O 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* N₂O emissions were assessed. Overall, our results showed that the *nosZ*II clade of N₂O-

reducers was more sensitive to environmental factors and agricultural practices than the *nosZI* clade. Tillage increased the diversity of this clade while it did not affect the diversity of the *nosZI* clade. To better access the contribution of different factors to the *in situ* N₂O 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 *nosZII* clade abundance and diversity across agricultural systems were negatively correlated with the *in situ* N₂O 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 N₂O is emitted from soils, with agricultural soils being the main source of anthropogenic N₂O 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 (NO₃- and NO₂-) are reduced into gaseous forms (NO, N₂O and N₂). The reduction of soluble NO₂⁻ to gaseous forms is catalyzed by the nitrite reductase encoded by the *nirK* or *nirS* genes. The other denitrification step important for N₂O-emissions is the reduction of N₂O to N₂, which is catalyzed by the N₂O 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 N₂O reductase and therefore produce N₂O as final denitrification product (Philippot et al., 2011) or can only reduce N₂O without producing it (Sanford et al., 2012), and are

therefore a potential sink for this GHG. Recently a new N₂O 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 N₂O 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 N₂O 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 N₂O mitigation strategy.

Several studies have investigated the influence of agriculture practices on microorganisms involved in N₂O 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 N₂O 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 N₂O 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 N₂O sources both nitrifiers and denitrifiers) and sinks (N₂O-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* N₂O 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* N₂O fluxes.

Chapter III

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 KNO₃, 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 N₂O 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 N₂O 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 N₂O production or N₂O 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) ammoniaoxidizing and the denitrifying communities, respectively (Bru et al., 2011). The *nosZ*I and *nosZ*II genes were used to target the N₂O-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 N₂O-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 μ l 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 μ g N₂O-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 μ g N₂O-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 μ g NO₂-N g⁻¹ DW soil h⁻¹, respectively (Fig. S2). A much lower PNA rate of 0.01 μ g NO₂-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, N2O-producers and N2O-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 N₂O producers (Fig. S4) nor of N₂O reducers (Fig. S5) were affected by agricultural practices. However, contrasted trends were observed for the two clades of N₂O reducers, with the abundance of the *nosZ*I community decreasing with tillage intensity while the *nosZ*II community remained stable (Fig S5).

Site	Treatment	t Management	pH	$\% \text{ Clav}^2$	$\% \mathrm{Loam}^2$	$\% \text{ Sand}^2$	SOM^3	Total-C ³	Total-N ³	C:N	CEC ^{4,5}	% Water
	11		8.1^{a}	30.2^{abc}	36.7 ^e	30.7^{a}	16.4^{bcd}	9.5^{bcd}	$1.0^{\rm abcd}$	9.2	18.4^{abc}	17.2 ^{bc}
	ΓI	LOW N Input	+0.2	+ 1.6	+ 1.1	+ 6.1	+2.7	+ 1.6	+0.2	+0.5	+ 3.5	+ 0.4
			$7.4^{\rm abcd}$	$30.6^{\rm abc}$	38.1°	29.2^{a}	16.7^{cd}	$9.7^{\rm abcd}$	$1.0^{\rm cd}$	9.4	$16.8^{\rm abcd}$	18.2^{abc}
	L1.C	TOW IN INPUT & CC	+ 1.0	+ 3.7	+ 1.0	+ 7.8	+3.2	+ 1.9	+0.2	+ 0.1	+ 4.5	+ 1.2
	1 LL		6.3^{cd}	$29.4^{\rm abcd}$	35.6°	34.9^{a}	14.3^{d}	8.3^{d}	0.9^{d}	9.1	14.6 ^{abcde}	15.2°
A	VLI	very low in input	+0.4	+ 1.6	+ 2.9	+ 2.0	+ 1.4	+ 0.8	+ 0.1	+0.4	+ 2.5	+ 1.9
Auzeville		Vary low N innut & CC	6.5 ^{cd}	$29.0^{\rm abcd}$	35.5^{e}	35.3^{a}	15.5 ^{cd}	9.0^{cd}	1.0^{bcd}	9.3	14.5 ^{abcde}	15.6°
		very row in highlic co	+0.3	+ 2.6	+ 1.6	+ 1.3	+ 1.9	+ 1.1	+ 0.1	+0.3	+ 1.2	+ 1.1
	A.S.A.	Intercronning	8.1^{a}	32.8^{a}	35.9°	30.5^{a}	17.1 ^{abcd}	9.8^{abcd}	$1.0^{\rm abcd}$	9.5	20.5^{a}	$16.7^{\rm bc}$
		Surviv	+0.3	+2.4	+3.0	+ 6.3	+ 2.3	+ 1.3	+0.2	+0.3	+2.7	+3.2
		Interview & CC	$7.7^{\rm ab}$	32.5^{ab}	35.6°	29.8^{a}	17.1 ^{abcd}	$9.9^{\rm abcd}$	1.1 ^{abcd}	9.3	19.6^{ab}	16.6^{bc}
	2.6A	π	+0.7	+ 2.4	+ 3.4	+ 9.2	+ 1.5	+0.9	+ 0.1	+ 0.1	+ 3.8	+ 3.5
	MIN	Miscanthus late harvest & N innut	$7.2^{\rm abcd}$	20.2^{e}	74.1 ^{abc}	5.6°	19.9^{ab}	11.5^{abc}	1.1 ^{abcd}	10.6	11.2 ^{de}	20.6^{abc}
		inditi vi e icai inti anti chittinactiti	+0.6	+ 3.6	+ 3.4	+ 0.4	+1.9	+ 1.1	+0.1	+0.4	+3.0	+1.0
	MFN	Miscanthus early harvest & N innut	7.4^{abc}	20.9°	72.8^{abcd}	6.0°	18.9^{abc}	10.9^{abc}	1.1^{abcd}	10.2	11.6^{de}	19.5^{abc}
		man in a see mu finn munnerit	+0.6	+ 1.8	+ 2.2	+ 1:3	+ 1.1	+ 0.7	+0.0	+0.2	+ 1.1	+ 0.7
	NIS	Switchorass late harvest & N innut	7.4^{abc}	21.9^{de}	72.3^{abcd}	5.70	20.5^{a}	11.8^{ab}	1.2^{abc}	10.3	12.3 ^{cde}	22.6^{a}
Mons - \mathbf{R}		ownerstand have near the transfer of the transfer	+0.6	+ 2.5	+ 1.4	+ 1.1	+ 1.3	+ 0.8	+0.1	+0.2	+ 1.1	+2.2
	CEN	Switchorace early harvest & N input	6.8^{bcd}	20.1^{e}	73.8^{abc}	6.0^{b}	20.7^{a}	12.0^{ab}	1.1 ^{abcd}	10.8	10.8^{e}	18.3^{abc}
		DWINDIGIASS CALLY HALVEST & MILTING	+0.1	+ 3.4	+2.9	+0.5	+ 0.9	+ 0.5	+ 0.1	+0.2	+ 1.8	+ 1.5
	5	Switcharsee late harveet & no N innut	$7.1^{\rm abcd}$	20.9°	$73.6^{\rm abcd}$	5.5 ^b	21.4 ^a	12.4^{a}	$1.1^{\rm abcd}$	10.9	13.0^{abcde}	20.1^{abc}
	10	DWINDIGLASS LAIC HALVESI & INO IN INPUL	+0.5	+ 1.8	+ 1.8	+ 0.0	+ 1.2	+ 0.7	+ 0.1	+ 0.0 +	+ 1.8	+ 1.2
	GE	Switchgrass early harvest & no N	$7.3^{\rm abc}$	20.4^{e}	74.1 ^{abc}	5.4^{b}	19.6^{ab}	11.4 ^{abc}	1.1 ^{abcd}	10.8	12.7^{cde}	17.6^{abc}
	30	input	+0.5	+ 5.2	+ 4.9	+ 0.8	+ 0.8	+ 0.5	+ 0.1	+0.2	+ 2.8	+ 0.6
	T.1	High N input, full Inversion tillage,	7.9^{a}	21.1^{e}	74.1^{a}	4.5 ^b	18.5^{abc}	$10.7^{\rm abcd}$	1.1 ^{abcd}	10.1	12.5 ^{cde}	$20.2^{\rm abc}$
	11	straw return & nonlegume as CC	+0.3	+ 4.1	+ 3.4	+ 0.6	+ 1.4	+ 0.8	+ 0.0	+0.4	+ 2.0	+ 0.8
	ст	High N input, shallow tillage, straw	7.9^{a}	17.7 ^e	77.0^{a}	4.9^{b}	$18.6^{\rm abc}$	$10.8^{\rm abcd}$	1.1 ^{abcd}	10.3	$10.5^{\rm e}$	21.8^{ab}
	17	return & non legume as CC	+0.3	+ 0.5	+ 0.1	+ 0.5	+0.5	+0.3	+ 0.0	+0.3	+0.5	+ 0.8
	Τ2	High N input, shallow tillage, straw	7.9^{a}	20.5^{e}	74.4 ^{abc}	4.7 ^b	19.7^{ab}	11.4 ^{abc}	1.1 ^{abcd}	10.4	12.1 ^{cde}	$18.8^{\rm abc}$
Mons -	C1	export & non legume as CC	+0.3	+ 2.8	+3.2	+ 0.7	+ 1.4	+ 0.8	+ 0.1	+0.4	+ 1.5	+ 0.5
SOERE	T	Low N input, full inversion tillage,	8.0^{a}	19.8^{e}	75.1 ^{ab}	4.8^{b}	$18.6^{\rm abc}$	$10.7^{\rm abcd}$	$1.0^{\rm abcd}$	10.4	11.7^{de}	$20.9^{ m abc}$
	1	straw return & non legume as CC	+0.2	+ 3.3	+ 3.4	+ 0.2	+0.9	+ 0.5	+ 0.1	+0.2	+ 2.0	+ 3.4
	Τς	Low N input, full inversion tillage,	8.1^{a}	17.8 ^e	77.1^{a}	4.8^{b}	$18.4^{ m abc}$	$10.7^{\rm abcd}$	$1.0^{\rm abcd}$	10.3	10.4^{e}	21.8^{abc}
	C I	straw return & legume as CC	+ 0.1	+ 1.7	+ 1.3	+0.6	+ 0.7	+0.4	+ 0.1	+0.5	+ 1.0	+ 2.5
	Тб	Switchorass & no N added	8.2^{a}	17.5°	76.5^{a}	$5.7^{\rm b}$	17.9^{abcd}	$10.4^{\rm abcd}$	$1.0^{\rm abcd}$	10.6	11.7^{de}	20.1^{abc}
	10	DWILLIGIASS & IIU IN AUUCU	+ 0.1	+ 3.0	+ 3.4	+ 0.5	+ 1.4	+ 0.9	+ 0.1	+ 1.4	+ 1.6	+ 2.4
	БIТ	Eull invarion tillora	6.6 ^{cd}	25.1^{bcde}	66.4^{d}	8.4^{b}	20.4^{ab}	11.8^{abc}	1.1 ^{ab}	10.6	$15.6^{\rm abcde}$	20.1^{abc}
	11.1		+0.4	+ 2.7	+ 1.6	+ 1.1	+ 3.6	+ 2.5	+0.2	+0.7	+ 2.6	+ 0.4
Boingrilla	L	Shallow tillaga	6.2^{d}	25.0^{bcde}	$67.2^{\rm cd}$	7.8^{b}	19.8^{ab}	11.4^{ab}	$1.0^{\rm abc}$	10.8	$13.4^{\rm abcde}$	17.1^{bc}
DUILG	10	Juanow unage	+0.2	+ 1.7	+1.9	+0.4	+ 1.1	+ 0.6	+ 0.0	+0.3	+ 1.2	+ 1.9
	NT	No tillaga	6.2^{d}	24.1 ^{cde}	$68.1^{\rm bcd}$	7.8^{b}	23.1^{ab}	$13.4^{\rm abc}$	1.1^a	11.2	12.8^{bcde}	$19.7^{\rm abc}$
	1 11		+0.2	+0.5	+ 1.8	+ 1.5	+ 1.2	+ 0.7	+ 0.0	+0.4	+ 0.5	+ 3.5
Means and 95% indicated with di and October res	confidence ifferent lett	e interval of soil physicochemical properti ers: "a", "b", and "c" (anova followed by ² Size fractions based on um Atterbero sca	ies are giv y Tukey HS ile: Clav =	en per treati 5D test, P < 21m Loam	ment within 0.05). ¹ CC re = 2.50µm	each experii present cov and Sand =	<i>nental bloc</i> er crop, " <i>Lc</i> 50-2000.um	k. Significan ate" and "ec . ³ o ko ⁻¹ · 4(ıt difference ırly" harves Tation exchi	es between st corresp anoe canc	n treatments ond to Febr acity ⁵ cmol	are uary koʻl
alla Uliver, ici	spernvery,	and fruinnes under on put anerver some	ue, Uwy	Lpun, Loum	- 4-JUMIN,	nunc nur	undana z-ac	, × v× , ,	מווחוו בערומ	unse rupr	with, unu	



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

N₂O-reducers Diversity

To assess the influence of agricultural practices on the composition and structure of N₂Oreducers, 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 *nosZII 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* N₂O 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* N₂O 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* N₂O 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 in *situ* N₂O 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.

Chapter III

Discussion

Agricultural Practices Influence on Potential Activities and in situ N2O 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 N₂O fluxes, PDA and PNA was observed in our study (Fig. 1). There is no consensus regarding the tillage effect on N₂O emissions. While some studies showed that no-till or reduced tillage promotes N₂O emissions (Baggs et al.2003, Smith et al., 2008), others reported on the contrary that tillage increased N₂O 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 N₂O-producing and N₂O-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 (*nosZI* or *nosZII*). 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 *nos*ZII 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 nosZII community, which can potentially act as a soil N₂O sink.

Soil Properties Influence on N₂O 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* N₂O emissions (Tabs 3 and 4). Accordingly, a large body of literature highlighted the importance of soil pH for nitrification, denitrification and N₂O 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 *nosZI* (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 *nosZII* than for *nosZI*. Common explanatory variables for *nosZI* and *nosZII* community structure were pH, AOA, C/N ratio, while sand, total N, cec (cation exchange capacity) only correlated to the *nosZII* 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 N₂O-producing and reducing microbial communities, their corresponding potential activities and *in situ* N₂O fluxes

Abundances of N₂O-producers and N₂O-reducers as well as the diversity of the nosZIcommunity 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 nosZII-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 nosZIIcommunity were important drivers of the soil N₂O sink capacity. More recently, we found that the diversity of nosZII community was the strongest predictor of the N2O:N2 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 N₂O 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 N₂O emissions across all sites, we found that the diversity of the nosZII-community was negatively related to the N₂O emissions for all percentiles higher than 50%. A negative relationship between the nosZII-community and in situ emissions was also observed for the 75% percentile only. Morales et al. (2010) previously reported a positive correlation between N₂O 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 N₂O fluxes and the newly identified nosZII-community. This strengthen the hypothesis that the *nosZII*-community can act as a N₂O 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 N₂O 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 N₂O reducers and N₂O production. Thus, addition of a Dyadobacter fermetans, a nosZII strain, significantly reduced N₂O production by denitrification of up to 189% in more than 1/3 of the studied soils. Interestingly, we also found that in situ N₂O emissions were negatively related to the abundance of AOA (Tab. S4). This could be due to lower emissions of N₂O 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 N₂O 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 *nos*ZII 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 *nos*ZII- than the *nos*ZI-community to environmental factors. However, despite significant variations in the *nos*ZII 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 N₂O emissions.

Acknowledgements

This work was supported by the European Union (Marie Curie ITN NORA, FP7- 316472) and the French Agency for the Environment and Energy (ADEME) thought the project EFEMAIR-N₂O. The authors thank Céline Peyrard for collecting the soil samples.

References

- Amougou, N., Bertrand, I., Cadoux, S., Recous, S., 2012. Miscanthus x giganteus leaf senescence, decomposition and C and N inputs to soil. Global Change Biology Bioenergy 4, 698-707.
- Amougou, N., Bertrand, I., Machet, J.M., Recous, S., 2011. Quality and decomposition in soil of rhizome, root and senescent leaf from Miscanthus x giganteus, as affected by harvest date and N fertilization. Plant and Soil 338, 83-97.
- Baggs, E.M., Stevenson, M., Pihlatie, M., Regar, A., Cook, H., Cadisch, G., 2003. Nitrous oxide emissions following application of residues and fertiliser under zero and conventional tillage. Plant and Soil 254, 361-370.
- Baudoin, E., Philippot, L., Cheneby, D., Chapuis-Lardy, L., Fromin, N., Bru, D., Rabary, B., Brauman, A., 2009. Direct seeding mulch-based cropping increases both the activity and the abundance of denitrifier communities in a tropical soil. Soil Biology & Biochemistry 41, 1703-1709.
- Berry, D., Ben Mahfoudh, K., Wagner, M., Loy, A., 2011. Barcoded Primers Used in Multiplex Amplicon Pyrosequencing Bias Amplification. Applied and Environmental Microbiology 77, 7846-7849.
- Bissett, A., Abell, G.C.J., Brown, M., Thrall, P.H., Bodrossy, L., Smith, M.C., Baker, G.H., Richardsson, A.E., 2014. Land-use and management practices affect soil ammonia oxidiser community structure, activity and connectedness. Soil Biology & Biochemistry 78, 138-148.
- Bouwman, A.F., Beusen, A.H.W., Griffioen, J., Van Groenigen, J.W., Hefting, M.M., Oenema, O., Van Puijenbroek, P., Seitzinger, S., Slomp, C.P., Stehfest, E., 2013. Global trends and uncertainties in terrestrial denitrification and N₂O emissions. Philosophical Transactions of the Royal Society B-Biological Sciences 368.
- Bru, D., Ramette, A., Saby, N.P.A., Dequiedt, S., Ranjard, L., Jolivet, C., Arrouays, D., Philippot, L., 2011. Determinants of the distribution of nitrogen-cycling microbial communities at the landscape scale. Isme Journal 5, 532-542.

- Canfield, D.E., Glazer, A.N., Falkowski, P.G., 2010. The Evolution and Future of Earth's Nitrogen Cycle. Science 330, 192-196.
- Caporaso, J.G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F.D., Costello, E.K., Fierer, N., Pena, A.G., Goodrich, J.K., Gordon, J.I., Huttley, G.A., Kelley, S.T., Knights, D., Koenig, J.E., Ley, R.E., Lozupone, C.A., McDonald, D., Muegge, B.D., Pirrung, M., Reeder, J., Sevinsky, J.R., Tumbaugh, P.J., Walters, W.A., Widmann, J., Yatsunenko, T., Zaneveld, J., Knight, R., 2010. QIIME allows analysis of high-throughput community sequencing data. Nature Methods 7, 335-336.
- Chan, K.Y., Heenan, D.P., So, H.B., 2003. Sequestration of carbon and changes in soil quality under conservation tillage on light-textured soils in Australia: a review. Australian Journal of Experimental Agriculture 43, 325-334.
- Chapuis-Lardy, L., Wrage, N., Metay, A., Chotte, J.-L., Bernoux, M., 2007. Soils, a sink for N₂O? A review. Global Change Biology 13, 1-17.
- Chatskikh, D., Olesen, J.E., 2007. Soil tillage enhanced CO2 and N₂O emissions from loamy sand soil under spring barley. Soil and Tillage Research 97, 5-18.
- Clark, I.M., Buchkina, N., Jhurreea, D., Goulding, K.W.T., Hirsch, P.R., 2012. Impacts of nitrogen application rates on the activity and diversity of denitrifying bacteria in the Broadbalk Wheat Experiment. Philosophical Transactions of the Royal Society B-Biological Sciences 367, 1235-1244.
- Cui, P., Fan, F., Yin, C., Song, A., Huang, P., Tang, Y., Zhu, P., Peng, C., Li, T., Wakelin, S.A., Liang, Y., 2016. Long-term organic and inorganic fertilization alters temperature sensitivity of potential N₂O emissions and associated microbes. Soil Biology and Biochemistry 93, 131-141.
- Di, H.J., Cameron, K.C., Shen, J.P., Winefield, C.S., O'Callaghan, M., Bowatte, S., He, J.Z., 2010. Ammonia-oxidizing bacteria and archaea grow under contrasting soil nitrogen conditions. Fems Microbiology Ecology 72, 386-394.
- Domeignoz-Horta, L.A., Putz, M., Spor, A., Bru, D., Breuil, M.C., Hallin, S., Philippot, L., 2016. Non-denitrifying nitrous oxide-reducing bacteria - An effective N₂O sink in soil. Soil Biology and Biochemistry 103, 376-379.
- Domeignoz-Horta, L.A., Spor, A., Bru, D., Breuil, M.C., Bizouard, F., Leonard, J., Philippot, L., 2015. The diversity of the N₂O reducers matters for the N₂O:N-2 denitrification end-product ratio across an annual and a perennial cropping system. Frontiers in Microbiology 6.
- Edgar, R.C., 2010. Search and clustering orders of magnitude faster than BLAST. Bioinformatics 26, 2460-2461.
- Fortuna, A.M., Honeycutt, C.W., Vandemark, G., Griffin, T.S., Larkin, R.P., He, Z.Q., Wienhold, B.J., Sistani, K.R., Albrecht, S.L., Woodbury, B.L., Torbert, H.A., Powell, J.M., Hubbard, R.K., Eigenberg, R.A., Wright, R.J., Alldredge, J.R., Harsh, J.B., 2012. Links among Nitrification, Nitrifier Communities, and Edaphic Properties in Contrasting Soils Receiving Dairy Slurry. Journal of Environmental Quality 41, 262-272.
- Govaerts, B., Verhulst, N., Castellanos-Navarrete, A., Sayre, K.D., Dixon, J., Dendooven, L., 2009. Conservation Agriculture and Soil Carbon Sequestration: Between Myth and Farmer Reality. Critical Reviews in Plant Sciences 28, 97-122.
- Graf, D.R.H., Jones, C.M., Hallin, S., 2014. Intergenomic Comparisons Highlight Modularity of the Denitrification Pathway and Underpin the Importance of Community Structure for N₂O Emissions. PLoS One 9.

- Graf, D.R.H., Zhao, M., Jones, C.M., Hallin, S., 2016. Soil type overrides plant effect on genetic and enzymatic N₂O production potential in arable soils. Soil Biology and Biochemistry 100, 125-128.
- Gubry-Rangin, C., Nicol, G.W., Prosser, J.I., 2010. Archaea rather than bacteria control nitrification in two agricultural acidic soils. Fems Microbiology Ecology 74, 566-574.
- Gulden, R.H., Tenuta, M., Mitchell, S., Langarica Fuentes, A., Daniell, T.J., 2015. Preceding crop and weed management history affect denitrification and denitrifier community structure throughout the development of durum wheat. Agriculture, Ecosystems & Environment 212, 49-63.
- Hallin, S., Jones, C.M., Schloter, M., Philippot, L., 2009. Relationship between N-cycling communities and ecosystem functioning in a 50-year-old fertilization experiment. ISME J 3, 597-605.
- Hartmann, M., Frey, B., Mayer, J., Mader, P., Widmer, F., 2015. Distinct soil microbial diversity under long-term organic and conventional farming. Isme Journal 9, 1177-1194.
- Hink, L., Nicol, G.W., Prosser, J.I., 2016. Archaea produce lower yields of N₂O than bacteria during aerobic ammonia oxidation in soil. Environmental Microbiology, n/a-n/a.
- Hu, H.W., Chen, D., He, J.Z., 2015. Microbial regulation of terrestrial nitrous oxide formation: understanding the biological pathways for prediction of emission rates. Fems Microbiology Reviews 39, 729-749.
- Jones, C.M., Spor, A., Brennan, F.P., Breuil, M.C., Bru, D., Lemanceau, P., Griffiths, B., Hallin, S., Philippot, L., 2014. Recently identified microbial guild mediates soil N₂O sink capacity. Nature Climate Change 4, 801-805.
- Kandeler, E., 1995. Nitrate reductase activity. In F. Schinner, R. Öhlinger, E. Kandeler, and R. Margesin (ed.), Methods in soil biology. Springer, Berlin, Germany.
- Kong, A.Y.Y., Hristova, K., Scow, K.M., Six, J., 2010. Impacts of different N management regimes on nitrifier and denitrifier communities and N cycling in soil microenvironments. Soil Biology & Biochemistry 42, 1523-1533.
- Lal, R., 2004. Soil carbon sequestration impacts on global climate change and food security. Science 304, 1623-1627.
- Lopez-Gutierrez, J.C., Henry, S., Hallet, S., Martin-Laurent, F., Catroux, G., Philippot, L., 2004. Quantification of a novel group of nitrate-reducing bacteria in the environment by real-time PCR. Journal of Microbiological Methods 57, 399-407.
- Lu, X.D., Bottomley, P.J., Myrold, D.D., 2015. Contributions of ammonia-oxidizing archaea and bacteria to nitrification in Oregon forest soils. Soil Biology & Biochemistry 85, 54-62.
- Marland, G., McCarl, B.A., Schneider, U., 2001. Soil carbon: Policy and economics. Climatic Change 51, 101-117.
- Melero, S., Pérez-de-Mora, A., Murillo, J.M., Buegger, F., Kleinedam, K., Kublik, S., Vanderlinden, K., Moreno, F., Schloter, M., 2011. Denitrification in a vertisol under long-term tillage and no-tillage management in dryland agricultural systems: Key genes and potential rates. Applied Soil Ecology 47, 221-225.
- Mendiburu F. 2014. Agricolae: Statistical Procedures for Agricultural Research. R package version 1.2-1.Availableonlineat: http://CRAN.R-project.org/package=agricolae
- Morales, S.E., Cosart, T., Holben, W.E., 2010. Bacterial gene abundances as indicators of greenhouse gas emission in soils. Isme Journal 4, 799-808.
- Orellana, L.H., Rodriguez-R, L.M., Higgins, S., Chee-Sanford, J.C., Sanford, R.A., Ritalahti, K.M., Loffler, F.E., Konstantinidis, K.T., 2014. Detecting Nitrous Oxide Reductase

(nosZ) Genes in Soil Metagenomes: Method Development and Implications for the Nitrogen Cycle. Mbio 5.

- Oksanen, J., Blanchet, F.G., Kindt, R., Legendre, P., Minchin, R.P., O'Hara, R. B., etal. 2015. vegan: Community Ecology Package. R package version 2.2-1. Available on line at: http://CRAN.R-project.org/package=vegan
- Petersen, D.G., Blazewicz, S.J., Firestone, M., Herman, D.J., Turetsky, M., Waldrop, M., 2012. Abundance of microbial genes associated with nitrogen cycling as indices of biogeochemical process rates across a vegetation gradient in Alaska. Environmental Microbiology 14, 993-1008.
- Petric, I., Philippot, L., Abbate, C., Bispo, A., Chesnot, T., Hallin, S., Laval, K., Lebeau, T., Lemanceau, P., Leyval, C., Lindstrom, K., Pandard, P., Romero, E., Sarr, A., Schloter, M., Simonet, P., Smalla, K., Wilke, B.M., Martin-Laurent, F., 2011. Interlaboratory evaluation of the ISO standard 11063 "Soil quality - Method to directly extract DNA from soil samples". Journal of Microbiological Methods 84, 454-460.
- Philippot, L., Andert, J., Jones, C.M., Bru, D., Hallin, S., 2011. Importance of denitrifiers lacking the genes encoding the nitrous oxide reductase for N₂O emissions from soil. Global Change Biology 17, 1497-1504.
- Powlson, D.S., Stirling, C.M., Jat, M.L., Gerard, B.G., Palm, C.A., Sanchez, P.A., Cassman, K.G., 2014. Limited potential of no-till agriculture for climate change mitigation. Nature Clim. Change 4, 678-683.
- Prosser, J.I., Nicol, G.W., 2012. Archaeal and bacterial ammonia-oxidisers in soil: the quest for niche specialisation and differentiation. Trends in Microbiology 20, 523-531.
- Ravishankara, A.R., Daniel, J.S., Portmann, R.W., 2009. Nitrous Oxide (N₂O): The Dominant Ozone-Depleting Substance Emitted in the 21st Century. Science 326, 123-125.
- R CoreTeam.2013. R: A Language and Environment for Statistical Computing. Vienna: R Foundation for Statistical Computing.
- Robertson, G.P., Paul, E.A., Harwood, R.R., 2000. Greenhouse gases in intensive agriculture: Contributions of individual gases to the radiative forcing of the atmosphere. Science 289, 1922-1925.
- Rochette, P., 2008. No-till only increases N₂O emissions in poorly-aerated soils. Soil & Tillage Research 101, 97-100.
- Rochette, P., Janzen, H.H., 2005. Towards a Revised Coefficient for Estimating N₂O Emissions from Legumes. Nutrient Cycling in Agroecosystems 73, 171-179.
- Sanford, R.A., Wagner, D.D., Wu, Q.Z., Chee-Sanford, J.C., Thomas, S.H., Cruz-Garcia, C., Rodriguez, G., Massol-Deya, A., Krishnani, K.K., Ritalahti, K.M., Nissen, S., Konstantinidis, K.T., Loffler, F.E., 2012. Unexpected nondenitrifier nitrous oxide reductase gene diversity and abundance in soils. Proceedings of the National Academy of Sciences of the United States of America 109, 19709-19714.
- Schauss, K., Focks, A., Leininger, S., Kotzerke, A., Heuer, H., Thiele-Bruhn, S., Sharma, S., Wilke, B.M., Matthies, M., Smalla, K., Munch, J.C., Amelung, W., Kaupenjohann, M., Schloter, M., Schleper, C., 2009. Dynamics and functional relevance of ammonia-oxidizing archaea in two agricultural soils. Environmental Microbiology 11, 446-456.
- Schloss, P.D., Westcott, S.L., Ryabin, T., Hall, J.R., Hartmann, M., Hollister, E.B., Lesniewski, R.A., Oakley, B.B., Parks, D.H., Robinson, C.J., Sahl, J.W., Stres, B., Thallinger, G.G., Van Horn, D.J., Weber, C.F., 2009. Introducing mothur: Open-Source, Platform-Independent, Community-Supported Software for Describing and Comparing Microbial Communities. Applied and Environmental Microbiology 75, 7537-7541.

- Shcherbak, I., Millar, N., Robertson, G.P., 2014. Global metaanalysis of the nonlinear response of soil nitrous oxide (N₂O) emissions to fertilizer nitrogen. Proceedings of the National Academy of Sciences of the United States of America 111, 9199-9204.
- ŠImek, M., Cooper, J.E., 2002. The influence of soil pH on denitrification: progress towards the understanding of this interaction over the last 50 years. European Journal of Soil Science 53, 345-354.
- Six, J., Ogle, S.M., Jay breidt, F., Conant, R.T., Mosier, A.R., Paustian, K., 2004. The potential to mitigate global warming with no-tillage management is only realized when practised in the long term. Global Change Biology 10, 155-160.
- Smith, P., D. Martino, Z. Cai, D. Gwary, H. Janzen, P. Kumar, B. McCarl, S. Ogle, F. O'Mara, C. Rice, B. Scholes, O. Sirotenko, 2007. Agriculture. In Climate Change 2007: Mitigation. Contribution of Working Group III to the Fourth Assessment Report of the Intergovernmental Panel on Climate Change [B. Metz, O.R. Davidson, P.R. Bosch, R. Dave, L.A. Meyer (eds)], Cambridge University Press, Cambridge, United Kingdom and New York, NY, USA.
- Smith, P., Martino, D., Cai, Z., Gwary, D., Janzen, H., Kumar, P., McCarl, B., Ogle, S., O'Mara, F., Rice, C., Scholes, B., Sirotenko, O., Howden, M., McAllister, T., Pan, G., Romanenkov, V., Schneider, U., Towprayoon, S., Wattenbach, M., Smith, J., 2008. Greenhouse gas mitigation in agriculture. Philosophical Transactions of the Royal Society B: Biological Sciences 363, 789-813.
- Snyder, C.S., Bruulsema, T.W., Jensen, T.L., Fixen, P.E., 2009. Review of greenhouse gas emissions from crop production systems and fertilizer management effects. Agriculture, Ecosystems & Environment 133, 247-266.
- Sterngren, A.E., Hallin, S., Bengtson, P., 2015. Archaeal Ammonia Oxidizers Dominate in Numbers, but Bacteria Drive Gross Nitrification in N-amended Grassland Soil. Frontiers in Microbiology 6.
- Strudley, M.W., Green, T.R., Ascough, J.C., 2008. Tillage effects on soil hydraulic properties in space and time: State of the science. Soil & Tillage Research 99, 4-48.
- Sun, R., Guo, X., Wang, D., Chu, H., 2015. Effects of long-term application of chemical and organic fertilizers on the abundance of microbial communities involved in the nitrogen cycle. Applied Soil Ecology 95, 171-178.
- Tatti, E., Goyer, C., Burton, D.L., Wertz, S., Zebarth, B.J., Chantigny, M., Filion, M., 2015. Tillage Management and Seasonal Effects on Denitrifier Community Abundance, Gene Expression and Structure over Winter. Microbial Ecology 70, 795-808.
- Tian, H.Q., Lu, C.Q., Ciais, P., Michalak, A.M., Canadell, J.G., Saikawa, E., Huntzinger, D.N., Gurney, K.R., Sitch, S., Zhang, B.W., Yang, J., Bousquet, P., Bruhwiler, L., Chen, G.S., Dlugokencky, E., Friedlingstein, P., Melillo, J., Pan, S.F., Poulter, B., Prinn, R., Saunois, M., Schwalm, C.R., Wofsy, S.C., 2016. The terrestrial biosphere as a net source of greenhouse gases to the atmosphere. Nature 531, 225-+.
- van Kessel, C., Venterea, R., Six, J., Adviento-Borbe, M.A., Linquist, B., van Groenigen, K.J., 2013. Climate, duration, and N placement determine N₂O emissions in reduced tillage systems: a meta-analysis. Global Change Biology 19, 33-44.
- Verhamme, D.T., Prosser, J.I., Nicol, G.W., 2011. Ammonia concentration determines differential growth of ammonia-oxidising archaea and bacteria in soil microcosms. Isme Journal 5, 1067-1071.
- Wang, B.Z., Zhao, J., Guo, Z.Y., Ma, J., Xu, H., Jia, Z.J., 2015. Differential contributions of ammonia oxidizers and nitrite oxidizers to nitrification in four paddy soils. Isme Journal 9, 1062-1075.

- Yoshinari, T., Hynes, R., Knowles, R., 1977. Acetylene inhibition of nitrous oxide reduction and measurement of denitrification and nitrogen fixation in soil. Soil Biology and Biochemistry 9, 177-183.
- Zhang, L.M., Hu, H.W., Shen, J.P., He, J.Z., 2012. Ammonia-oxidizing archaea have more important role than ammonia-oxidizing bacteria in ammonia oxidation of strongly acidic soils. Isme Journal 6, 1032-1045.

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).

			n	osZI		nosZII							
Site	Treatment	OTUs	Chao1	PD	Simpson reciprocal	OTUs	Chao1	PD	Simpson reciprocal				
	тт	114	135.3	8.7	24.4	242	339.2	21.9	30.5				
	LI	± 10	± 7.9	± 0.7	± 9.3	± 68	± 103.6	± 2.2	± 31.5				
Auzeville Mons B&E Mons SOERE		113	142.1	8.3	20.0	230	328.0	21.2	23.7				
	LI.C	± 4	± 17.7	± 0.4	± 12.8	± 5	± 34.1	± 2.9	± 6.9				
	VLI	115	134.6	8.7	25.2	185	252.0	18.0	24.3				
		± 34	± 39.5	± 2.8	± 14.2	± 39	± 47.7	± 5.2	± 26.7				
		112	137.9	8.9	29.4	197	263.0	21.0	26.0				
	VLI.C	± 11	± 15.8	± 1.4	± 3.2	± 40	± 58.5	± 4.2	± 23.9				
		106	125.6	8.2	14.4	256	351.3	23.9	26.9				
	AS	± 22	± 32.4	± 1.0	± 7.4	± 14	± 20.4	± 3.8	± 3.1				
		122	147.8	9.0	22.1	238	299.3	22.6	52.4				
	AS.C	± 28	± 36.1	± 1.8	± 7.2	± 17	± 35.7	± 2.9	± 20.9				
	MIN	94	108.6	7.3	19.1	224	319.8	22.4	16.6				
	IVILIN	± 20	± 17.9	± 1.2	± 7.5	± 44	± 72.2	± 4.8	± 6.4				
	MEN	92	108.6	7.3	19.7	233	344.6	22.9	19.5				
	IVIEIN	± 6	± 15.5	± 0.1	± 3.8	± 34	± 18.2	± 3.0	± 12.5				
	SUN	100	131.1	8.2	11.0	216	328.6	22.2	18.6				
	SLIN	± 11	± 49.2	± 1.2	± 4.2	± 2	± 7.1	± 2.0	± 7.7				
	SEN	106	127.4	8.4	27.6	197	308.1	20.5	11.8				
		± 34	± 46.6	± 2.2	± 14.2	± 14	± 58.2	± 0.7	± 4.2				
	SL	123	153.0	9.1	26.1	248	393.4	24.4	19.9				
		± 33	± 46.7	± 2.3	± 15.7	± 83	± 104.5	± 7.3	± 14.3				
	SE	105	122.4	8.2	15.5	238	368.7	23.1	16.0				
	51	±16	± 27.4	± 1.0	± 7.2	± 38	± 25.6	± 3.2	± 9.8				
	Т1	97	124.4	7.7	16.9	249	387.4	22.4	29.1				
	11	± 7	± 20.3	± 0.2	± 4.7	± 8	± 26.1	± 0.5	± 7.3				
	Т2	88	100.3	7.2	15.8	261	354.2	22.7	41.0				
	12	± 14	± 15.5	± 0.7	± 3.0	± 17	± 45.0	± 1.2	± 15.2				
	Т3	89	103.3	7.4	19.6	235	374.2	22.3	26.6				
	15	± 13	± 16.3	± 0.8	± 8.5	± 18	± 80.1	± 1.5	± 8.8				
	Τ4	91	119.8	7.5	17.2	227	319.1	20.2	25.2				
	T4	± 10	± 16.3	± 0.8	± 7.24	± 22	± 46.6	± 0.7	± 2.3				
	Т5	97	135.4	7.7	16.78	242	370.3	21.4	32.9				
	T5	± 23	± 11.6	± 1.2	± 2.03	± 28	± 82.1	± 2.8	± 14.5				
	Т6	105	126.9	8.1	21.5	230	334.4	20.5	35.7				
	T6	± 16	± 12.2	± 1.0	± 7.4	± 47	± 84.9	± 2.4	± 19.8				
	FIT	126	153.9	9.4	24.6	246 ^a	335.6 ^a	25.8^{a}	45.4				
	111	± 15	± 17.5	± 1.0	± 2.2	± 37	± 68.3	± 1.5	± 19.2				
Rojonavilla	SТ	112	128.9	10.1	26.1	188^{ab}	236.7 ^{ab}	20.4 ^b	36.9				
Doighevine	51	± 16	± 20.9	± 1.5	± 11.6	± 22	± 32.8	± 2.0	± 8.2				
	NT	120	144.2	9.6	27.4	173 ^b	223.1 ^b	19.4 ^b	24.0				
	1 N 1	± 36	± 42.0	± 0.6	± 8.8	± 26	± 24.1	± 2.0	± 17.5				

__TT			
Community	Soil Physic-chemical	Abundance	Diversity
Structure			
nosZI	pH, C/N	AOA, nosZI, nosZII, nosZI/nosZII	nosZI (PD)
nosZII	pH, C/N, Total N, cec, sand	AOA, AOB, nosZII	nosZI (PD)

Tab S2. Significant explanatory variables for nosZI and nosZII community structure.

Tab S3. Correlation coefficients between potential denitrification and nitrification activities, and soil properties, functional guilds abundances and N₂O-reducers diversity.

Potential Activities	pН	Clay	Sand	Loam	SOM	q.AOA	q.AOB	q. <i>nirK</i>	q. <i>nirS</i>	q. <i>nosZ</i> I	q. <i>nosZ</i> II	nosZI PD	nosZII PD	<i>nosZ</i> I SR	<i>nosZ</i> 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 N₂O emissions and soil properties, abundance and diversity of microorganisms.

N ₂ O "in situ"	nЦ	Clay	Sand	Loom	SOM	a 101		a wirV	a wir C	a nos7I	a mos711	nosZI	nosZII	nosZI	nosZII
fractions"	рп	Clay	Sanu	Loain	SOM	q.AOA	q.AOD	q. <i>mr</i> K	q. <i>m</i> /s	q.noszi	q.noszn	PD	PD	SR	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).

Chapter III



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)

Chapter III



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)

Chapter III



Fig S5. Abundance of *nos***ZI and** *nos***ZII.** 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 nosZII N₂O-reducers matters for soil N₂O reduction

In **Chapter I** we evaluated the influence of altered proportions of *nosZ*II N₂O 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 N₂O reductase. Our results showed that increasing abundance of *Dyadobacter fermentans* resulted in a decrease of the N₂O production in 1/3 of the tested soils. Previously, Philippot et al. (2011) had manipulated the proportion of N₂O-producers by inoculating *Agrobacterium tumefaciens*, a strain producing N₂O but not capable to reduce it, into three Swedish soils. They showed an increase in N₂O production with higher *A. tumefaciens* inoculation levels. However, in one of the three tested soils, the N₂O/N₂ 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 N₂O produced by *A. tumefaciens*. Altogether these two studies investigating respectively the importance of denitrifiers lacking *nosZ* and of N₂O-reducers can be of importance for explaining soil N₂O production.

In our study, the N₂O 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 N₂O sink. The reduction of N₂O by a few other *nos*ZII strains was evaluated in recent studies. Similarly to D. fermentans, Anaeromyxobacter dehalogenans was also able to grow using N₂O 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 N₂O-reductase may operate with higher efficiency than the respiratory machinery of a complete denitrifier. Yoon et al. (2016) also compared the growth yield and N₂O consumption kinetics between two nosZI and three nosZII 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 N₂Oreducers clades regarding Michaelis-Menten parameters Ks and Vmax under growth with N₂O was observed. nosZI strains presented a higher Vmax than nosZII (ranging between 0.44 and 4.16 against 0.017 to 0.461 µmol min⁻¹ mg biomass⁻¹, for nosZI and nosZII, respectively). Regarding Ks, nosZI strains showed also a higher range with 7.0 to 35.5 µM (N₂O), while nosZII strains recorded from 0.32 to 1.34 µM (N₂O). The authors suggested that the nosZI clade could be considered as an r-strategist and *nosZII* 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 nosZI clade has a relatively low diversity and is composed by alpha-, beta-, and gamma-, proteobacteria. In contrast, the nosZII 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 N₂O 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 N₂O-reducers may act as a N₂O 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 N₂O in the different soils. Accordingly, Liu et al. (2014) suggested that low soil pH diminishes N2O reduction due to an unsuccessful assembly of the N2O reductase. pH has been shown by various studies as a major variable controlling the proportion of N₂O produced by denitrification (Šimek & Cooper 2002) and N₂O 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 strainspecific pH range for performing N₂O-reduction. The other soil property that was related to the capacity of D. fermentans to reduce N₂O 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 N₂O. It is also worth noticing that the C/N ratio was one of the soil properties driving the nosZII community in Jones et al. 2014.

Our study was the first to show that a nosZII strain inoculated in soils can reduce N₂O produced by the indigenous microbial communities. Previous studies investigated the capacity of plant-symbiotic bacteria having the nosZI gene to reduce N₂O 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 N₂O consumption in the treatments with the functional nosZ. Interestingly, the inoculums with B. japonicum were able to reduce N₂O under both anaerobic and aerobic conditions, suggesting that this could be an efficient solution to reduce N₂O in the field with low dependence on the O₂ partial pressure. These results were confirmed at a field scale by Itakura et al. (2013), who showed that postharvest N₂O emissions due to soybean nodules degradation could be mitigated by the inoculation of B. japonicum with enhanced N2O-reductase activity. The potential of free living denitrifying nosZI strains from the genus Azospirillum sp. (gamma-proteobacteria) and Herbaspirillum sp. (beta-proteobacteria) to reduce N₂O production was also shown, with most of the strains reducing N₂O 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 N₂O. 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 N₂O 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 N₂O-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 N₂O 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 N₂O-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 nosZII and although not significantly, it seemed to enhance the abundance of this clade while it did not change nosZI diversity or abundance. Comparing our results for the nosZII clade to the literature is impossible because, to our knowledge, no other study is available. We will therefore restrain our comparisons to the nosZI clade. Melero et al. (2011) studied the abundance of nosZI 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 nosZI 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 nosZI 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 *nosZI* 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 N₂O 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 N₂O emissions. Thus, some studies indicated tillage promotes N₂O 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 N₂O 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 pO₂, 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 N₂O emissions in heavy clay soil compared to NT. Venterea et al. (2005) showed that NT effect on N₂O emissions was also dependent on fertilizer type with increased N₂O 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 N₂O fluxes.

An agricultural practice which had no effect on the microbial communities but impacted the in situ N₂O emissions and the N₂O:N₂ ratio was the harvest date in the B&E experimental site. We observed a trend of higher in situ N₂O 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 N2O 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 N₂O production. Peyrard et al. (2016a) investigated N₂O emissions in Miscanthus early and late harvest at the same experimental site and observed that harvest date had the stronger effect on N₂O 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 N₂O:N₂ ratio. In particular, early harvest of *M. giganteus* showed a significantly higher proportion of N₂O produced during denitrification than late harvest. Previous studies reported that production of N₂O relative to N2 during denitrification in soils is influenced by carbon availability and pO2 (Firestone et al., 1980; Murray and Knowles, 2004; Giles et al., 2012;). The proportion of N₂O produced during denitrification in soils was shown to increase with increasing pO₂ (Firestone et al., 1980), which is in accordance with our results as the N₂O:N₂ 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 N₂O fluxes, which highlights the importance of the agricultural practices for avoiding high N₂O 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 nosZII community was more diverse in the ORE compared to B&E, while nosZI diversity was not impacted. Similarly, the abundance of nosZII was higher in the B&E experiment than in the ORE, but we did not observe any difference in nosZI 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 N₂O production we did not observe significant differences in the potential N₂O production or PDA. However, we showed a higher proportion of N₂O produced during denitrification (N₂O:N₂) in the perennial site compared to the annual one. Measurements of daily N₂O emissions by Thompson et al. (2016) showed significantly lower emissions in the perennial fields compared to the annual ones (1.81 ng N₂O-N m⁻² s⁻¹ and 10.01 ng N₂O-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 N₂O fluxes or nosZI 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 nosZI 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(NO₃)₂), ammonium sulfate ((NH₄)₂SO₄), 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 (NH₄)₂SO₄ and sewage sludge reduced the abundance of nosZI compared to Ca(NO₃)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 (NH₄NO₃) 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 nosZI abundance, nor on N₂O reducers community structure. These results suggested that the type of N may be more important than the rate of fertilization for N₂O-reducers. The different fertilization rates did not influence the N₂O 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 N2O 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 N₂O-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 control microcosms and 50.9% of the denitrifier 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 N₂O 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 N₂O-reducers diversity and abundance for soil N₂O 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 N₂O:N₂ ratio variation and did not correlate to the PDA or potential N₂O production. In **Chapter III**, we expanded the approach from **Chapter II** to multiple sites from different locations. 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 ~ $\frac{1}{4}$), 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 N₂O. Similarly, Morales et al (2010) reported a positive relationship between *nirS* gene abundance minus *nosZI* gene abundance and the N₂O emissions. To our knowledge, this study is the first to study the relationships between the newly identified *nosZ*II community and *in situ* N₂O emissions, which lead to the identification of a negative correlation between *nosZ*II abundance and diversity and *in situ* N₂O emissions. Interestingly, we also observed that *in situ* N₂O 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 N₂O 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 the *in situ* N₂O emissions, the observed negative relationship between AOA and *in situ* N₂O 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 N₂O 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 N₂O-reducers clade for GHG emissions. Our main findings were:

- Addition of a non-denitrifying *nos*ZII strain to 11 different soils significantly reduced N₂O production in more than 1/3 of the soils. Some soils even became potential sinks of N₂O. Therefore our results provide unambiguous evidence that the overlooked non-denitrifying NosZII-type bacteria can contribute to N₂O consumption in soil.
- Among the studied agricultural practices, the only practice which impacted the N₂Oreducing 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 N₂O emitted as end-product of denitrification and the *in situ* N₂O 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 N₂O produced as end-product of denitrification and was the stronger predictor of its variation (26%). Similarly, subdivision of the 70000 *in situ* N₂O 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 *nos*ZII-community might be of importance for mitigating N₂O emissions. However, little is known about the *nos*ZII community and further research is needed for understanding their ecophysiology. Thus, the N₂O-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 N₂O-reductase in both clades. To better understand the relevance of N₂O-reducers to the fate of N₂O 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 N₂O 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 N₂O-reductase within each group, as we showed that increasing this proportion has the potential to decrease the soil N₂O 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 N₂O-reducers from *nos*ZI and *nos*ZII 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 N₂O-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 N₂O 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 N₂O reduction.

8 References

Agreste, 2014. Dossier Fertilisation Dossier N°21.

http://www.agreste.agriculture.gouv.fr/IMG/pdf/dossier21_fertilisation.pdf.

- Akiyama, H., Yan, X., Yagi, K., 2010. Evaluation of effectiveness of enhanced-efficiency fertilizers as mitigation options for N₂O and NO emissions from agricultural soils: meta-analysis. Global Change Biology 16, 1837-1846.
- Alder, N.N., Theg, S.M., 2003. Energetics of Protein Transport across Biological Membranes: A Study of the Thylakoid ΔpH-Dependent/cpTat Pathway. Cell 112, 231-242.
- Amougou, N., Bertrand, I., Machet, J.M., Recous, S., 2011. Quality and decomposition in soil of rhizome, root and senescent leaf from Miscanthus x giganteus, as affected by harvest date and N fertilization. Plant and Soil 338, 83-97.
- Amougou, N., Bertrand, I., Cadoux, S., Recous, S., 2012. Miscanthus x giganteus leaf senescence, decomposition and C and N inputs to soil. Global Change Biology Bioenergy 4, 698-707.
- Angers, D.A., Eriksen-Hamel, N.S., 2008. Full-inversion tillage and organic carbon distribution in soil profiles: A meta-analysis. Soil Science Society of America Journal 72, 1370-1374.
- Attard, E., Poly, F., Commeaux, C., Laurent, F., Terada, A., Smets, B.F., Recous, S., Roux, X.L., 2010. Shifts between Nitrospira- and Nitrobacter-like nitrite oxidizers underlie the response of soil potential nitrite oxidation to changes in tillage practices. Environmental Microbiology 12, 315-326.
- Attard, E., Recous, S., Chabbi, A., De Berranger, C., Guillaumaud, N., Labreuche, J., Philippot, L., Schmid, B., Le Roux, X., 2011. Soil environmental conditions rather than denitrifier abundance and diversity drive potential denitrification after changes in land uses. Global Change Biology 17, 1975-1989.
- Aulakh, M.S., Doran, J.W., Mosier, A.R., 1991. Field-Evaluation of 4 Methods for measuring denitrification. Soil Science Society of America Journal 55, 1332-1338.
- Baggs EM, Philippot L., 2010., Microbial terrestrial pathways to nitrous oxide. In:Smith K (ed) Nitrous oxide and climate change. Earthscan, London, pp 4–35
- Baggs, E.M., Smales, C.L., Bateman, E.J., 2010. Changing pH shifts the microbial sourceas well as the magnitude of N₂O emission from soil. Biology and Fertility of Soils 46, 793-805.
- Bates B., Kundzewicz Z., Wu S., Palutikof J., 2008. Climate change and water, in Technical Paper of the Intergovernmental Panel on Climate Change (Geneva: IPCC).
- Braker G, and Conrad R., 2011. Diversity, structure, and size of N₂O-producing microbial communities in soils-What matters for their functioning? Adv Appl Microbiol 75: 33–70.
- Bakken, L.R., 1988. Denitrification under different cultivated plants effects of soil-moisture tension, nitrate concentration, and photosynthetic activity. Biology and Fertility of Soils 6, 271-278.
- Bandibas, J., Vermoesen, A., Degroot, C.J., Vancleemput, O., 1994. The effect of different moisture regimes and soil characteristics on nitrous-oxide emission and consumption by different soils. soil science 158, 106-114.
- Barton, L., Schipper, L.A., 2001. Regulation of nitrous oxide emissions from soils irrigated with dairy farm effluent. Journal of Environmental Quality 30, 1881-1887.

- Basche, A.D., Miguez, F.E., Kaspar, T.C., Castellano, M.J., 2014. Do cover crops increase or decrease nitrous oxide emissions? A meta-analysis. Journal of Soil and Water Conservation 69, 471-482.
- Baudoin, E., Philippot, L., Chèneby, D., Chapuis-Lardy, L., Fromin, N., Bru, D., Rabary, B., Brauman, A., 2009. Direct seeding mulch-based cropping increases both the activity and the abundance of denitrifier communities in a tropical soil. Soil Biology and Biochemistry 41, 1703-1709.
- Benckiser, G., Schartel, T., Weiske, A., 2015. Control of NO₃- and N₂O emissions in agroecosystems: A review. Agronomy for Sustainable Development 35, 1059-1074.
- Bobbink, R., Hicks, K., Galloway, J., Spranger, T., Alkemade, R., Ashmore, M., Bustamante, M., Cinderby, S., Davidson, E., Dentener, F., Emmett, B., Erisman, J.W., Fenn, M., Gilliam, F., Nordin, A., Pardo, L., De Vries, W., 2010. Global assessment of nitrogen deposition effects on terrestrial plant diversity: a synthesis. Ecological Applications 20, 30-59.
- Bouwman, A.F., Boumans, L.J.M., Batjes, N.H., 2002a. Emissions of N₂O and NO from fertilized fields: Summary of available measurement data. Global Biogeochemical Cycles 16, 6-1-6-13.
- Bru, D., Ramette, A., Saby, N.P.A., Dequiedt, S., Ranjard, L., Jolivet, C., Arrouays, D., Philippot, L., 2011. Determinants of the distribution of nitrogen-cycling microbial communities at the landscape scale. Isme Journal 5, 532-542.
- Butterbach-Bahl, K., Breuer, L., Gasche, R., Willibald, G., Papen, H., 2002. Exchange of trace gases between soils and the atmosphere in Scots pine forest ecosystems of the northeastern German lowlands 1. Fluxes of N₂O, NO/NO₂ and CH₄ at forest sites with different N-deposition. Forest Ecology and Management 167, 123-134.
- Butterbach-Bahl, K., Baggs, E.M., Dannenmann, M., Kiese, R., Zechmeister-Boltenstern, S., 2013. Nitrous oxide emissions from soils: how well do we understand the processes and their controls? Philosophical Transactions of the Royal Society B-Biological Sciences 368.
- Cadoux, S., Ferchaud, F., Demay, C., Boizard, H., Machet, J.M., Fourdinier, E., Preudhomme, M., Chabbert, B., Gosse, G., Mary, B., 2014. Implications of productivity and nutrient requirements on greenhouse gas balance of annual and perennial bioenergy crops. Global Change Biology Bioenergy 6, 425-438.
- Canfield, D.E., Glazer, A.N., Falkowski, P.G., 2010. The Evolution and Future of Earth's Nitrogen Cycle. Science 330, 192-196.
- Chapuis-Lardy, L., Wrage, N., Metay, A., Chotte, J.-L., Bernoux, M., 2007. Soils, a sink for N₂O? A review. Global Change Biology 13, 1-17.
- Che, J., Zhao, X.Q., Zhou, X., Jia, Z.J., Shen, R.F., 2015. High pH-enhanced soil nitrification was associated with ammonia-oxidizing bacteria rather than archaea in acidic soils. Applied Soil Ecology 85, 21-29.
- Cheneby, D., Perrez, S., Devroe, C., Hallet, S., Couton, Y., Bizouard, F., Iuretig, G., Germon, J.C., Philippot, L., 2004. Denitrifying bacteria in bulk and maize-rhizospheric soil: diversity and N₂O-reducing abilities. Canadian Journal of Microbiology 50, 469-474.
- Clark, I.M., Buchkina, N., Jhurreea, D., Goulding, K.W.T., Hirsch, P.R., 2012. Impacts of nitrogen application rates on the activity and diversity of denitrifying bacteria in the Broadbalk Wheat Experiment. Philosophical Transactions of the Royal Society B-Biological Sciences 367, 1235-1244.
- Coleman D.C., C.D.A.J.H.P.F., 2004. Fundamentals of soil ecology. Elsevier Academic Press.

- Cuhel, J., Simek, M., Laughlin, R.J., Bru, D., Cheneby, D., Watson, C.J., Philippot, L., 2010. Insights into the Effect of Soil pH on N₂O and N₂ Emissions and Denitrifier Community Size and Activity. Applied and Environmental Microbiology 76, 1870-1878.
- Cuypers, H., Viebrocksambale, A., Zumft, W.G., 1992. NOSR, a membrane-bound regulatory component necessary for expression of nitrous-oxide reductase in denitrifying pseudomonas-stutzeri. Journal of Bacteriology 174, 5332-5339.
- Daims, H., Lebedeva, E.V., Pjevac, P., Han, P., Herbold, C., Albertsen, M., Jehmlich, N., Palatinszky, M., Vierheilig, J., Bulaev, A., Kirkegaard, R.H., von Bergen, M., Rattei, T., Bendinger, B., Nielsen, P.H., Wagner, M., 2015. Complete nitrification by Nitrospira bacteria. Nature 528, 504-509..
- Dambreville, C., Hallet, S., Nguyen, C., Morvan, T., Germon, J.C., Philippot, L., 2006. Structure and activity of the denitrifying community in a maize-cropped field fertilized with composted pig manure or ammonium nitrate. Fems Microbiology Ecology 56, 119-131.
- Dandie, C.E., Burton, D.L., Zebarth, B.J., Henderson, S.L., Trevors, J.T., Goyer, C., 2008. Changes in Bacterial Denitrifier Community Abundance over Time in an Agricultural Field and Their Relationship with Denitrification Activity. Applied and Environmental Microbiology 74, 5997-6005.
- Davidson, E.A., Kanter, D., 2014. Inventories and scenarios of nitrous oxide emissions. Environmental Research Letters 9, 105012.
- de Gannes, V., Eudoxie, G., Hickey, W.J., 2014. Impacts of Edaphic Factors on Communities of Ammonia-Oxidizing Archaea, Ammonia-Oxidizing Bacteria and Nitrification in Tropical Soils. PLoS One 9 (2):e99568.
- de Klein, C.A.M., Vanlogtestijn, R.S.P., 1994. Denitrification in the top-soil of managed grasslands in the netherlands in relation to soil type and fertilizer level. Plant and Soil 163, 33-44.
- de Klein C., N.R.A., Ogle, S., Smith, K. A., Rochette P., Wirth T. C., 2006. N₂O Emissions from Managed Soils, and CO2 Emissions from Lime and Urea Application IPCC Guidelines for National Greenhouse Gas Inventories Volume 4: Agriculture, Forestry and Other Land Use
- Di, H.J., Cameron, K.C., Shen, J.P., Winefield, C.S., O'Callaghan, M., Bowatte, S., He, J.Z., 2010. Ammonia-oxidizing bacteria and archaea grow under contrasting soil nitrogen conditions. Fems Microbiology Ecology 72, 386-394.
- Dong, L.F., Smith, C.J., Papaspyrou, S., Stott, A., Osborn, A.M., Nedwell, D.B., 2009. Changes in Benthic Denitrification, Nitrate Ammonification, and Anammox Process Rates and Nitrate and Nitrite Reductase Gene Abundances along an Estuarine Nutrient Gradient (the Colne Estuary, United Kingdom). Applied and Environmental Microbiology 75, 3171-3179.
- Drewer, J., Finch, J.W., Lloyd, C.R., Baggs, E.M., Skiba, U., 2012. How do soil emissions of N₂O, CH₄ and CO₂ from perennial bioenergy crops differ from arable annual crops? GCB Bioenergy 4, 408-419.
- El Titi, A., 2003. Soil Tillage in Agroecosystems. (ed. A. El Titi). CRC Press, Boca Raton, FL.
- Eisenhauer, N., Beßler, H., Engels, C., Gleixner, G., Habekost, M., Milcu, A., Partsch, S., Sabais, A.C.W., Scherber, C., Steinbeiss, S., Weigelt, A., Weisser, W.W., Scheu, S., 2010. Plant diversity effects on soil microorganisms support the singular hypothesis. Ecology 91, 485-496.

- Enwall, K., Philippot, L., Hallin, S., 2005. Activity and composition of the denitrifying bacterial community respond differently to long-term fertilization. Applied and Environmental Microbiology 71, 8335-8343.
- Erguder, T.H., Boon, N., Wittebolle, L., Marzorati, M., Verstraete, W., 2009. Environmental factors shaping the ecological niches of ammonia-oxidizing archaea. Fems Microbiology Reviews 33, 855-869.
- Fenn, M.E., Poth, M.A., Johnson, D.W., 1996. Evidence for nitrogen saturation in the San Bernardino Mountains in southern California. Forest Ecology and Management 82, 211-230.
- Fierer, N., Ladau, J., Clemente, J.C., Leff, J.W., Owens, S.M., Pollard, K.S., Knight, R., Gilbert, J.A., McCulley, R.L., 2013. Reconstructing the Microbial Diversity and Function of Pre-Agricultural Tallgrass Prairie Soils in the United States. Science 342, 621-624.
- Firestone, M.K., Firestone, R.B., Tiedje, J.M., 1980. Nitrous-oxide from soil denitrification factors controlling its biological production. Science 208, 749-751.
- Flippo, T.S., Holder, W.D., 1993. Neurologic degeneration associated with nitrous-oxide anesthesia in patients with vitamin-b-12 deficiency. Archives of Surgery 128, 1391-1395.
- Gaba, S., Lescourret, F., Boudsocq, S., Enjalbert, J., Hinsinger, P., Journet, E.P., Navas, M.L., Wery, J., Louarn, G., Malezieux, E., Pelzer, E., Prudent, M., Ozier-Lafontaine, H., 2015. Multiple cropping systems as drivers for providing multiple ecosystem services: from concepts to design. Agronomy for Sustainable Development 35, 607-623.
- Galloway, J.N., Cowling, E.B., 2002. Reactive nitrogen and the world: 200 years of change. Ambio 31, 64-71.
- Galloway, J.N., Aber, J.D., Erisman, J.W., Seitzinger, S.P., Howarth, R.W., Cowling, E.B., Cosby, B.J., 2003. The nitrogen cascade. Bioscience 53, 341-356.
- Gao, N., Shen, W., Kakuta, H., Tanaka, N., Fujiwara, T., Nishizawa, T., Takaya, N., Nagamine, T., Isobe, K., Otsuka, S., Senoo, K., 2016. Inoculation with nitrous oxide (N₂O)-reducing denitrifier strains simultaneously mitigates N₂O emission from pasture soil and promotes growth of pasture plants. Soil Biology & Biochemistry 97, 83-91.
- Geisseler, D., Lazicki, P.A., Scow, K.M., 2016. Mineral nitrogen input decreases microbial biomass in soils under grasslands but not annual crops. Applied Soil Ecology 106, 1-10.
- Giles, M., Morley, N., Baggs, E., Daniell, T., 2012. Soil nitrate reducing processes drivers, mechanisms for spatial variation, and significance for nitrous oxide production. Frontiers in Microbiology 3:407.
- Glockner, A.B., Jungst, A., Zumft, W.G., 1993. Copper-containing nitrite reductase from pseudomonas-aureofaciens is functional in a mutationally cytochrome-cd(1)-free background (nirs-) of pseudomonas-stutzeri. Archives of Microbiology 160, 18-26.
- Grace, P.R., Robertson, G.P., Millar, N., Colunga-Garcia, M., Basso, B., Gage, S.H., Hoben, J., 2011. The contribution of maize cropping in the Midwest USA to global warming: A regional estimate. Agricultural Systems 104, 292-296.
- Graf, D.R.H., Jones, C.M., Hallin, S., 2014. Intergenomic Comparisons Highlight Modularity of the Denitrification Pathway and Underpin the Importance of Community Structure for N₂O Emissions. PLoS One 9 (12):e1141118.

- Graf, D.R.H., Zhao, M., Jones, C.M., Hallin, S., 2016. Soil type overrides plant effect on genetic and enzymatic N₂O production potential in arable soils. Soil Biology and Biochemistry 100, 125-128.
- Graham, E.B., Knelman, J.E., Schindlbacher, A., Siciliano, S., Breulmann, M., Yannarell, A., Bemans, J.M., Abell, G., Philippot, L., Prosser, J., Foulquier, A., Yuste, J.C., Glanville, H.C., Jones, D.L., Angel, F., Salminen, J., Newton, R.J., Burgmann, H., Ingram, L.J., Hamer, U., Siljanen, H.M.P., Peltoniemi, K., Potthast, K., Baneras, L., Hartmann, M., Banerjee, S., Yu, R.Q., Nogaro, G., Richter, A., Koranda, M., Castle, S.C., Goberna, M., Song, B., Chatterjee, A., Nunes, O.C., Lopes, A.R., Cao, Y.P., Kaisermann, A., Hallin, S., Strickland, M.S., Garcia-Pausas, J., Barba, J., Kang, H., Isobe, K., Papaspyrou, S., Pastorelli, R., Lagomarsino, A., Lindstrom, E.S., Basiliko, N., Nemergut, D.R., 2016. Microbes as Engines of Ecosystem Function: When Does Community Structure Enhance Predictions of Ecosystem Processes? Frontiers in Microbiology 7.
- Griffis, T.J., Lee, X., Baker, J.M., Russelle, M.P., Zhang, X., Venterea, R., Millet, D.B., 2013. Reconciling the differences between top-down and bottom-up estimates of nitrous oxide emissions for the US Corn Belt. Global Biogeochemical Cycles 27, 746-754.
- Gruber, N., Galloway, J.N., 2008. An Earth-system perspective of the global nitrogen cycle. Nature 451, 293-296.
- Gubry-Rangin, C., Nicol, G.W., Prosser, J.I., 2010. Archaea rather than bacteria control nitrification in two agricultural acidic soils. Fems Microbiology Ecology 74, 566-574.
- Habekost, M., Eisenhauer, N., Scheu, S., Steinbeiss, S., Weigelt, A., Gleixner, G., 2008. Seasonal changes in the soil microbial community in a grassland plant diversity gradient four years after establishment. Soil Biology and Biochemistry 40, 2588-2595.
- Hallin, S., Jones, C.M., Schloter, M., Philippot, L., 2009. Relationship between N-cycling communities and ecosystem functioning in a 50-year-old fertilization experiment. ISME J 3, 597-605.
- Harper, W.F., Takeuchi, Y., Riya, S., Hosomi, M., Terada, A., 2015. Novel abiotic reactions increase nitrous oxide production during partial nitrification: Modeling and experiments. Chemical Engineering Journal 281, 1017-1023.
- Harter, J., Weigold, P., El-Hadidi, M., Huson, D.H., Kappler, A., Behrens, S., 2016. Soil biochar amendment shapes the composition of N₂O-reducing microbial communities. Science of The Total Environment 562, 379-390.
- Hartmann, M., Frey, B., Mayer, J., Mader, P., Widmer, F., 2015. Distinct soil microbial diversity under long-term organic and conventional farming. Isme Journal 9, 1177-1194.
- He, J.-Z., Hu, H.-W., Zhang, L.-M., 2012. Current insights into the autotrophic thaumarchaeal ammonia oxidation in acidic soils. Soil Biology and Biochemistry 55, 146-154.
- Henault, C., Revellin, C., 2011. Inoculants of leguminous crops for mitigating soil emissions of the greenhouse gas nitrous oxide. Plant and Soil 346, 289-296.
- Henry, S., Texier, S., Hallet, S., Bru, D., Dambreville, C., Cheneby, D., Bizouard, F., Germon, J.C., Philippot, L., 2008. Disentangling the rhizosphere effect on nitrate reducers and denitrifiers: insight into the role of root exudates. Environmental Microbiology 10, 3082-3092.
- Hink, L., Nicol, G.W., Prosser, J.I., 2016. Archaea produce lower yields of N₂O than bacteria during aerobic ammonia oxidation in soil. Environmental Microbiology,00(00),00-00.

- Hoben, J.P., Gehl, R.J., Millar, N., Grace, P.R., Robertson, G.P., 2011. Nonlinear nitrous oxide (N₂O) response to nitrogen fertilizer in on-farm corn crops of the US Midwest. Global Change Biology 17, 1140-1152.
- Hooper, D.U., Chapin, F.S., Ewel, J.J., Hector, A., Inchausti, P., Lavorel, S., Lawton, J.H., Lodge, D.M., Loreau, M., Naeem, S., Schmid, B., Setala, H., Symstad, A.J., Vandermeer, J., Wardle, D.A., 2005. Effects of biodiversity on ecosystem functioning: A consensus of current knowledge. Ecological Monographs 75, 3-35.
- Hu, H.-W., Xu, Z.-H., He, J.-Z., 2014. Chapter Six Ammonia-Oxidizing Archaea Play a Predominant Role in Acid Soil Nitrification, In: Donald, L.S. (Ed.), Advances in Agronomy. Academic Press, pp. 261-302.
- Hu, H.W., Chen, D., He, J.Z., 2015. Microbial regulation of terrestrial nitrous oxide formation: understanding the biological pathways for prediction of emission rates. Fems Microbiology Reviews 39, 729-749.
- Huang, Y., Zou, J., Zheng, X., Wang, Y., Xu, X., 2004. Nitrous oxide emissions as influenced by amendment of plant residues with different C:N ratios. Soil Biology and Biochemistry 36, 973-981.
- Hussain, I., Olson, K.R., Ebelhar, S.A., 1999. Long-term tillage effects on soil chemical properties and organic matter fractions. Soil Science Society of America Journal 63, 1335-1341.
- Kuypers, M.M.M., 2015. A division of labour combined. Nature 528, 487-488.
- IFA (International Fertilizer Industry Association), FAO (Food and Agriculture Organisation of the United Nations), 2001. Global estimates of gaseous emissions of NH₃, NO and N₂O from agricultural land. FAO, Rome, Italy.
- IPCC, 2013: Summary for Policymakers. In: Climate Change 2013: The Physical Science Basis.Contribution of Working Group I to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change [Stocker, T.F., D. Qin, G.-K. Plattner, M. Tignor, S. K. Allen, J. Boschung, A. Nauels, Y. Xia, V. Bex and P.M. Midgley (eds.)]. Cambridge University Press, Cambridge, United Kingdom and New York, NY, USA.
- Inselsbacher, E., Hinko-Najera Umana, N., Stange, F.C., Gorfer, M., Schuller, E., Ripka, K., Zechmeister-Boltenstern, S., Hood-Novotny, R., Strauss, J., Wanek, W., 2010. Short-term competition between crop plants and soil microbes for inorganic N fertilizer. Soil Biology & Biochemistry 42, 360-372.
- Itakura, M., Uchida, Y., Akiyama, H., Hoshino, Y.T., Shimomura, Y., Morimoto, S., Tago, K., Wang, Y., Hayakawa, C., Uetake, Y., Sanchez, C., Eda, S., Hayatsu, M., Minamisawa, K., 2013. Mitigation of nitrous oxide emissions from soils by Bradyrhizobium japonicum inoculation. Nature Clim. Change 3, 208-212.
- Jiang, X., Xin, X., Li, S., Zhou, J., Zhu, T., Müller, C., Cai, Z., Wright, A.L., 2015. Effects of Fe oxide on N transformations in subtropical acid soils. Scientific Reports 5, 8615.
- Jones, C.M., Welsh, A., Throbäck, I.N., Dörsch, P., Bakken, L.R., Hallin, S., 2011. Phenotypic and genotypic heterogeneity among closely related soil-borne N₂ and N₂O-producing Bacillus isolates harboring the nosZ gene. Fems Microbiology Ecology 76, 541-552.
- Jones, C.M., Graf, D.R.H., Bru, D., Philippot, L., Hallin, S., 2013. The unaccounted yet abundant nitrous oxide-reducing microbial community: a potential nitrous oxide sink. Isme Journal 7, 417-426.
- Jones, C.M., Spor, A., Brennan, F.P., Breuil, M.C., Bru, D., Lemanceau, P., Griffiths, B., Hallin, S., Philippot, L., 2014. Recently identified microbial guild mediates soil N₂O sink capacity. Nature Climate Change 4, 801-805.

- Jung, M.Y., Well, R., Min, D., Giesemann, A., Park, S.J., Kim, J.G., Kim, S.J., Rhee, S.K., 2014. Isotopic signatures of N₂O produced by ammonia-oxidizing archaea from soils. Isme Journal 8, 1115-1125.
- Kammann, C., Muller, C., Grunhage, L., Jager, H.J., 2008. Elevated CO2 stimulates N₂O emissions in permanent grassland. Soil Biology & Biochemistry 40, 2194-2205.
- Kern, M., Simon, J., 2009. Electron transport chains and bioenergetics of respiratory nitrogen metabolism in Wolinella succinogenes and other Epsilonproteobacteria. Biochimica Et Biophysica Acta-Bioenergetics 1787, 646-656.
- Kiese, R., Hewett, B., Graham, A., Butterbach-Bahl, K., 2003. Seasonal variability of N₂O emissions and CH₄ uptake by tropical rainforest soils of Queensland, Australia. Global Biogeochemical Cycles 17.
- Killham, K., 1990. Nitrification in coniferous forest soils. Plant and Soil 128, 31-44.
- Kim, D.-G., Hernandez-Ramirez, G., Giltrap, D., 2013. Linear and nonlinear dependency of direct nitrous oxide emissions on fertilizer nitrogen input: A meta-analysis. Agriculture, Ecosystems & Environment 168, 53-65.
- Kong, A.Y.Y., Hristova, K., Scow, K.M., Six, J., 2010. Impacts of different N management regimes on nitrifier and denitrifier communities and N cycling in soil microenvironments. Soil Biology & Biochemistry 42, 1523-1533.
- Konneke, M., Bernhard, A.E., de la Torre, J.R., Walker, C.B., Waterbury, J.B., Stahl, D.A., 2005. Isolation of an autotrophic ammonia-oxidizing marine archaeon. Nature 437, 543-546.
- Koskinen, W.C., Keeney, D.R., 1982. Effect of pH on the rate of gaseous products of denitrification in a silt loam soil. Soil Science Society of America Journal 46, 1165-1167.
- Kulmatiski, A., Beard, K.H., 2008. Decoupling plant-growth from land-use legacies in soil microbial communities. Soil Biology and Biochemistry 40, 1059-1068.
- Lal, R., 2004. Soil carbon sequestration impacts on global climate change and food security. Science 304, 1623-1627.
- Lauber, C.L., Hamady, M., Knight, R., Fierer, N., 2009. Pyrosequencing-Based Assessment of Soil pH as a Predictor of Soil Bacterial Community Structure at the Continental Scale. Applied and Environmental Microbiology 75, 5111-5120.
- Le Roux, X., Poly, F., Currey, P., Commeaux, C., Hai, B., Nicol, G.W., Prosser, J.I., Schloter, M., Attard, E., Klumpp, K., 2008. Effects of aboveground grazing on coupling among nitrifier activity, abundance and community structure. Isme Journal 2, 221-232.
- Ledgard, S.F., Penno, J.W., Sprosen, M.S., 1999. Nitrogen inputs and losses from clover/grass pastures grazed by dairy cows, as affected by nitrogen fertilizer application. Journal of Agricultural Science 132, 215-225.
- Lee, P.A., Tullman-Ercek, D., Georgiou, G., 2006. The Bacterial Twin-Arginine Translocation Pathway. Annual review of microbiology 60, 373-395.
- Legg, T.M., Zheng, Y., Simone, B., Radloff, K.A., Mladenov, N., Gonzalez, A., Knights, D., Siu, H.C., Rahman, M.M., Ahmed, K.M., McKnight, D.M., Nemergut, D.R., 2012. Carbon, metals, and grain size correlate with bacterial community structure in sediments of a high arsenic aquifer. Frontiers in Microbiology 3.
- Liu, B.B., Frostegard, A., Bakken, L.R., 2014. Impaired Reduction of N₂O to N-2 in Acid Soils Is Due to a Posttranscriptional Interference with the Expression of nosZ. Mbio 5.
- Logsdon, S.D., Karlen, D.L., 2004. Bulk density as a soil quality indicator during conversion to no-tillage. Soil & Tillage Research 78, 143-149.

- Sutton, M. A., W. Erisman, C.M.H., J G. Billen, A. Bleeker, P. Grennfelt, H. Van Grinsven and B. Grizzetti., 2011. The european nitrogen assessment: Sources, effects and policy perspectives. Cambridge university press, UK.
- Maeda, K., Spor, A., Edel-Hermann, V., Heraud, C., Breuil, M.C., Bizouard, F., Toyoda, S., Yoshida, N., Steinberg, C., Philippot, L., 2015. N₂O production, a widespread trait in fungi. Scientific Reports 5.
- Mallon, C.A., Poly, F., Roux, X.L., Marring, I., Elsas, J.D.v., Salles, J.F., 2015. Resource pulses can alleviate the biodiversity-invasion relationship in soil microbial communities. Ecology 96, 915-926.
- Marland, G., McCarl, B.A., Schneider, U., 2001. Soil carbon: Policy and economics. Climatic Change 51, 101-117.
- Matson, P.A., McDowell, W.H., Townsend, A.R., Vitousek, P.M., 1999. The globalization of N deposition: ecosystem consequences in tropical environments. Biogeochemistry 46, 67-83.
- McSwiney, C.P., Robertson, G.P., 2005. Nonlinear response of N₂O flux to incremental fertilizer addition in a continuous maize (Zea mays L.) cropping system. Global Change Biology 11, 1712-1719.
- Melero, S., Pérez-de-Mora, A., Murillo, J.M., Buegger, F., Kleinedam, K., Kublik, S., Vanderlinden, K., Moreno, F., Schloter, M., 2011. Denitrification in a vertisol under long-term tillage and no-tillage management in dryland agricultural systems: Key genes and potential rates. Applied Soil Ecology 47, 221-225.
- Miller, M.N., Zebarth, B.J., Dandie, C.E., Burton, D.L., Goyer, C., Trevors, J.T., 2008. Crop residue influence on denitrification, N₂O emissions and denitrifier community abundance in soil. Soil Biology and Biochemistry 40, 2553-2562.
- Mohan, S. B. and Cole, J. A., 2007. The dissimilatory reduction of nitrate to ammonia by anaerobic bacteria, in: Biology of the Nitrogen Cycle, edited by: Bothe, H., Ferguson, S. J., and Newton, W. E., Elsevier, Amsterdam, The Netherlands, 93– 106.
- Montzka, S.A., Dlugokencky, E.J., Butler, J.H., 2011. Non-CO2 greenhouse gases and climate change. Nature 476, 43-50.
- Morales, S.E., Cosart, T., Holben, W.E., 2010. Bacterial gene abundances as indicators of greenhouse gas emission in soils. Isme Journal 4, 799-808.
- Mosquera, J., Hol, J.M.G., Rappoldt, C., Dolfing, J., 2007. Precise soil management as a tool to reduce CH₄ and N₂O emissions from agricultural soils. Animal Sciences Group, Lelystad.Report 28.
- Mothapo, N., Chen, H.H., Cubeta, M.A., Grossman, J.M., Fuller, F., Shi, W., 2015. Phylogenetic, taxonomic and functional diversity of fungal denitrifiers and associated N₂O production efficacy. Soil Biology & Biochemistry 83, 160-175.
- Murray, R.E., Knowles, R., 2004. Trace amounts of O₂ affect NO and N₂O production during denitrifying enzyme activity (DEA) assays. Soil Biology & Biochemistry 36, 513-517.
- Nagele, W., Conrad, R., 1990. Influence of soil-ph on the nitrate-reducing microbialpopulations and their potential to reduce nitrate to no and N₂O. Fems Microbiology Ecology 74, 49-57.
- Natale, P., Brüser, T., Driessen, A.J.M., 2008. Sec- and Tat-mediated protein secretion across the bacterial cytoplasmic membrane—Distinct translocases and mechanisms. Biochimica et Biophysica Acta (BBA) - Biomembranes 1778, 1735-1756.
- Nemergut, D.R., Cleveland, C.C., Wieder, W.R., Washenberger, C.L., Townsend, A.R., 2010. Plot-scale manipulations of organic matter inputs to soils correlate with shifts in

microbial community composition in a lowland tropical rain forest. Soil Biology & Biochemistry 42, 2153-2160.

- Nemergut, D.R., Shade, A., Violle, C., 2014. When, where and how does microbial community composition matter? Frontiers in Microbiology 5:497.
- Nishizawa, T., Quan, A.H., Kai, A., Tago, K., Ishii, S., Shen, W.S., Isobe, K., Otsuka, S., Senoo, K., 2014. Inoculation with N-2-generating denitrifier strains mitigates N₂O emission from agricultural soil fertilized with poultry manure. Biology and Fertility of Soils 50, 1001-1007.
- Orellana, L.H., Rodriguez-R, L.M., Higgins, S., Chee-Sanford, J.C., Sanford, R.A., Ritalahti, K.M., Loffler, F.E., Konstantinidis, K.T., 2014. Detecting Nitrous Oxide Reductase (nosZ) Genes in Soil Metagenomes: Method Development and Implications for the Nitrogen Cycle. Mbio 5(3):e01193-14.
- Palmer, T., Berks, B.C., 2012. The twin-arginine translocation (Tat) protein export pathway. Nat Rev Micro 10, 483-496.
- Pell, M., Stenberg, B., Stenstrom, J., Torstensson, L., 1996. Potential denitrification activity assay in soil - With or without chloramphenicol? Soil Biology & Biochemistry 28, 393-398.
- Pastorelli, R., Vignozzi, N., Landi, S., Piccolo, R., Orsini, R., Seddaiu, G., Roggero, P.P., Pagliai, M., 2013. Consequences on macroporosity and bacterial diversity of adopting a no-tillage farming system in a clayish soil of Central Italy. Soil Biology and Biochemistry 66, 78-93.
- Petersen, D.G., Blazewicz, S.J., Firestone, M., Herman, D.J., Turetsky, M., Waldrop, M., 2012. Abundance of microbial genes associated with nitrogen cycling as indices of biogeochemical process rates across a vegetation gradient in Alaska. Environmental Microbiology 14, 993-1008.
- Peyrard, C., Ferchaud, F., Mary, B., Gréhan, E., Léonard, J., 2016a. Management Practices of Miscanthus × giganteus Strongly Influence Soil Properties and N₂O Emissions Over the Long Term. BioEnergy Research, 1-17.
- Peyrard, C., Mary, B., Perrin, P., Véricel, G., Gréhan, E., Justes, E., Léonard, J., 2016b. N₂O emissions of low input cropping systems as affected by legume and cover crops use. Agriculture, Ecosystems & Environment 224, 145-156.
- Philippot, L., Piutti, S., Martin-Laurent, F., Hallet, S., Germon, J.C., 2002. Molecular analysis of the nitrate-reducing community from unplanted and maize-planted soils. Applied and Environmental Microbiology 68, 6121-6128.
- Philippot L, Hallin S, Schloter M., 2007. Ecology of denitrifying prokaryotes in agricultural soil. Advances in Agronomy, Vol 96, 249–305.
- Philippot, L., Čuhel, J., Saby, N.P.A., Chèneby, D., Chroňáková, A., Bru, D., Arrouays, D., Martin-Laurent, F., Šimek, M., 2009. Mapping field-scale spatial patterns of size and activity of the denitrifier community. Environmental Microbiology 11, 1518-1526.
- Philippot, L., Andert, J., Jones, C.M., Bru, D., Hallin, S., 2011. Importance of denitrifiers lacking the genes encoding the nitrous oxide reductase for N₂O emissions from soil. Global Change Biology 17, 1497-1504.
- Philippot, L., Hallin, S., 2011. Towards food, feed and energy crops mitigating climate change. Trends in Plant Science 16, 476-480.
- Phillips, R., Grelet, G., McMillan, A., Song, B., Weir, B., Palmada, T., Tobias, C., 2016. Fungal denitrification: Bipolaris sorokiniana exclusively denitrifies inorganic nitrogen in the presence and absence of oxygen. Fems Microbiology Letters 363.

- Prendergast-Miller, M.T., Baggs, E.M., Johnson, D., 2011. Nitrous oxide production by the ectomycorrhizal fungi Paxillus involutus and Tylospora fibrillosa. Fems Microbiology Letters 316, 31-35.
- Prosser, J., 2005. Nitrification. Nitrogen in Soils. The Encyclopedia of Soils in the Environment. Elsevier Ltd. All Rights Reserved.
- Prosser, J.I., Nicol, G.W., 2012. Archaeal and bacterial ammonia-oxidisers in soil: the quest for niche specialisation and differentiation. Trends in Microbiology 20, 523-531.
- Ravishankara, A.R., Daniel, J.S., Portmann, R.W., 2009. Nitrous Oxide (N₂O): The Dominant Ozone-Depleting Substance Emitted in the 21st Century. Science 326, 123-125.
- Reay, D.S., Edwards, A.C., Smith, K.A., 2009. Importance of indirect nitrous oxide emissions at the field, farm and catchment scale. Agriculture, Ecosystems & Environment 133, 163-169.
- Reay, D.S., Davidson, E.A., Smith, K.A., Smith, P., Melillo, J.M., Dentener, F., Crutzen, P.J., 2012. Global agriculture and nitrous oxide emissions. Nature Climate Change 2, 410-416.
- Risgaard-Petersen, N., Langezaal, A.M., Ingvardsen, S., Schmid, M.C., Jetten, M.S.M., Op den Camp, H.J.M., Derksen, J.W.M., Pina-Ochoa, E., Eriksson, S.P., Peter Nielsen, L., Peter Revsbech, N., Cedhagen, T., van der Zwaan, G.J., 2006. Evidence for complete denitrification in a benthic foraminifer. Nature 443, 93-96.
- Robertson, G.P., Paul, E.A., Harwood, R.R., 2000. Greenhouse gases in intensive agriculture: Contributions of individual gases to the radiative forcing of the atmosphere. Science 289, 1922-1925.
- Robertson, G.P., Vitousek, P.M., 2009. Nitrogen in Agriculture: Balancing the Cost of an Essential Resource. Annual Review of Environment and Resources 34, 97-125.
- Rocca, J.D., Hall, E.K., Lennon, J.T., Evans, S.E., Waldrop, M.P., Cotner, J.B., Nemergut, D.R., Graham, E.B., Wallenstein, M.D., 2015. Relationships between proteinencoding gene abundance and corresponding process are commonly assumed yet rarely observed. Isme Journal 9, 1693-1699.
- Rochette, P., 2008. No-till only increases N₂O emissions in poorly-aerated soils. Soil & Tillage Research 101, 97-100.
- Rochette, P., Janzen, H.H., 2005. Towards a Revised Coefficient for Estimating N₂O Emissions from Legumes. Nutrient Cycling in Agroecosystems 73, 171-179.
- Rochette, P., van Bochove, E., Prévost, D., Angers, D.A., C oté, D., Bertrand, N., 2000. Soil Carbon and Nitrogen Dynamics Following Application of Pig Slurry for the 19th Consecutive Year II. Nitrous Oxide Fluxes and Mineral Nitrogen. Soil Science Society of America Journal 64, 1396-1403.
- Roling, W.F.M., 2007. Do microbial numbers count? Quantifying the regulation of biogeochemical fluxes by population size and cellular activity. Fems Microbiology Ecology 62, 202-210.
- Roobroeck, D., Butterbach-Bahl, K., Brueggemann, N., Boeckx, P., 2010. Dinitrogen and nitrous oxide exchanges from an undrained monolith fen: short-term responses following nitrate addition. European Journal of Soil Science 61, 662-670.
- Rütting, T., Boeckx, P., Müller, C., Klemedtsson, L., 2011. Assessment of the importance of dissimilatory nitrate reduction to ammonium for the terrestrial nitrogen cycle. Biogeosciences 8, 1779-1791.
- Saggar, S., Jha, N., Deslippe, J., Bolan, N.S., Luo, J., Giltrap, D.L., Kim, D.G., Zaman, M., Tillman, R.W., 2013. Denitrification and N₂O:N2 production in temperate grasslands: Processes, measurements, modelling and mitigating negative impacts. Science of The Total Environment 465, 173-195.

- Samarkin, V.A., Madigan, M.T., Bowles, M.W., Casciotti, K.L., Priscu, J.C., McKay, C.P., Joye, S.B., 2010. Abiotic nitrous oxide emission from the hypersaline Don Juan Pond in Antarctica. Nature Geoscience 3, 341-344.
- Sanford, R.A., Wagner, D.D., Wu, Q.Z., Chee-Sanford, J.C., Thomas, S.H., Cruz-Garcia, C., Rodriguez, G., Massol-Deya, A., Krishnani, K.K., Ritalahti, K.M., Nissen, S., Konstantinidis, K.T., Loffler, F.E., 2012. Unexpected nondenitrifier nitrous oxide reductase gene diversity and abundance in soils. Proceedings of the National Academy of Sciences of the United States of America 109, 19709-19714.
- Schlesinger, W.H., 2013. An estimate of the global sink for nitrous oxide in soils. Global Change Biology 19, 2929-2931.
- Scholefield, D., Hawkins, J.M.B., Jackson, S.M., 1997. Use of a flowing helium atmosphere incubation technique to measure the effects of denitrification controls applied to intact cores of a clay soil. Soil Biology and Biochemistry 29, 1337-1344.
- Shcherbak, I., Millar, N., Robertson, G.P., 2014. Global metaanalysis of the nonlinear response of soil nitrous oxide (N₂O) emissions to fertilizer nitrogen. Proceedings of the National Academy of Sciences of the United States of America 111, 9199-9204.
- Shoun, H., Fushinobu, S., Jiang, L., Kim, S.W., Wakagi, T., 2012. Fungal denitrification and nitric oxide reductase cytochrome P450nor. Philosophical Transactions of the Royal Society B-Biological Sciences 367, 1186-1194.
- Silver, W.L., Herman, D.J., Firestone, M.K., 2001. Dissimilatory nitrate reduction to ammonium in upland tropical forest soils. Ecology 82, 2410-2416.
- Silver, W.L., Thompson, A.W., Reich, A., Ewel, J.J., Firestone, M.K., 2005. Nitrogen cycling in tropical plantation forests: Potential controls on nitrogen retention. Ecological Applications 15, 1604-1614.
- Šimek, M., Cooper, J.E., 2002. The influence of soil pH on denitrification: progress towards the understanding of this interaction over the last 50 years. European Journal of Soil Science 53, 345-354.
- Simon, J., Einsle, O., Kroneck, P.M.H., Zumft, W.G., 2004. The unprecedented nos gene cluster of Wolinella succinogenes encodes a novel respiratory electron transfer pathway to cytochrome c nitrous oxide reductase. Febs Letters 569, 7-12.
- Six, J., Elliott, E.T., Paustian, K., 1999. Aggregate and soil organic matter dynamics under conventional and no-tillage systems. Soil Science Society of America Journal 63, 1350-1358.
- Six, J., Ogle, S.M., Jay breidt, F., Conant, R.T., Mosier, A.R., Paustian, K., 2004. The potential to mitigate global warming with no-tillage management is only realized when practised in the long term. Global Change Biology 10, 155-160.
- Skiba, U., Smith, K.A., 2000. The control of nitrous oxide emissions from agricultural and natural soils. Chemosphere Global Change Science 2, 379-386.
- Skiba, U., Drewer, J., Tang, Y.S., van Dijk, N., Helfter, C., Nemitz, E., Famulari, D., Cape, J.N., Jones, S.K., Twigg, M., Pihlatie, M., Vesala, T., Larsen, K.S., Carter, M.S., Ambus, P., Ibrom, A., Beier, C., Hensen, A., Frumau, A., Erisman, J.W., Brüggemann, N., Gasche, R., Butterbach-Bahl, K., Neftel, A., Spirig, C., Horvath, L., Freibauer, A., Cellier, P., Laville, P., Loubet, B., Magliulo, E., Bertolini, T., Seufert, G., Andersson, M., Manca, G., Laurila, T., Aurela, M., Lohila, A., Zechmeister-Boltenstern, S., Kitzler, B., Schaufler, G., Siemens, J., Kindler, R., Flechard, C., Sutton, M.A., 2009. Biosphere–atmosphere exchange of reactive nitrogen and greenhouse gases at the NitroEurope core flux measurement sites: Measurement strategy and first data sets. Agriculture, Ecosystems & Environment 133, 139-149.

Smil, V., 2002. Nitrogen and food production: Proteins for human diets. Ambio 31, 126-131.

- Smith, M.S., Tiedje, J.M., 1979. Phases of denitrification following oxygen depletion in soil. Soil Biology & Biochemistry 11, 261-267.
- Smith, K.A., Thomson, P.E., Clayton, H., McTaggart, I.P., Conen, F., 1998. Effects of temperature, water content and nitrogen fertilisation on emissions of nitrous oxide by soils. Atmospheric Environment 32, 3301-3309.
- Smith, P., D. Martino, Z. Cai, D. Gwary, H. Janzen, P. Kumar, B. McCarl, S. Ogle, F. O'Mara, C. Rice, B. Scholes, O. Sirotenko, 2007. Agriculture. In Climate Change 2007: Mitigation. Contribution of Working Group III to the Fourth Assessment Report of the Intergovernmental Panel on Climate Change [B. Metz, O.R. Davidson, P.R. Bosch, R. Dave, L.A. Meyer (eds)], Cambridge University Press, Cambridge, United Kingdom and New York, NY, USA.
- Smith, K.A., Mosier, A.R., Crutzen, P.J., Winiwarter, W., 2012. The role of N₂O derived from crop-based biofuels, and from agriculture in general, in Earth's climate. Philosophical Transactions of the Royal Society B: Biological Sciences 367, 1169-1174.
- Smith, P., Martino, D., Cai, Z., Gwary, D., Janzen, H., Kumar, P., McCarl, B., Ogle, S., O'Mara, F., Rice, C., Scholes, B., Sirotenko, O., Howden, M., McAllister, T., Pan, G., Romanenkov, V., Schneider, U., Towprayoon, S., Wattenbach, M., Smith, J., 2008. Intergovernmental Panel on Climate Change [B. Metz, O.R. Davidson, P.R. Bosch, R. Dave, L.A. Meyer (eds)], Cambridge UniversityPress, Cambridge, United Kingdom and New York, NY, USA. Greenhouse gas mitigation in agriculture. Philosophical Transactions of the Royal Society B: Biological Sciences 363, 789-813.
- Snyder, C.S., Bruulsema, T.W., Jensen, T.L., Fixen, P.E., 2009. Review of greenhouse gas emissions from crop production systems and fertilizer management effects. Agriculture, Ecosystems & Environment 133, 247-266.
- Steffen, W., Richardson, K., Rockstrom, J., Cornell, S.E., Fetzer, I., Bennett, E.M., Biggs, R., Carpenter, S.R., de Vries, W., de Wit, C.A., Folke, C., Gerten, D., Heinke, J., Mace, G.M., Persson, L.M., Ramanathan, V., Reyers, B., Sorlin, S., 2015. Planetary boundaries: Guiding human development on a changing planet. Science 347.
- Song, H., Che, Z., Cao, W.C., Huang, T., Wang, J.G., Dong, Z.R., 2016. Changing roles of ammonia-oxidizing bacteria and archaea in a continuously acidifying soil caused by over-fertilization with nitrogen. Environmental Science and Pollution Research 23, 11964-11974.
- Spott, O., Russow, R., Stange, C.F., 2011. Formation of hybrid N₂O and hybrid N-2 due to codenitrification: First review of a barely considered process of microbially mediated N-nitrosation. Soil Biology & Biochemistry 43, 1995-2011.
- Stange, C.F., Spott, O., Arriaga, H., Menéndez, S., Estavillo, J.M., Merino, P., 2013. Use of the inverse abundance approach to identify the sources of NO and N₂O release from Spanish forest soils under oxic and hypoxic conditions. Soil Biology and Biochemistry 57, 451-458.
- Stehfest, E., Bouwman, L., 2006. N₂O and NO emission from agricultural fields and soils under natural vegetation: summarizing available measurement data and modeling of global annual emissions. Nutrient Cycling in Agroecosystems 74, 207-228.
- Stief, P., Fuchs-Ocklenburg, S., Kamp, A., Manohar, C.S., Houbraken, J., Boekhout, T., de Beer, D., Stoeck, T., 2014. Dissimilatory nitrate reduction by Aspergillus terreus isolated from the seasonal oxygen minimum zone in the Arabian Sea. BMC Microbiology 14.

- Su, F., Takaya, N., Shoun, H., 2004. Nitrous oxide-forming codenitrification catalyzed by cytochrome P450nor. Bioscience Biotechnology and Biochemistry 68, 473-475.
- Subbarao, G.V., Ito, O., Sahrawat, K.L., Berry, W.L., Nakahara, K., Ishikawa, T., Watanabe, T., Suenaga, K., Rondon, M., Rao, I.M., 2006. Scope and strategies for regulation of nitrification in agricultural systems-challenges and opportunities. Critical Reviews in Plant Sciences 25, 303-335.
- Subbarao, G.V., Nakahara, K., Hurtado, M.P., Ono, H., Moreta, D.E., Salcedo, A.F., Yoshihashi, A.T., Ishikawa, T., Ishitani, M., Ohnishi-Kameyama, M., Yoshida, M., Rondon, M., Rao, I.M., Lascano, C.E., Berry, W.L., Ito, O., 2009. Evidence for biological nitrification inhibition in Brachiaria pastures. Proceedings of the National Academy of Sciences of the United States of America 106, 17302-17307.
- Subbarao, G.V., Sahrawat, K.L., Nakahara, K., Ishikawa, T., Kishii, M., Rao, I.M., Hash, C.T., George, T.S., Rao, P.S., Nardi, P., Bonnett, D., Berry, W., Suenaga, K., Lata, J.C., 2012. Biological nitrification inhibition-a novel strategy to regulate nitrification in agricultural systems, In: Sparks, D.L. (Ed.), Advances in Agronomy, Vol 114, pp. 249-302.
- Sullivan, M.J., Gates, A.J., Appia-Ayme, C., Rowley, G., Richardson, D.J., 2013. Copper control of bacterial nitrous oxide emission and its impact on vitamin B-12dependent metabolism. Proceedings of the National Academy of Sciences of the United States of America 110, 19926-19931.
- Tanimoto, T., Hatano, K., Kim, D.H., Uchiyama, H., Shoun, H., 1992. Co-denitrification by the denitrifying system of the fungus fusarium-oxysporum. Fems Microbiology Letters 93, 177-180.
- Tatti, E., Goyer, C., Burton, D.L., Wertz, S., Zebarth, B.J., Chantigny, M., Filion, M., 2015. Tillage Management and Seasonal Effects on Denitrifier Community Abundance, Gene Expression and Structure over Winter. Microbial Ecology 70, 795-808.
- Taylor, A.E., Vajrala, N., Giguere, A.T., Gitelman, A.I., Arp, D.J., Myrold, D.D., Sayavedra-Soto, L., Bottomley, P.J., 2013. Use of Aliphatic n-Alkynes To Discriminate Soil Nitrification Activities of Ammonia-Oxidizing Thaumarchaea and Bacteria. Applied and Environmental Microbiology 79, 6544-6551.
- Thangarajan, R., Bolan, N.S., Tian, G.L., Naidu, R., Kunhikrishnan, A., 2013. Role of organic amendment application on greenhouse gas emission from soil. Science of The Total Environment 465, 72-96.
- Thompson, K.A., Bent, E., Abalos, D., Wagner-Riddle, C., Dunfield, K.E., 2016. Soil microbial communities as potential regulators of in situ N₂O fluxes in annual and perennial cropping systems. Soil Biology and Biochemistry 103, 262-273.
- Thomson, A.J., Giannopoulos, G., Pretty, J., Baggs, E.M., Richardson, D.J., 2012. Biological sources and sinks of nitrous oxide and strategies to mitigate emissions. Philosophical Transactions of the Royal Society B-Biological Sciences 367, 1157-1168.
- Tian, H.Q., Lu, C.Q., Ciais, P., Michalak, A.M., Canadell, J.G., Saikawa, E., Huntzinger, D.N., Gurney, K.R., Sitch, S., Zhang, B.W., Yang, J., Bousquet, P., Bruhwiler, L., Chen, G.S., Dlugokencky, E., Friedlingstein, P., Melillo, J., Pan, S.F., Poulter, B., Prinn, R., Saunois, M., Schwalm, C.R., Wofsy, S.C., 2016. The terrestrial biosphere as a net source of greenhouse gases to the atmosphere. Nature 531, 225.
- Trautmann, N.M.; Porter, K.S.; Wagenet, R.J.. 1989. Nitrogen: The Essential Element. Cornell Cooperative Extension. Fact Sheet 400.06.
- Trinsoutrot, I., Recous, S., Bentz, B., Lineres, M., Cheneby, D., Nicolardot, B., 2000. Biochemical quality of crop residues and carbon and nitrogen mineralization

kinetics under nonlimiting nitrogen conditions. Soil Science Society of America Journal 64, 918-926.

- Trolldenier, G., 1988. Visualisation of oxidizing power of rice roots and of possible participation of bacteria in iron deposition. Zeitschrift für Pflanzenernährung und Bodenkunde 151, 117-121.
- Trudinger P. A., W.M.R., 1980. Biogeochemistry of Ancient and Modern Environments. Springer-Verlag Berlin Heidelberg.
- Valentine, D.L., 2007. Adaptations to energy stress dictate the ecology and evolution of the Archaea. Nature Reviews Microbiology 5, 316-323.
- van Kessel, M., Speth, D.R., Albertsen, M., Nielsen, P.H., Op den Camp, H.J.M., Kartal, B., Jetten, M.S.M., Lucker, S., 2015. Complete nitrification by a single microorganism. Nature 528-555.
- Venterea, R.T., Burger, M., Spokas, K.A., 2005. Nitrogen oxide and methane emissions under varying tillage and fertilizer management. Journal of Environmental Quality 34, 1467-1477.
- Venterea, R.T., Halvorson, A.D., Kitchen, N., Liebig, M.A., Cavigelli, M.A., Grosso, S.J.D., Motavalli, P.P., Nelson, K.A., Spokas, K.A., Singh, B.P., Stewart, C.E., Ranaivoson, A., Strock, J., Collins, H., 2012. Challenges and opportunities for mitigating nitrous oxide emissions from fertilized cropping systems. Frontiers in Ecology and the Environment 10, 562-570.
- Vitousek, P. and R. Howarth., 1991. Nitrogen limitation on land and in the sea: How can it occur? Biogeochemistry 13(2): 87–115.
- Vitousek, P.M., Mooney, H.A., Lubchenco, J., Melillo, J.M., 1997. Human Domination of Earth's Ecosystems. Science 277, 494-499.
- Walker, C.B., de la Torre, J.R., Klotz, M.G., Urakawa, H., Pinel, N., Arp, D.J., Brochier-Armanet, C., Chain, P.S.G., Chan, P.P., Gollabgir, A., Hemp, J., Hugler, M., Karr, E.A., Konneke, M., Shin, M., Lawton, T.J., Lowe, T., Martens-Habbena, W., Sayavedra-Soto, L.A., Lang, D., Sievert, S.M., Rosenzweig, A.C., Manning, G., Stahl, D.A., 2010. Nitrosopumilus maritimus genome reveals unique mechanisms for nitrification and autotrophy in globally distributed marine crenarchaea. Proceedings of the National Academy of Sciences of the United States of America 107, 8818-8823.
- Wang, B.Z., Zhao, J., Guo, Z.Y., Ma, J., Xu, H., Jia, Z.J., 2015. Differential contributions of ammonia oxidizers and nitrite oxidizers to nitrification in four paddy soils. Isme Journal 9, 1062-1075.
- Wasser, I.M., de Vries, S., Moenne-Loccoz, P., Schroder, I., Karlin, K.D., 2002. Nitric oxide in biological denitrification: Fe/Cu metalloenzyme and metal complex NOx redox chemistry. Chemical Reviews 102, 1201-1234.
- Weier, K.L., Macrae, I.C., Myers, R.J.K., 1993. Denitrification in a clay soil under pasture and annual crop - losses from 15n-labeled nitrate in the subsoil in the field using c2h2 inhibition. Soil Biology & Biochemistry 25, 999-1004.
- Whitney, K.D., Gabler, C.A., 2008. Rapid evolution in introduced species, 'invasive traits' and recipient communities: challenges for predicting invasive potential. Diversity and Distributions 14, 569-580.
- Wijler J, Delwiche CC., 1954: Investigations on the denitrifying process in soil. Plant Soil, 5, 155–169.
- Winogradsky, S. 1890., Recherches sur les organismes de la nitrification. Ann. Inst. Pasteur (Paris) 4, 213–231.

- Xu, Y.C., Shen, Q.R., Li, M.L., Dittert, K., Sattelmacher, B., 2004. Effect of soil water status and mulching on N₂O and CH4 emission from lowland rice field in China. Biology and Fertility of Soils 39, 215-217.
- Yao, H., Gao, Y., Nicol, G.W., Campbell, C.D., Prosser, J.I., Zhang, L., Han, W., Singh, B.K., 2011. Links between Ammonia Oxidizer Community Structure, Abundance, and Nitrification Potential in Acidic Soils. Applied and Environmental Microbiology 77, 4618-4625.
- Yang, C., Hamel, C., Gan, Y.T., 2015. Incongruous variation of denitrifying bacterial communities as soil N level rises in Canadian canola fields. Applied Soil Ecology 89, 93-101.
- Yoon, S., Nissen, S., Park, D., Sanford, R.A., Loffler, F.E., 2016. Nitrous Oxide Reduction Kinetics Distinguish Bacteria Harboring Clade I NosZ from Those Harboring Clade II NosZ. Applied and Environmental Microbiology 82, 3793-3800.
- Zhang, J., Müller, C., Cai, Z., 2015. Heterotrophic nitrification of organic N and its contribution to nitrous oxide emissions in soils. Soil Biology and Biochemistry 84, 199-209.
- Zhou, X., Fornara, D., Wasson, E.A., Wang, D.M., Ren, G.D., Christie, P., Jia, Z.J., 2015. Effects of 44 years of chronic nitrogen fertilization on the soil nitrifying community of permanent grassland. Soil Biology & Biochemistry 91, 76-83.
- Zumft, W.G., 1997. Cell biology and molecular basis of denitrification. Microbiology Molecular Biology Reviews 61.
- Zumft, W.G., 2005. Nitric oxide reductases of prokaryotes with emphasis on the respiratory, heme-copper oxidase type. Journal of Inorganic Biochemistry 99, 194-215.

9 Acknowledgements

My Family - I simply have no words to thank my family for their unconditional support and for their belief that I could achieve my dreams. If close or across the ocean they were always there when I most needed them. I owe them this last achievement and dedicate to them my Thesis. I could not be licker with the sister and brother that I have. They are simply able to irradiate happiness. My Mother always had the most encouraging words and kept me motivated through my studies. My Father is the one who planted the seed of science into me, always trying to understand nature.

Laurent Philippot - It is difficult to describe how I appreciate everything that you have done for me in the last three years. A very, very big thanks for your patience, guidance, and for sharing your knowledge. I learned a lot during these last years and you are the main responsible for that.

Aymé Spor – I am also very grateful for your guidance, Aymé. A special thanks for the statistical and R lessons, and also for respecting my timing and showing me that I was able to do it!

The EMFI Group – Thanks to all of you for your kind words and making the working atmosphere a nice place to be. This Group is very special, and when I leave I will be sad but I will keep all the good memories of these three years. A very big thanks to **Marie-Christine**! I did not meet another technician so deeply involved. Thanks also for sharing your knowledge with me! I am very grateful to **David**, not only as a work colleague but also as a friend with whom I spent some of the nicest moments in Dijon and I sincerely hope that we restart the guitar lessons one day. I am so glad to share the office with possibly the nicest officemate! **Sara**, you were very important for me in this chapter of my life. I am very lucky to have you as a friend. A very big thanks for keeping your big smile always even in the most hard moments! I told you, I will come to visit you in Spain very soon! **Veronika**, hard to find words here, it was really nice to accomplish this step in our lives together! You are an

awesome flatmate, workmate, and specially friend! Your positive energy makes things possible! Keep that flame on and you can achieve all your dreams! **Ilonka**, how many awesome moments did we spend together these last years!? You can keep trying to be the "hard" person, but we all know that you are very soft inside! I hope that we will still do a lot of triathlons together! **Kadiya**, it is not always possible to meet people with such a nice heart! Our friendship will still have a lot of chapters in México or Brazil or somewhere else! **Jérémie** it was really nice to meet you, I expect that we can still have a lot of nice evenings, with a lot of nice food and movies! Thanks also to all the other group members who somehow contributed to that I accomplished this step, a very sincere thanks to **Fabrice**, **Marion**, **Romain**, **Céline**, **Chloé**, **Sana**, **Nadine** and **Florian**.

The NORA Partners – A special thanks to the NORA partners, especially to **Sara Hallin** who gave me a warm welcome in her group. The months that I stayed in your Group were a stimulating period during my thesis. **Tina**, it was a big pleasure to work with you, to do some sport and enjoy a glass of wine in the evenings. I hope we can work together in the future, and if not continue good friends. A special thanks also to all the group partners who made my stay there very pleasant: **Lea**, **Jaanis**, **Gérman**, **Chris** and **Carlos**. Thanks also to **Jim Prosser** for receiving me in his group, and for **Linda** for sharing her knowledge on nitrifiers with me.

I would also like to thank ALL my Friends spread over the world that contributed to made me the person I am now and made me strong to face this challenge. A very, very big thanks to Claudia, Isadora, Heera, Wolle, Ina, Hannah, Dani, Katrin, Philipp, Hannes, Sia, Grace, Norbert, Vinicius, Edgar, Luis, Ana, Flavia, Roberto, Leandro, Olivio and Alessandro.