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DR. LAURENT PHILIPPOT (Orcid ID : 0000-0003-3461-4492)

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TITLE: Peaks of *in situ* N₂O emissions are influenced by N₂O producing and reducing microbial communities across arable soils

Domeignoz-Horta¹ L.A., Philippot L.^{1*}, Peyrard C.², Bru D.¹, Breuil M.C.¹, Bizouard F.¹,
Justes E.³, Mary B.², Léonard J.², Spor A.¹

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

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

³AGIR, Université de Toulouse, INPT, INP-PURPAN, INRA, 31320 Castanet Tolosan,
France

*Corresponding author

laurent.philippot@inra.fr

Tel: 33 (0) 3 80 69 33 46

Fax: 33 (0) 3 80 69 32 24

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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 known biological process eliminating this greenhouse gas. Recent studies showed that a previously unknown clade of N₂O-reducers (*nosZII*) was related to the potential 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-producers and N₂O-reducers were affected by agricultural practices across a range of cropping systems in order to evaluate the consequences for N₂O emissions. The abundance of both ammonia oxidizers and denitrifiers was quantified by real-time qPCR, and the diversity of *nosZ* clades was determined by 454 pyrosequencing. Denitrification and nitrification potential activities as well as *in situ* N₂O emissions were also assessed. Overall, greatest differences in microbial activity, diversity and abundance were observed between sites rather than between agricultural practices at each site. To better understand the contribution of abiotic and biotic factors to the *in situ* N₂O emissions, we subdivided more than 59.000 field measurements into fractions from low to high rates. We found that the low N₂O emission rates were mainly explained by variation in soil properties (up to 59%), while the high rates were explained by variation in abundance and diversity of microbial communities (up to 68%). Notably, the diversity of the *nosZII* clade but not of the *nosZI* clade was important to explain the variation of *in situ* N₂O emissions. Altogether, these

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results lay the foundation for a better understanding of the response of N₂O reducing bacteria to agricultural practices and how it may ultimately affect 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 CO₂ or CH₄ but seldom for N₂O consumption (Six *et al.*, 2004; Chapuis-Lardy *et al.*, 2007; Tian *et al.*, 2016). Nevertheless, N₂O is an important potent greenhouse gas with a global warming potential (GWP) over 100 years of about 298 and 11.9 times that of CO₂ and CH₄, respectively. N₂O is also the dominant ozone depleting substance after the suppression of CFCs by the Kyoto Protocol (Ravishankara *et al.*, 2009). Anthropogenic nitrogen (N) input, mainly via industrial N₂ fixation to produce fertilizers contributes double the natural rate of terrestrial nitrogen fixation (Canfield *et al.*, 2010). As a result, N₂O concentration is estimated to increase of up to 60% by 2050 compared to the beginning of last century (Bouwman *et al.*, 2013).

At the global scale, most of N₂O is emitted from soils, with agricultural soils being the main source and an estimated contribution of 59% of total emissions by 2030 (Hu *et al.*, 2015). N₂O emissions are, to a great extent, the result of microbial driven processes such as denitrification and nitrification (Hu *et al.*, 2015; Snyder *et al.*, 2009). Nitrification is the oxidation of ammonia to nitrate by some microorganisms. Bacteria produce N₂O during the first step, *i.e.* the oxidation of ammonia into nitrite via hydroxylamine (Prosser & Nicol, 2012). The mechanisms by which ammonia oxidizing archaea produce N₂O are unclear (Walker *et al.*, 2010) but recent studies suggested that they produce lower yields of N₂O than their bacterial counterpart (Hink *et al.*, 2016). Denitrification is a respiratory process during which soluble nitrogen oxides (NO₃⁻ and NO₂⁻) are sequentially reduced into gaseous forms

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(NO, N₂O and N₂). A key step is the reduction of soluble NO₂⁻ to gaseous forms, which is catalyzed by nitrite reductases encoded by the *nirK* or *nirS* genes. The other denitrification step important for N₂O fluxes is the reduction of N₂O to N₂ catalyzed by the nitrous oxide reductase encoded by the *nosZ* gene, representing the only known sink for this GHG (Chapuis-Lardy *et al.*, 2007). Denitrification is described as a modular process (Graf *et al.*, 2014). Thus, some organisms are able to perform the complete pathway while others either (i) lack the nitrate reductase, (ii) lack the nitrous oxide reductase and therefore produce N₂O as final denitrification product (Philippot *et al.*, 2011) or (iii) only reduce N₂O without producing it (Sanford *et al.*, 2002), and are therefore a potential sink for this GHG. Recently a new N₂O reducing clade has been identified (Hallin *et al.*, 2017 and references therein), herein named *nosZII*, which is diverse and abundant in soils (Domeignoz-Horta *et al.*, 2015; Jones *et al.*, 2014; Orellana *et al.*, 2014). An analysis of the sequenced genomes of N₂O reducers' strains revealed that about half of *nosZII* harbor the nitrous oxide reductase but none of the nitrite reductases, a percentage that reaches only about 17% of *nosZI* (Graf *et al.*, 2014). This highlight the potential importance of *nosZII* 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 *nosZII* community were the main drivers of soil N₂O sink capacity. Such results stress 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 agricultural practices on microorganisms involved in N₂O production (Kong *et al.*, 2010; Bissett *et al.*, 2014; Hallin *et al.*, 2009; Hartmann *et al.*, 2015; Thompson *et al.*, 2016). Fertilization, which is a major driver of N₂O emissions (Shcherbak *et al.*, 2014; Smith, 2007), was for example shown to also affect the denitrifier community (Chen *et al.*, 2010; Clark *et al.*, 2012; Hallin *et al.*,

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2009; Tatti *et al.*, 2013). Other practices highly debated in the climate change context such as no-tillage (Smith *et al.*, 2007; Six *et al.*, 2004; Powlson *et al.*, 2014) can influence both N₂O emissions and the corresponding microbial communities (Smith *et al.*, 2007; Six *et al.*, 2004; Tatti *et al.*, 2015; Antle & Ogle, 2011). However, our knowledge of the interactions between land use practices, microbial communities and N₂O emissions still remains limited (Butterbach-Bahl *et al.*, 2013), and this is especially true for the *nosZII* community due to its relatively recent identification.

Here we assessed how the microbial communities responsible for N₂O source (N₂O-producers encompassing both nitrifiers and denitrifiers), and sink (N₂O-reducers) were affected by 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, with a special focus on N₂O reducers. We hypothesized in particular that soil properties and agricultural practices would differentially affect the N₂O producers and reducers and that the *nosZII* community rather than the *nosZI* would be negatively related to *in situ* N₂O fluxes.

Material and methods

Experimental design and sampling

Soil samples were collected in April 2014 from 4 experimental sites in France: the SOERE ACBB (Mons-SOERE, 49.522° N, 3.153° E) and Biomass & Environment (Mons-B&E, 49.521° N, 3.047° E) experiments in Estrées-Mons, the soil tillage experiment in Boigneville (Boigneville, 48.327° N, 2.381° E), and the Mic-Mac experiment in Auzeville (Auzeville, 43.527° N, 1.506° E). These long-term experiments undergo a large range of agricultural practices (Table 1). In all cases, each treatment has at least three replicate samples in

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randomized blocks. Briefly, the Mons-SOERE experiment, which has 6 treatments (T1–T6), was set up in 2010 to study the effect of soil tillage, crop residue management, fertilization rate, legume cover crops and of a perennial crop. The Mons-B&E experiment was set up in 2006 to compare the productivity and environmental impacts of different energy crops including *Miscanthus giganteus* (Miscanthus, M) and *Panicum virgatum* (Switchgrass, S) together with different management practices such as early (E) or late harvest (L) and with (N) or without N-fertilization. The Auzeville experiment, which has 6 different treatments, was set up in 2010 to compare 3 low N input alternatives to the conventional durum wheat-sunflower crop rotation (Low Input, LI; Very Low Input with legumes, VLI; intercropping, AS), each one with or without cover crops (C) (Table 1). The Boigneville soil tillage experiment was set up in 1970 to study the effect of three tillage management modalities: Full Inversion Tillage (FIT), Shallow Tillage (ST) and No-Tillage (NT) (Table 1).

Three replicate samples were collected for all treatments, each being a composite sample of five subsamples (soil cores diameter 2.5 cm, height 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 S1).

Potential denitrification and nitrification activities and in situ N_2O emissions

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

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then added to reach 0.1 atm partial pressure. Flasks were incubated at 25°C under agitation at 175 rpm and gas samples were taken every 30 min for 150 min (Pell *et al.*, 1996). The N₂O concentration at each time was determined using a gas chromatograph equipped with an EC-detector (Trace GC Ultra, Thermo Scientific). Potential nitrification activity (PNA) measurement was performed according to ISO 15685. Briefly, 1.4 mM ammonium sulfate was added to 10g of fresh weight soil supplemented with 500 mM of sodium chlorate solution 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 *et al.*, 1995). In addition to the potential activities, more than 59.000 daily values of *in situ* N₂O emissions from the different treatments collected before and during the year of sampling were used (18 treatments for 32.5 months on average). These measurements relied on automatic chambers that closed for 20 min 4 times a day. During that time, N₂O concentration was measured every 10 s by an infra-red gas analyzer (Thermo Instruments, 46C) as described by Peyrard *et al.* (2016a). The N₂O flux was then derived by fitting a linear or exponential function to the concentration vs. time relationship. Daily N₂O emissions (expressed in g N₂O-N ha⁻¹ day⁻¹) were finally calculated as the average of the 4 measurements a day. For each treatment, the 25th, 50th, 75th, 90th and 95th percentiles of N₂O emission were calculated. They constitute indicators of *in situ* emission levels, from basal emissions (25th) to emission peaks (95th).

Nucleic acid 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.

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Abundances 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) ammonia-oxidizing and the denitrifying communities, respectively (Bru *et al.*, 2011) while the *nosZI* and *nosZII* genes were used to target the N₂O-reducers (Jones *et al.*, 2013). qPCR Reactions were carried out in a Step One Plus (Life Technologies, Carlsbad, CA, USA) with 15 µl reaction volume containing 1 ng of DNA, 7.5 µl of SYBRgreen PCR Master Mix (Absolute qPCR SYBR GreenRox, Thermo, Courtaboeuf, France), 1 µM of each primer, 250 ng of T4 gene 32 (QBiogene, Illkrich, France). Three independent qPCR assays were performed for each gene. No-template controls giving null or negligible values were run for each 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 97% (Table S2).

Assessment of the diversity of the N₂O-reducers

The diversity of *nosZI* and *nosZII* communities was analyzed by 454 pyrosequencing. Briefly, *nosZ* fragments were amplified using the *nosZI* and *nosZII* primers (Jones *et al.*, 2014) and sequencing adapters and multiplex identifiers were added by a second amplification round (Berry *et al.*, 2011). 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.

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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 *nosZI* and *nosZII*, 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 SRP105364. 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 analyses were performed using the R statistical software and the agricolae (Mendiburu, 2014) and vegan (Oksanen *et al.*, 2015) packages. Significant differences were 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 the significance of soil properties, community abundances and diversity as 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, $P < 0.05$). Significant explanatory variables for the different percentiles were chosen by linear regression and model selection (backwards) and

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by minimizing the Akaike Information Criterion (AIC). Statistical significance was tested by 1000 permutations of the reduced model. The variables, which significantly explained each percentile (Table 2) were used to calculate their relative contribution to percentiles' variation using the function *varpart* (Peres-Neto *et al.*, 2006).

Results

Potential denitrification activity ($N_2O + N_2$), potential nitrification activity and in situ N_2O emissions.

Strong between-sites differences were observed for both PDA and PNA. The highest PDA were observed for the two experiments located in Estrées-Mons with average rates of 1.2 and 1.1 $\mu\text{g } N_2O\text{-N g}^{-1} \text{ DW soil h}^{-1}$ for SOERE and B&E respectively. Average PDA rates at Auzeville and Boigneville ranged between 0.3 and 0.5 $\mu\text{g } N_2O\text{-N g}^{-1} \text{ DW soil h}^{-1}$ (Fig. S1). The SOERE and B&E experiments also showed higher PNA with average rates of 0.3 and 0.2 $\mu\text{g } NO_2\text{-N g}^{-1} \text{ DW soil h}^{-1}$, respectively. Much lower PNA rates (0.01 $\mu\text{g } NO_2\text{-N g}^{-1} \text{ DW soil h}^{-1}$ and below) were observed at Auzeville and Boigneville. Significant difference in PNA between treatments (i.e. associated to variations in agricultural practices) were observed at Auzeville only, with the intercropping treatment showing higher potential nitrification rates than the very low input treatment, either with or without cover crop (Fig. S2). No significant differences were observed between treatments within a given experimental site for the PDA.

In situ N_2O emission levels tended to be lower in the Mons-SOERE and Auzeville compared to Boigneville and Mons-B&E (Fig.1). No clear trend was observed between agricultural practices within most experimental sites except for the harvest date at Mons-B&E (Fig. 1). Thus, for both bioenergy crops (*i.e.* Miscanthus and Switchgrass), N_2O emissions were lower for early harvest than late harvest whatever the percentile (Fig. 1). Moreover, at

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Mons-SOERE the treatments receiving higher N input (100 kg N h⁻¹ y⁻¹; T1, T2 and T3) showed higher peak emissions in comparison with the treatments with lower N input (40 kg N h⁻¹ y⁻¹; T4, T5 and T6) (Fig. 1).

Abundance of N₂O-producers and N₂O-reducers

Across the 63 soil samples, significant differences were observed between sites but not between treatments within a site for the N₂O-producers (both denitrifiers and ammonia oxidizers). For example, significantly lower abundances of AOA and AOB were found in Auzeville compared to other sites. In all 63 soil samples, AOA were more abundant than AOB, with lowest abundances of 1.18 x 10³ gene copy number ng⁻¹ DNA (CI_{95%}= 1.81 x 10² – 2.18 x 10³) and 9.63 x 10¹ gene copy number ng⁻¹ DNA (CI_{95%}= 5.48 x 10¹ – 1.38 x 10²), and highest abundances reaching 1.24 x 10⁴ gene copy number ng⁻¹ DNA (CI_{95%}= 6.79 x 10³ – 1.80 x 10⁴) and 9.02 x 10² gene copy number ng⁻¹ DNA (CI_{95%}= 3.72 x 10² – 1.43 x 10³) for AOA and AOB, respectively (Fig. S3). Regarding denitrifying N₂O producers, we observed higher abundances of *nirK* than *nirS* in all sampled soils with lowest abundance reaching 1.12 x 10⁴ nbc ng⁻¹ DNA (CI_{95%}= 1.29 x 10⁴ – 9.58 x 10⁴) and 3.29 x 10⁴ nbc ng⁻¹ DNA (CI_{95%}= 2.89 x 10³ – 3.69 x 10³) for *nirK* and *nirS*, respectively.

Contrarily to N₂O-producers, the abundance of N₂O-reducers was affected by agricultural practices. The *nosZI* community abundance significantly decreased with tillage intensity at Boigneville while the abundance of the *nosZII* community was not significantly different between treatments (Fig. S5). We observed higher or equal abundances of *nosZI* compared to *nosZII* in all sampled soils with *nosZI/nosZII* ratios ranging from 1.2 to 2.9.

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N₂O-reducers diversity

To assess the influence of agricultural practices on the composition and structure of N₂O-reducers, the diversity of *nosZI* and *nosZII* communities was determined by 454 pyrosequencing. After quality checking, 125590 and 240642 sequences were found for *nosZI* and *nosZII*, respectively. 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 (Table S3). 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 treatment at Boigneville had a significant effect on the *nosZII* community diversity. Thus, full inversion tillage increased the diversity of *nosZII*, with a PD (phylogenetic diversity index) of 25.8 compared to 19.4 and 20.4 in ST and NT treatments (Table S3). Both OTUs richness and *chao1* were also significantly higher in FIT than in NT for the *nosZII* community (Table S3). An ANalysis Of SIMilarity (ANOSIM) showed an *R* coefficient of 0.30 and 0.67 for *nosZI* and *nosZII*, respectively ($P < 0.001$), which indicates that the clustering of all soil samples by site was significant for both communities but higher for the *nosZII* community (Fig. 2).

Soil properties, abundance and diversity of microbial communities in relation to the potential activities and in situ N₂O emissions

Some soil properties were correlated both to PDA and PNA. Thus, pH was positively related to both PDA and PNA ($r = 0.4$, $P < 0.001$ and $r = 0.81$, $P < 0.0001$, respectively), while a negative relationship was found between clay content and both potential activities ($r = -0.54$,

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$P < 0.001$ and $r = -0.41$, $P < 0.01$ for PDA and PNA, respectively) (Table S4). The abundance of ammonia oxidizing archaea (AOA) but not of their bacterial counterpart was significantly related to PNA. Similarly, the abundances of *nirK*-, *nirS*-, *nosZI*- and *nosZII*-communities were positively related to PDA (Table S4). Neither PDA nor PNA were related to *in situ* emissions.

Variance partitioning was used to determine the relative contribution of different groups of variables to the variation of *in situ* N₂O emissions. The soil physical and chemical characteristics, the abundance of N₂O-producers and N₂O-reducers, and the diversity of N₂O-reducers were separated into three groups of explanatory variables and used to explain each fraction of N₂O emissions. Soil properties were found to explain mostly basal N₂O emissions, contributing to up to 56% of the observed variance (Fig. 3). They remained important for median level of emissions (50th), explaining 19% of the variance but their influence decreased drastically for high levels of emissions (Fig. 3). In contrast, variations in abundance and diversity of the studied functional guilds mainly influenced high levels of emissions (Fig. 3). Microbial abundances actually explained only 9% and 5% of the two lower levels of emissions, (25th and 50th) while it explained 23% of the 95th percentile. Similarly, the diversity of N₂O-reducers explained only 2% and 6% of the 25th and 50th percentiles, respectively but 11 to 25% of the higher levels of emissions (75th, 90th and 95th). Interestingly, the interaction between diversity and abundance was a significant determinant of the highest emissions levels only (95th). Multiple linear regressions coupled with model selection allowed identifying the variables within each group that best explained the observed variance (Table 2). Among soil variables, soil pH, SOM and C/N were important predictors for several percentiles. Among microbial properties, only the abundance of N₂O-producers (denitrifiers and nitrifiers) contributed to explain the variance in N₂O

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emissions. Finally, the diversity of the *nosZII* community was a significant determinant of all fractions except the lowest one, which was explained by the diversity of the *nosZI* community only (Table 2).

Discussion

Responses of N₂O-producing, N₂O-reducing microbial communities and of potential activities, in situ N₂O emissions to agricultural practices

While it is broadly accepted that agricultural practices influence N-fluxes, the size of the effects reported vary considerably across the soils examined (Rochette, 2008; Akiyama *et al.*, 2010; Shcherbak *et al.*, 2014; Charles *et al.*, 2017) and the contribution of N-cycling microbial guilds to this variability remains contentious (Attard *et al.*, 2011; Graham *et al.*, 2014). *In situ* N₂O emission rates observed in our study are in agreement with measurements made by other authors in arable soils (Bouwman *et al.*, 2002; Van Groeningen *et al.*, 2010). Comparison of agricultural practices showed a consistent trend of harvest date on *in situ* N₂O emissions at Estrées-Mons B&E with higher N₂O 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 the 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.*, 2012, 2011) and creates a mulch layer, which increases soil moisture by reducing soil evaporation. Such conditions are known to be favorable to N₂O emissions by denitrification as recently reported with Miscanthus (Peyrard *et al.*, 2016b). 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

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sampling while *in situ* N₂O emissions were monitored over 3 years. Differences between early and late harvest treatments could also affect the N₂O:N₂ denitrification end product ratio rather than PDA, as previously observed (Domeignoz-Horta *et al.*, 2015). The minor changes in the soil properties observed between agricultural practices in our study may explain why many had a low or inexistent effect on the measured microbial activities and *in situ* N₂O emissions (Table S1).

Differences in agricultural practices were also not reflected in the abundances of N₂O-producers (*nirS*- and *nirK*- denitrifiers and AOA and AOB) (Fig. S3 and S4). In fact, the greatest differences in microbial abundances occurred between sites rather than between agricultural practices at each site. There are large discrepancies between the 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 (Chen *et al.*, 2010; Cui *et al.*, 2016; Kong *et al.*, 2010; Sun *et al.*, 2015). All types of mineral and organic fertilizers affected the *nirK*-community in a long-term fertilization experiment in Sweden while the *nirS*-community showed a negative response only to the ammonium sulfate and sewage sludge treatments (Hallin *et al.*, 2009). In another fertilization experiment, Cui *et al.* (2015) observed that *nirS*-, *nirK*- and *nosZ*-communities only increased in the two treatments fertilized with pig manure alone or in combination with mineral fertilizers. Other practices such as direct seeding, mulch-based cropping and weediness were shown to affect the abundance of denitrifier communities (Baudoin *et al.*, 2009; Gulden *et al.*, 2015). Similarly to the measured activities, 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 between studied practices at our experimental sites (Table S1). However, in contrast to N₂O-producing communities, we did find significant differences in the abundance and diversity of the N₂O-reducing communities according to the

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tillage regime at the Boigneville site. Thus, the *nosZI* clade significantly increased in abundance in the no tillage treatment, while the diversity of *nosZII* was the highest with full inversion tillage (Fig. S5; Table S3). Tillage *per se* consist of soil inversion, which leads to a redistribution of plant residues in deeper soil horizon and may causes changes in soil properties such as aggregate size and porosity (Chan *et al.*, 2003; Govaerts *et al.*, 2009; Strudley *et al.*, 2008). These changes may in turn impact soil water flow and aeration, which can also influence soil microorganisms. While our study was not designed to identify which changes in soil properties due to tillage were driving the studied communities, our results suggest that full tillage is fostering the diversity of the *nosZII* community compared to shallow or no tillage.

Soil properties influence on N₂O producing and reducing microbial and their activities

Comparison of all plots across the four different experimental sites showed that pH was significantly related to PDA and PNA (Table S4). Accordingly, a large body of literature highlighted the importance of soil pH for nitrification, denitrification and N₂O fluxes (Enwall *et al.*, 2007; Gubry-Rangin *et al.*, 2010; Nicol *et al.*, 2008; Šimek *et al.*, 2002). 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.*, 2012).

The analysis of the structure of the N₂O reducing community shows a stronger cluster by site for *nosZII* than for *nosZI*. A larger number of significant explanatory variables was found for *nosZII* community structure. Thus, common explanatory variables for the

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community structure of both *nosZ* clades were pH and C/N ratio, while sand content, total N and the cation exchange capacity (CEC) only explained the *nosZII* community structure. This supports previous work suggesting that the recently discovered clade of N₂O reducers is more sensitive to environmental factors than *nosZI* and provide additional evidence for the presence of niche differentiation between these two clades (Jones *et al.*, 2014; Domeignoz-Horta *et al.*, 2015) (Fig. 2).

Relationships between N₂O-producing and reducing microbial communities, their corresponding potential activities and in situ N₂O emissions

Relationships between abundances or diversity of microbial communities and the corresponding processes are unclear and contradictory results have been reported (Graham *et al.* 2016; Rocca *et al.* 2015). We found that abundances of denitrifying N₂O-producers and N₂O-reducers as well as the diversity of the *nosZI*-community only were related to the PDA (Table S4). 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 (Graf *et al.*, 2016; Orellana *et al.*, 2014; Samad *et al.*, 2016). In a previous work, we also found that diversity of the *nosZII* community did not explain the variation in PDA, while it was the strongest predictor of the N₂O:N₂ ratio measured by a potential activity assay (Domeignoz-Horta *et al.*, 2015). This might be due to the fact that about 51% of the bacterial genomes having *nosZII* lack either *nirK* or *nirS* (Graf *et al.*, 2014) and are therefore contributing only to N₂O reduction but not to PDA. Potential nitrification activity was significantly related to the abundance of AOA but not to AOB. Similarly to our findings, several studies show 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). However, the relative contribution of

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bacteria and archaea to the first step of nitrification is still unclear and it has been proposed that both groups of ammonia oxidizers have different ecological niches and that no single factor can discriminate between these two groups (Prosser & Nicol., 2012; Schleper, 2010; Sterngren *et al.*, 2015).

When analyzing the relationships between the studied microbial communities and the measurements of about 59 000 *in situ* N₂O emissions across all sites, we found that abiotic properties (i.e. soil pH, SOM, C/N) were the predominant explanatory factors of the basal emissions, while biotic factors (i.e. abundance and diversity of microorganisms) explained variation of the higher emissions fractions. To our knowledge, this is the first study disentangling the relative contribution of biotic and abiotic factors to baseline and peak *in situ* N₂O emissions. Such result has implications for modeling N₂O emissions and suggests that the understanding of microbial communities might help to better simulate N₂O peak emissions but is of little importance for baseline emissions. In accordance with our hypothesis, we also found that diversity of the *nosZII*-community rather than that of the *nosZI* community was negatively correlated to variation of the higher *in situ* N₂O emissions. A recent study reported a negative relationship between the *nosZII*-community and N₂O emissions measured in a laboratory incubation experiment (Samad *et al.*, 2016) but our work is the first one showing a negative link between N₂O fluxes measured *in situ* and this newly identified N₂O reducing community. This strengthens the hypothesis that the *nosZII*-community can act as a N₂O sink as suggested both by soil surveys (Jones *et al.*, 2014; Domeignoz-Horta *et al.*, 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) showed that inoculation of a non-denitrifying *nosZII* strain can contribute to lowering net soil potential N₂O production by soil denitrifiers.

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The recent discovery of the previously unaccounted *nosZII* clade of N₂O-reducers opened up new possibilities to mitigate the emissions of this greenhouse gas by, for example, selecting agricultural practices fostering this clade. Our results highlight the higher sensitivity of the *nosZII*- than *nosZI*-community to environmental factors. However, despite significant variations in the *nosZII* community across the sites examined, only a few of the studied agricultural practices resulted in shifts on the diversity of this community. Nevertheless, comparison of all plots across the different sites showed for the first time that a higher diversity of the *nosZII* community was concomitant with lower *in situ* fluxes. Moreover, our work also indicates that microbial communities were more important for explaining variations in high than in low N₂O emissions. This work emphasizes the consideration that the N₂O-reducing community should have when addressing process-related N₂O fluxes, particularly in studies aiming at mitigating emissions.

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Figure Legends

Fig. 1. *In situ* N₂O emissions. Representation of low emissions percentile (25th) and high emissions percentiles (75th and 95th).

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

Fig. 3. Variation partitioning of *in situ* N₂O emissions. Variance of *in situ* N₂O emissions was partitioned into soil physicochemical properties (S), abundance of N₂O-producers and abundance of N₂O-reducers (A), diversity of N₂O-reducers (D), and by combinations of predictors (a). Geometric areas are proportional to the respective percentages of explained variation. The edges of the triangles depict the variation explained by each factor alone, while percentages of variation explained by interactions of two or all factors are indicated on the sides and in the middle of the triangles, respectively. Variance partitioning of basal *in situ* N₂O emissions (b; 25th), variance partitioning of median *in situ* N₂O emissions (c; 50th), (d),

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(e), (f) and (g) correspond to the variation partitioning of high N₂O emissions of 75th, 90th and 95th, respectively. All numbers represent percentages. Only variance fractions $\geq 5\%$ are shown. The variables used for each variation partitioning are indicated in the Table 2.

Legend supplemental figures.

Fig S1. Potential denitrification activity (N₂O+N₂; PDA). Significant differences between treatments are indicated with different letters (anova followed by Tukey HSD test, $P < 0.05$)

Fig S2. Potential nitrification activity (PNA). Significant differences between treatments are indicated with different letters (anova followed by Tukey HSD test, $P < 0.05$).

Fig S3. Abundance of ammonia-oxidizers bacteria (a) and archaea (b). Significant differences between treatments are indicated with different letters (anova followed by Tukey HSD test, $P < 0.05$).

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

Fig S5. Abundance of *nosZI* (a) and *nosZII* (b). Significant differences between treatments are indicated with different letters (anova followed by Tukey HSD test, $P < 0.05$)

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Table 1. Management practices at the different sites

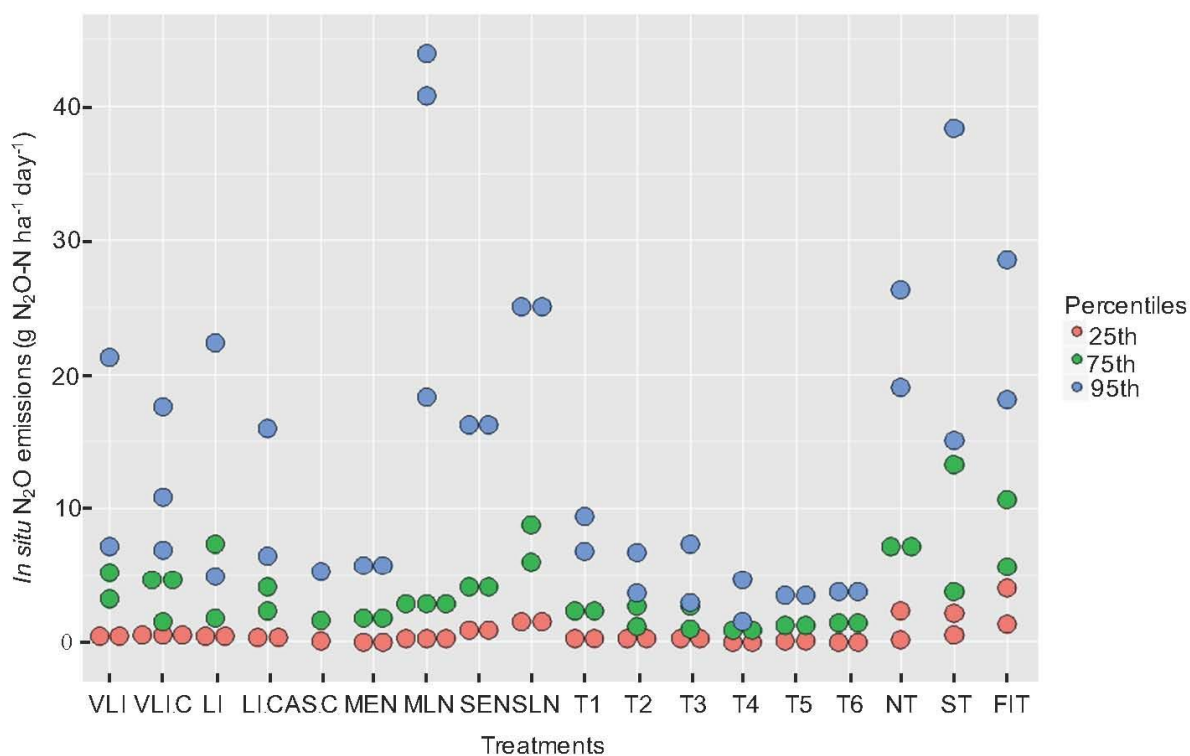
Site	Treatment	Management
Auzeville	LI	Low N input
	LIC	Low N input & cover crop
	VLI	Very low N input
	VLIC	Very low N input & cover crop
	AS	Intercropping
	AS.C	Intercropping & cover crop
Mons - B&E	MLN	Miscanthus late harvest & N input
	MEN	Miscanthus early harvest & N input
	SLN	Switchgrass late harvest & N input
	SEN	Switchgrass early harvest & N input
	SL	Switchgrass late harvest & no N input
	SE	Switchgrass early harvest & no N input
Mons - SOERE	T1	High N input, full Inversion tillage, straw return & non legume as cover crop
	T2	High N input, shallow tillage, straw return & non legume as cover crop
	T3	High N input, shallow tillage, straw export & non legume as cover crop
	T4	Low N input, full inversion tillage, straw return & non legume as cover crop
	T5	Low N input, full inversion tillage, straw return & legume as cover crop
	T6	Switchgrass late harvest & N input
Boigneville	FIT	Full inversion tillage
	ST	Shallow tillage
	NT	No tillage

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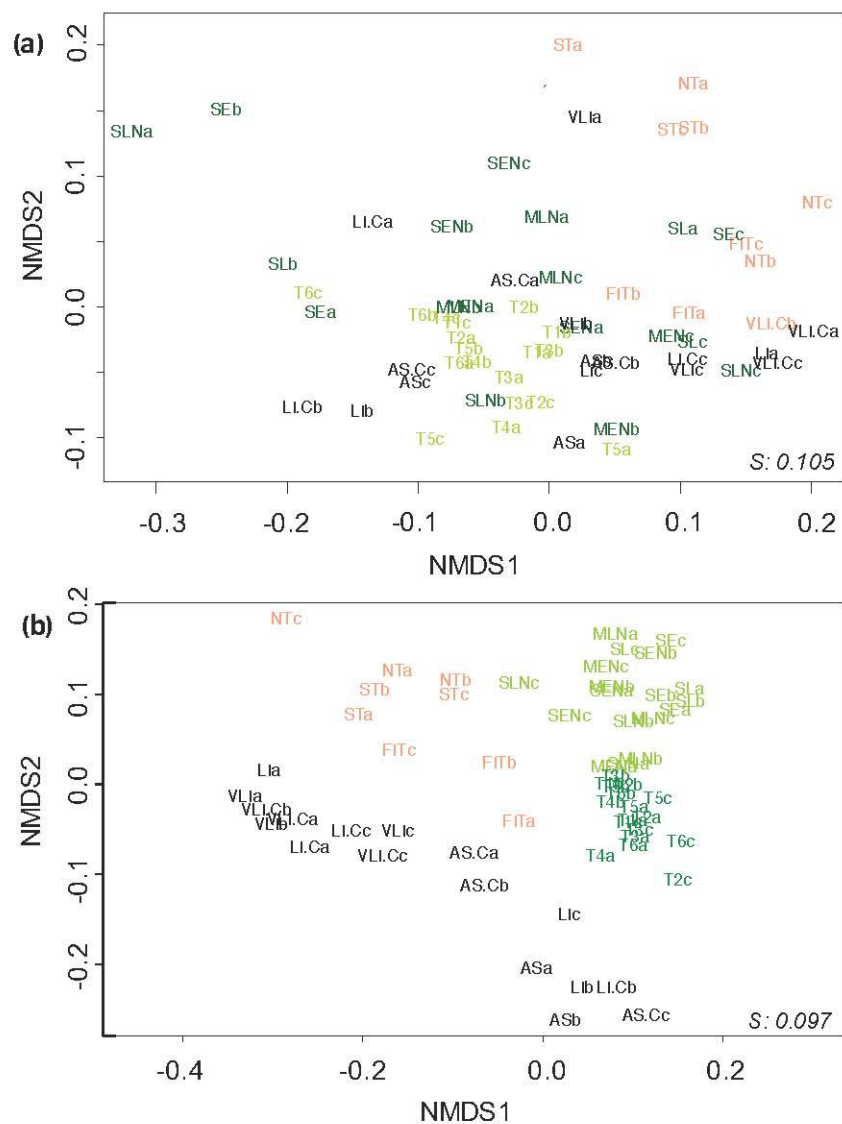
Table 2. Selected explaining variables for the variation partitioning analyses

N ₂ O percentile (th)	Soil Properties ¹	Abundance ²	Diversity indexes ³
25	pH(-), SOM(+), C/N(-)	AOB(+), <i>nirK/nirS</i> (+)	<i>nosZI</i> PD(-), <i>nosZI</i> SR(-)
50	pH(-), SOM(+), C/N(-)	AOB(+)	<i>nosZII</i> PD(-), <i>nosZII</i> SR(-)
75	pH(-), sand (+), CEC(+)	AOB(+), <i>nirK/nirS</i> (+)	<i>nosZII</i> PD(-), <i>nosZII</i> SR(-)
90	pH(-)	AOA(-)	<i>nosZII</i> PD(-), <i>nosZII</i> SR(-)
95	sand(+), C/N(-)	AOA(-), AOB(+)	<i>nosZI</i> SR(-), <i>nosZII</i> PD(-), <i>nosZII</i> SR(-)

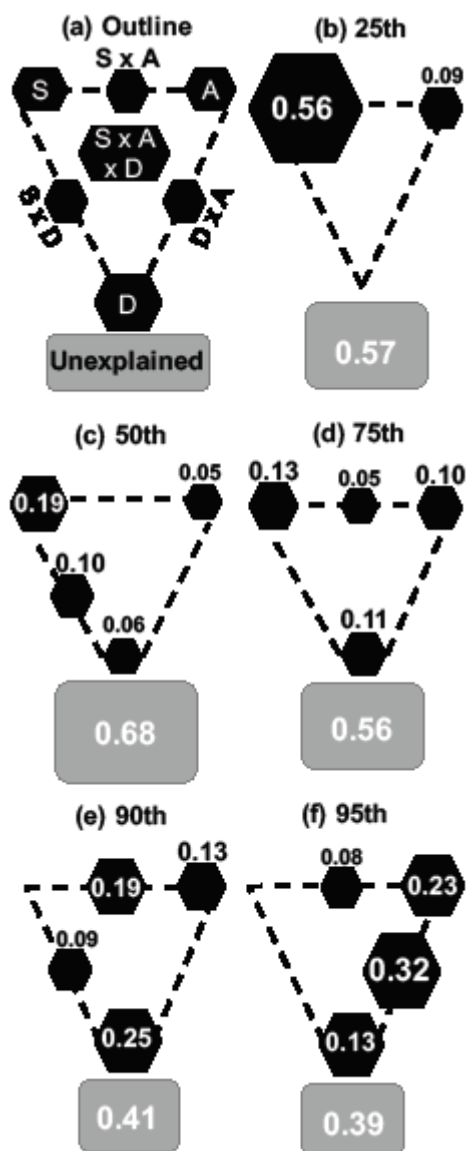
¹SOM and CEC correspond to soil organic matter (g kg⁻¹) and cation exchange capacity (cmolc kg⁻¹), respectively. Abundance of N₂O producers and reducers is expressed in nbc ng DNA⁻¹. ²PD and SR correspond to Faith's PD index and Simpson reciprocal index, respectively. The direction of correlation is shown between parenthesis.



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