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

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To cite this version:
Luiz Domeignoz Horta, Laurent Philippot, Céline Peyrard, David Bru, Marie-Christine Breuil, et al.. Peaks of in situ N2O emissions are influenced by N2O producing and reducing microbial communities across arable soils. Global Change Biology, Wiley, 2017, online (1), 34 p. 10.1111/gcb.13853. hal-01603115

HAL Id: hal-01603115
https://hal.archives-ouvertes.fr/hal-01603115

Submitted on 27 May 2020

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TITLE: Peaks of in situ N$_2$O emissions are influenced by N$_2$O producing and reducing microbial communities across arable soils

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Running head: N$_2$O reducers and N$_2$O emissions in arable soils

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1111/gcb.13853

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

Abstract

Agriculture is the main source of terrestrial N\textsubscript{2}O emissions, a potent greenhouse gas and the main cause of ozone depletion. The reduction of N\textsubscript{2}O into N\textsubscript{2} by microorganisms carrying the nitrous oxide reductase gene (\textit{nosZ}) is the only known biological process eliminating this greenhouse gas. Recent studies showed that a previously unknown clade of N\textsubscript{2}O-reducers (\textit{nosZII}) was related to the potential capacity of the soil to act as a N\textsubscript{2}O sink. However little is known about how this group responds to different agricultural practices. Here, we investigated how N\textsubscript{2}O-producers and N\textsubscript{2}O-reducers were affected by agricultural practices across a range of cropping systems in order to evaluate the consequences for N\textsubscript{2}O emissions.

The abundance of both ammonia oxidizers and denitrifiers was quantified by real-time qPCR, and the diversity of \textit{nosZ} clades was determined by 454 pyrosequencing. Denitrification and nitrification potential activities as well as \textit{in situ} N\textsubscript{2}O 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 \textit{in situ} N\textsubscript{2}O emissions, we subdivided more than 59,000 field measurements into fractions from low to high rates. We found that the low N\textsubscript{2}O 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 \textit{nosZII} clade but not of the \textit{nosZI} clade was important to explain the variation of \textit{in situ} N\textsubscript{2}O emissions. Altogether, these
results lay the foundation for a better understanding of the response of N\textsubscript{2}O reducing bacteria to agricultural practices and how it may ultimately affect N\textsubscript{2}O 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\textsubscript{2} or CH\textsubscript{4} but seldom for N\textsubscript{2}O consumption (Six *et al*., 2004; Chapuis-Lardy *et al*., 2007; Tian *et al*., 2016). Nevertheless, N\textsubscript{2}O is an important potent greenhouse gas with a global warming potential (GWP) over 100 years of about 298 and 11.9 times that of CO\textsubscript{2} and CH\textsubscript{4}, respectively. N\textsubscript{2}O 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 N\textsubscript{2} fixation to produce fertilizers contributes double the natural rate of terrestrial nitrogen fixation (Canfield *et al*., 2010). As a result, N\textsubscript{2}O 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 N\textsubscript{2}O 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). N\textsubscript{2}O 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 N\textsubscript{2}O 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 N\textsubscript{2}O are unclear (Walker *et al*., 2010) but recent studies suggested that they produce lower yields of N\textsubscript{2}O than their bacterial counterpart (Hink *et al*., 2016). Denitrification is a respiratory process during which soluble nitrogen oxides (NO\textsubscript{3}\textsuperscript{-} and NO\textsubscript{2}\textsuperscript{-}) are sequentially reduced into gaseous forms.
(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.,...
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
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 N₂Ot 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 KNO₃, 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 N₂O reductase, acetylene was
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.
Abundances of microbial communities potentially involved in N$_2$O production or N$_2$O 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 N$_2$O-reducers (Jones et al., 2013). qPCR Reactions were carried out in a Step One Plus (Life Technologies, Carlsbad, CA, USA) with 15µl reaction volume containing 1 ng of DNA, 7.5 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, Illkirch, 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 N$_2$O-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.
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
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\textsubscript{2}O + N\textsubscript{2}), potential nitrification activity and in situ N\textsubscript{2}O 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 µg N\textsubscript{2}O-N g\textsuperscript{-1} DW soil h\textsuperscript{-1} for SOERE and B&E respectively. Average PDA rates at Auzeville and Boigneville ranged between 0.3 and 0.5 µg N\textsubscript{2}O-N g\textsuperscript{-1} DW soil h\textsuperscript{-1} (Fig. S1). The SOERE and B&E experiments also showed higher PNA with average rates of 0.3 and 0.2 µg NO\textsubscript{2}-N g\textsuperscript{-1} DW soil h\textsuperscript{-1}, respectively. Much lower PNA rates (0.01 µg NO\textsubscript{2}-N g\textsuperscript{-1} DW soil h\textsuperscript{-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\textsubscript{2}O 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\textsubscript{2}O emissions were lower for early harvest than late harvest whatever the percentile (Fig. 1). Moreover, at...
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 \(\text{N}_2\text{O}\)-producers and \(\text{N}_2\text{O}\)-reducers**

Across the 63 soil samples, significant differences were observed between sites but not between treatments within a site for the \(\text{N}_2\text{O}\)-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 \(1.18 \times 10^3\) gene copy number ng\(^{-1}\) DNA (CI\(_{95\%}\) = \(1.81 \times 10^2 – 2.18 \times 10^3\)) and \(9.63 \times 10^1\) gene copy number ng\(^{-1}\) DNA (CI\(_{95\%}\) = \(5.48 \times 10^1 – 1.38 \times 10^2\)), and highest abundances reaching \(1.24 \times 10^4\) gene copy number ng\(^{-1}\) DNA (CI\(_{95\%}\) = \(6.79 \times 10^3 – 1.80 \times 10^4\)) and \(9.02 \times 10^2\) gene copy number ng\(^{-1}\) DNA (CI\(_{95\%}\) = \(3.72 \times 10^2 – 1.43 \times 10^3\)) for AOA and AOB, respectively (Fig. S3). Regarding denitrifying \(\text{N}_2\text{O}\) producers, we observed higher abundances of \(\text{nirK}\) than \(\text{nirS}\) in all sampled soils with lowest abundance reaching \(1.12 \times 10^4\) nbc ng\(^{-1}\) DNA (CI\(_{95\%}\) = \(1.29 \times 10^4 – 9.58 \times 10^4\)) and \(3.29 \times 10^4\) nbc ng\(^{-1}\) DNA (CI\(_{95\%}\) = \(2.89 \times 10^3 – 3.69 \times 10^3\)) for \(\text{nirK}\) and \(\text{nirS}\), respectively.

Contrarily to \(\text{N}_2\text{O}\)-producers, the abundance of \(\text{N}_2\text{O}\)-reducers was affected by agricultural practices. The \(\text{nosZI}\) community abundance significantly decreased with tillage intensity at Boigneville while the abundance of the \(\text{nosZII}\) community was not significantly different between treatments (Fig. S5). We observed higher or equal abundances of \(\text{nosZI}\) compared to \(\text{nosZII}\) in all sampled soils with \(\text{nosZI}/\text{nosZII}\) ratios ranging from 1.2 to 2.9.
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 (CI₉₅% = 106 - 120), 103 (CI₉₅% = 94 - 112), 94 (CI₉₅% = 89 – 99), and 119 (CI₉₅% = 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 (CI₉₅% = 206 - 242), 226 (CI₉₅% = 209 - 243), 241 (CI₉₅% = 231 - 251), and 202 (CI₉₅% = 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,
$P < 0.001$ 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 N$_2$O emissions. The soil physical and chemical characteristics, the abundance of N$_2$O-producers and N$_2$O-reducers, and the diversity of N$_2$O-reducers were separated into three groups of explanatory variables and used to explain each fraction of N$_2$O emissions. Soil properties were found to explain mostly basal N$_2$O 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 N$_2$O-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 N$_2$O-producers (denitrifiers and nitrifiers) contributed to explain the variance in N$_2$O emissions.
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
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
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
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
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 N$_2$O 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 N$_2$O emissions. Such result has implications for modeling N$_2$O emissions and suggests that the understanding of microbial communities might help to better simulate N$_2$O 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 N$_2$O emissions. A recent study reported a negative relationship between the nosZII-community and N$_2$O emissions measured in a laboratory incubation experiment (Samad et al., 2016) but our work is the first one showing a negative link between N$_2$O fluxes measured in situ and this newly identified N$_2$O reducing community. This strengthens the hypothesis that the nosZII-community can act as a N$_2$O 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 N$_2$O 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 N$_2$O production by soil denitrifiers.
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.

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. This work has relied on the SOERE ACBB experimental facility and the Biomass & Environment experiment in Estrées-Mons, the MICMAC cropping system experiment in Auzeville and the long-term soil tillage experiment in Boigneville.
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Figure Legends

Fig. 1. *In situ* N$_2$O 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* N$_2$O emissions. Variance of *in situ* N$_2$O emissions was partitioned into soil physicochemical properties (S), abundance of N$_2$O-producers and abundance of N$_2$O-reducers (A), diversity of N$_2$O-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* N$_2$O emissions (b; 25th), variance partitioning of median *in situ* N$_2$O emissions (c; 50th), (d),
(e), (f) and (g) correspond to the variation partitioning of high N$_2$O 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 (N$_2$O+N$_2$; 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, $P < 0.05$).
Table 1. Management practices at the different sites

| Site       | Treatment | Management                                                                 |
|------------|-----------|-----------------------------------------------------------------------------|
| Auzeville  | LI        | Low N input                                                                |
|            | LI.C      | Low N input & cover crop                                                   |
|            | VLI       | Very low N input                                                           |
|            | VLI.C     | 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                                                                 |
Table 2. Selected explaining variables for the variation partitioning analyses

| N₂O percentile (th) | Soil Properties¹ | Abundance² | Diversity indexes³ |
|---------------------|------------------|------------|--------------------|
| 25                  | pH(-), SOM(+), C/N(-) | AOB(+), nirK/nirS(+) | nosZI PD(-), nosZI SR(-) |
| 50                  | pH(-), SOM(+), C/N(-) | AOB(+)     | nosZI PD(-), nosZI SR(-) |
| 75                  | pH(-), sand (+), CEC(+) | AOB(+), nirK/nirS(+) | nosZI PD(-), nosZI SR(-) |
| 90                  | pH(-)             | AOA(-)     | nosZI PD(-), nosZI SR(-) |
| 95                  | sand(+), C/N(-)   | AOA(-), AOB(+) | nosZI SR(-), nosZI PD(-), nosZI 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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Domeignoz Horta, L., Philippot, L. (Auteur de correspondance), Peyrard, C., Bru, D., Breuil, M.-C., Bizouard, F., Justes, E., Mary, B., Leonard, J., Spor, A. (2018). Peaks of in situ N₂O emissions are influenced by N₂O producing and reducing microbial communities across arable soils. Global Change Biology, 24 (1), 360-370. DOI: 10.1111/gcb.13853
Peaks of in situ $\text{N}_2\text{O}$ emissions are influenced by $\text{N}_2\text{O}$ producing and reducing microbial communities across arable soils. Global Change Biology, 24 (1), 360-370. DOI: 10.1111/gcb.13853
