# Recently identified microbial guild mediates soil N$_2$O sink capacity

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Nitrous oxide (N$_2$O) is the predominant ozone-depleting substance and contributes approximately 6% to overall global warming[1,2]. Terrestrial ecosystems account for nearly 70% of total global N$_2$O atmospheric loading, of which at least 45% can be attributed to microbial cycling of nitrogen in agriculture[3]. The reduction of N$_2$O to nitrogen gas by microorganisms is critical for mitigating its emissions from terrestrial ecosystems, yet the determinants of a soil's capacity to act as a source or sink for N$_2$O remain uncertain[4]. Here, we demonstrate that the soil N$_2$O sink capacity is mostly explained by the abundance and phylogenetic diversity of a newly described N$_2$O-reducing microbial group[5,6], which mediate the influence of edaphic factors. Analyses of interactions and niche preference similarities suggest niche differentiation or even competitive interactions between organisms with the two types of N$_2$O reductase. We further identified several recurring communities comprised of co-occurring N$_2$O-reducing bacterial genotypes that were significant indicators of the soil N$_2$O sink capacity across different European soils.

Disturbance of the natural nitrogen (N) cycle by human activity has resulted in atmospheric N$_2$O concentrations increasing at a rate of nearly 0.8 ppb per year[7], prompting calls for better accounting of the mechanisms driving its production and consumption in soils[4]. In contrast to the other major greenhouse gases CO$_2$ and CH$_4$, the underlying controls of soil N$_2$O sink capacity have seldom been studied despite N$_2$O consumption in soil being frequently reported[8]. The only known sink for N$_2$O in the biosphere is its enzymatic reduction to dinitrogen (N$_2$) by N$_2$O reductase[9,10]. This protein is found among microorganisms capable of complete denitrification, which is the anaerobic respiration of nitrate (NO$_3^-$) or nitrite (NO$_2^-$) to N$_2$ through N$_2$O. However, truncated versions of this respiratory pathway are common. A significant proportion of denitrifying microorganisms produce N$_2$O as a terminal product owing to the absence of the *nosZ* gene encoding the catalytic subunit of the N$_2$O reductase[11]. On the other hand, several microorganisms with a N$_2$O reductase that can use exogenous N$_2$O as the sole electron acceptor do not possess the preceding steps in the denitrification pathway[6,12]. Recent studies revealed that the abundance and diversity of these potential N$_2$O consumers has been underestimated[5], and their environmental role, as well as that of denitrifiers having *nosZ*, in net N$_2$O emissions remains undefined.

To examine the contribution of the microbial populations in determining the potential of soils to act as a sink for N$_2$O, we undertook a survey of 47 soils across Europe (Supplementary Methods and Table 1). The soils' N$_2$O sink capacity was investigated by manipulating the abundance of denitrifiers producing N$_2$O
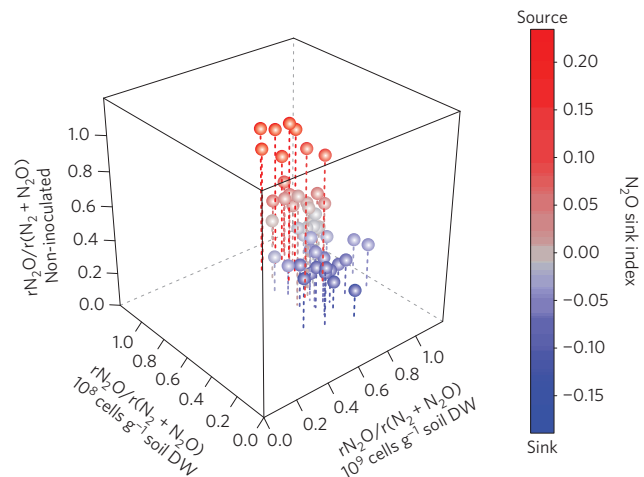
Figure 1 | Range of potential soil N$_2$O sink capacity. Points show the ratio of potential N$_2$O production (rN$_2$O) to total denitrification activity (r(N$_2$O + N$_2$)) in soil microcosms with different inoculation levels of *A. tumefaciens* C58. Colour corresponds to relative N$_2$O sink index, calculated as a function of rN$_2$O/r(N$_2$ + N$_2$O) at all three inoculation levels (Supplementary Methods and Fig. 2). Negative values (blue) indicate soils with a greater capacity to consume excess N$_2$O produced by inoculated *A. tumefaciens* cells, whereas positive values (red) indicate soils that are potential net sources of N$_2$O. DW: dry weight.

by adding different amounts of the bacterium *Agrobacterium tumefaciens* C58 in soil microcosms[13]. This strain of *A. tumefaciens* lacks the *nosZ* gene[14] and thereby produces only N$_2$O under denitrifying conditions, allowing for addition of N$_2$O directly into the soil matrix during incubation under optimal denitrifying conditions. The activity of indigenous denitrifying communities in the non-inoculated microcosms varied substantially across the different soils, with values of 0.06–10.2 and 0.2–28.9 µg N$_2$O-N g^{-1} soil dry weight h^{-1} for potential N$_2$O emission and total denitrification activity, respectively (Supplementary Fig. 1). The proportion of N$_2$O emitted by denitrification, calculated as the ratio of the rate of potential N$_2$O production and total denitrification activity (rN$_2$O/r(N$_2$O + N$_2$)), ranged from 0.1 to 0.95 in the non-inoculated microcosms (Fig. 1 and Supplementary Fig. 1) and was not dependent on overall denitrifier community functioning, as no significant correlation was observed with N$_2$O production or total denitrification rates. The addition of 10^8 *A. tumefaciens* cells led to an increase in potential N$_2$O emissions up to 45-fold,
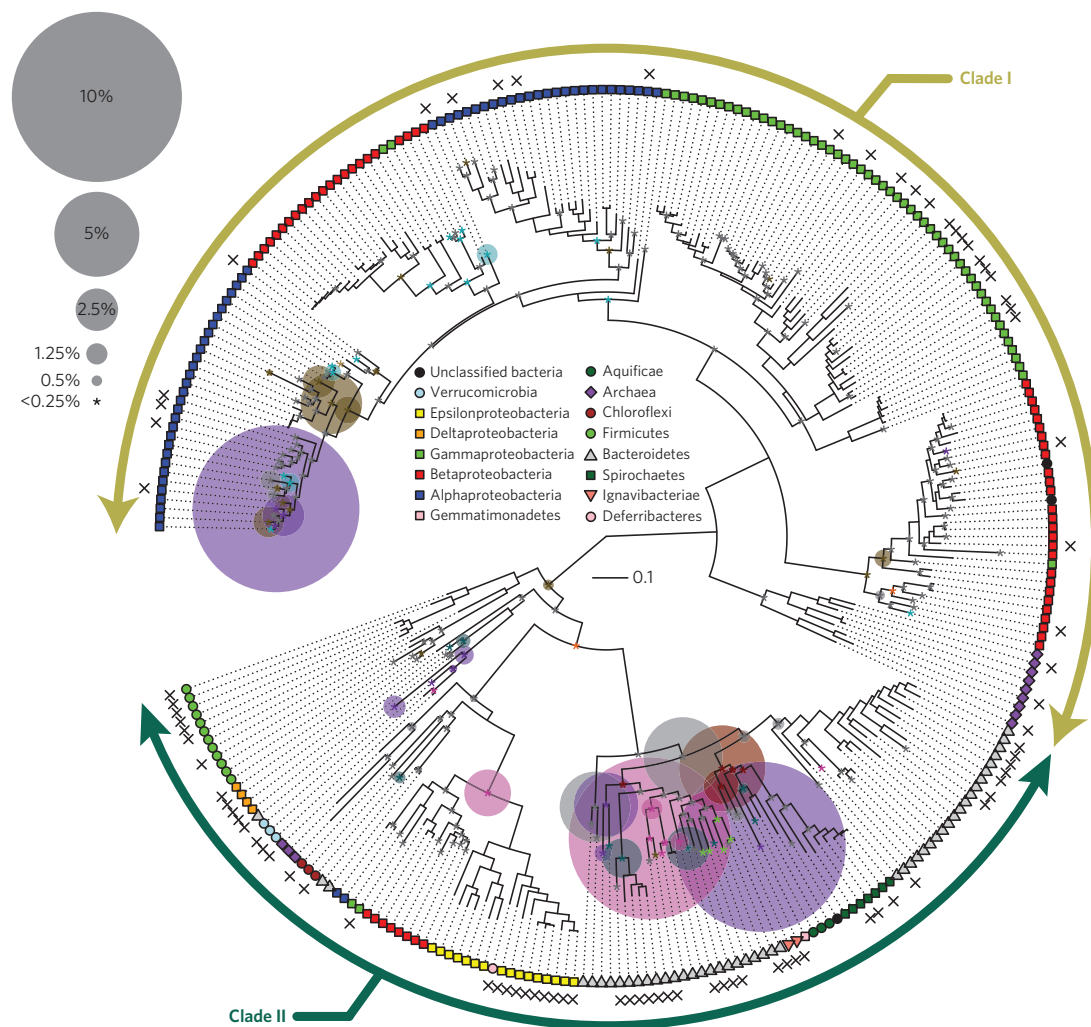
Figure 2 | Phylogenetic placement of *nosZ* pyrosequencing reads within a reference phylogeny. Phylogeny was inferred using maximum likelihood analysis of full-length *nosZ* amino acid sequences obtained from microbial genomes. Circles plotted on internal or terminal edges within the phylogeny show placements of reads using the pplacer algorithm[25], with size indicating the number of reads relative to the total number of *nosZ* reads obtained from all samples. Coloured circles denote membership of reads, grouped by edge placement, to different *nosZ* communities inferred in Fig. 4. Symbols projecting from the tips of the phylogeny denote taxonomic affiliation of source organisms for reference *nosZ* sequences, and genomes that lack genes for either of the dissimilatory nitrite reductases involved in denitrification (*nirS* or *nirK*) are indicated (cross). Scale bar indicates amino-acid substitution rate per site, and bootstrap confidence levels are shown in Supplementary Fig. 6.

whereas the 10^9 inoculation level resulted in N_2O emission rates that were nearly 155 times higher than that of the non-inoculated soils. Whereas the $rN_2O/r(N_2O + N_2)$ ratios were typically higher in the inoculated microcosms, small changes were observed for several soils despite addition of the N_2O-producing *A. tumefaciens*, resulting in these soils having a negative N_2O exchange index (Fig. 1 and Supplementary Fig. 2 for calculation of index), and hence being characterized as N_2O sinks. Indeed, almost half the soils were capable of reducing more than one-third of the N_2O produced by the introduction of 10^8 *A. tumefaciens* cells.

As the abundance of functional groups has been shown to be useful for predicting potential N-cycling rates[15], we quantified the abundances of functional genes as proxies for the microorganisms involved in N_2O production and reduction. Organisms that can produce N_2O by denitrification were quantified by real-time PCR of the *nirS* and *nirK* genes that encode the two types of dissimilatory nitrite reductase. These genes are mutually exclusive in the genomes of organisms that perform denitrification and represent two ecologically distinct denitrifying communities[16,17]. The abundance of organisms that reduce N_2O was quantified by targeting the

nosZ gene, which consists of two distinct clades[5] hereafter referred to as clades I and II. The latter has recently been recognized as a previously unaccounted clade of 'atypical' nitrous oxide reducers found in a variety of different ecosystems[5,6,12]. Across the different soils, the relative abundance of *nirS* and *nirK* genes ranged from 2.6% to 25.9% of the total 16S ribosomal RNA gene copy number (Supplementary Table 4), with *nirK* being equally or up to 3.8 times more abundant than *nirS* in 35 out of 47 soils. In accordance with previous studies[18,19], the abundance of *nosZ* genes was lower, comprising 0.8–15.1% of the bacterial community, and largely consisted of organisms found within *nosZ* clade I (Supplementary Table 4). Nevertheless, the N_2O sink capacity increased with the ratio of clade II/clade I *nosZ* abundance (Spearman's $\rho = 0.51$, $P < 0.01$), suggesting that the predominance of either *nosZ* clade may have substantial consequences for net soil N_2O emissions. This concurs with the higher percentage of bacterial genomes lacking either of the *nir* genes within *nosZ* clade II (47%) compared with clade I (17%), and therefore being potential N_2O sinks (Fig. 2 and Supplementary Fig. 6). The N_2O sink capacity of the soils was also related to the proportion between the *nir* genes ($\rho = -0.49$,
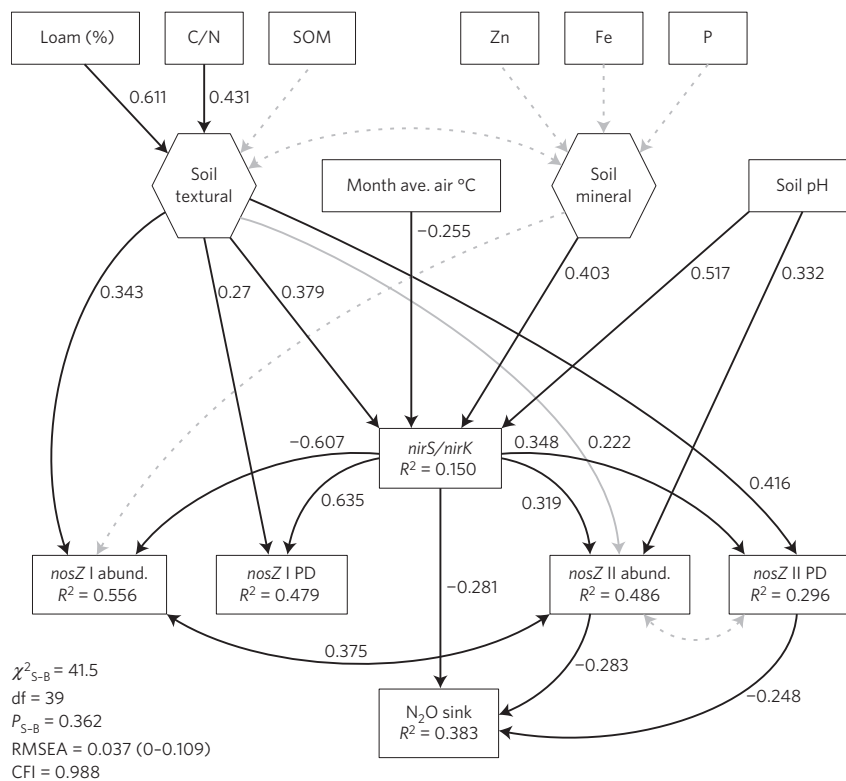
Figure 3 | Structural equation model showing the relative influence of soil abiotic and denitrifier community factors on the soil N_2O sink capacity. Soil textural and mineral factors are shown as composite latent variables (hexagons), representing the collective effect of the set of soil abiotic factors assigned to each. Paths that could not be constrained to zero without significant reduction in model fit ($P(\chi^2) < 0.05$ in nested model comparisons) are shown, along with standardized path coefficients. Non-significant relationships are shown as dotted grey paths, whereas marginal ($0.1 > P > 0.05$) and significant ($P < 0.05$) relationships are shown as solid grey and black paths, respectively. Amount of variance explained by the model (R^2) is listed for each response variable, and measures of overall model fit are shown in the lower left. SOM: soil organic matter; PD: phylogenetic diversity; RMSEA: root mean square error of approximation; CFI: comparative fit index.

$P < 0.01$), with a higher N_2O consumption with increasing $nirS$ to $nirK$ ratio. This reflects a previous observation that $nosZ$ among denitrifiers occurs more sporadically in the genomes of strains with $nirK$ than those with $nirS$ (ref. 11).

To further address whether the composition and structure of the N_2O-reducing communities matters for N_2O consumption in soil, we analysed the diversity of $nosZ$ genes amongst the different soils by pyrosequencing. Phylogenetic placement of $nosZ$ sequences amplified using clade I and clade II specific primers resulted in all reads mapping to 249 of 578 internal and terminal edges throughout the reference $nosZ$ phylogeny (Fig. 2); a distribution that reflects a comprehensive coverage of the known diversity of the $nosZ$ gene. Nearly 40% were similar to $nosZ$ sequences from Bacteroidetes, Chloroflexi and Gemmatimonadetes within clade II, whereas 29% mapped to various lineages of $nosZ$ from α-proteobacteria within clade I, with 15% bearing the greatest degree of similarity to $nosZ$ from *Bradyrhizobium* spp. Approximately 20% of the sequences mapped to deeper internal edges, indicating that there remains a large degree of $nosZ$ diversity that is not represented in available genomic databases. Following clustering of sequences at 97% nucleotide similarity, we observed that the phylogenetic diversity of the resulting operational taxonomic units (OTUs) for each $nosZ$ clade differed by a factor of 2 and 3 across all sites for clade I and II, respectively (Supplementary Fig. 3). Whereas no significant correlation was observed between the phylogenetic diversity values of clade I and II, soils with a greater capacity to reduce excess N_2O also had higher levels of diversity in both clades ($\rho = -0.39, P < 0.01$ and $\rho = -0.36, P = 0.01$ for clade I and clade II, respectively). This effect of diversity is consistent with recent work showing that soil denitrification

activity was also affected by diversity loss[20]. Interestingly, the diversity of both clades was also significantly positively correlated to the ratio of $nirS$ to $nirK$ across soils ($\rho = 0.68, P < 0.01$ and $\rho = 0.35, P = 0.02$ for clade I and clade II, respectively), suggesting that soils with low $nosZ$ diversity are more likely to be dominated by $nirK$ type denitrifiers. Together, these results demonstrate that the soil N_2O sink capacity is mediated by N_2O-reducing microorganisms, especially the recently identified clade II.

To determine the degree to which the phylogenetic diversity and abundance of each N_2O-reducing community, as well as the ratio of $nirK$ and $nirS$ type denitrifiers, mediate the influence of different abiotic factors on the relative N_2O sink capacity, a structural equation model was constructed (Fig. 3 and Supplementary Figs 4 and 5). We further specified the microbial community block of the model by asserting that the ratio of $nirS/nirK$ abundance will influence the phylogenetic diversity and abundance of the two different $nosZ$ clades, as well as the N_2O sink capacity. The abiotic factors were reduced into four categories; the soil mineral and textural factors, soil pH, and average air temperature for the month before sampling (Supplementary Tables 2 and 3). Although soil pH is commonly cited as the controlling variable in determining denitrification end-product ratios[21–23], our structural equation model indicates that the relative abundance and phylogenetic diversity of the $nosZ$ clade II community were the strongest drivers of soil N_2O sink capacity, together with the ratio of $nirS/nirK$ type denitrifiers (Fig. 3). Nevertheless, the abundance of the $nosZ$ clade II was more influenced by pH than that of clade I, which in contrast responded more to differences in soil textural properties (Fig. 3). The phylogenetic diversity of both clades was also significantly
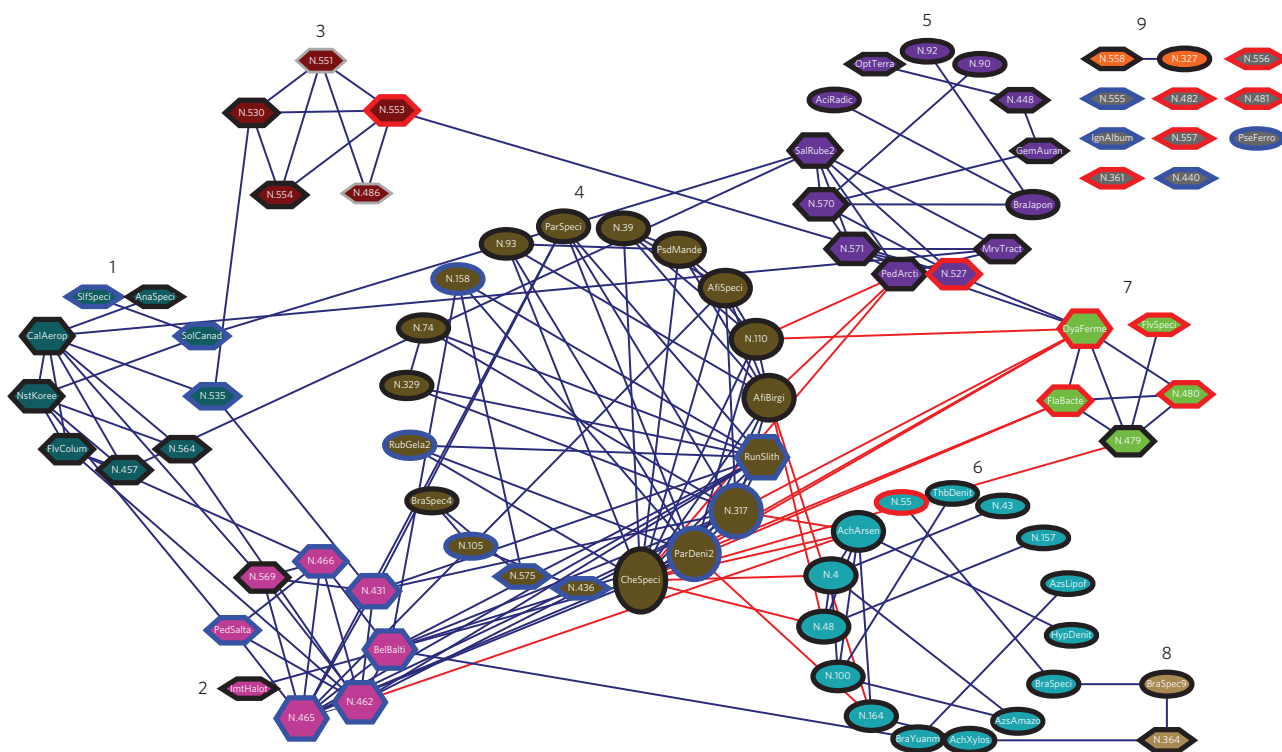
Figure 4 | Network analysis of *nosZ* sequence groups identifying N$_2$O-reducing communities associated with soils acting as potential N$_2$O sinks.
Nodes correspond to phylogenetic placements of *nosZ* reads shown in Fig. 2 (see Supplementary Fig. 6 for location of group names within the reference phylogeny). Circles and hexagons represent groups found in either *nosZ* clade I or clade II, respectively. Communities detected using networks analysis combined with modulated modularity clustering[29] are numbered, with node size proportional to degree. Connections between nodes indicate strong associative (blue) or exclusionary relationships (red) as defined by Spearman's $\rho > 0.6$ or $\rho < -0.6$, respectively. Nodes with coloured borders correspond to *nosZ* groups found to be significant predictors of soil potential N$_2$O sink capacity based on variable importance analysis, indicating whether the relative abundance of each group increases (blue) or decreases (red) in soils acting as N$_2$O sinks (Spearman's ρ, $P < 0.05$; Supplementary Fig. 7 and Table 7). Grey node borders indicate groups above the variable importance threshold with non-significant correlations, and singleton nodes that were not significant predictors of N$_2$O sink capacity were excluded.

influenced by soil textural properties; however, a substantially larger effect was observed on the diversity of clade II. These results suggest niche partitioning between the two *nosZ* clades, as previously observed for *nirS* and *nirK* denitrifying communities[16–18], and changes in edaphic factors will thereby influence the relative proportion and diversity of the microorganisms with the potential to reduce N$_2$O to N$_2$.

As the model showed that the genetic diversity of the entire *nosZ* community, that is, both clade I and II, was an important component explaining the soil's ability to reduce N$_2$O, we further analysed potential interactions between *nosZ* groups and how they relate to the soil N$_2$O sink capacity. Using the grouping of *nosZ* sequences delimited by phylogenetic placement (Fig. 2 and see Supplementary Fig. 6 for group names), specific *nosZ* communities were identified using co-occurrence analysis and network clustering of groups of coexisting organisms. We identified 9 distinct *nosZ* communities that largely consisted of groups from a single clade (Fig. 4 and Supplementary Figs 7 and 8), which suggests similarity in niche preference amongst organisms with either *nosZ* type. The exclusionary interactions ($\rho < -0.6$) observed between several nodes from different clades further suggest possible niche differentiation or even competitive interactions between organisms with different *nosZ* types. The communities identified in the network analysis were remarkably consistent with the results of variable importance analysis, which identified 35 groups as being indicative of soil N$_2$O sink capacity owing to either increasing or decreasing relative abundance (Supplementary Fig. 9 and Table 7). Groups that were identified as significant indicators of soil N$_2$O

sink capacity were predominant in clade II communities 2 and 7, which consisted of groups that had either lower or higher relative abundance in soils that reduced excess N$_2$O. Notably, group N.465 (*nosZ* community 2), which was found in high abundance in soils with negative sink indices, is associated with *nosZ* clade II lineages from organisms that lack either *nir* gene (Fig. 2 and Supplementary Fig. 6) and probably function as N$_2$O sinks.

Although it is frequently suggested that microbial communities mediating Earth's biogeochemical cycling are functionally redundant, we demonstrated a significant relationship between the relative abundance and the phylogenetic diversity of the *nosZ* community and the ability of the soil to consume N$_2$O, as well as distinct intra-clade patterns of co-associations of key guilds. Our findings reveal that abundance and phylogenetic diversity of previously unaccounted N$_2$O-reducing microorganisms as well as community membership is critical for the soil N$_2$O sink capacity. Information is now needed to determine how changes in land use and management affect *nosZ* communities and thereby favour or hamper N$_2$O mitigation strategies.

Methods

Supplementary Fig. 1). To quantify the capacity of the indigenous microbial community in each soil to consume the excess N_2O generated by the inoculated *A. tumefaciens* strain, we developed an index of soil N_2O exchange that discriminates between soils that are potential sinks or sources for N_2O based on the $rN_2O/r(N_2O + N_2)$ ratio measured for each inoculation level (Supplementary Methods and Fig. 2). Negative values denote a greater capacity to consume excess N_2O, whereas positive values indicate soils that are net N_2O producers. A full description of sampling details, microcosm set-up and determination of potential N_2O and denitrification rates are provided in the Supplementary Methods.

Quantification of 16S rRNA and the different denitrification genes was performed using previously described methods, as detailed in Supplementary Methods. To target *nirS* and *nirK* denitrifiers, we used available *nirS* and *nirK* primer sets that, although not covering the extant genetic diversity of each group, still allows for a comparative analysis of the relative abundance of each across the different soils samples by sampling a standard subset of each group for which denitrification functionality is verified[24]. The abundance and diversity of N_2O-reducing organisms were determined by using primers sets that encompass the known diversity of the *nosZ* gene, allowing for a complete assessment of the N_2O-reducing community. Pyrosequencing of the *nosZ* gene resulted in 399,263 reads after quality filtering, and maximum likelihood placement of reads within a reference *nosZ* phylogeny was performed using the pplacer algorithm[25]. To determine the phylogenetic diversity of each *nosZ* clade within samples, all sequences were clustered at 97% nucleotide similarity, resulting in 2,129 and 10,118 OTUs for clades I and II, respectively. Representative sequences from each OTU were then used to generate an amino-acid phylogeny, from which Faith's phylogenetic diversity[26] was calculated. All sequence data were submitted to NCBI under BioProject accession number PRJNA223232, and full details on sequence processing and analysis are provided in the Supplementary Methods.

Structural equation modelling was performed using the 'lavaan' package[27] within the R statistical programming environment. For soil mineral and textural factors, we combined the measured variables that were determined to be significant for predicting soil N_2O sink capacity (Supplementary Methods and Fig. 4) into composite latent variables, allowing us to examine the joint effects of the different soil variables within each group as well as to manage model complexity[28]. Model estimation was performed using the Satorra–Bentler maximum likelihood procedure, and model fit was assessed using χ^2, root mean square error of approximation, and comparative fit indices. Co-occurrence networks of *nosZ* groups delimited by pplacer were based on strong absolute Spearman correlations ($\rho > 0.6$, false discovery rate-corrected $P < 0.01$). Additional community structure within the main network was detected using modulated modularity clustering[29]. Ranking of *nosZ* groups according to their ability to predict the N_2O sink index was determined by conditional variable importance analysis[30] based on the random forests algorithm. Full details on the structural equation modelling procedure and network analysis are provided in the Supplementary Methods.

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Author contributions

C.M.J., A.S., S.H. and L.P. designed the study, analysed the data and compiled the manuscript with the help of B.G. and P.L. Soil samples were collected by D.B., F.P.B., C.M.J., A.S. and L.P. with support from the EcoFINDERS project. Microcosm set-up and gas analysis was performed by M-C.B., D.B., F.P.B. and C.M.J., and soil DNA extractions, real-time PCR and 454 sequencing was performed by A.S., M-C.B. and D.B.

Additional information

Supplementary information is available in the online version of the paper. Reprints and permissions information is available online at www.nature.com/reprints. Correspondence and requests for materials should be addressed to S.H.

Competing financial interests

The authors declare no competing financial interests.