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Organic fertilization reduces nitrous oxide emission by altering nitrogen cycling microbial guilds favouring complete denitrification at soil aggregate scale

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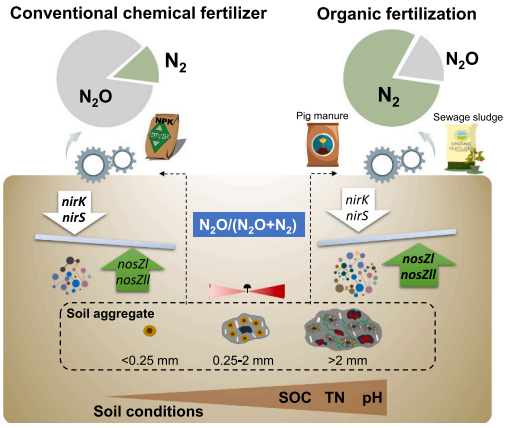
HIGHLIGHTS

- N_2O production and reduction hotspots occurred in smaller soil aggregates.
- Organic fertilization reduced N_2O emissions by promoting complete denitrification.
- Reduction is linked to a dynamic community with reduced NO_3^- to N_2O-reducer ratios.
- Emission is linked to interaction between soil condition and denitrifies selection.

GRAPHICAL ABSTRACT



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ABSTRACT

Agricultural management practices can induce changes in soil aggregation structure that alter the microbial nitrous oxide (N_2O) production and reduction processes occurring at the microscale, leading to large-scale consequences for N_2O emissions. However, the mechanistic understanding of how organic fertilization affects these context-dependent small-scale N_2O emissions and associated key nitrogen (N) cycling microbial communities is lacking. Here, denitrification gas (N_2O, N_2) and potential denitrification capacity $N_2O/(N_2O + N_2)$ were assessed by automated gas chromatography in different soil aggregates (>2 mm, 2–0.25 and <0.25 mm), while associated microbial communities were assessed by sequencing and qPCR of N_2O-producing (*nirK* and *nirS*) and reducing (*nosZ clade* I and II) genes. The results indicated that organic fertilization reduced N_2O emissions by

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enhancing the conversion of N_2O to N_2 in all aggregate sizes. Moreover, potential N_2O production and reduction hotspots occurred in smaller soil aggregates, with the degree depending on organic fertilizer type and application rate. Further, significantly higher abundance and diversity of *nosZ* clades relative to *nirK* and *nirS* revealed complete denitrification promoted through selection of denitrifying communities at microscales favouring N_2O reduction. Communities associated with high and low emission treatments form modules with specific sequence types which may be diagnostic of emission levels. Taken together, these findings suggest that organic fertilizers reduced N_2O emissions through influencing soil factors and patterns of niche partitioning between N_2O-producing and reducing communities within soil aggregates, and selection for communities that overall are more likely to consume than emit N_2O. These findings are helpful in strengthening the ability to predict N_2O emissions from agricultural soils under organic fertilization as well as contributing to the development of net-zero carbon strategies for sustainable agriculture.

1. Introduction

Acute concerns about current and future global climate change have prompted greater attention to the rise in nitrous oxide (N_2O) emissions from agricultural soils (Cui et al., 2021; Reay et al., 2012; Tian et al., 2020). N_2O is a potent and long-lived greenhouse gas with a global warming potential approximately 300 times greater than carbon dioxide (CO_2) over a 100-year time period (IPCC, 2013; Jain, 2023). The last step of denitrification is considered to be the dominant sink for the conversion of reactive nitrogen (N) to inert N_2 in agricultural ecosystems, but incomplete denitrification is the main source of N_2O emissions (Jones et al., 2022). Developing agricultural management strategies to reduce overall denitrification and/or encourage complete denitrification are important to control atmospheric N_2O concentrations.

Organic fertilization constitutes one of the core strategies for sustainable nutrient cycling that can enhance soil carbon (C) stocks and mitigate climate change, but these benefits can be offset by N_2O emissions (Duan et al., 2024; Guenet et al., 2021). Organic fertilizers exert great influence on the microbial processes involved in N_2O emissions, resulting in different patterns and magnitude of emissions than those from conventional chemical fertilizers, but are difficult to predict (Lazcano et al., 2021). N_2O emissions depend on the balance between N_2O production and reduction processes and are the outcomes of complex interactions between soil properties and the microbial communities present (Jones et al., 2014; Saggar et al., 2013). This complexity means that organic fertilizers altering the same soil property under different contexts may have opposing effects on N_2O emissions, which impedes the development of optimal management practices for organic fertilizers. For example, studies have reported that organic amendments, such as manure application, increased N_2O emissions attributed to the additional C input from organic fertilizers providing abundant energy for heterotrophic denitrifiers (Shakoor et al., 2021; Wang et al., 2023). Alternatively, other studies have shown that increases in denitrifier population sizes and potential denitrification rates do not necessarily lead to higher N_2O emissions, as increased organic C content can reduce N_2O emission by favouring the reduction of N_2O to N_2 (Curtright and Tiemann, 2023; Li et al., 2021). Organic fertilizer type and application rate may also play a crucial role in soil N_2O emissions, leading to inconsistent results (Zhang et al., 2020).

Besides the intricate mechanisms involved, organic fertilizer can induce changes in soil aggregation structure that alter the N_2O production and reduction processes occurring at the microscale. Soil aggregates, as the essential structure and functional unit of soil, have complex hierarchical structures that create spatially heterogeneous microhabitats and niches for microbial mediated processes (Hartmann and Six, 2023). Large soil aggregates are usually well-ventilated with relatively rapid turnover rates of soil organic matter enhancing the activity of aerobic microbes and potentially disfavouring complete denitrification (Luo et al., 2021). In contrast, small aggregates are characterized by stronger water holding capacity and provide a protective microenvironment for the growth of anaerobic microbes and are thus likely to favour complete denitrification (Jayarathne et al., 2021; Six et al., 2004). The changes in community gene complementation of nitrite reductase genes (*nirK* and *nirS*) or N_2O reductase genes (*nosZ* clade I and II) may result in differential effects on N_2O emissions (Jones et al., 2022). Increasing evidence indicates that niche differentiation exists between N_2O-producing and reducing communities, potentially causing imbalances between N_2O production and consumption in many environments (Assémien et al., 2019; Xu et al., 2024b). Uptake of organic amendments affects soil physicochemical properties, including the formation and the stability of soil aggregates (Situ et al., 2022; Sonsri and Watanabe, 2023). This is likely to drive differential selection and co-occurrence patterns of N_2O producing and reducing communities at a soil aggregate scale, thus affecting emissions. Although the functional and metabolic potential of N-cycling microbial communities under organic fertilization has been investigated at field, inter-zone, or larger scales (Korbel et al., 2022; Tang et al., 2022), the related scales of soil aggregates and particles have not been addressed. How organic fertilizers affect the linkages between soil aggregate and microbiome functions and their interactions with their abiotic and biotic environments have not been adequately discussed.

To address these uncertainties, an automated helium incubation system was used to directly measure the denitrification end products N_2O and N_2 under controlled microcosm conditions using field soils with a history of partial substitution of chemical fertilizers with organic forms (25% and 50% pig manure or municipal sludge). The objectives of this study were to determine the denitrification processes in different soil aggregate fractions following organic amendments, with an emphasis on N_2O production and reduction processes. Additionally, we assessed the abundance, community structure, and co-association patterns of N_2O-producing and -reducing communities, aiming to understand linkage between N_2O production and reduction processes with the relevant microbial communities. We hypothesized that 1) organic fertilizer amendments will reduce N_2O emissions by promoting complete denitrification, depending on organic fertilizer type, application rate, and soil aggregate size, and 2) the size and composition of N_2O producing and reducing communities in different aggregate fraction sizes will be altered following organic fertilizer amendments due to the spatial and nutrient heterogeneity of the aggregates.

2. Materials and methods

2.1. Site description and soil sampling

Soil samples were collected from a field experiment site in the Zhangxi catchment, Zhejiang Province, China (29°47′34″N, 121°21′48″E). This area experiences a subtropical monsoon climate with an average annual temperature of 17.4 °C and an annual precipitation of 1460 mm. Sampling took place during the 8th experimental crop rotation after harvest of an amaranth (*Amaranthus tricolor* L.) crop two years after experimental initiation (Tang et al., 2022). Prior to the start of the experimental vegetable rotation the site was cultivated using conventional chemical N fertilization and other practices with a rice-wheat rotation typical for this region. The soil is classified as Hydragric Anthrosols (Howard, 2017; WRB, 1998) and has a silty loam texture with 38.8% sand, 41.6% silt, and 19.6% clay, a pH of 5.6, 2.6% organic

C, and 0.3% total N (TN). The organic amendment treatments aimed to balance fertilization on the basis of total N added as follows: ConN (conventional chemical fertilizer as urea with an application rate of 180 kg N ha^{-1} $crop^{-1}$); M25 (25% substitution of chemical fertilizer N with pig manure); M50 (50% substitution with pig manure); S25 (25% substitution with municipal sludge) and S50 (50% substitution of chemical fertilizer with municipal sludge). All treatments with four replicates were in a randomized block design, with each plot measuring 6 m × 7 m. The pig manure was composted with 20% sawdust/rice husks, with 2.1% TN, 27.9% TC and pH 7.9 and was purchased from Ningbo Huanying Agricultural Technology Co., Ltd. The municipal sludge was composted with additional 15–20% plant ash and rice straw, with 0.5% TN, 7.0% TC and pH 8.0, and was purchased from Jiangsu Hongyang Soil Co., Ltd.

Soil was collected from 5 points within each plot to a depth of 20 cm, and the soil samples were homogenized after the removal of plant roots and stones to form a composite bulk sample of approximately 1 kg. Aggregates were isolated using the optimal-moisture sieving approach according to the procedure modified from Kristiansen et al. (2006) and Dorodnikov et al. (2009). The rationale for choosing this method was because it was considered to be gentler than conventional wet/dry sieving techniques, with minimal mechanical effects on the biological properties of the aggregate fractions (Kristiansen et al., 2006; Li et al., 2019). Field moist samples were spread into a thin layer in a ventilation box (room temperature 22 °C) and, when they reached the desired condition, gently broken apart along the natural weakness points and crumbled to <8 mm. The soils were then separated using a set of sterilised sieves with mesh openings ranging between 2 mm and 0.25 mm by shaking for 1 min at approximately 200 rpm on a sieve shaker (CSI Scientific, Easton, PA). The distribution of aggregate fractions was determined by combining aliquot weights. Three size classes were used in this study: large macro-aggregates (LM) (>2 mm), small macro-aggregates (SM) (0.25–2 mm) and micro-aggregates (MI) (<0.25 mm).

2.2. Soil incubation under anoxic conditions

The soil incubations were performed in triplicate. Twenty grams of soil (dry weight equivalent) from each aggregate size class was transferred to 120-ml serum flasks and pre-incubated for one week in the dark under aerobic conditions at 45% water filled pore space (WFPS) and 25 °C constant temperature to allow recovery of normal activity. Flasks were then adjusted to 85% WFPS and sealed with air-tight, butyl-rubber septa and aluminium crimp caps. Displacement of N_2 in the serum flasks was achieved by evacuating (0.1 kPa) and refilling with 99.999% helium. After five repeated rinses, the pressure inside the flasks was adjusted to 101.3 kPa. All samples were pre-incubated at 25 °C for 24 h to stabilize the serum flask headspace system. An automatic helium incubation system was used to monitor the headspace of gases (N_2O, N_2) at an 8-h sampling interval (Molstad et al., 2007). After each sampling, an equal volume of ultrapure helium was automatically injected to maintain constant pressure inside the vials. The N_2O production index (IN_2O) is based on the concentration changes in the headspace using the area under the N_2O curve divided by the area of the $N_2 + N_2O$ curve (Qu et al., 2014), using the following formula:

$$IN2O = \int_0^T N2O(t)dt \Big/ \int_0^T [N2O(t) + N2(t)]dt$$

At the end of incubation, soil ammonium (NH_4^+-N) and nitrate (NO_3^--N) was determined following 2 M KCl extraction with a Discrete Auto Chemistry Analyzer (Westco Smartchem 200, Rome, Italy). Soil pH was measured using a pH meter (PHS − 3C, Shanghai, China) from slurries of 5:1 deionized water to soil. Dissolved soil organic C (DOC) was measured in CO_2 free distilled water extracts after filtering through ash-free cellulose filters on a TOC/TN analyzer (Multi NC 3100, JENA, Germany). Soil organic C (SOC) was measured following the H_2SO_4-$K_2Cr_2O_7$ digestion method (Nelson and Sommers, 1996), and TN was determined using a Vario MAX CNS elemental analyzer (Elementar, Germany).

2.3. Quantitative PCR and amplicon sequencing

Total DNA was extracted from the incubated soils (0.5 g) using FastDNA Spin Kits (MP Biomedicals, CA, USA) with the addition of a mutated template to allow relative real-time quantitative PCR (Daniell et al., 2012). The quantity and quality of DNA were determined by NanoDrop 2000 spectrophotometry (Thermo Fisher Scientific, USA). Relative real-time PCR and Illumina amplicon sequencing were used to assess changes in community size and structure for the general bacterial gene 16S rRNA and functional genes involved in denitrification pathways (*nir*: *nirK* and *nirS*, *nos*: *nosZ* clade I and II). Quantitative PCR was performed using the SYBR-Green detection chemistry in a QuantStudio™3 Real Time PCR System (Thermo Fisher Scientific Inc., Waltham, MA, USA) PCR conditions are shown in Table S1. Negative controls (sterile water) were included in each amplification run. Standard curves were obtained using linearized plasmid with target insert of known copy number and serially diluted in Tris-EDTA buffer solution. Correlation coefficients (R^2) were > 0.99 and the amplification efficiencies ranged between 90% and 110%.

Amplification for Illumina sequencing was performed in 20 μL reactions containing 1 unit of TransStart® FastPfu DNA Polymerase 1× FastPfu Buffer, 0.25 mM dNTPs (TransGen Biotech, Bejing, China). 2 μM of each primer and 10 ng of template DNA. Detailed reaction conditions are described in Table S3. Barcoding and sequencing were performed on an Illumina MiSeq platform, v3, 2 × 300 bp (Majorbio Company in Shanghai, China). Raw sequence data were demultiplexed, and barcodes and primers were removed using cutadapt (version 1.8.1).

Bioinformatic analysis of the sequencing data was processed with QIIME2 (version 2020.11). Quality control, chimera removal, trimming and sequence pairing were performed using the DADA2 pipeline (Callahan et al., 2016). *NosZ* clade I and II were assessed as single end reads as PCR fragments of ca. 700 bp reads could not be merged; the reverse read was used due to higher average phred scores. Paired-end reads of 16S rRNA, *nirS* and *nirK* gene sequences were rarefied to 15578, 3288, 1169 sequences per sample, respectively; *nosZ* clade I and II were rarefied to 5927 and 7528 sequences per sample, respectively. Both alpha-diversity and beta-diversity were calculated using QIIME2. Representative sequences of 16S rRNA genes were used for taxonomic assignment based on the SILVA v132 database at 99% similarity by training a Naïve Bayes classifier with the QIIME2 q2-feature-classifier plugin. Representative sequences of functional genes were identified based on the FunGene database using the Ribosomal Database Project (Cole et al., 2014; Fish et al., 2013). BLAST was used to search the non-redundant nucleotide database in the NCBI to further identity representative sequences.

2.4. Statistical analyses

All statistical analyses were conducted in R v.4.2.1. The differences in soil properties and the abundance and diversity of studied genes were assessed by two-way ANOVAs to test the main and interactive effects of fertilizer treatments and soil aggregates. Post-hoc tests were performed using least square means and Tukey's multiple comparisons adjustment using the *lsmeans* package (Lenth, 2017), with statistical significance tested at $p < 0.05$. Main effects and interactions of fertilizer, aggregate size and sampling time on gas emissions were analyzed by three-way repeated measures ANOVAs. Bonferroni post-hoc tests were conducted when a significant main effect was present to identify where the differences occurred. Bacterial and functional gene community structures were assessed by calculating weighted UniFrac distance matrices and then ordinated using principal coordinates analysis (PCoA). PERMANOVA (999 permutations) was conducted using the *adonis* function in

vegan. Pearson correlation analysis was conducted to test the correlations of soil properties, abundance and diversity of microbial communities, denitrification gaseous emission and $N_2O/(N_2O + N_2)$ product ratios. Random forest analysis was employed to identify the dominant predictor for $N_2O/(N_2O + N_2)$ product ratios when accounting for microbial attributes and soil properties using the *Boruta* package (Kursa and Rudnicki, 2010).

Network analysis was performed to investigate the co-occurrence of communities based on nitrite and N_2O reductase data to identify the main ecological clusters of strongly associated sequence types. *NirK* and *nirS,* and *nosZ* clade I and II ASVs were merged to form *nir* and *nos* tables, and the dominant ASVs (relative abundance >0.05%) selected to build co-occurrence networks. Links that strongly co-occurred (Spearman's correlation coefficient >0.70) and significant (p value <0.05) were considered. The correlation between Z score normalised relative abundance of each module (ecological cluster) and $N_2O/(N_2O + N_2)$ product ratios were tested. The main ecological clusters (modules) of the co-occurrence networks were visualized with Gephi (Bastian et al., 2009).

3. Results

3.1. Effects of organic amendments on soil properties

Organic fertilization significantly altered the mass distribution of the soil aggregates (Fig. S1a). Compared to ConN, the mass proportion of large macro-aggregates (>2 mm) in M50 and S50 was significantly increased by 31.5% and 40.2%, while that of micro-aggregates (<0.25 mm) significantly decreased by 25.7% and 33.9%, respectively. The proportion of small macro-aggregates (0.25–2 mm) showed no clear trend across treatments. There were significant effects of fertilizer treatment, aggregate size and in some cases their interaction on soil physicochemical properties (Fig. 1, Table S1). Soil pH was increased significantly by 14%–43% under organic fertilization, with micro-aggregates showing a small but significant increase over large macro-aggregates. The SOC and TN contents were significantly increased by organic fertilizers, with increased application rates showing larger effects. Furthermore, SOC and TN increased significantly with decreasing aggregate size (Fig. 1). DOC (except for M25) was significantly affected by fertilization, but not by aggregate size. NO_3^--N levels fell significantly over the course of the incubation, whilst NH_4^+-N was relatively stable (Fig. S1).

3.2. Characteristics of N_2O and N_2 emissions

Across the incubation period, N_2O emissions increased rapidly over time in all aggregate fractions and basically leveled off by the end of incubation, except for S50, which remained at constantly low levels (below 160 nmol N g^{-1} soil) (Fig. S2a). N_2O emissions in S50 showed either a minor increase, remained level or even decreased over time. Cumulative N_2O emissions were highly variable among fertilizer treatments ($p < 0.001$) and aggregate fractions ($p < 0.001$) (Fig. 2a, Table S1). Emissions were significantly reduced under organic fertilization, with reductions of 16%–20% for pig manure treatments, 38%–94% for municipal sludge, and a near total removal of N_2O emissions in S50 (Fig. 2a). Emissions also decreased with increasing aggregate size, with this affected by a significant interaction with higher emissions from microaggregates compared to large macroaggregates in M25 and S25 (Fig. S2d).

N_2 emissions increased linearly over time in all treatments, with the patterns varying by fertilization treatment and aggregate size (Fig. S2b). The emission rates of ConN ranged from 1.78 to 3.52 nmol N g^{-1} h^{-1}, which were clearly lower than those of the organic fertilizer treatments, with 5.56–9.03 nmol N g^{-1} h^{-1} for the municipal sludge treatments and 1.92–4.28 nmol N g^{-1} h^{-1} for the pig manure treatments. Cumulative N_2 emissions were significantly increased by organic fertilizers compared with ConN, with the largest increases of 78%–654% in large macro-aggregates, followed by 36%–378% in small macro-aggregates and 12%–252% in micro-aggregates (Fig. 2b). The significant interaction observed was due to the greater separation of all aggregate size classes in the municipal sludge treatments in contrast to pig manure and ConN treatments and differences over time, with this separation significant between treatments (Fig. S2b).

The cumulated production of $N_2O + N_2$ increased significantly with decreasing aggregate size, although no consistent pattern was observed

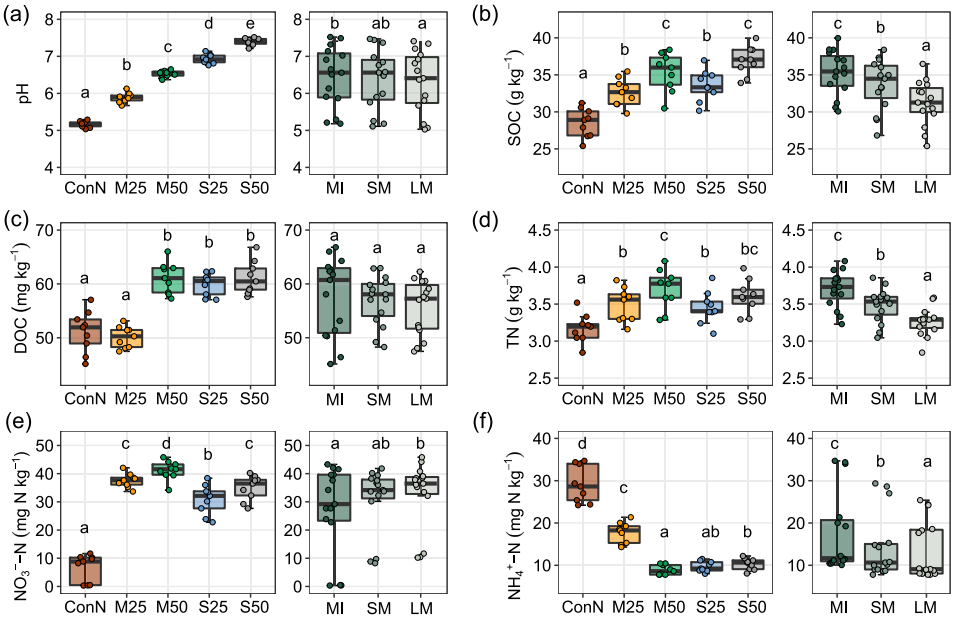

Fig. 1. Soil properties under the influences of fertilization treatments and soil aggregate sizes. pH (a), soil organic carbon, SOC (b), soil dissolved organic carbon, DOC (c), total nitrogen, TN (d), nitrate, NO_3^--N (e), ammonium, NH_4^+-N (f). ConN: conventional fertilization, M25: 25% of total N as composted pig manure, M50: 50% of total N as composted pig manure, S25: 25% of total N as composted sewage sludge, S50: 50% of total N as composted sewage sludge. MI: microaggregates (<0.25 mm), SM: small macro-aggregates (0.25–2 mm), LM: large macro-aggregates (>2 mm). Values not represented by the same letter differ significantly at $P <$ 0.05 by two-way ANOVA and Tukey's post hoc test. Data shows means and standard errors.

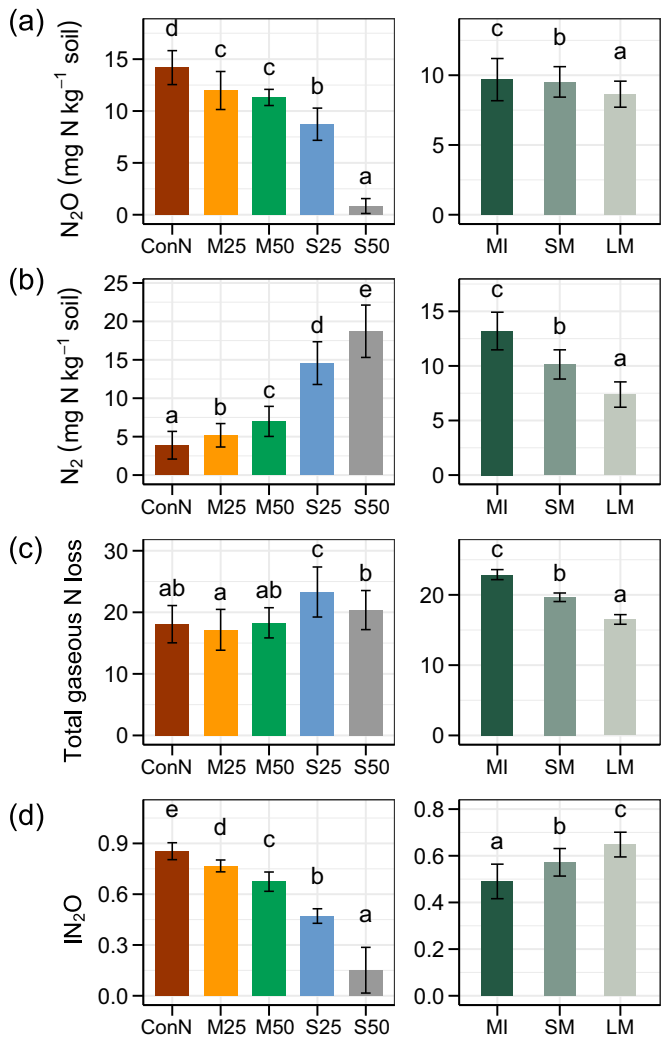

Fig. 2. Cumulative N_2O (a), N_2 (b) emissions, total gaseous N loss ($N_2O + N_2$) (c) and denitrification N_2O production index (IN_2O) (d) in soil samples from different fertilizer treatments and soil aggregate sizes after 72 h incubation. Values denoted by different letters differ significantly by two-way ANOVA and Tukey's post hoc test. Data shows means and standard errors. Fertilizer treatment and soil aggregate abbreviations are as described in Fig. 1.

between fertilizer treatments (Fig. 2c). The $N_2O/(N_2O + N_2)$ product ratio decreased with incubation period and the pattern varied with fertilization treatment and aggregate size (Fig. S2c). The $N_2O/(N_2O + N_2)$ product ratio of large macro-aggregates in ConN remained at the highest level (>0.87) throughout the incubation, and followed by those of M25, M50, S25 and S50. The N_2O production index (IN_2O) was significantly reduced by 10%–21% for pig manure treatments and 45%–82% for municipal sludge treatments compared to ConN, with increased application ratios showing larger effects (Fig. 2d). IN_2O increased with aggregate size, with the interaction driven by more pronounced decreases in smaller classes in S50 than other treatments (Fig. S2g).

3.3. The abundance of general bacteria, N_2O-producers and N_2O-reducers

All target genes showed higher abundance in smaller aggregate classes (Fig. 3). The abundance of bacterial 16S rRNA and functional genes involved in N_2O reduction (*nos*: *nosZ* clades I and II) were significantly increased by 32%–57%, 39%–81% and 17%–34%, respectively, under organic fertilizer treatments, with increases more pronounced in municipal sludge treatments than for pig manure,

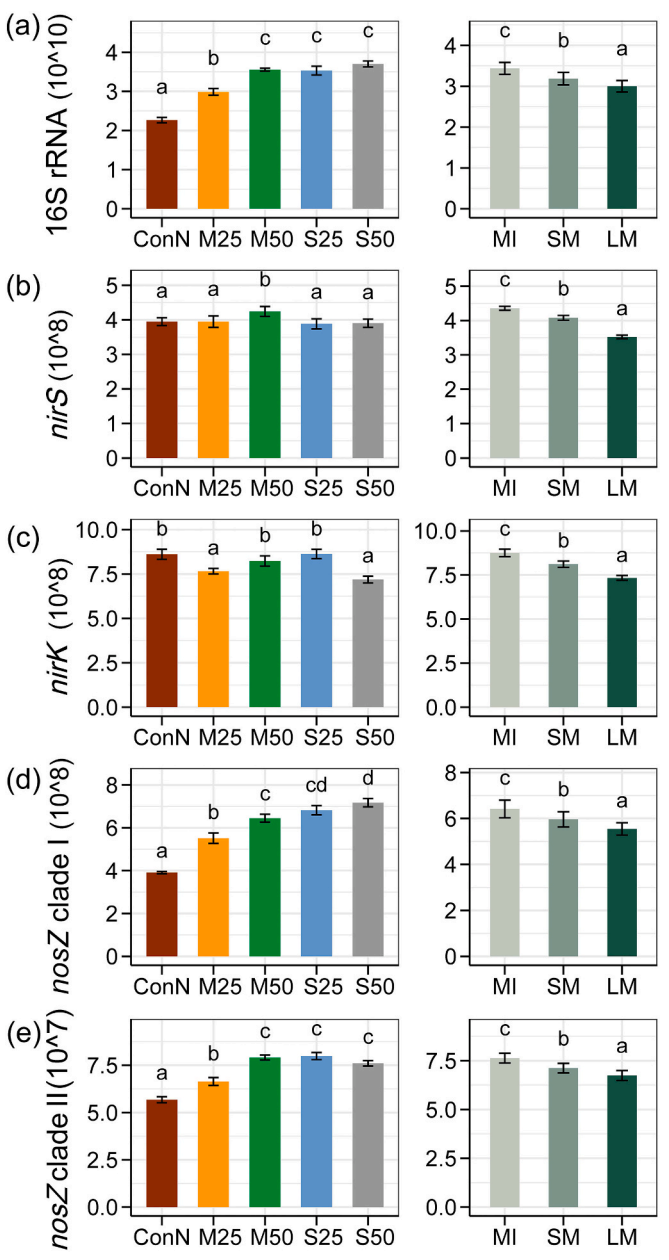

Fig. 3. The abundance of bacterial and denitrifying genes under the main effect of fertilizer treatment and soil aggregate size. 16S rRNA (a), *nirS* (b) and *nirK* genes (c) encoding for nitrite reductases in denitrifiers, and *nosZ* clade I (d) and *nosZ* clade II (e) encoding for N_2O reductases (copies g^{-1} dry weight soil). Different letters indicate significant differences, $P < 0.05$. Data shows means and standard errors. Abbreviations are as described in Fig. 1.

especially in smaller aggregates (Fig. 3a, d and e). In contrast, differences in functional genes involved in N_2O production (*nir*: *nirK* and *nirS*), although significant, were relatively minor and showed no consistent pattern (Fig. 3b and c). The *nir*/16S rRNA gene ratio decreased significantly with organic amendment, and varied between treatment levels, indicating that a lower proportion of the community was capable of denitrification after organic amendment (Fig. S3a). In contrast, the proportion of the population with *nosZ* clade I increased with amendment although the shift with pig manure treatments were not significant (Fig. S3b). The relationship between *nir* and *nosZ* clade I and II gene abundance showed a significant reduction in ratio between ConN and amended treatments, with this effect greater in the municipal sludge than pig manure treatments (Fig. S3d and g). There were increased

ratios in the smaller and micro-aggregates than the large macro-aggregates in both ratios, with this effect driven by the interaction for clade I, with the aggregate effect only observed in ConN (Fig. S3f and h).

3.4. Diversity and composition of general bacterial and N_2O producing and reducing communities

Bacterial community diversity (richness, Shannon index and phylogenetic diversity) were increased in organic amended treatments ($p < 0.001$) (Fig. S4a, Table S1). The functional genes responded differently to the fertilizer treatments. The diversity of *nirK* showed decreases in M25 compared to other treatments; whilst *nirS* and *nosZ* clade I and II generally showed increases with organic fertilizer treatments, with pig manure treatments often intermediate (Fig. S4 b-e). Where *nosZ* clade I and II diversity values varied with aggregate, they were typically higher in smaller aggregates with municipal sludge treatments driving the significant interactions observed for *nosZ* clade II (Fig. S5c, d and f).

Variations in all community structures were significantly explained by both fertilizer treatment and aggregate size, with significant interactions for these variables only for the general bacterial and *nirS* communities (Fig. 4). The community structure of *nosZ* clades I and II showed a similar pattern to that of general bacteria, with PC1 and PC2 together explaining 66.4%, 56.5%, and 61.0% of the variation, respectively (Fig. 4a, d and e). ConN and M25 samples were distinct clusters from the other amended samples in PC1. PC2 showed clear separation between ConN and M25 samples, and between M50 and S50 samples. Large macro-aggregates were obviously separated from other aggregates in ConN samples with other treatments showing unsystematic responses to aggregate size explaining the interaction observed with the PERMANOVA (Fig. 4a). *NirS* showed a clear separation between ConN, M25, M50 and municipal sludge samples in PC1 and clear separation for aggregate in PC2 for the ConN samples which are less distinct in the amended treatments potentially explaining the interaction observed in the PERMANOVA analysis (Fig. 4b). *NirK* also showed weaker clustering

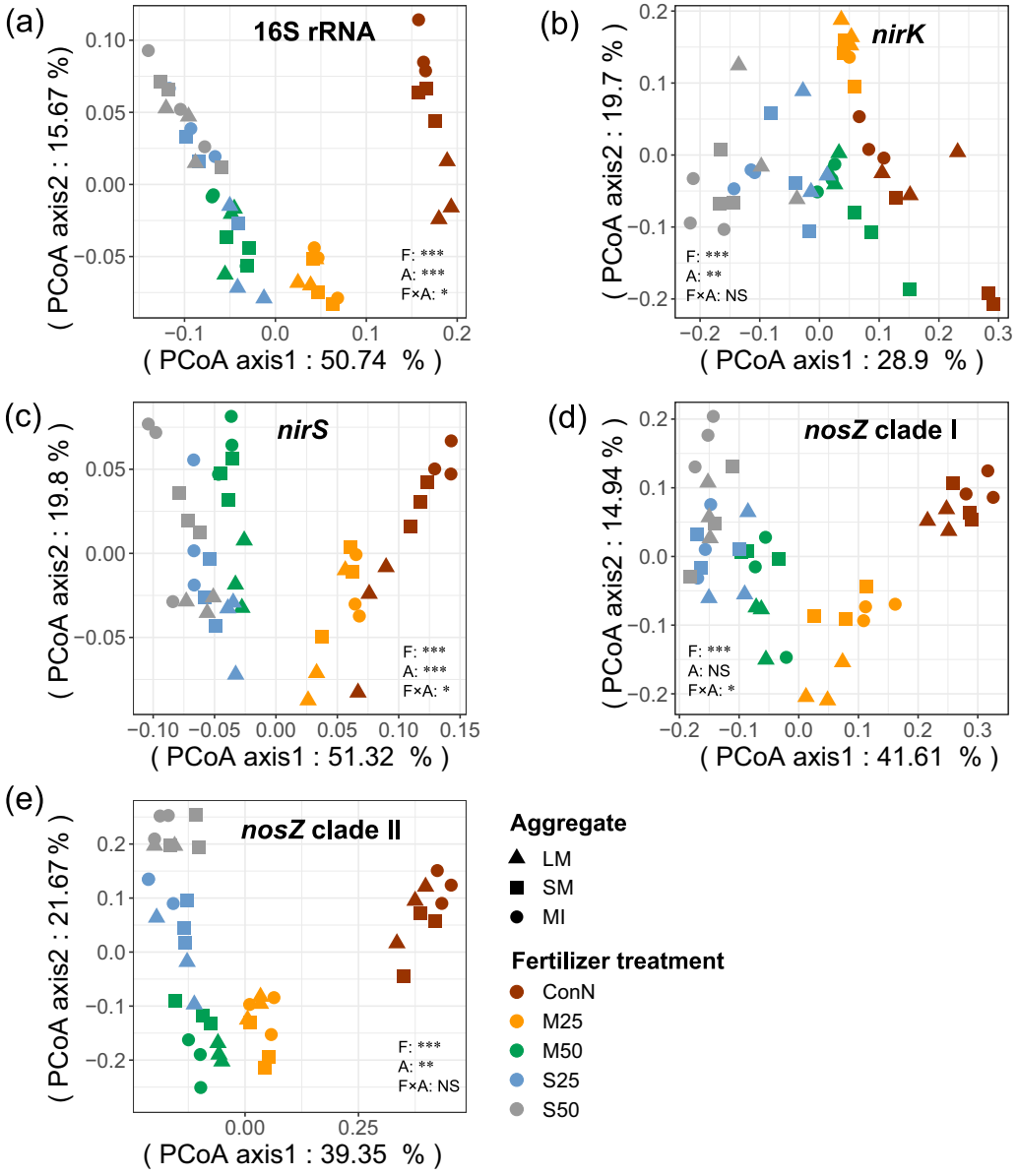

Fig. 4. Assessment of general bacterial and denitrifying community structures in different soil aggregate sizes following different fertilizer regimes. General bacterial 16S rRNA gene (a), and the functional genes associated with nitrite reduction and N_2O reduction processes, including: *nirS* (b) and *nirK* genes (c) encoding for nitrite reductases in denitrifiers and *nosZ* clade I (d) and *nosZ* clade II (e) encoding for N_2O reductases. Different colours represent different fertilization treatments, and different symbols indicate different aggregate classes. Abbreviations are as described in Fig. 1.

of fertilizer treatments explaining a lower proportion of the variation in the first dimension and no clear separation of aggregates in either of the first two dimensions (Fig. 4c).

3.5. Co-occurrence networks of N_2O-producing and reducing communities

Four ecological clusters were observed for both *nir* and *nos* ASVs (Fig. 5a and 6a). The relative abundance of module 1 ASVs strongly increased in organic amended treatments and negatively correlated with $N_2O/(N_2O + N_2)$ product ratios (Fig. 5b and 6b). In contrast, the relative abundance of module 2 ASVs was reduced by organic amendment, particularly under sewage sludge additions, and positively correlated with $N_2O/(N_2O + N_2)$ (Fig. 5c and 6c). The other two modules showed no correlation with $N_2O/(N_2O + N_2)$ (data not shown). Although substantial portions of sequences most closely matched those from uncultured bacteria in the sequence database, among those that could be identified in module 1, *Pseudomonas* and *Rhodanobacter*, and *Bradyrhizobium* and *Aquibium* were identified as the dominant species of *nirS*- and *nirK*-harbouring bacteria respectively. In module 2, *Rhizobacter* and *Rhodopseudomonas* were the dominant species of *nirS*- and *nirK*-harbouring bacteria respectively (Fig. 5d, e). Among the identifiable ASVs, *nosZ* I-harbouring *Sinorhizobium* and *Bradyrhizobiaceae* and *nosZ* II-harbouring *Bacteroidetes* and *Gemmatimonadetes* were dominant in module 1. *Frateuria, Thiothrix* and *Azospirillum* harbouring *nosZ* I, and *Chryseolinea* and *Gemmatirosa* harbouring *nosZ* II species were highly prevalent in module 2 (Fig. 6d, e). Phylogenetic analysis of sequences demonstrated strong clustering of module 1 and 2 ASVs associated with high and low emissions for each gene with, for example, distinct clusters of *nosZ* II harbouring *Gemmatimonadetes* and *nosZ* I containing *Rhizobiaceae* associated with both module 1 and 2 (Fig. S6).

3.6. The critical factor that determines N_2O production and consumption

Variable importance analysis based on random forest modelling showed that several factors are significantly linked to variation in emission with soil pH the best predictor for $N_2O/(N_2O + N_2)$, followed by the abundance ratios of *nir/nos* and *nosZ* clade I (Fig. 7). N_2O, IN_2O and $N_2O/(N_2O + N_2)$ strongly and positively correlated with abundance ratios of *nir/nos*, and negatively correlated to pH, SOC, DOC, the abundance and phylogenetic diversity of 16S rRNA, *nosZ* clade I and II, while the opposite was true for N_2 (Fig. S7).

4. Discussion

4.1. Soil aggregate size affected potential N_2O emissions and denitrification

Soil N_2O emissions are characterized by high spatial and temporal variability (Tian et al., 2020). Spatially heterogeneous habitats in soil aggregates can shape the distribution of microbes involved in N-cycling, resulting in the occurrence of hotspots and hot moments of N_2O production and consumption (Zhang et al., 2024). In this study, the hotspots for potential N_2O emissions were found to occur at the smallest fraction classes (<0.25 mm). In line with previous studies, increased N_2O emission was higher in smaller soil aggregates than in larger aggregates (Jayarathne et al., 2021; Li et al., 2020). This result indicates that the microbial capacity to produce or consume N_2O is not distributed randomly across either taxonomic groups or environments. Soil

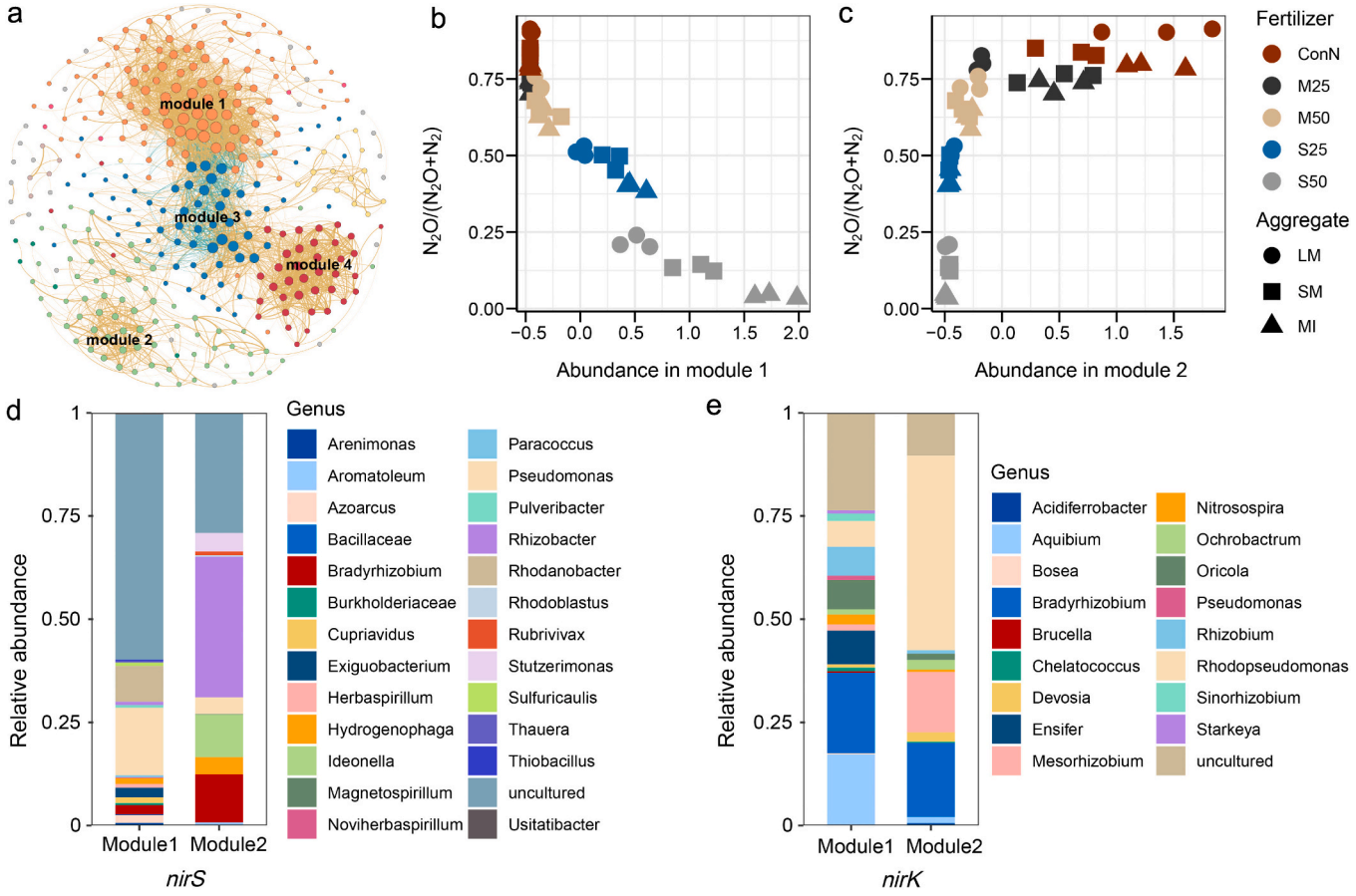

Fig. 5. The co-occurrence network of N_2O-producing (*nir* gene-based) communities. Network diagram with nodes coloured according to each of the main ecological clusters (a). Regressions between the denitrification end-product ratio $N_2O/(N_2O + N_2)$ and the *Z*-score normalised relative abundance of the main ecological clusters (b-c). Relative abundance of the dominant species in modules 1 and 2 separated for *nirS* (d) and *nirK* (e). Abbreviations are as described in Fig. 1.

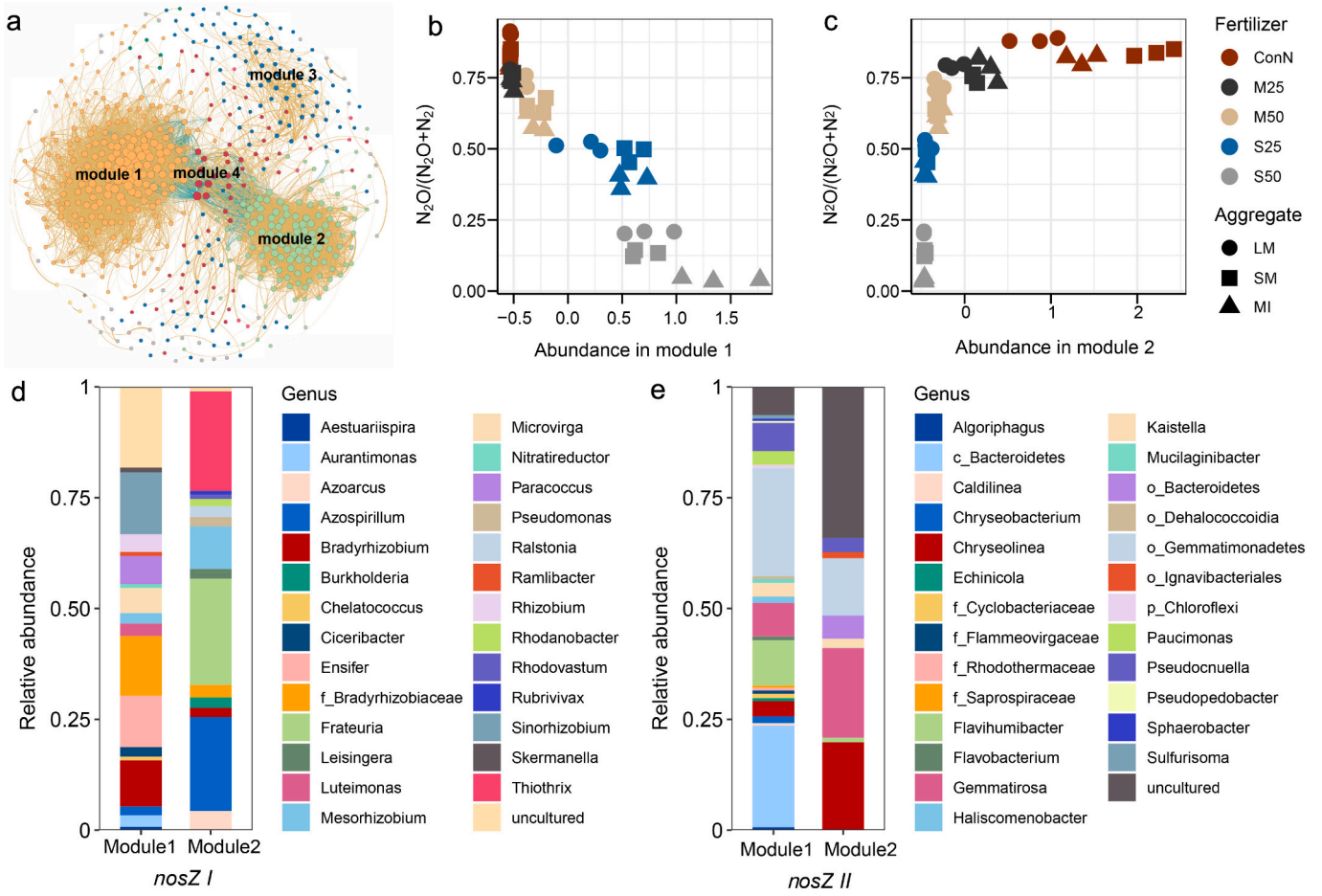

Fig. 6. The co-occurrence network of N_2O-reduing (*nos* gene-based) communities. Network diagram with nodes coloured according to each of the main ecological clusters (a). Regressions between the denitrification end-product ratio $N_2O/(N_2O + N_2)$ and the Z-score normalised relative abundance of the main ecological clusters (b, c). Relative abundance of the dominant species in modules 1 and 2 separated for *nosZ* clade I (d) and *nosZ* clade II (e).

aggregates represent the scale of the key functions of the soil microbiome in agroecosystems, determining both resource availability in their habitats and the bio-interactions that drive community dynamics and assembly (Hartmann and Six, 2023). The differences in emissions among aggregate fractions can be attributed to differences in nutrient resources, physicochemical conditions and microbial communities in these aggregate fractions (Zhang et al., 2021). Finer aggregates are considered to be more favourable for faster microbial growth due to increased surface accessibility of organic compounds directly available to microbes and extracellular enzymes (Mo et al., 2021; Trivedi et al., 2015). Furthermore, compared to macro-aggregates, micro-aggregates are enriched with more organic mineral complexes dominated by low molecular weight and N-rich compounds (Daly et al., 2021). Given its low C/N ratio, it has been demonstrated that in cultivated soils, the N in organic mineral complexes can be more easily mineralized into forms that are available to microbes and plants or lost as N-gas (Jilling et al., 2020). In this study, soil nutrients (SOC, DOC and TN) were negatively correlated with soil aggregate size under different fertilization treatments. The excess electron donors provided by organic C is preferentially beneficial to the last step of denitrification, as soil N_2O-reducing bacteria might be induced to use N_2O as a terminal electron acceptor (Pan et al., 2013).

4.2. *Organic fertilization regulated the denitrification end-product ratio at soil aggregate scale*

Organic fertilizer amendments are key determinants of soil structure formation, and can enhance soil aggregation and porosity, thereby affecting context-dependent processes at small scales (Fan et al., 2023; Loaiza Puerta et al., 2018). N_2O emissions were significantly decreased in the organic fertilizer treatments at all aggregate size classes, with the effect being more pronounced at higher application rates and in larger aggregates. This is consistent with previous findings that substituting organic fertilizers for chemical fertilizers decreased N_2O emissions by promoting complete denitrification with further reduction of N_2O to N_2 (Xu et al., 2024a). In contrast, other studies indicated that organic fertilizers emitted more N_2O by stimulating denitrification, due to the increased availability of organic C compounds in the soil providing energy sources for denitrifiers (Saha et al., 2021). These studies for different organic fertilizer characteristics, soil properties and climatic conditions may have led to different conclusions about the magnitude and pattern of N_2O emissions from organic fertilization. In this study, although organic fertilization did not significantly affect denitrification total N emissions, the ratio of denitrification end-products was significantly changed in all aggregate classes. N_2 emissions increased significantly by organic fertilizers compared to conventional chemical fertilizers, with the largest increases occurring in large macroaggregates, whereas IN_2O and $N_2O/(N_2O + N_2)$ were significantly decreased, with municipal sludge treatments being more effective than pig manure (Fig. 2). These indicate an effective reduction of N_2O during subsequent denitrification steps, which may include the following two aspects. First, the increase in pH was more pronounced in soils treated with municipal sludge than in pig manure. Soil pH is a strong edaphic variable controlling denitrification end-product ratios through enzyme sensitivity (Cao et al., 2021; Deveautour et al., 2022). N_2O reductase

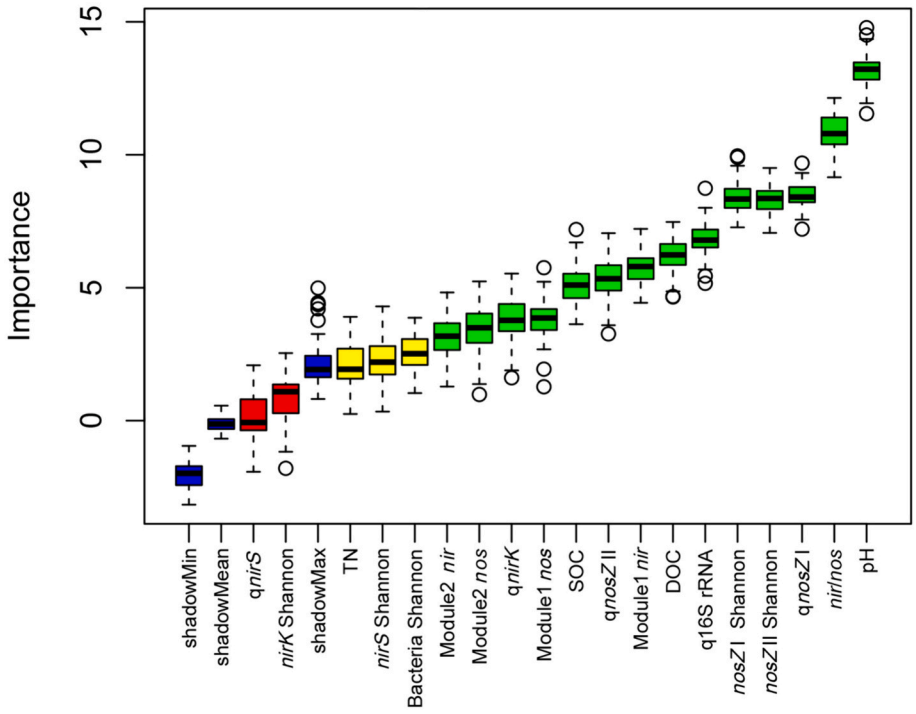

Fig. 7. Relative importance of input variables for predicting $N_2O/(N_2O + N_2)$ product ratio, as measured by performing feature selection. The variables are sorted in decreasing order of the importance value. Boxplots show mean decrease in accuracy across 100 individual runs. Blue boxplots correspond to minimal, average and maximum Z score of a shadow attribute. Red, yellow and green boxplots represent Z scores of rejected, tentative and confirmed attributes respectively. *nir/nos*: gene abundance ratio of (*nirK* + *nirS*)/(*nosZ* clade I + II); q16S rRNA, q*nirS*, q*nirK*, q*nosZ*I and q*nosZ*II: gene abundance of general bacterial 16S rRNA, *nirS* and *nirK* (encoding the nitrite reductase in denitrifiers), and *nosZ* clade I and II (coding for the clade I and II-type N_2O reductase), respectively; Bacteria, *nirS*, *nirK*, *nosZ*I and *nosZ*II Shannon: Shannon index of bacterial 16S rRNA, *nirS*, *nirK*, *nosZ* clade I and II genes; module 1 and 2 *nir*, module 1 and 2 *nos*, representing the relative abundance of their network modules, respectively.

synthesis and assembly are severely impaired under acidic conditions, resulting in N_2O being the main product of denitrification (Blum et al., 2018; Liu et al., 2010). Microbes possessing N_2O reductases (*nosZ*) are the only known biotic environmental sink of N_2O, and their diversity has been shown to be strongly linked to N_2O release from soils (Bahram et al., 2022; Thomson et al., 2012). In support of this, the abundance and diversity of N_2O-reducers (containing *nosZ* clade I or II) were significantly promoted by organic amendments relative to N_2O-producers (containing *nirS* or *nirK*), and were more pronounced in municipal sludge than in pig manure. Second, higher SOC, TN and DOC concentrations and lower NO_3^--N content in the municipal sludge treatments potentially modulated the $N_2O/(N_2O + N_2)$ product ratio of denitrification. The availability of substrate C and N in the soil is essential reflects the electron donor/acceptor ratio in the heterotrophic denitrification process (Pan et al., 2013). It was found that when the supply of C source was adequate, i.e., sufficient electron donors, more complete denitrification process could be proceeded and more products were emitted as N_2 (Fu et al., 2022).

4.3. Soil key N cycling microbial consortiums at the soil aggregate scale

Exploring the diagnostic taxa that drive either complete or truncated denitrification pathways is critical for the identification of agronomic treatments favouring these consortia and reducing N_2O emissions. Co-occurrence network analysis has been widely used to explore potential interactions among complex microbial communities, reveal niche structure, and yield insights into microbiome functioning (Gao et al., 2022). Using this approach, potential interactions between N_2O-producing and reducing communities were further analyzed by building co-occurrence networks incorporating the detected dominant phylotypes. We identified large differences between fertilizer treatments and aggregate fractions in the relative abundance of four ecological clusters, with fertilizer treatments exerting a stronger influence than aggregate fraction. By altering the environmental constraints that define shared niche preferences among species, changes in fertilization practice have been indicated to drive the formation of consortia that can either synergistically or individually alter the fate of N (De Corato et al., 2024; Kumawat et al., 2022). In both the *nir* and *nos* networks two clusters of co-occurring ASVs associated with high and low emissions were identified (Figs. 5 and 6), suggesting that selection of distinct communities was driven by change in fertilization application occurring within a two-year period. Microbial communities with different genetic potentials are subjected to ecological processes to assemble into various functional modules, thus affecting their function (Hallin et al., 2018; Romdhane et al., 2022).

These clusters consisted of a diverse range of bacterial species, which can be regarded as sub-communities associated by shared niche space and putative biotic interactions (Ma et al., 2020). These clusters suggest that different groups of organisms are selected and are diagnostic of high and low emission communities. These included groups within the same taxonomic level including phylum (e.g., *Gemmatimonadetes* and *Bacteroidetes* for *nosZ* clade II) or genus (e.g., *Bradyrhizobium* for *nirK*). These findings are consistent with the assumption that similarity of niche preference among phylogenetically different but functionally equivalent organisms, drives assembly of consortia which work cooperatively (Hallin et al., 2018; Han et al., 2020). Notably, many of the bacteria in modules of *nir* and *nos* gene-based networks that correlate with N_2O reduction are known to be important in complete denitrification including *Pseudomonas* and *Rhizobium* (Pishgar et al., 2019; Zhang et al., 2022), although the phylogenetics suggests that other closely related species are neutral or associated with high emission. It can be inferred that the form of fertilization applied in this study drove a separation in community assembly to form modules associated with the fertilizer type applied and the level of emissions. This is consistent with the changing

ratios of N_2O producing and consuming genes, suggesting a selection for communities that overall are more likely to consume than emit N_2O, forming distinct co-occurrence clusters in the network analysis.

5. Conclusion

Our study provided experimental evidence at the soil aggregate level that partial substitution of chemical fertilizers with pig manure and municipal sludge decreased soil N_2O emissions by favouring complete denitrification (conversion of N_2O to N_2). Moreover, municipal sludge treatments were more effective in reducing N_2O emissions than pig manure. We attributed the increased N_2 emissions and decreased denitrification end-product ratio $N_2O/(N_2O + N_2)$ from smaller aggregates under organic fertilizer treatments to altered soil properties and development of microbial communities with higher genetic potential to reduce N_2O relative to producing it. We confirmed that organic amendments are a promising option for mitigating N_2O emissions by manipulating the balance of N_2O producing and reducing communities, and their activity, at microscale in soil aggregates. These results provide new understanding of the mechanisms of N_2O emissions from soils under organic fertilization, thus potentially offering opportunities to reduce emissions. Future research to bridge micro- and field- scales are needed to inform agricultural management decision-making to develop net-zero carbon strategies, and to utilize soil microbiota as a nature-based sustainable agricultural solution.

CRediT authorship contribution statement

Quan Tang: Writing – review & editing, Writing – original draft, Visualization, Funding acquisition, Data curation, Conceptualization. **Sara Moeskjær:** Writing – review & editing, Software, Formal analysis, Data curation. **Anne Cotton:** Writing – review & editing, Visualization, Methodology, Investigation, Data curation. **Wenxia Dai:** Visualization, Software, Investigation. **Xiaozhi Wang:** Writing – review & editing, Funding acquisition, Formal analysis. **Xiaoyuan Yan:** Writing – review & editing, Supervision, Formal analysis, Conceptualization. **Tim J. Daniell:** Writing – review & editing, Visualization, Supervision, Software, Methodology, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

The sequencing data used in this study have been submitted to the Genome Sequence Archive in the BIG Data Center (http://bigd.big.ac.cn/gsa) under accession number CRA005713.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2024.174178.

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