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Using adaptive and aggressive N_2O-reducing bacteria to augment digestate fertilizer for mitigating N_2O emissions from agricultural soils

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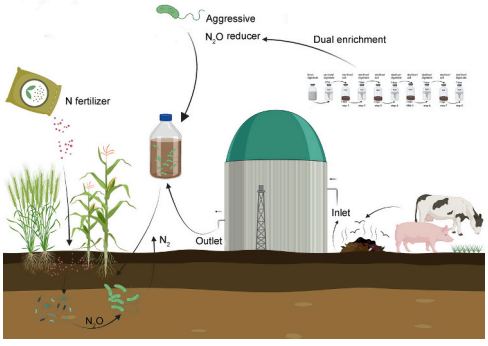
HIGHLIGHTS

- Diverse N_2O-reducing bacteria exist in the anaerobic digestate.
- Dual enrichment can enrich the digestate-soil generalists.
- Adaptive and aggressive N_2O-reducing bacteria were enriched and isolated.
- *nosZ* clade II isolates are more promising for mitigating N_2O emissions.
- The N_2O-reducing bacteria augmented digestate can reduce N_2O emissions from soil.

GRAPHICAL ABSTRACT



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ABSTRACT

Nitrous oxide (N_2O) emitted from agricultural soils destroys stratospheric ozone and contributes to global warming. A promising approach to reduce emissions is fertilizing the soil using organic wastes augmented by non-denitrifying N_2O-reducing bacteria (NNRB). To realize this potential, we need a suite of NNRB strains that fulfill several criteria: efficient reduction of N_2O, ability to grow in organic waste, and ability to survive in farmland soil. In this study, we enriched such organisms by sequential anaerobic batch incubations with N_2O and reciprocating inoculation between the sterilized substrates of anaerobic manure digestate and soils. 16S rDNA amplicon sequencing and metagenomics analysis showed that a cluster of bacteria containing *nosZ* genes encoding N_2O-reductase, was enriched during the incubation process. Strains of several dominant members were then isolated and characterized, and three of them were found to harbor the *nosZ* gene but none of the other denitrifying genes, thus qualifying as NNRB. The selected isolates were tested for their capacities to reduce N_2O emissions from three different typical Chinese farmland soils. The results indicated the significant mitigation effect of these isolates, even in very acidic red soil. In conclusion, this study demonstrated a strategy to engineer the soil microbiome with promising NNRB with high adaptability to livestock manure digestate as well as

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different agricultural soils, which would be suitable for developing novel fertilizer for farmland application to efficiently mitigate the N_2O emissions from agricultural soils.

1. Introduction

According to IPCC, constraining global warming to no >1.5 °C above pre-industrial levels must be pursued (IPCC, 2018) by reducing the anthropogenic emissions of greenhouse gases to the atmosphere. Nitrous oxide (N_2O) is a potent and long-lasting greenhouse gas with a stronger global warming potential than that of carbon dioxide (CO_2) and methane (CH_4) (Montzka et al., 2011; Pulles and van Amstel, 2010). Soil is a major source of N_2O emissions, including both natural soils and agricultural soils (Tian et al., 2020). With the continuous growth of the global population and intensive land use, the rapidly increasing amount of nitrogen fertilizers in agriculture has significantly increased N_2O emissions (Bahram et al., 2022; Davidson and Kanter, 2014).

Among the pathways involved in the turnover of N_2O in soils, microbial nitrification and denitrification are the most important (Sanchez and Minamisawa, 2019). Nitrification is a net source of N_2O, which releases N_2O as a byproduct by oxidizing ammonium to nitrite (Stein, 2020), while denitrification is both a source and sink for N_2O depending on the balance of its production and reduction. The mitigation of N_2O emissions from agricultural soil could be based on reducing the production of N_2O or promoting the reduction of N_2O to N_2. Various agronomic measures have been proposed to reduce the emission of N_2O from agricultural soils, such as improving the nitrogen use efficiency of the system (Hassan et al., 2022), enhancing the expression of functional N_2O-reductase by increasing soil pH (liming or application of biochar) (Aamer et al., 2020; He et al., 2019; Shaaban et al., 2020; Vazquez et al., 2020), or inhibiting nitrification (Das et al., 2022; Lin and Hernandez-Ramirez, 2020). None of these measures are likely to substantially reduce emissions; however, inconsistent effects have been observed (Liu et al., 2020; Scheer et al., 2011; Yanai et al., 2007). Except for the inhibition of N_2O production via nitrification inhibitors or optimized fertilization, the synthesis of nitrous oxide reductase (Nos) in the soil microbial community is of utmost importance because it is the only known biological sink of N_2O in the biosphere (Bakken and Frostegard, 2017; Hu et al., 2015; Liu et al., 2022). The effective synthesis of Nos in bacteria containing the *nosZ* gene would be beneficial to the reduction of N_2O in the soil.

The application of functional microorganisms to reduce N_2O emissions from soil has been reported in several studies, in which different N_2-generating denitrifier strains were successfully used to mitigate N_2O emissions from soil (Akiyama et al., 2016; Gao et al., 2016; Nishizawa et al., 2014; Obando et al., 2019). When aggressive N_2O reducers were artificially augmented in the soil microbial community, N_2O emissions were greatly reduced without sacrificing fertilizer inputs (Bakken and Frostegard, 2020; Henault et al., 2022). To date, it has been proven that inoculation of soils with N_2O-reducing bacteria can reduce N_2O emissions, but this is not feasible in agricultural practice due to the high cost of requiring large amounts of inoculants. Jonassen et al. reported an interesting low-cost alternative by using organic wastes as substrates and vectors for enriching and carrying nitrous oxide-reducing bacteria (NRB) (Jonassen et al., 2022a). Anaerobic digestion is a microbial-mediated process and is widely applied in organic waste treatment coupled with bioenergy production (Kim et al., 2021). The residual digestates contain nutrients and are destined for agricultural soils as fertilizers (Moller and Muller, 2012). The latest research has shown that it is feasible to reduce N_2O emissions from soil by fertilizing with digestate in which N_2O-reducing bacteria have been cultured, and this method has broad application prospects (Jonassen et al., 2022a). To make this technology more practical, an urgent task is to identify more suitable bacteria that can grow to high cell densities in digestates, survive for a minimum of time after being transferred to soil, and, more importantly, are effective in reducing N_2O.

Microorganisms lacking all denitrification genes except the *nosZ* gene (known as non-denitrifying N_2O-reducing bacteria (NNRB)) are more attractive than organisms with a full-fledged denitrification pathway because they will not generate N_2O and thus can act as a net sink of N_2O, which would be more promising for bioaugmentation in reducing N_2O emissions but with less nitrogen loss from soil. The first attempts to achieve this goal by enrichment culturing (anoxic with N_2O) in digestates were only partly successful (Jonassen et al., 2022a). Although the isolated organisms included strains that were strong sinks for N_2O due to regulatory traits, they were all equipped with a full-fledged denitrification pathway and were more efficiently growing in the digestate rather than the soil. Therefore, they are not suitable for soil inoculation due to the nitrogen loss they may cause and their poor growth in the soil. In subsequent experiments, a dual enrichment procedure was developed, switching between sterilized digestate and sterilized soil as substrate, and this resulted in the isolation of promising strains that lacked all denitrification genes other than *nosZ* (Jonassen et al., 2022b). This proof of concept warrants more research using dual enrichment to isolate more powerful N_2O 'sink' organisms as candidates for bioaugmentation that grow well in digestate and adapt to soils.

In the present study, we used the digestate from the fermentation of wastes of livestock that was different from the municipal wastewater digestate used by Jonassen et al. (2022a&b). The livestock digestate could more easily meet the needs of food safety than the wastewater digestate due to the potential harmful pollutants contained in the latter. By using multiple soils, we successfully enriched and screened out the N_2O-reducing bacteria that are better adapted to the different farmland soils. During the study, both high-throughput sequencing of the 16S rRNA gene and metagenomic sequencing were used to uncover the active microbial populations that may be responsible for N_2O reduction and to find target bacteria for isolation. This study is a step forward from the study of Jonassen et al. (2022a&b) and not only obtained aggressive and adaptive bacteria for N_2O sinks but also provides a real simulation for actual agricultural soil N_2O emission reduction. This study could promote the application of a novel strategy to manipulate the soil microbiome to mitigate climate forcing.

2. Materials and methods

2.1. Sample collection

The digestate was taken from a tank of anaerobic digestion (Supplementary Methods 1 for further details). Four typical farmland soils were collected from different regions of China with significant differences in pH and the types of crops cultivated (Supplementary Methods 2). The pH of each soil was measured in a slurry with distilled water (2.5 mL/g dry soil) (Table 1).

2.2. Dual enrichment experiment

We followed the same scheme (Jonassen et al., 2022b) for dual

Table 1
pH of different raw materials.

	Substrates used in this study					
	Digestate (DG)	Fluvo-aquic soil (FS)	Black soil (BS)	Red soil A (RSA)	Red soil B (RSB)	1:1 mixture of FS and RSA (FRS)
pH	7.2	8.4	6.2	5.5	4.8	7.4

enrichment to enrich and isolate N_2O-reducing organisms that can grow both in digestate and soil environments, but with different soils, combinations of soils and more agronomically relevant digestates (manure derived). Enrichment microcosms were established in 120 mL serum vials filled with 30 mL of newly collected digestate. The headspace gas was replaced with helium to establish anoxic conditions. Then, 5 mL of N_2O was added to the vials. The reduction of N_2O and the production of N_2 were continuously monitored during the incubation at 25 °C by a robotized incubation system (Molstad et al., 2007).

After 275 h of enrichment incubation with continuous N_2O addition, 2 mL of enriched digestate was inoculated into 20 g gamma-ray sterilized soils (Fig. 1). Sterilized water was then used to adjust the water content to 30 % (*w*/w), and the vials were incubated for 7 days at 25 °C, supplemented with 5 mL N_2O once a day. Then, 3 g of the cultured soil was transferred to 30 mL of sterilized digestate, and incubated anaerobically for 7 days with daily injections of N_2O. The culture was transferred back and forth between the soil and digestate for four rotations, for a total of eight sequential batch cultures for enrichment. The addition of N_2O was reduced in the 7th and 8th batch cultures, during which N_2O was injected into the vials every second day. The reciprocal inoculations between sterile soil and digestate were performed in four parallel series. These four series used different soils or mixtures as substrates. In detail, the DR series used red soil A; the DF series used fluvo-aquic soil; the DM series used a soil mixture of red soil A and fluvo-aquic soil; and the DRF series used red soil A and fluvo-aquic soil alternatingly, with cyclic inoculation from red soil A to digestate then to fluvo-aquic soil.

2.3. Sequencing of 16S rRNA gene amplicon

The V3-V4 regions in the 16S rRNA gene sequencing library was established by two-step PCR amplification according to a modified version of the instructions provided by the manufacturer as previously described with modified primers (Qin et al., 2019). For the bioinformatics and sequencing data analysis, the QIIME2 software (v2018.11) (Bolyen et al., 2019) was used to process and analyze the 16S rRNA gene sequence data (Ghosh et al., 2021). More details about the sequencing are given in Supplementary Methods 3.

2.4. Metagenome sequencing and binning

To gain a better understanding of the microbial community, the samples of fresh digestate (FD), enriched digestate (ED), red soil series (DR-1, DR-2, DR-7, DR-8), fluvo-aquic soil series (DF-1, DF-2, DF-7, DF-8), were selected for shotgun metagenomic sequencing. Sequencing procedure of genomic DNA on Illumina NovaSeq with PE150 strategy and the methods for metagenome binning are described in detail in Supplementary Methods 4.

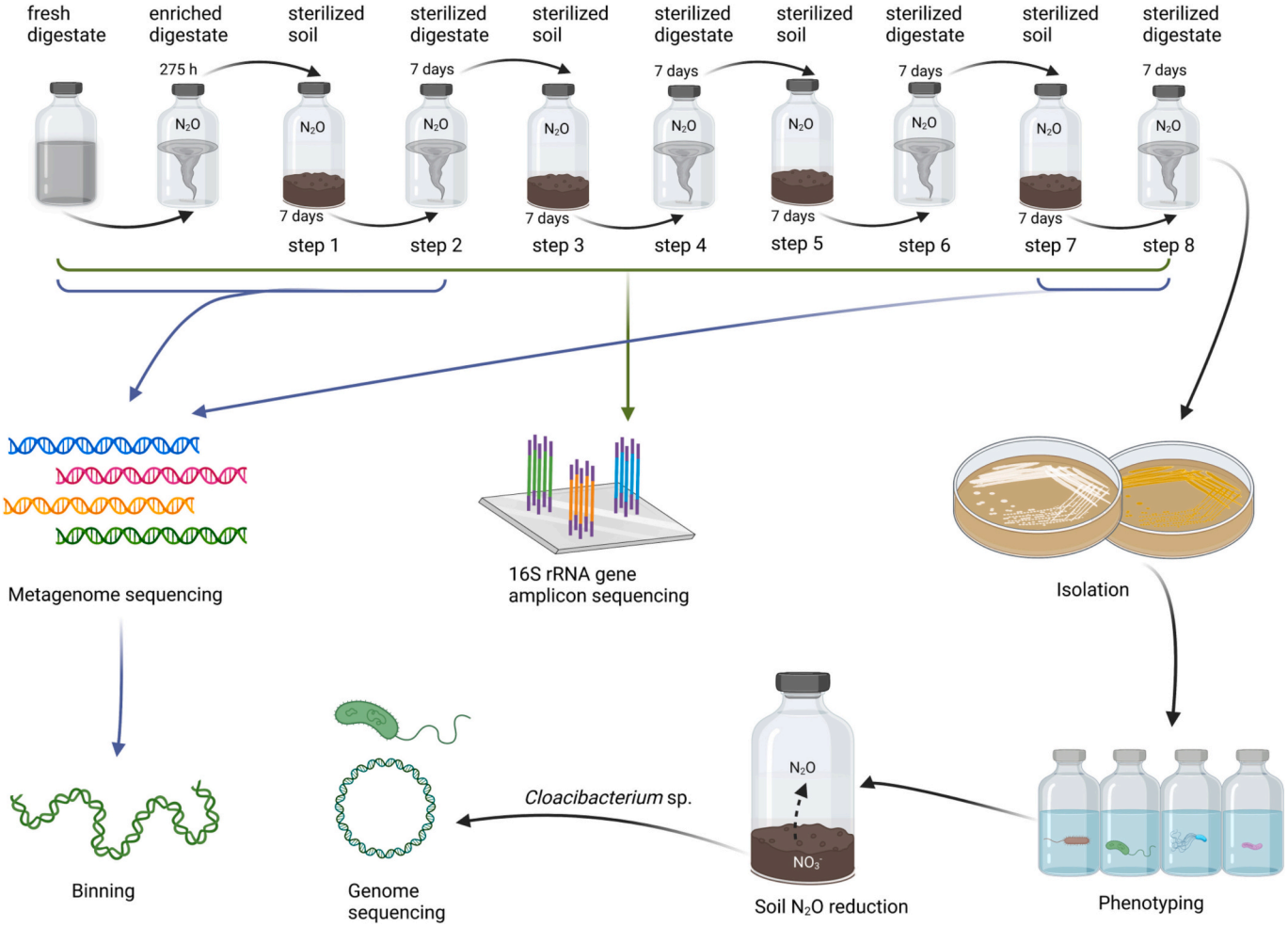

Fig. 1. Graphical summary of the experimental design and workflow. The fresh digestate was taken from a tank of anaerobic fermentation pig manure. After anaerobic culturing with N_2O, the enriched digestate was then inoculated into sterilized soil in four parallel series using different soils or mixtures of soils , and then transfer back to sterile digestate. After 4 rounds of inoculation and incubation, the samples were performed the 16S rRNA gene amplicon sequencing and metagenomics analyses to uncover the key N_2O-reducing bacteria adapted to both substrates. This information was further used for the guided target organism isolation. The well performed isolates for N_2O reduction were characterized by genome sequencing analysis.

2.5. Genome sequencing of Cloacibacterium sp. TD35

The genome of *Cloacibacterium* sp. TD35 was sequenced using a combination of PacBio RS and Illumina sequencing platforms. More details about the sequencing and genome annotation are described in Supplementary Methods 5.

2.6. Isolation and identification of bacteria

Two enriched cultures were spread on 1/2 TSA and 1/2 R2A medium (Supplementary Methods 6), 24 plates of each, in three different dilutions and four replicates for each dilution. All plates were incubated at 30 °C aerobically, and colonies with different morphology were picked every day (up to 7 days), to get the most diverse bacterial isolates as possible. The selected colonies were purified on a new plate of the same type of medium. The amplification of *nirK* and *nirS* was performed as described (Yang et al., 2017). Primer sets nirS cd3A/R3cd (AACGYSAAGGARACSGG/GASTTCGGRTGSGTCTTGA) and nirK1040/F1aCu (GCCTCGATCAGRTTRTGGTT/ATCATGGTSCTGCCGCG) were used. The primers used for *nosZ* amplification were nosZ1mod-F/nosZ1mod-R (WCSYTSTTCMTSGAYAGCCAG/ATRTCGAsTSARCTGVKCRTTYTC) for clade I and NosZ-II-F/NosZ-II-R (CTIGGICCIYTKCAYAC/GCIGARCARAAITCBGTRC) for clade II (Chee-Sanford et al., 2020). The details of PCR are given in Supplementary Methods 7. ERIC-PCR (Supplementary Methods 8) was used for typing all isolates. The bacterial strains with different ERIC-PCR band profiles were grown in vials with modified 1/2 R2A medium and tested for whether they could anaerobically reduce nitrate, nitrite or nitrous oxide.

2.7. Soil microcosm experiment for assessing mitigation of N_2O emissions

300 μL of each selected isolates in pure culture was inoculated and grown in 30 mL of sterilized digestate for two days under anaerobic conditions, respectively. Then, 3 mL of cultured digestate was mixed with 30 g of fluvo-aquic soil, black soil and red soil B. Soil mixed with 3 mL of uncultured sterilized digestate served as the control for the bacterial effect. The water content of the mixture was then adjusted to 30 % (*w*/w) by sterilized water. All the treatments were conducted with three replicates and incubated under aerobic conditions for one day at 25 °C. Then, 3 mg of NO_3^- and 30 mg of corn stover powder were added to the vials to simulate denitrification. Finally, all the vials were sealed, and the production and reduction of N_2O were monitored in a helium atmosphere. To assess the capacity of the strains to survive in soil, aerobic incubation (soil with NRB-enriched digestate) was extended to 30 days, before monitoring N_2O turnover in response to the addition of nitrate and corn stover powder. The N_2O production index (I_{N2O}) was used to assess the effect of different isolates in three soils on N_2O emission reduction (Liu et al., 2010; Samad et al., 2016). The formula to calculate the N_2O production index is as follows:

$$IN2O = \int_0^T \mathrm{N2O(t)dt} \Big/ \left[\int_0^T \mathrm{N2O(t)} + \int_0^T \mathrm{N2(t)} \right] \mathrm{dt}.$$

where the integrals are the area under the curve for N_2O and N_2 production, respectively. In this study, we considered 48 h as T to compare the N_2O reduction ability of each isolate in different soils.

2.8. Statistical analysis

Graphpad Prism (v8.4.2) and R (v4.3.0) were used to perform the statistical analysis. The Shannon indices and permutational multivariate analysis of variance (PERMANOVA) tests were undertaken using R package vegan (v2.6–4). Ordinary one-way analysis of variance (ANOVA) followed by a Fisher's Least Significant Difference (LSD) test was used to compare the statistical significances of the Shannon indices and gene abundances. Microbial compositional variation was calculated by Bray–Curtis distance metrics and visualized by principal-coordinate analysis (PCoA). The package of pheatmap (v1.0.12) was used to visualize data and construct the heatmaps. The phylogeny trees were constructed by IQ-TREE (v2.2.0.3) (Nguyen et al., 2015) and visualized on iTOL (https://itol.embl.de/).

3. Results

3.1. N_2O reduction in the fresh digestate

To enrich the N_2O-reducing bacteria, the freshly collected digestate was incubated in sealed vials with continuous supplementation of N_2O in the headspace (helium atmosphere). The activities of N_2O-reducing bacteria were confirmed by monitoring N_2O reduction and N_2 production. After 10 rounds of N_2O additions (Fig. 2a), the N_2O reduction rate of the digestate increased gradually from the initial 6.6 μmol N_2O h^{-1} to a maximum of 25.3 μmol N_2O h^{-1} during the 275-h incubation (Fig. 2b), and a total of approximately 3045 μmol N_2O was reduced (Fig. 2c).

3.2. Shifts in microbial community structures during dual enrichment

To characterize the population development throughout the dual enrichments, alternating between digestate and soils, samples were taken at the end of each 7-day batch incubation for genomic analysis. In addition, fresh digestate (FD) and N_2O-enriched digestate (ED) were also collected in triplicate for genomic analyses. In total, 102 samples were sequenced for the V3-V4 regions of the 16S rRNA gene. A total of 4,265,653 high-quality reads and 3973 amplicon sequence variants (ASVs) were obtained, with an average of 41,820 ± 5323 reads per sample. For metagenomic sequencing, in total, 2.3 billion reads were produced from Illumina NovaSeq sequencing with an average of 77,340,278 reads of 150 bp for each sample (Table S1). All samples had >97 % nucleotides with base accuracy >99 % (Q20) and > 93 % nucleotides with base accuracy >99.9 % (Q30). After quality control, 793.87 Gb of high-quality metagenomic data were generated. For the assembly of contigs, the maximum scaffold length ranged from 165 kb (ED-2) to 1140 kb (DR-1-3) (Table S2).

16S rRNA gene amplicon sequencing data (Fig. 3a) showed that the first incubation with N_2O reduced the diversity (ED versus FD) and that the diversity continued to decline throughout the subsequent series of enrichment batches. However, the diversity increased somewhat in the 7th and 8th batches, which received less N_2O than the previous batches. The metagenomic sequencing results showed different patterns (Fig. 3b), in which the Shannon indices increased when cultured with N_2O. Metagenomic sequencing resulted in 34,322 species identified in total, much more than the 3973 ASVs identified by 16S rRNA gene amplicon sequencing.

The principal coordinate analysis (PCoA) plot based on Bray–Curtis distances from the 16S rRNA gene amplicon sequencing data showed that the FD and ED samples separated from each other, indicating that anoxic incubation with N_2O addition changed the bacterial community structure in fresh digestate. The samples of the four series at the same step of dual enrichment showed similar microbial community structures. However, the soil and digestate samples were separated into three different clusters on the PCoA plots (Fig. 3c and d), both for 16S rRNA gene amplicons and metagenomes. The results of permutational analysis of variance (PERMANOVA) were in agreement with those of the PCoA in that dual enrichment had a significant impact on the microbial communities ($P < 0.001$).

The three most abundant microbial phyla were Proteobacteria, Bacteroidetes and Firmicutes (Fig. S1a). During the enrichment, the abundances of Proteobacteria and Bacteroidetes increased to >94 %, whereas the abundance of Firmicutes decreased. Except for a small percentage of Epsilonbacteraeota in the DF series, there were few differences in the microbial composition between the four series, showing the same patterns for microbial shift. The relative abundances of

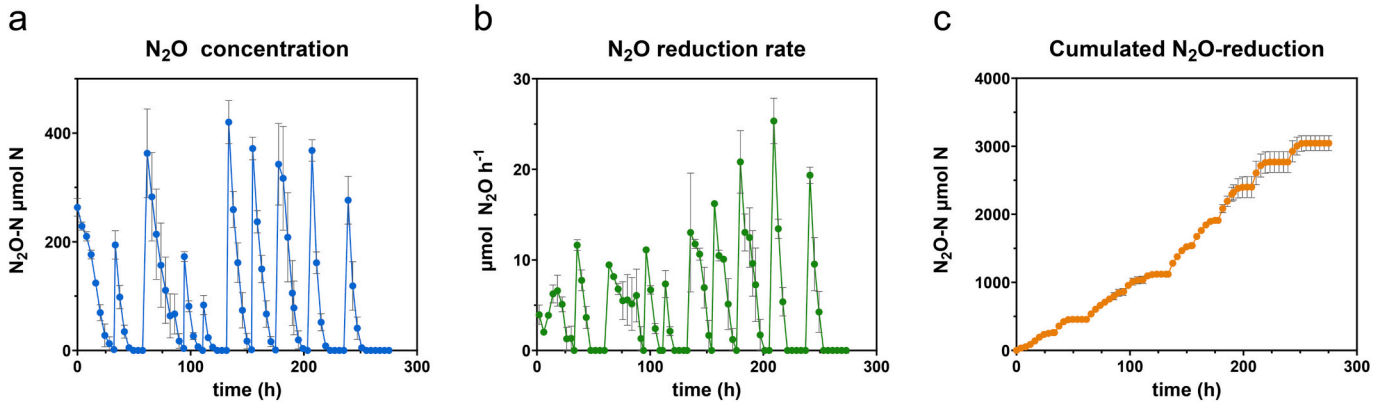

Fig. 2. Gas kinetics during anaerobic enrichment in fresh digestate. Panel **a** shows the concentration of N_2O in the vials (µmol N $vial^{-1}$), which was replenished by repeated injections. Panel **b** shows the calculated N_2O reduction rate in fresh digestate. Panel **c** shows the cumulated reduced N_2O during the 275-h enrichment. All the data are expressed as the mean ± SEM at each timepoint (n = 3).

Proteobacteria and Bacteroidetes similarly increased in the metagenomic sequencing results (Fig. S1b). In addition, the abundances of Chloroflexi and Euryarchaeota decreased with N_2O enrichment in both the digestate and soil.

Focusing on the genus level, as shown in Fig. 4a, compared with the fresh digestate, the abundances of *Azonexus, Pseudomonas*, and *Cloacibacterium* increased after N_2O enrichment, and they maintained higher abundances in all samples from step 1 to step 8. *Macellibacteroides* and a genus of Rikenellaceae maintained relatively stable abundances. However, *Azonexus* had a high abundance in the digestate, and its abundance decreased after being transferred to the soil, indicating that it was a digestate specialist. In contrast, *Pseudomonas* and *Cloacibacterium* were more adaptive to the soil. The heatmap of the top 50 genera resulting from metagenomic sequencing further revealed shifts in the microbial community (Fig. 4b). Based on the dynamic change in the relative abundances of these genera across all samples, they were clustered into five clades. The genera in clade I had high abundances in the fresh digestate, but they were diluted out from the cultures after transfer to soil. Clade II included the digestate specialists with only high abundances in the digestate samples. In contrast, clade III contained the soil specialists. Clade IV and clade V members seemed to adapt well to both soil and digestate, although they were enriched differently in these two substrates.

The shifts in the abundances of most ASVs showed similar trends in the four different treatments. For example, ASV_1, ASV_4 and ASV_8, belonging to *Azonexus*, had higher abundances in digestate than in soil. In contrast, ASV_3 and ASV_5 belonging to *Pseudomonas*, had higher abundances in soil than in digestate (Fig. S1c). From the view of identified species by metagenomic sequencing analysis, the abundance of *Cloacibacterium normanense* was significantly increased, especially, in the soil samples where its abundance reached 15 % or more (Fig. S1d). During enrichment, alternately incubated in soil and digestate, the microorganisms related to fermentation and methanogenesis were gradually diluted out, such as *Brevefilum fermentans* and *Methanothrix soehngenii* (Jetten et al., 1992; Mcllroy et al., 2017), which were the two species with the highest abundances in the original digestate collected from an anaerobic fermentation tank (Fig. S1d).

3.3. Shift in functional genes during dual enrichment

The metagenomic sequencing data analysis indicated that N_2O enrichment increased the diversity of identified KOs (KEGG Orthologues) (Fig. 5a). Additionally, the diversities of soil samples were higher than those of digestate samples. However, the carbohydrate-active enzyme gene diversities in the digestate were higher than that in the soil according to the results obtained using the CAZy database (Fig. 5b). The KEGG annotation indicated that the transcripts per million (TPM) value of the *nosZ* gene increased after N_2O enrichment (Fig. 5c). The taxonomic identities of the taxa harboring *nosZ* were mainly affiliated with Proteobacteria, Bacteroidetes and Others.

The functional potential of methane metabolism and nitrogen metabolism in the microbial communities was also investigated. After N_2O enrichment in the soil, the abundances of genes related to methane metabolism (*mcr*) and nitrogen fixation (*nif*) obviously decreased (Fig. S2). The abundances of some denitrification genes, such as *nirS*, *norB*, *norC*, and *nosZ*, increased in all samples with incubation time. In the DF series in which fluvo-aquic soil and digestate were used as substrates, increased abundances of genes related to ammonia oxidation (*amo*) were also observed. The other genes, *nar*, *nir*, *nap*, and *nas*, showed a strong correlation with soil since they were only enriched in soil samples.

The results of CAZy annotation (Fig. 5d, Fig. S3) also showed that the gene abundances of enzymes related to carbohydrate hydrolysis, glycoside hydrolases (GHs), polysaccharide lyases (PLs), carbohydrate-binding modules (CBMs), in the digestate were higher, while the gene abundances of glycosyl transferases (GTs), carbohydrate esterases (CEs), auxiliary activities (AAs) were higher in the soil.

3.4. The recovered genomes of denitrifying bacteria

After binning of metagenome reads of dual enrichment samples, 296 non-redundant bins with completeness >80 % and contamination <10 % were obtained, including 290 bacterial bins and 6 archaeal bins. Among the bacterial bins, there were 104 Bacteroidetes, 65 Proteobacteria and 60 Firmicutes (Fig. S4a). After identifying genes by using the KEGG Orthology databases, 62 bins were identified as carriers of the *nosZ* gene. The genome information and taxonomy of each *nosZ* gene-containing bin are shown in Table S3. Among these, bins affiliated with *Cloacibacterium*, *Sediminibacterium* and Desulfobacterota were found to contain only *nosZ*, without dissimilatory nitrate/nitrite reduction genes, and were thus considered as non-denitrifying N_2O-reducing bacteria (NNRB). In contrast, the bins containing strains within *Azonexus*, *Pseudomonas* and *Flavobacterium* have other denitrification genes, such as *napA/narG/nirK/nirS*. The phylogenetic tree of the *nosZ* genes was constructed by the sequences recovered from metagenome bins together with 10 *nosZ* clade I and 10 *nosZ* clade II reference sequences (downloaded from the Uniprot database) (Sanford et al., 2012) (Fig. S4b). The results showed that, except for *Azonexus*, all the bacteria belonging to Proteobacteria contained *nosZ* clade I, while the rest of the bacteria harbored *nosZ* clade II.

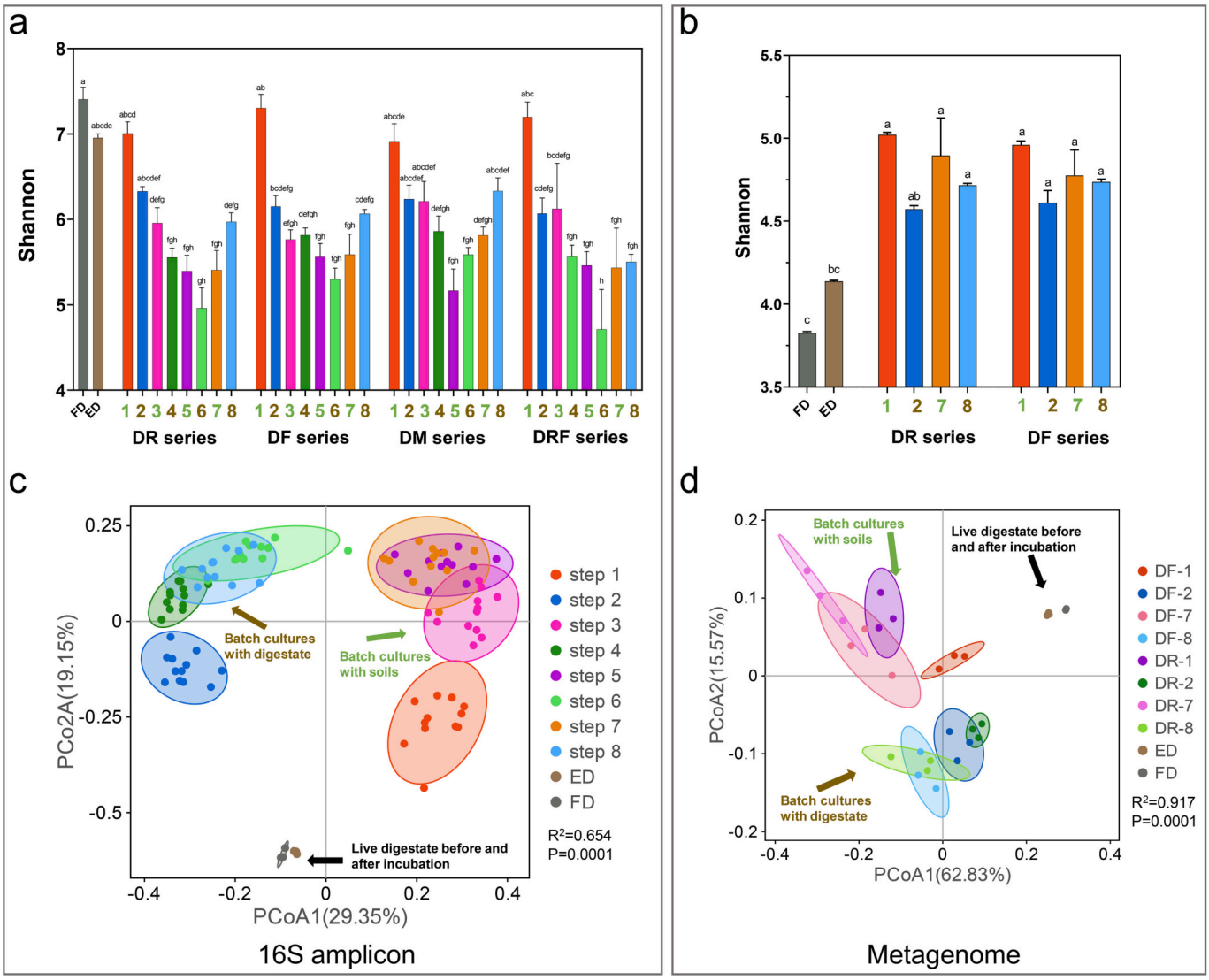

Fig. 3. Changes in the microbial community during dual enrichment. The alpha diversities of each enrichment series (i.e., with different soils) were measured by the Shannon index of the 16S rRNA gene amplicon sequencing (Panel **a**) and metagenomic sequencing (Panel **b**). The indices are expressed as the mean ± SEM with ordinary one-way analysis of variance (ANOVA) followed by Fisher's least significant difference (LSD) test. FD and ED represent fresh digestate and enriched digestate, respectively. The dual N_2O enriched samples were collected after each 7-day incubation, and the samples collected at the same timepoints in different series were divided into steps. Steps 1, 3, 5, and 7 were soil samples, while steps 2, 4, 6, and 8 were digestate samples. Panels **c** and **d** show the results of principal coordinate analysis (PCoA) of the microbial communities measured by 16S rRNA gene amplicon sequencing results and metagenomic sequencing results along with corresponding PERMANOVA comparisons (9999 permutations) for each sample. The ordinations were calculated based on the Bray–Curtis distances. The four parallel enrichment series (DR: red soil–digestate transfer; DF: fluvo-aquic soil–digestate transfer; DM: soil mixture–digestate transfer; DRF: red soil–digestate–fluvo-aquic soil–digestate transfer) are shown with the same symbol at each timepoint. Ellipses with different colors circumscribe different categories (indicated in the Panel **c**, **d**).

3.5. Selection of N_2O-reducing bacterial isolates as potential N_2O sinks

Cloacibacterium was enriched in the dual enrichment experiment with N_2O addition and reached very high abundances in both the soil and digestate. The assembled *Cloacibacterium* bins harbored the *nosZ* gene clade II without dissimilatory nitrate/nitrite reduction genes, suggesting that the bacteria belonging to *Cloacibacterium* could be potential high-efficiency nitrous oxide reducers. Isolating these bacteria would be of great benefit. After screening 350 isolates that were identified into 32 genera by 16S rRNA gene sequencing (Table S4) and by the amplification of nitrogen cycle-related genes (Table S5), the bacteria affiliated with three genera, *Cloacibacterium*, *Bacillus* and *Chryseobacterium,* were recognized as potential NNRB because these bacteria only contained *nosZ* clade II. These NNRB isolates together with the other representative isolates, which were shown to contain *nosZ* and abundant in the enrichment, such as *Pseudomonas* and *Azonexus*, etc. (Fig. S4, Table S3), were selected for the denitrification and N_2O reduction measurements. Finally, seven isolates were found to be putatively aggressive N_2O-reducing bacteria (N_2O sink) (Fig. S5), including three non-denitrifying N_2O-reducing bacteria and four denitrifying N_2O-reducing bacteria. The genotypes and phenotypes of these strains are shown in Table 2.

3.6. Comparison of *Cloacibacterium* strains isolated from different digestates

The whole genome sequences of an isolate of TD35 were assembled with a total of 2,595,287 bp and an average GC content of 33.08 % (Table S6), and this isolate was affiliated with *Cloacibacterium normanense* by 16S rRNA gene sequence analysis (Fig. S6). A total of 1185

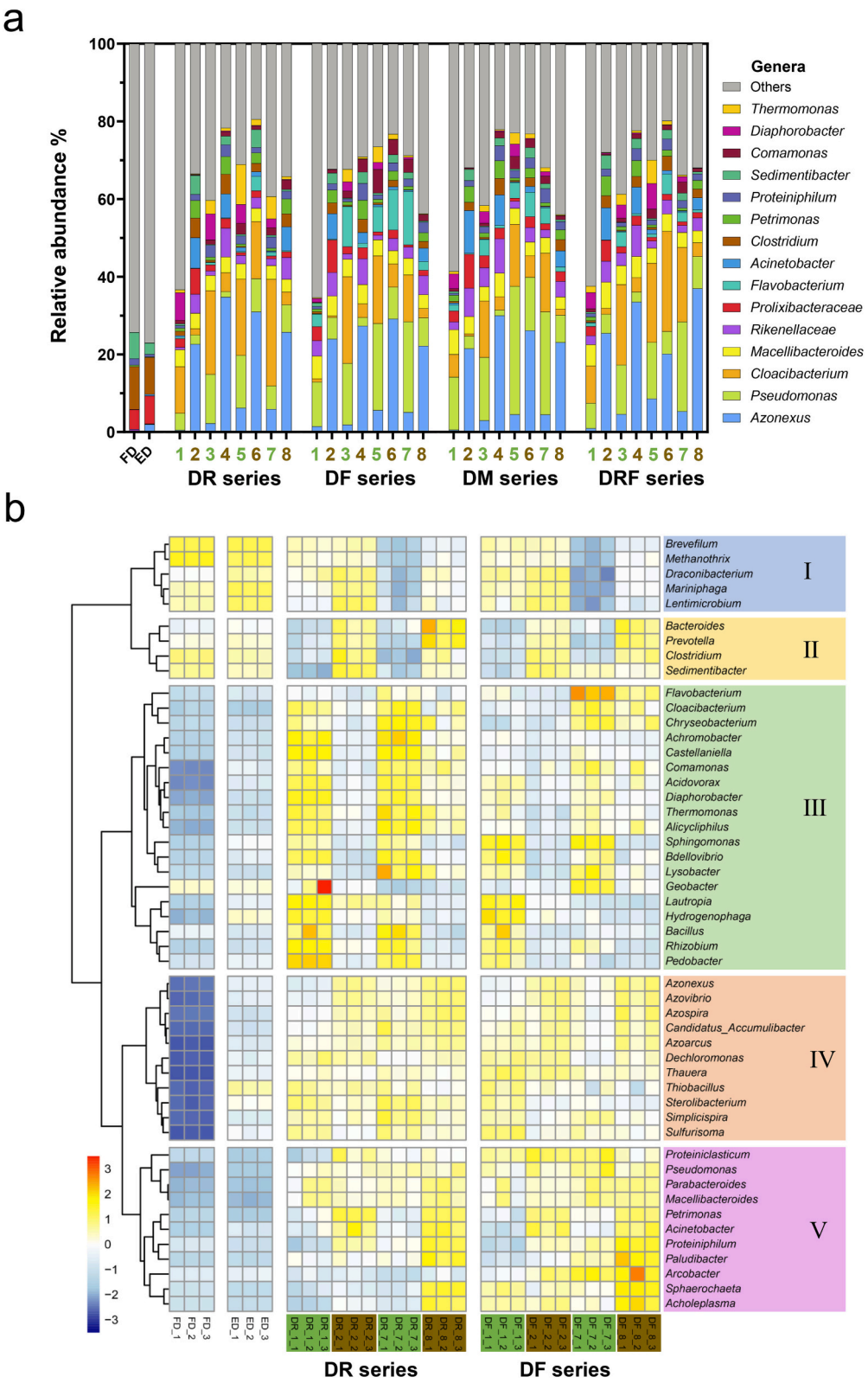

Fig. 4. Comparison of microbial community composition at the genus level. **(a)** Taxonomic composition based on 16S rRNA gene amplicon sequencing data, presented as the relative abundances of major microbial genera. **(b)** Heatmapping and clustering of the top 50 genera with the highest abundances identified by metagenomic sequencing data. These genera were clustered into five clades (I, II, III, IV, V) based on shifts in their relative abundances throughout the different samples. Soil samples are highlighted in green while digestate samples are highlighted in brown. FD and ED represent fresh digestate and enriched digestate, respectively. DR series: red soil–digestate transfer; DF series: fluvo-aquic soil–digestate transfer; DM series: soil mixture–digestate transfer; DRF series: red soil–digestate–fluvo-aquic soil–digestate transfer.

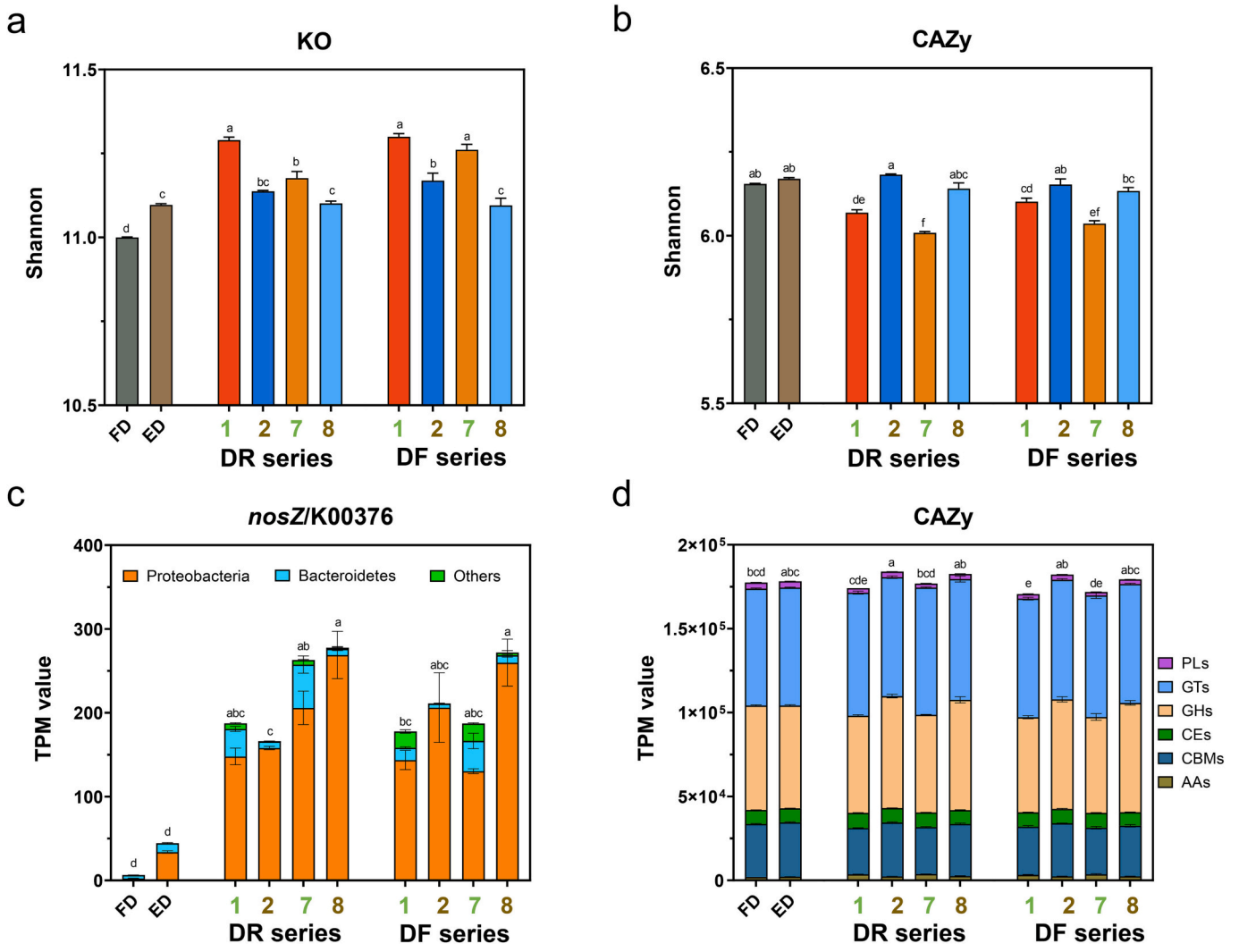

Fig. 5. Diversities and abundances of functional genes. (**a**) Shannon diversity based on identified KOs (KEGG Orthologues). (**b**) Shannon diversity based on identified carbohydrate-active enzymes in the CAZy database. (**c**) Abundances (as TPM values) and classifications of the *nosZ* genes encoding nitrous-oxide reductase, the entry of which in the KEGG Orthology databases is K00376. (**d**) Abundances (as TPM values) of genes encoding six carbohydrate-active enzymes. Auxiliary activities (AAs), carbohydrate-binding modules (CBMs), carbohydrate esterases (CEs), glycoside hydrolases (GHs), glycosyl transferases (GTs), polysaccharide lyases (PLs). Soil samples are highlighted in green while digestate samples are highlighted in brown. FD and ED represent fresh digestate and enriched digestate, respectively. DR series: red soil–digestate transfer; DF series: fluvo-aquic soil–digestate transfer.

Table 2
The property of selected N_2O-reducing bacterial isolates.

Isolates	Taxonomy	Clusters of 16S rRNA gene	Genotype (gene amplification)				Phenotype		
			nirK	*nirS*	*nosZ I*	*nosZ II*	Nitrate reduction	Nitrite reduction	N_2O reduction
TD35	*Cloacibacterium*	ASV_2				✔	×	×	✔
TS69	*Bacillus*	/				✔	×	×	✔
TS38	*Chryseobacterium*	ASV_34				✔	×	×	✔
RD87	*Azonexus*	/		✔			×	✔	✔
TD77	*Paracoccus*	ASV_987			✔		×	✔	✔
RS36	*Rhizobium*	ASV_206	✔	✔	✔		×	✔	✔
RS30	*Denitrobacter*	/			✔		×	✔	✔

annotated protein-coding genes confirmed that *Cloacibacterium* sp. TD35 only harbors the *nosZ* gene but lacks dissimilatory nitrate/nitrite reduction genes. The genome of this anaerobic pig slurry digestate-derived *Cloacibacterium* bacteria was compared with the genomes of two other *Cloacibacterium* strains isolated from dual substrate anaerobic enrichments with N_2O, using municipal wastewater and clay loam (Jonassen et al., 2022b): *Cloacibacterium caeni* CB01 (GenBank assembly accession: GCA_907163125) and *Cloacibacterium caeni* CB03 (GenBank assembly accession: GCA_907163105). The results show that the nucleotide sequence identity of the three *nosZ* genes is >94.37. These three genomes have 832 KOs in common, but TD35 contains 33 unique KOs (Table S7, Fig. S7).

3.7. The selected isolates reduce N_2O emissions in soil

The capacity of the isolates to act as sinks for N_2O was assessed in different soils by measuring N_2 and N_2O kinetics during anaerobic incubation. The soils were amended with digestates in which the different isolates had been grown to high cell density. Some of the N_2O-reducing bacteria augmented the digestate-enhanced mitigation effect (Fig. 6, Fig. S8), but their effects appeared to depend on soil type. The NNRB-strains *Chryseobacterium* and *Bacilllus* were effective in all soils, while the third NNRB-strain *Cloacibacterium* had an effect only in the very acidic red soil (pH = 4.8). Among the denitrifying strains (i.e., with *nirS/K*), the N_2O indices were lower (compared to the soil with sterilized digestate) in the fluvo-aquic soil (pH = 8.4) and the black soil (pH = 6.2) but not in the very acidic red soil. After a month of aerobic incubation in the microcosms, however, the N_2O reduction effects were no longer statistically significant in any of the soils (Figs. S9 and S10).

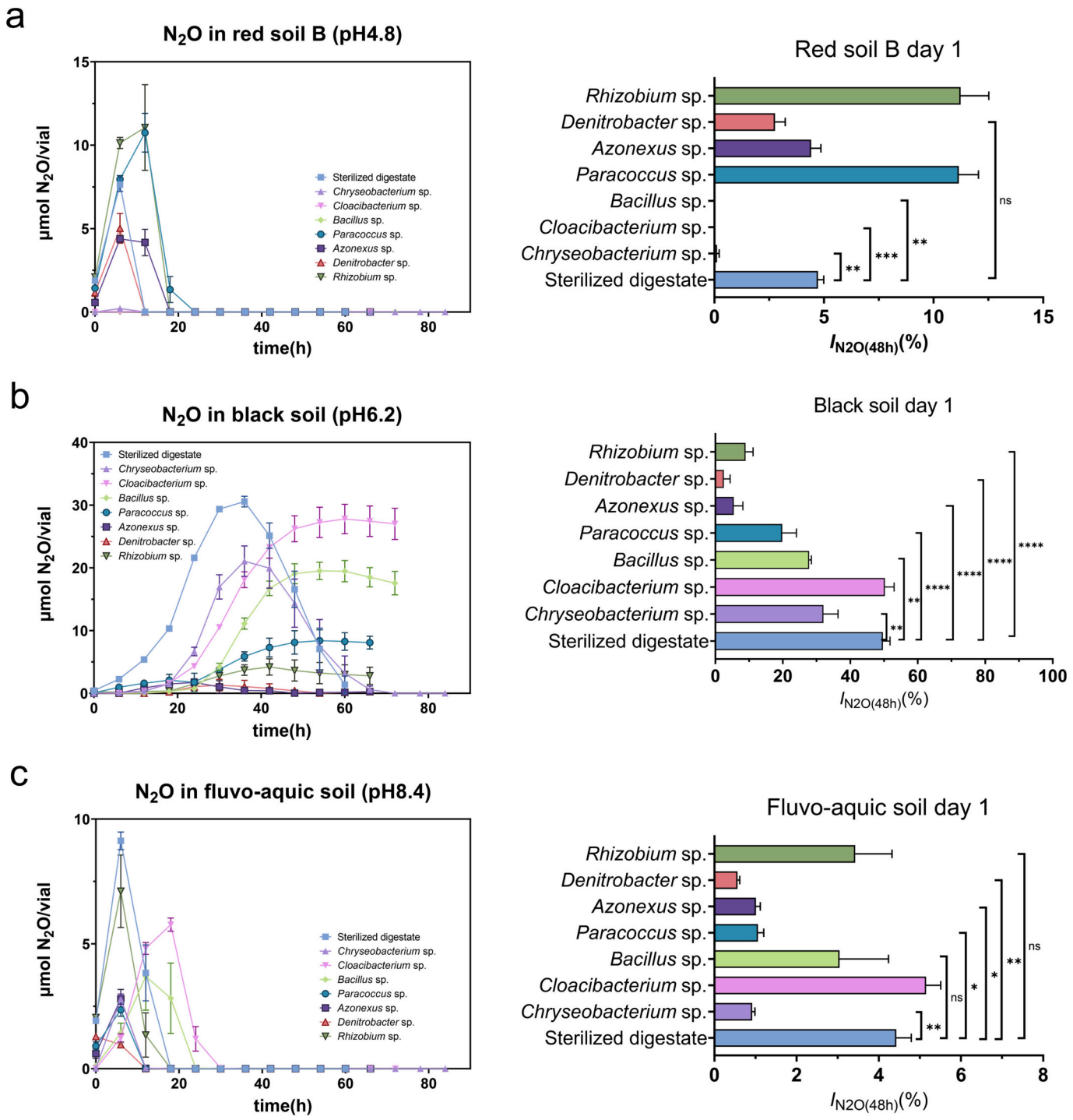

Fig. 6. Effects of enriched digestate addition on N_2O accumulation in three types of soil after one-day aerobic incubation. The main graphs (Panels **a**, **b** and **c**) show the N_2O kinetics in different soils amended with various pretreated digestates and one control (sterilized digestate addition) immediately after digestate addition. The data are expressed as the mean ± SEM for each treatment (n = 3). The bar graphs to the right show the N_2O indices expressed as a percentage (average I_{N2O} for each treatment), and statistical significance was assessed by one-way ANOVA followed by Tukey's multiple comparisons test (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$). I_{N2O} is the area under the N_2O curve as a percentage of the area under the $N_2O + N_2$ curve of the first 48 h after NO_3^- addition.

4. Discussion

4.1. Utilization of digestate as biofertilizer

The application of manure and digestates to soils increases the organic content and thus is beneficial to improve the quality of soil. However, it also normally induces a transient increase in N_2O emission due to stimulated respiration and denitrification. Therefore, reducing emissions from the organic fertilization is also an urgent task, especially, in the context of the global response to climate change and the demand for control of greenhouse gas emissions.

Recent studies have shown that the application of N_2O-reducing bacteria can reduce N_2O emissions from agricultural soil. Using agricultural waste digestate carrying nitrous oxide-reducing bacteria (NRB) to fertilize the soil could be the best choice for a double win strategy. Digestate from an anaerobic digester fed with sewage sludge as the carrier being used to enrich the nitrous oxide-reducing bacteria was first reported by Jonassen and colleagues (Jonassen et al., 2022a&b). Anaerobic digestate (AD) uses various wastes as fermentation substrates (Liu et al., 2021). Sewage sludge may not be suitable for agricultural applications due to organic and heavy metal pollutants and possibly other hazardous materials, such as antibiotics and endocrine disrupters (Gu et al., 2020; Yang et al., 2020).

Agricultural residue and animal waste digestate are rich in nutrients and widely used as biofertilizers for agriculture production (McDowell et al., 2020; Wang et al., 2021a). Using animal waste digestate to cultivate the nitrous oxide-reducing bacteria would be more favorable to the practical application. In this study, we followed the same scheme as Jonassen et al. used (Jonassen et al., 2022b), but with a more agronomically relevant digestate (pig slurry digestate), expanded it to include several different types of farmland soils as substrates, intending to enrich, identify, isolate and apply the aggressive N_2O-reducing bacteria, which are expected to adapt to both digestate and various type of agricultural soils.

4.2. Bacteria adapted to soil and digestate

Microbial species can be divided into habitat generalists and habitat specialists (Muller, 2019; Peralta et al., 2014; Szekely and Langenheder, 2014). Habitat generalists have broad environmental tolerance and can survive in many habitats, while habitat specialists are more limited to specific habitats (Hu et al., 2019; Wu et al., 2018). Generally, under changing environmental conditions, habitat generalists are more competitive, while habitat specialists are apt to decline in numbers and eventually fade out. In this study, a dual enrichment procedure in which the microbial community was repeatedly transferred between the digestate and different sterile soils was conducted. The results showed that the enrichment cultures in the different soils converged with respect to the composition of their communities. These enriched microorganisms might be generalists considering that they were competent for different types of soils and survived in both soil and digestate.

Metagenomic analysis was performed in this study to better understand the microbial enrichment process. From the perspective of identified KOs, the diversities of soil samples were higher than that in the digestate. However, the CAZy annotation results showed that carbohydrate activity genes were more diverse in digestate than in soil. The possible reason is that liquid digestate substrate in constant stirring created a relatively uniform environment with diverse carbohydrate resources (Guilayn et al., 2020). Thus, the complexity of the microbial community in the digestate was low and dominated by bacteria relying on the degradation of carbohydrates, which contributed to the high gene abundances of glycoside hydrolases (GHs), polysaccharide lyases (PLs), carbohydrate-binding modules (CBMs) (Fig. S3).

4.3. N_2O-reducing bacteria and nitrous oxide reduction

By shotgun metagenomic sequencing and binning of MAGs, we identified some potential functional generalist N_2O-reducing bacteria in the enriched samples (Wang et al., 2021b). N_2O-reducing bacteria were isolated by using the identified genomic information as a guide (Mao et al., 2010). Among them, seven isolated strains were identified as N_2O sinks due to their strong N_2O reduction ability. One of the three NNRB belongs to the genus *Cloacibacterium*, and N_2O-reducing bacteria from the same genus were also isolated in the study of Jonassen et al. (2022b), although the digestate used in the two studies had nothing in common. However, the two strains isolated from wastewater sludge by Jonassen et al. (2022b) were later found to grow poorly in animal manure or manure digestate (Jonassen, personal communication), indicating that they cannot be used for manufacturing biofertilizer by animal manure. Further comparative genome analysis for three isolates indicated that the *hipO* gene, which encodes hippurate hydrolase (Table S7), an enzyme utilizing the hippurate in the manure digestate, was carried only by the TD35 genome but not by the other two genomes.

The microcosm experiment showed that all seven strains were effective N_2O-sinks in at least one of the three soils (Fig. 6). All NNRB strains (*Cloacibacterium*, *Chryseobacterium* and *Bacillus*) effectively reduced the N_2O/N_2 product ratio in the acid red soil, while in the less acidic black soil and the alkaline fluvio-aquic soil, the only effective NNRB-strain was *Chryseobacteriunm*. The other strains (i.e., with functional NirK/S) showed the opposite trend, reducing the N_2O/N_2 product ratio in the black- and fluvo-aquic soils, but not in the acid red soils. This lack of effect in the acid soil is plausibly due to hampered synthesis of functional N_2O-reductase at low pH, as demonstrated for a number of model strains (Bakken and Frostegård, 2020). The NNRB strains showed the opposite pattern, by being most effective in the acidic soil. This could be taken to suggest that the synthesis of N_2O-reductase in these strains requires low pH, as demonstrated for a strain identified as *Rhodanobacter* (Lycus et al., 2017). However, the failure of an isolate to reduce N_2O in a particular soil can be due to other factors than pH: since the dual enrichment technique used gamma-sterilized soil (not live soils), there is no selection for the ability to withstand the suppressive effect of the indigenous soil microbiota. It may well be, therefore, that the variable performance of the strains, as dependent on the soil type, is due to specific suppressive effects of the indigenous microbiota in the different soil.

Although the four bacterial isolates containing with *nosZ* clade I could function well to reduce N_2O emissions in the black soil and fluvo-aquic soil, they were all able to reduce nitrite, which means that they could enhance nitrogen loss via denitrification. In this respect, NNRB strains are much to prefer because they can only respire N_2O produced by denitrifying organisms in the soil, thus affecting the N_2O/N_2 product of denitrification in soil, but not the rate of denitrification.

Fertilizing with digestates generally induces a transient burst of N_2O emission (Baral et al., 2017), and reducing N_2O emissions during this period is of great importance. The experiments demonstrated that this was achieved with all strains in at least one of the soils, while the measured effects 30 days after application was marginal, suggesting that the strains are unable to survive for long time in soil.

4.4. nosZ II plays a crucial role in N_2O reducing

Another finding in this study is that bacteria containing *nosZ* clade II, such as *Cloacibacterium*, *Azonexus*, and *Flavobacterium*, were enriched more than organisms with *nosZ* clade I during dual enrichment. In fact, the majority of known *nosZ* clade II microbes are considered as non-denitrifying N_2O reducers due to the lack of the *nir* gene (Graf et al., 2014; Hallin et al., 2018). Moreover, *nosZ* clade II has a wider distribution among microbial taxa (Chee-Sanford et al., 2020; Jones et al., 2014; Shan et al., 2021), and these microorganisms are widely found in different ecosystems (Bertagnolli et al., 2020; Domeignoz-Horta et al.,

2016). Compared with *nosZ* clade I carrying N_2O reducers, *nosZ* clade II organisms appear to have a higher affinity for N_2O, which provides them with a selective advantage when competing for limited N_2O *in situ* in the micro-environment of soil (Yoon et al., 2016). All these studies suggest that *nosZ* clade II-carrying microorganisms may have better N_2O reduction potential than *nosZ* clade I-carrying microorganisms. Studies have already performed some enrichment and isolation of *nosZ* clade II carrying bacteria, such as *Dechloromonas* and *Azospira* which were proven to be N_2O sinks (Conthe et al., 2018; Suenaga et al., 2019). Based on these results, *nosZ* clade II bacteria are more promising for use in agriculture to reduce N_2O emissions because these microorganisms have higher growth yield, higher N_2O affinity, and better resilience to oxygen exposure (Qi et al., 2021). In this study, *nosZ* clade II bacteria accounted for the majority of isolated *nosZ*-containing bacteria. Interestingly, most of the enriched bacteria in Jonassen's work also belonged to *nosZ* clade II-carrying bacteria (Jonassen et al., 2022b). This implies that the varying environment of the alternating incubation in farmland soil and digestate during dual enrichment with N_2O may be more favorable for *nosZ* clade II-carrying bacteria.

5. Conclusion

Enhancing the abundances and activities of functional bacterial N_2O sinks could be a promising way to reduce N_2O emissions from agricultural soils. In this study, we intended to screen highly adaptive N_2O sink bacteria that could adapt well to various soils and be cultured in manure digestate as a type of biofertilizer to achieve the goal of mitigating N_2O emissions during fertilization in farmland. The results showed that the digestate cultured with selected NNRB strains significantly reduced N_2O emissions after being applied to three different farmland soils. This study provides a comprehensive understanding of N_2O reducers and their adaptivity in digestate and different soils. This further indicates that the application of agricultural waste digestate-derived organic fertilizer containing highly efficient N_2O-reducing bacteria could be a promising strategy for mitigating N_2O emissions from agricultural soils.

CRediT authorship contribution statement

Xinhui Wang: Methodology, Investigation, Data analysis, Draft writing and editing.

Baoyu Xiang: Resources, Data analysis.

Ji Li: Investigation, Methodology.

Menghui Zhang: Methodology, Data analysis.

Asa Frostegard: Conceptualization, Writing– review & editing.

Lars Bakken: Conceptualization, Writing– review & editing.

Xiaojun Zhang: Conceptualization, Methodology, Resources, Writing– review & editing, Funding acquisition, Project administration.

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

All the raw sequence reads reported in this study, including 16S rRNA gene amplicon sequencing, metagenome sequencing, whole genome sequencing, and the assembled *Cloacibacterium* sp. TD35 genome were deposited at the NCBI GenBank under the BioProject accession number PRJNA871363.

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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.2023.166284.

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