Nitrous oxide respiring bacteria in biogas digestates for reduced agricultural emissions

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INTRODUCTION

Nitrous oxide is an intermediate in the nitrogen cycle and a powerful greenhouse gas emitted in large volumes from agricultural soils, accounting for ~1/3 of total anthropogenic N2O emissions [1]. Reduced emissions can be achieved by minimizing the consumption of fertilizer nitrogen through improved agronomic practice and reduction of meat consumption [2, 3], but such measures are unlikely to do more than stabilize the global consumption of fertilizer-N [4]. This calls for more inventive approaches to reduce N2O emissions, targeting the microorganisms involved in production and consumption of N2O in soil [5].

N2O turnover in soil involves several metabolic pathways, controlled by a plethora of fluctuating physical and chemical variables [7–9]. Heterotrophic denitrification is the dominant N2O source in most soils, while autotrophic ammonia oxidation may dominate in well-drained calcareous soils and references therein [10]. Heterotrophic denitrifying organisms are both sources and sinks for N2O because N2O is a free intermediate in their stepwise reduction of nitrate to dinitrogen (NO3− → NO2− → NO → N2O → N2). Denitrification involves four enzymes collectively referred to as denitrification reductases: nitrate reductase (Nar/Nap), nitrite reductase (Nir), nitric oxide reductase (Nor) and nitrous oxide reductase (Nos), encoded by the genes nar/nap, nir, nor and nosZ, respectively. Oxygen is a strong repressor of denitrification, both at the transcriptional and the metabolic level [11, 12]. Many organisms have truncated denitrification pathways, lacking from one to three of the four reductase genes [13, 14], and truncated denitrifiers can thus act as either N2O producers (organisms without nosZ) or N2O reducers (organisms with nosZ only). The organisms with nosZ only, coined non-denitrifying N2O-reducers [15], have attracted much interest as N2O sinks in the environment [16]. Of note, organisms with a full-fledged denitrification pathway may also be strong N2O sinks depending on the relative activities and regulation of the various enzymes in the denitrification pathway [17, 18]. Despite their promise, feasible ways to utilize N2O-reducing organisms to reduce N2O emissions have not yet emerged.

A soil with a strong N2O-reducing capacity will emit less N2O than one dominated by net N2O producing organisms, as experimentally verified by Domeignoz-Horta et al. [19], who showed that soils emitted less N2O if inoculated with large numbers (107–108 cells g−1 soil) of organisms expressing Nos as their sole denitrification reductase. As a standalone operation, the large-scale production and distribution of N2O-respiring bacteria would be prohibitively expensive and impractical. However, the use of N2O-respiring bacteria could become feasible if adapted to an existing fertilization pipeline, such as fertilization with the nitrogen- and phosphate-rich organic waste (digestate) generated by biogas production in anaerobic digesters. Anaerobic digestion (AD) is already a core technology for treating urban organic wastes, and is expected to treat an increasing proportion of the much larger volumes of waste produced by the agricultural sector.
producing biogas from sludge produced by a wastewater treatment plant. The sludge was a poly-aluminum chloride (PAX-XL61™, Kemira) and ferric chloride (PIX318™, Kemira) precipitated municipal wastewater sludge, with an organic matter content of 5.6% (w/w). Both digesters reduced the organic matter by approximately 60%, producing digestates containing ~2.1% organic matter, 1.8–1.9 g NH₄⁺·N L⁻¹, ~16 and 32 Meq VFA L⁻¹, pH = 7.6–7.8 and 8.2; mesophilic and thermophilic, respectively (see Supplementary Methods 1 for further details). The digestates were transported to the laboratory in 1 L insulated steel-vessels and used for incubation experiments 3–6 h after sampling.

The robotized incubation system developed by Molstad et al. [21, 22] was used in all experiments where gas kinetics was monitored. The system hosts 30 parallel stirred batches in 120 mL serum vials, crimp sealed with gas tight butyl rubber septa, which are monitored for headspace concentration of O₂, N₂, N₂O, NO, NO₂, CO₂ and CH₄ by frequent sampling. After each sampling, the system returns an equal volume of He, and elaborated routines are used to account for the gas loss by sampling to calculate the production/consumption-rate of each gas for each time interval between two samplings. More details are given in Supplementary Methods 2.

Enrichment culturing of N₂O-respiring bacteria (NRB) in digestate was done as stirred (300 rpm) batches of 50 mL digestate per vial. Prior to incubation, the headspace air was replaced with Helium by repeated evacuation and He-filling [21] and supplemented with N₂O. The N₂O in the headspace was sustained by repeated injections in response to depletion. Liquid samples (1 mL) were taken by syringe, for metagenomic and metaproteomic analyses, and for quantification of volatile fatty acids (VFA) and 16S rRNA gene abundance. The samples were stored ~80 °C before analyzed. The growth of NRB in the enrichments was modeled based on the N₂O reduction kinetics. The modeling and the analytic methods (quantification of VFA and 16S rRNA gene abundance) are described in detail in Supplementary Methods 3.

Metagenomics and metaproteomics

Sequencing of DNA (Illumina Hiseq4000), and the methods for Metagenome-Assembled Genome (MAG) binning, and the phylogenetic placement of the MAGs is described in detail in Supplementary Methods 4. Proteins were extracted and digested to peptides, which were analyzed by nanolC-MS/MS, and the acquired spectra were inspected, using the metagenome-assembled genomes (149 MAGs) as a scaffold (Supplementary Methods 5).

Isolation of N₂O-respiring bacteria (NRB) (Supplementary Methods 6). NRB present in the enrichment cultures were isolated by spreading diluted samples on agar plates with different media composition, then incubated in an anaerobic atmosphere with N₂O. Visible colonies were re-streaked and subsequently cultured under aerobic conditions, and 16s-sequenced. Three isolates, AS (Azospira sp), AN (Azonexus sp) and SP (Pseudomonas sp) (names based on their 16s sequence), were selected for genome sequencing, characterization of their denitrification phenotypes, and for testing their effect as N₂O sinks in soil.

Genome sequencing and phenotyping of isolates

Three isolates were genome sequenced and compared with MAG’s of the enrichment culture (Supplementary Methods 7). The isolates’ ability to utilize various organic C substrates was tested on BIOLOG Phenotype MicroArray™ microtitre plates, and their characteristic regulation of denitrification was tested through a range of incubation experiments as in previous investigations [17, 18, 23, 24], by monitoring the kinetics of O₂, N₂, N₂O, NO and NO₂⁻ throughout the cultures’ depletion of O₂ and transition from aerobic to anaerobic respiration in stirred batch cultures with He + O₂ (±NH₄Cl) in the headspace (Supplementary Methods 8). The kinetics of electron flow through the oxic and anoxic phase in these experiments were used to assess if the organisms were bet hedging, as demonstrated for Paracoccus denitrificans [17], i.e. that only a minority of cells express nitrate- and/or nitrite-reductase, while all express Nos, in response in response to oxygen depletion. Putative bet hedging was corroborated by measuring the abundance of nitrate-, nitrite- and nitrous oxide reductase (Supplementary Methods 9).

N₂O mitigation experiments (Supplementary Methods 10). To assess the capacity of the isolates to reduce the N₂O emission from soil, they were grown aerobically in sterilized digestate, which was then added to soil in microcosms, for measuring the NO-, N₂O- and N₂ kinetics of denitrification in the soil. For comparison, the experiments included soils amended with sterilized digestate, live digestate (no pretreatment), and digestate in

MATERIALS AND METHODS

The digestates were taken from two anaerobic digesters, one mesophilic (37 °C) and one thermophilic (52 °C), which were running in parallel,
which N₂O-reducing bacteria had been enriched by anaerobic incubation with N₂O (as for the initial enrichment culturing).

RESULTS AND DISCUSSION
Enrichment of indigenous nitrous oxide respiring bacteria (NRB) in digestates
We hypothesized that suitable organisms could be found in anaerobic digesters fed with sewage sludge, since such sludge contains a diverse community of denitrifying bacteria stemming from prior nitrification/denitrification steps [25]. We further hypothesized that these bacteria could be selectively enriched in digestates by anaerobic incubation with N₂O. We decided to enrich at 20 °C, rather than at the temperatures of the anaerobic digesters (37 and 52 °C), to avoid selecting for organisms unable to grow within the normal temperature range of soils.

The digestates were incubated anaerobically as stirred batch cultures with N₂O in the headspace (He atmosphere), and the activity and apparent growth of N₂O reducers was assessed by monitoring the N₂O-reduction to N₂. Figure 2A shows the results of the enrichment experiment where N₂O-depletion (as seen at 100–140 h in panel A) was avoided, to allow more precise assessment and modeling of growth kinetics. Panel B: N₂O concentration in the digestate (μM N₂O), rate of N₂-production (V₉₂) and N₂ produced (mmol N₂ mL⁻¹ digestate, all log scaled. The curved black line shows the modeled V₉₂ assuming two populations, one growing exponentially (μ = 0.1 h⁻¹), and one whose activity was dying out gradually (rate = −0.03 h⁻¹). The dotted black line is the activity of the exponentially growing population extrapolated to time = 0. Panel C shows the modeled density (cells mL⁻¹) of cells growing by N₂O respiration, extrapolated back to t = 0 h (dashed line), and the respiratory electron flow rate per cell (Vₑ, fmol electrons cell⁻¹ h⁻¹), which declined gradually after 110 h. Standard deviations (n = 3) are shown as vertical lines. Supplementary Fig. S1 provides additional data for the experiment depicted in Panel A, as well as a detailed description of the modeling procedures and their results.

![Image](55x59 to 283x478)

**Fig. 2** Gas kinetics in anaerobic enrichment cultures with digestate. Panel A shows results for the enrichment culture (tripllicate culture vials) sampled for metagenomics, metaproteomics, quantification of volatile fatty acids (VFAs) and 16S rRNA gene abundance (sampling times = 0, 115 and 325 h). The top panel shows the amounts of N₂O produced (mmol N₂ L⁻¹ digestate, log scale) and 16S rRNA gene copy numbers. The mid panel shows the concentration of N₂O in the digestate (log scale), which was replenished by repeated injections (20 mL N₂O resulting in 5 mM N₂O in the liquid) from t = 140 h and onwards (indicated by black arrows). The bottom panel shows the rate of methane production. Standard deviations (n = 3) are shown as vertical lines in all panels. Panels B and C show the results of a repeated enrichment experiment where N₂O-depletion (as seen at t = 100–140 h in panel A) was avoided, to allow more precise assessment and modeling of growth kinetics. Panel B: N₂O concentration in the digestate (μM N₂O), rate of N₂-production (V₉₂) and N₂ produced (mmol N₂ mL⁻¹ digestate), all log scaled. The curved black line shows the modeled V₉₂ assuming two populations, one growing exponentially (μ = 0.1 h⁻¹), and one whose activity was dying out gradually (rate = −0.03 h⁻¹). The dotted black line is the activity of the exponentially growing population extrapolated to time = 0. Panel C shows the modeled density (cells mL⁻¹) of cells growing by N₂O respiration, extrapolated back to t = 0 h (dashed line), and the respiratory electron flow rate per cell (Vₑ, fmol electrons cell⁻¹ h⁻¹), which declined gradually after 110 h. Standard deviations (n = 3) are shown as vertical lines. Supplementary Fig. S1 provides additional data for the experiment depicted in Panel A, as well as a detailed description of the modeling procedures and their results.

The provision of substrate for the N₂O-respiring bacteria can be understood by considering the enrichment culture as a continuation of the metabolism of the anaerobic digester (AD), albeit slowed down by the lower temperature (20 °C versus 37 °C in the digester). In AD, organic polymers are degraded and converted to CO₂ and CH₄ through several steps, conducted by separate guilds of the methanogenic microbial consortium: 1) hydrolysis of polysaccharides to monomers by organisms with carbohydrate-active enzymes, 2) primary fermentation of the resulting monomers to volatile fatty acids (VFAs), 3) secondary fermentation of VFAs to acetate, H₂ and CO₂, and 4) methane production from acetate, CO₂, H₂, and methylated compounds. By providing N₂O to
this (anaerobic) system, organisms that respire N\textsubscript{2}O can tap into the existing flow of carbon, competing with the methanogenic consortium for intermediates, such as monomeric sugars, VFAs (such as acetate) and hydrogen [26]. Thus, the respiration and growth of the N\textsubscript{2}O-respiring bacteria is plausibly sustained by a flow of carbon for which the primary source is the depolymerization of organic polymers. It is possible that the retardation of growth after \(-100\) h of enrichment was due to carbon becoming limiting. Thus, at this point, the population of N\textsubscript{2}O-respiring organisms may have reached high enough cell densities to reap most of the intermediates produced by the consortium.

Parallel incubations of digestates without N\textsubscript{2}O confirmed the presence of an active methanogenic consortium, sustaining a methane production rate of \(\sim 0.2\) \(\mu\)mol CH\textsubscript{4} mL\(^{-1}\) h\(^{-1}\) throughout (Supplementary Fig. S3). Methane production was inhibited by N\textsubscript{2}O, and partly restored in periods when N\textsubscript{2}O was depleted (Fig. 2A, Supplementary Figs. S3 and S4). We also conducted parallel incubations with O\textsubscript{2} and NO\textsubscript{3}\textsuperscript{-} as electron acceptors. These incubations showed that methanogenesis was completely inhibited by NO\textsubscript{3}\textsuperscript{-}, and partly inhibited by O\textsubscript{2} (concentration in the liquid ranged from 20 to 90 \(\mu\)M O\textsubscript{2} (Supplementary Fig. S3). The rates of O\textsubscript{2} and NO\textsubscript{3}\textsuperscript{-} reduction indicated that the digestate contained a much higher number of cells able to respire O\textsubscript{2} and NO\textsubscript{3}\textsuperscript{-} than cells able to respire N\textsubscript{2}O (Supplementary Fig. SSA–C).

During the enrichment culturing with NO\textsubscript{3}\textsuperscript{-} almost all reduced NO\textsubscript{3}\textsuperscript{-} N appeared in the form of N\textsubscript{2}O-N during the first 50 h (Supplementary Fig. S5E), another piece of evidence that in the digestate, prior to enrichment culturing, the organisms reducing NO\textsubscript{3}\textsuperscript{-} to N\textsubscript{2}O outnumbered those able to reduce N\textsubscript{2}O to N\textsubscript{2}. The measured production of CH\textsubscript{4} and electron flows to electron acceptors deduced from measured gases (N\textsubscript{2}O, O\textsubscript{2} and CO\textsubscript{2}) were used to assess the effect of the three electron acceptors (N\textsubscript{2}O, NO\textsubscript{3}\textsuperscript{-} and O\textsubscript{2}) on C-mineralization. While oxygen appeared to have a marginal effect, NO\textsubscript{3}\textsuperscript{-} and N\textsubscript{2}O caused severe retardation of C-mineralization during the first 50 and 100 h, respectively (Supplementary Fig. SSA–D). This retarded mineralization is plausibly due to the inhibition of methanogenesis, causing a transient accumulation of H\textsubscript{2} and VFAs until the N\textsubscript{2}O-reducing bacteria reach a cell density that allowed them to effectively reap these compounds. This was corroborated by measurements of H\textsubscript{2} and VFAs (Supplementary Fig. S13).

To track the origin of the enriched N\textsubscript{2}O-respiring bacteria in the digestate, we considered the possibility that these are indigenous wastewater-sludge bacteria that survive the passage through the anaerobic digester, which had a retention time of 20–24 days. We assessed survival of N\textsubscript{2}O-respiring bacteria in the sludge survived the passage (Supplementary Fig. S6). We also did enrichment culturing with a digestate from a thermophilic digester (52 °C) operated in parallel with the mesophilic digester (52 °C) operated in parallel with the mesophilic digester (52 °C) to explore the effect of the anaerobic incubation with N\textsubscript{2}O on the entire microbial consortium, and to identify the organisms growing by N\textsubscript{2}O reduction. Metagenomic sequences were assembled and resultant contigs assigned to 278 metagenome-assembled genomes (MAGs), of which 149 were deemed to be of sufficient quality (completeness > 50% and contamination < 20%, Supplementary Data S1) for downstream analysis. The phylogenetic relationship and the relative abundance of the MAGs throughout the enrichment are summarized in Fig. 3, which also shows selected features revealed by the combined metagenomic and metaproteomic analyses, including information about genes and detected proteins involved in N\textsubscript{2}O reduction, other denitrification steps, methanogenesis, syntrophic acetate oxidation and methane oxidation.

Closer inspections of the abundance of individual MAGs, based on their coverage in the metagenome and metaproteome, showed that the majority of the MAGs had a near constant population density throughout the incubation, while two MAGs (260 and 268) increased substantially (Fig. 4; further analyses in Supplementary Section B, Supplementary Figs. S8–S11). The stable abundance of the majority indicates that the methanogenic consortium remained intact despite the downshift in temperature (20 °C versus 37 °C) and the inhibition of methanogenesis by N\textsubscript{2}O. Only 9 MAGs showed a consistent decline in abundance throughout the enrichment (Supplementary Table S1). These MAGs could theoretically correspond to microbes whose metabolism is dependent on efficient H\textsubscript{2} scavenging by methanogens [27], but we found no genomic evidence for this, and surmise that organisms circumscribed by the declining MAGs were unable to adapt to the temperature downshift from 37 °C to 20 °C.

Six MAGs, including the two that were clearly growing (MAG260 & MAG268) contained the nosZ gene and thus had the genetic potential to produce N\textsubscript{2}O-reductase (Nos) (Fig. 4). Nos proteins originating from five of these MAGs were detected in the metaproteome. Importantly, while all but one of these MAGs contained genes encoding the other denitrification reductases, none of these were detected in the metaproteome, suggesting that the organisms can regulate the expression of their denitrification machinery to suit available electron acceptors, in this case N\textsubscript{2}O. Three of the MAGs with detectable Nos in the proteome (MAG004, MAG059, MAG248) appeared to be non-growing during the enrichment. The detected levels of their Nos proteins remained more or less constant, and their estimated abundance in the metagenome and proteome did not increase (Fig. 4B). It is conceivable that these three MAGs belong to the initial population of N\textsubscript{2}O reducers whose N\textsubscript{2}O-reduction activity was present initially but gradually decreased during the early phase of the enrichment (Fig. 2A). The two growing MAGs (MAG260 and MAG268) showed increasing Nos levels and increasing abundance both in terms of coverage and metaproteomic detection (Fig. 4B, in proportion with the N\textsubscript{2}O produced (Supplementary Fig. S11). MAG260 reached the highest abundance of the two and accounted for 92% of the total detectable Nos pool at the final time point. MAG260 is taxonomically most closely affiliated with the genus \textit{Dechloromonas} (GTDB, 97.9% amino acid similarity). Interestingly, Nap rather than Nar takes the role of nitrate reductase in MAG260 (Fig. 4), which makes it a promising organism for N\textsubscript{2}O mitigation since organisms with Nap only (lacking Nar) preferentially channel electrons to N\textsubscript{2}O rather than to NO\textsubscript{3}\textsuperscript{-} [18]. MAG260, MAG004 and MAG088 contain a clade II nosZ, characterized by a sec-dependent signal peptide, in contrast to the more common \textit{tat}-dependent clade I nosZ [16] The physiological implications of clade I versus clade II nosZ remains unclear. Organisms with nosZ Clade II have high growth yield and high affinity (low \(k\)) for N\textsubscript{2}O, in proportion with the N\textsubscript{2}O Clade I [28], suggesting a key role of nosZ Clade II organisms for N\textsubscript{2}O reduction in soil, but this was contested by Conthe et al. [29], who found that Clade I organisms had higher “catalytic efficiency” (\(V_{\text{max}}/k\)) than those with Clade II. The apparent inhibition of methanogenesis by N\textsubscript{2}O seen in the present study has been observed frequently [30] and is probably due to inhibition of coenzyme M methyltransferase [31], which is a membrane bound enzyme essential for methanogenesis and common to all methanogenic archaea [32]. The gas kinetics demonstrate that the inhibition was reversible, being partly
restored whenever N2O was depleted (Fig. 2). In the enrichment culture where metagenomics and metaproteomics was monitored, several such incidents of N2O depletion occurred (Fig. 2A) and during these periods CH4 accumulated to levels amounting to 10% of levels in control vials without N2O (Supplementary Fig. S4B). These observations suggest that methanogens would be able to grow, albeit sporadically, during the enrichment, which is corroborated by the sustained detection of the complete methanogenesis pathway, including the crucial coenzyme M methyl-transferase, of *Methanothrix* (MAG025), *Methanoregulaceae* (MAG014) and *Methanobacterium* (MAG124) at high levels in the metaproteome. In fact, both MAG coverage data and 16S rRNA gene copy numbers assessed by ddPCR suggested that the majority of the original methanogenic consortium continued to grow (Supplementary Section B). A tentative map of the metabolic flow of the methanogenic consortium, including the reaping of intermediates (monosaccharides, fatty acids, acetate and H2) by N2O-respiring bacteria is shown in Supplementary Fig. S12. Since methane production was inhibited from the very beginning of the incubation, while it took ~100 h for the N2O-respiring bacteria to
reach high enough numbers to become a significant sink for intermediates (Fig. 2), one would expect transient accumulation of volatile fatty acids and H₂, which was corroborated by measurements of these metabolites (Supplementary Fig. S13).

Of note, we detected methane monooxygenase and methanol dehydrogenase proteins from MAG087 and MAG059, respectively, in the metaproteome. This opens up the tantalizing hypothesis of N₂O-driven methane oxidation, a process only recently suggested [33, 34]. However, a close inspection of the N₂O- and CH₄-kinetics indicated that N₂O-driven methane oxidation played a minor role (Supplementary Fig. S4CD).

In a follow-up experiment, 7 parallel enrichment cultures were analyzed by 16S rRNA gene amplicon sequencing, demonstrating reproducibility of the selective enrichment of organisms circumscribed by MAG 260 (Fig. S14).

**Isolation of N₂O-respiring bacteria and their geno- and phenotyping**

Whilst this enrichment culture could be used directly as a soil amendment, this approach is likely to have several disadvantages. First, it would require the use of large volumes of N₂O for enrichment, a process which would be costly and require significant infrastructure. An alternative approach would be to introduce an axenic or mixed culture of digestate-derived, and likely digestate-adapted, N₂O-respiring bacteria to sterilized/sanitized digestates. This approach has multiple benefits: (1) it would remove the need for N₂O enrichment on site as isolates could be grown aerobically in the digestate material, (2) one could chose organisms with favorable denitrification genotypes and regulatory phenotypes, (3) the sanitation would eliminate the methanogenic consortium hence reducing the risk of methane emissions from anoxic micro-niches in the amended soil, and (4) sanitation of digestates aligns with current practices that require such a pretreatment prior to use for fertilization. For these reasons an isolation effort was undertaken to obtain suitable digestate-adapted N₂O-respiring microorganisms from the N₂O-enrichment cultures (Supplementary Material and Methods, Chapter 6). These efforts resulted in the recovery of three axenic N₂O-respiring bacterial cultures, which were subjected to subsequent genomic and phenotypic characterization.

The isolates were phylogenetically assigned to *Pseudomonas* sp. (PS), *Azospira* sp. (AS) and *Azonexus* sp. (AN) (working names in bold) based on full length 16S rRNA genes obtained from the sequenced genomes (accessions ERR4842639 – 40, Supplementary Table S2, phylogenetic trees shown in Supplementary Fig. S15). All were equipped with genes for a complete denitrification pathway (Fig. 4C). AN and AS carried napAB, encoding the periplasmic nitrate reductase (Nap) and nosZ clade.
II, whilst PS carried genes for the membrane bound nitrate reductase (Nar), encoded by narG, and nosZ clade I. All had nirS and norBC, coding for nitrite reductase (NirS) and nitric oxide reductase (Nor), respectively. Pairwise comparison of average nucleotide identities (ANI) with MAGs from the enrichment metagenomes showed that the isolate AN matched the *Dechloromonas*-affiliated MAG260 with 98.2% ANI, suggesting the isolate is circumscribed by MAG260 [35]. Given the GTDB phylogeny of AN and MAG260 and the 16S rRNA gene homology of AN (95.2% sequence identity to *Azonexus hydrophilus* DSM23864, Supplementary Fig. S15C), we conclude that AN likely represents a novel species within the *Azonexus* lineage. Unfortunately, the 16S rRNA gene was not recovered in MAG260, preventing direct comparison with related populations. No significant ANI matches in our MAG inventory were identified for the genomes of PS and AS indicating they were not captured via our metagenomic approaches, which highlights the complementarity of applying culture-dependent methods in parallel.

The carbon catabolism profiles of the isolates were assayed using Biolog PM1 and PM2 microplates, to screen the range of carbon sources utilized (Supplementary Section E: Supplementary Table S3). PS utilized a wide spectrum of carbon sources (amino acids, nucleic acids, volatile fatty acids (VFA), alcohols, sugar alcohols, monosaccharides and amino sugars), but only one polymer (laminarin). AN and AS could only utilize small VFAs (e.g. acetate, butyrate), intermediates in the TCA cycle and/or the β-oxidation/methyl malonyl-CoA pathways of fatty acid degradation (e.g. malate, fumarate, succinate), and a single amino acid (glutamate). Thus, all three would be able to grow in a live digestate by reaping the VFAs produced by the methanogenic consortium. While the utilization of VFAs as C-substrates is one of several options for PS, AN and AS appear to depend on the provision of VFAs. This was confirmed by attempts to grow the three isolates in an autoclaved digestate: while PS grew well and reached high cell densities without any provision of extra carbon sources, AN and AS showed early retardation of growth unless provided with an extra dose of suitable carbon source (glutamate, acetate, pyruvate or ethanol) (Supplementary Figs. S26 and S27). A high degree of specialization and metabolic streamlining may thus explain the observed dominance of AN (MAG260) during enrichment culturing.

To evaluate the potentials of these isolates to act as sinks for N₂O, we characterized their denitrification phenotypes, by monitoring kinetics of oxygen depletion, subsequent denitrification and transient accumulation of denitrification intermediates (N₂O−, NO, N₂O). The experiments were designed to assess properties associated with strong N₂O reduction such as 1) bet hedging, i.e. that all cells express N₂O reductase while only a fraction of the cells express nitrite- and/or nitrate-reductase, as demonstrated for *Paracoccus denitrificans* [17]; 2) strong metabolic preference for N₂O-reduction over NO −reduction, as demonstrated for organisms with periplasmic nitrate reductase [18]. Supplementary section F (Supplementary Figs. S16–S25) provides the results of all the experiments and a synopsis of the findings. In short: *Azonexus* sp. (AN) had a clear preference for N₂O over NO −reduction, but not over NO −reduction, ascribed to bet hedging with respect to the expression of nitrate reductase (a few cells express Nap, while all cells express Nos), which was corroborated by proteomics: the Nos/Nap abundance ratio was ~25 during the initial phase of denitrification (Supplementary Fig. S18). *Azospira* sp. (AS) had a similar preference for N₂O over NO −reduction, albeit less pronounced than in AN, and no preference for N₂O over NO −. *Pseudomonas* sp. (PS) showed a phenotype resembling that of *Paracoccus denitrificans* [17], with denitrification kinetics indicating that Nir is expressed in a majority of cells in response to O₂ depletion, while all cells appeared to express N₂O reductase. This regulation makes PS a more robust sink for N₂O than the two other isolates, since it kept N₂O extremely low even when provided with NO −.

In summary, PS appeared to be the most robust candidate as a sink for N₂O in soil for two reasons; 1) it can utilize a wide range of carbon substrates, and 2) its N₂O sink strength is independent of the type of nitrogen oxycan present (NO −or N₂O−). In contrast, AN and AS appear to be streamlined for harvesting intermediates produced by anaerobic consortia, hence their metabolic activity in soil could be limited. In addition, they could be sources rather than sinks for N₂O if provided with NO −, which is likely to happen in soils, at least in soils of neutral pH, during hypoxic/anoxic spells [36].

**Effects on N₂O emissions**

To assess if fertilization with digestates containing N₂O-reducing bacteria could reduce N₂O emissions from denitrification in soil, we conducted a series of incubation experiments with soils fertilized with digestates with and without N₂O-reducing bacteria. The fertilized soils were incubated in closed culture vials containing He + 0.5 vol % O₂, and O₂, NO, N₂O and N₂ were monitored during oxygen depletion and subsequent denitrification. The experiments included soils amended with digestates in which indigenous N₂O-reducing bacteria had been enriched by anaerobic incubation with N₂O (Fig. 2), as well as autoclaved digestates in which the isolates from the current study had been grown by aerobic cultivation (see Supplementary Figs. S26 and S27 for cultivation details). The experiments included three types of control digestates: digestate (directly from the digester), digestate heated to 70 °C for 2 h (to eliminate most of the indigenous consortium), and autoclaved digestate in which the strain PS had been grown aerobically and then heated to 70 °C for 2 h, to kill PS. In all cases, 3 mL of digestate was added to 10 g of soil. Since soil acidity has a pervasive effect on the synthesis of functional N₂O reductase [24], we tested the digestates with two soils from a liming experiment [37] with different pH (pH_CaCl₂ = 5.5 and 6.6).

The transient N₂O accumulation during denitrification was generally higher in the acid than in the near-neutral soil (Fig. 5), which was expected since the synthesis of functional Nos is hampered by low pH [23, 24]. Based on the kinetics of both N₂ and N₂O (see Supplementary Figs. S28 and S29), we calculated the N₂O-index (IN₂O) which is a measure of the molar amounts of N₂O relative to N₂ + N₂O in the headspace for a specific period (0–T), see equation at top of Fig. 5. Low values of IN₂O indicate efficient N₂O-reduction. In this case, we calculated IN₂O for the incubation period until 40% of the available NO − had been recovered as N₂ + N₂O (≡IN₂O_40) and for the incubation period until 100% was recovered (IN₂O_100). Statistical analyses showed significant effects of digestate treatments and soil pH, and the interaction between the two (p < 0.001).

Extremely low IN₂O values were recorded for the treatments with digestate in which N₂O-reducing bacteria were enriched by anaerobic incubation with N₂O, even in the acid soil. This is in line with the current understanding of how pH affects N₂O-reduction: low pH slows down the synthesis of functional Nos, but once synthesized, it remains functional even at low pH [23]. Functional Nos had already been expressed during the enrichment and was evidently activeafter amendment to the soils.

The presence of the isolates in the digestates had clear but variable effects on IN₂O. The most relevant control-treatment for evaluating the effect of the isolates would be PS_70 °C, because this digestate had been treated exactly the same way as that with metabolically active isolates present (autoclaved, aerated and aerobic cultivation). These contrasts in IN₂O for PS_70 °C versus PS, AN and AN) were all statistically significant (confidential intervals did not overlap), thus all strains reduced IN₂O compared to PS_70 °C. AN and AS resulted in much higher IN₂O in the acid than in the neutral, suggesting that the expression of functional N₂O reductase in these strains was hampered by low pH. In contrast
Fig. 5 Soil incubations, effects on N$_2$O emission. N$_2$O kinetics during incubation of soils amended with seven different digestates and a control (soil only); panel A: pH 5.5 soil, panel B: pH 6.6 soil. The digestates treatments are: Soil = soil without any amendment; Digestate = digestate directly from the anaerobic digester; Dig$_{70}^{\circ}$C = digestate heat treated to 70°C for 2 h; PS, AN and AS = autoclaved digestate in which isolates PS, AN and AS (respectively) had been grown aerobically (see Supplementary Figs. S26 and S27); PS$_{70}^{\circ}$C = digestate in which PS had been grown (as for the PS treatment), subsequently heated to 70°C for 2 h, N$_2$O enr. = digestate in which indigenous N$_2$O-respiring bacteria had been enriched (as shown in Fig. 2). The left panels show measured N$_2$O throughout soil incubations; the insets with altered scaling show N$_2$O levels for treatments that accumulated very little N$_2$O. The right panels show the N$_2$O indexes ($I_{N2O}$) expressed as % (equation shown in the panel), with confidential intervals (ANOVA + Tukey’s range test). $I_{N2O}$ is a proxy for potential N$_2$O emission from denitrification in soil [56]. Two $I_{N2O}$ values are shown: one for the timespan until 40% of the NO$_3^-\text{-N}$ was recovered as N$_2$ + N$_2$O + NO ($I_{N2O 40}$), and one for 100% recovery ($I_{N2O 100}$). More details (including N$_2$ and NO kinetics) are shown in Supplementary Figs. S28 and S29.
to AN and AS, PS resulted in very low $I_{\text{N}_2\text{O}}$ values in both soils, suggesting that this organism has an exceptional capacity to synthesize functional Nos at low pH.

These results show that the emission of $\text{N}_2\text{O}$ from soil fertilized with digestates can be manipulated by tailoring the digestate microbiome. Interestingly, measurements of methane in these soil incubations showed that the methanogenic consortia in digestates that had not been heat-treated (i.e. the live digestate and the $\text{N}_2\text{O}$ enrichment) remained metabolically intact in the soil, and started producing methane as soon as $\text{N}_2\text{O}$ and nitrogen oxycyanides had been depleted, while no methane was produced in the soils amended with autoclaved digestate, and that heated to 70 °C (Supplementary Fig. S30).

In an effort to determine the survival of the $\text{N}_2\text{O}$-scavenging capacity of a digestate enriched with $\text{N}_2\text{O}$ reducers, we also tested its effect on soil $\text{N}_2\text{O}$ emissions after a 70-h aerobic storage period (in soil or as enrichment culture, at 20 °C). These experiments demonstrated a sustained beneficial effect on $I_{\text{N}_2\text{O}}$ after 70 h of aerobic storage (Supplementary Fig. S31). This result indicates that the enrichment strategies discussed here are robust, although long-lasting storage experiments as well as field trials are needed.

**CONCLUDING REMARKS**

This feasibility study identifies an avenue for large scale cultivation of $\text{N}_2\text{O}$ reducers for soil application, which could be low cost if implemented as an add-on to biogas production systems. Further efforts should be directed towards selecting organisms that are both strong sinks for $\text{N}_2\text{O}$ and able to survive and compete in soil, to secure long-lasting effects on $\text{N}_2\text{O}$ emissions. A tantalizing added value would be provided by selecting organisms (or consortia of organisms) that are not only strong $\text{N}_2\text{O}$-sinks, but also promote plant growth and disease resistance [38, 39].

Gas kinetics, metagenomics and metaproteomics revealed that the methanogenic consortium of the digestate remains active during anaerobic incubation with $\text{N}_2\text{O}$, and that bacteria with an anaerobic respiratory metabolism grew by harvesting fermentation intermediates. The inhibition of methanogenesis by $\text{N}_2\text{O}$ implies that the respiring organisms would have immediate access to the electron donors that would otherwise be used by the methanogens, i.e. acetate and $\text{H}_2$, while they would have to compete with fermentative organisms for the “earlier” intermediates such as alcohols and VFA. The importance of fermentation intermediates as a carbon source for the $\text{N}_2\text{O}$-respiring bacteria would predict a selective advantage for organisms with a streamlined (narrow) catabolic capacity, i.e. limited to short fatty acids, and our results lend some support to this: the catabolic capacity of the organism that became dominant (MAG260, isolate AN) was indeed limited, as was also the case for isolate AS. Such organisms are probably not ideal $\text{N}_2\text{O}$-sinks in soil because their ability to survive in this environment would be limited. Organisms with a wider catabolic capacity, such as the isolated *Pseudomonas* sp. (PS), are stronger candidates for long term survival and $\text{N}_2\text{O}$-reducing activity in soil. The ideal organisms are probably yet to be found, however, and refinements of the enrichment culturing process are clearly needed.

The digestate used in this study contained $\text{N}_2\text{O}$-respiring bacteria, most likely survivors from the raw sludge, which however, were clearly outnumbered by bacteria that are net producers of $\text{N}_2\text{O}$. We surmise that the relative amounts of $\text{N}_2\text{O}$-producers and $\text{N}_2\text{O}$-reducers in digestates may vary, depending on the feeding material and configuration for the anaerobic digestion. This could explain the observed variable effects of digestates on $\text{N}_2\text{O}$ emission from soils [40, 41]. The high abundance of both $\text{NO}_2^-$ - and $\text{O}_2$-respiring organisms in digestates has practical implications for the attempts to grow isolated strains in digestates: they could be outnumbered by the indigenous $\text{NO}_3^-$ - and $\text{O}_2$-respiring organisms (Supplementary Fig. S5). Hence, we foresee that future implementation of this strategy will require a brief heat treatment or other sanitizing procedure. A bonus of such sanitation is that it eliminates methane production by the digestate in soil.

We failed to enrich organisms lacking all other denitrification genes than nosZ; the only reconstructed genome with nosZ only (MAG004) did not grow at all. Failure to selectively enrich such organisms by anaerobic incubation with $\text{N}_2\text{O}$ was also experienced by Conthe et al. [29]. The organisms that did grow by respiring $\text{N}_2\text{O}$ in our enrichment, were all equipped with genes for the full denitrification pathway, although the only denitrification enzyme expressed/detected during the enrichment was Nos. This agrees with the current understanding of the gene regulatory network of denitrification; nosZ is the only gene whose transcription does not depend on the presence of $\text{N}_2\text{O}$, $\text{NO}_2^-$ or NO [42], which were all absent during the enrichment. Two of the reconstructed MAGs had genes encoding periplasmic nitrate reductase (nap), as was the case for two of the three isolates (AN and AS). This in itself would predict preference for $\text{N}_2\text{O}$- over $\text{NO}_3^-$ reduction at a metabolic level [43], but otherwise their potential for being $\text{N}_2\text{O}$ sinks cannot be predicted by their genomes. The phenotyping of the isolates revealed conspicuous patterns of *bet hedging* as demonstrated for *Paracoccus denitrificans* [17]. The *bet hedging* in *P. denitrificans* is characterized by expression of *Nir* (and *Nor*) in a minority of the cells, while Nos is expressed in all cells, in response to oxygen depletion, hence the population as a whole is a strong sink for $\text{N}_2\text{O}$. The isolated *Pseudomonas* sp. (PS) displayed denitrification kinetics that closely resembles that of *P. denitrificans*. The two other isolates (AN and AS) showed indications of *bet hedging* as well, but of another sort: Nap appears to be expressed in a minority of the cells. This different regulatory phenotype had clear implications for the ability of organisms to function as $\text{N}_2\text{O}$-sinks: while all isolates were strong $\text{N}_2\text{O}$ sinks when provided with $\text{NO}_3^-$ only, AN and AS accumulated large amounts of $\text{N}_2\text{O}$ if provided with $\text{N}_2\text{O}^2$.

The $\text{N}_2\text{O}$ sink capacity of the organisms was tested by fertilizing soils with digestates with and without the organisms, and monitoring the gas kinetics in response to oxygen depletion, thus imitating the hot spots/hot moments of hypoxia/anoxia [44]. Since the isolates were raised by aerobic growth in autoclaved digestates, they would have to synthesize all denitrification enzymes in the soil, hence the synthesis of functional Nos was expected to be hampered by low pH [24]. The results for isolates AS and AN lend support to this (high $I_{\text{N}_2\text{O}}$ in the soil with pH $= 5.5$). AN was also dominating in the digestate enrichment culture, and in this case the organism had a strong and pH-independent effect on $\text{N}_2\text{O}$ emission, plausibly due synthesis of Nos prior to incorporation into the soils.

In summary, we have demonstrated that a digestate from biogas production can be transformed into an effective agent for mitigating $\text{N}_2\text{O}$ emission from soil, simply by allowing the right bacteria to grow to high cell densities in the digestate prior to fertilization. The technique is attractive because it can be integrated in existing biogas production systems, and hence is scalable. If we manage to treat a major part of waste materials in agroecosystems by AD, the resulting digestates would suffice to treat a large share of total farmland, as illustrated by Fig. 1. Estimation of the potential $\text{N}_2\text{O}$-mitigation effect is premature, but the documented feasibility and the scalability of the approach warrant further refinement as well as rigorous testing under field condition. Our approach suggests one avenue for a much needed valorization of organic wastes [45] via anaerobic digestion. Future developments of this approach could extend beyond the scope of climate change mitigation and include the enrichment of microbes for pesticide- and other organic pollutant degradation [46], plant growth promotion [47] and inoculation of other plant symbiotic bacteria [48].
REFERENCES

1. Tian H, Xu R, Canadell JG, Thompson RL, Winiwarter W, Suntharalingam P, et al. Nitrous oxide emissions increase under the Chinese plain. Environ Sci Technol. 2018;52:12504–12513.

2. Snyder CS, Davidson EA, Smith P, Venterea RT. Agriculture: sustainable crop and animal production to help mitigate nitrous oxide emissions.Curr Opin Environ Sustain. 2019;49:46–54.

3. Sutton MA, Oenema O, Erisman JW, Leip A, Van Grinsven H, Winiwarter W. Too much of a good thing. Nature. 2014;127:159–166.

4. Erisman JW, Sutton MA, Oenema O, Erisman JW, Leip A, Van Grinsven H, Winiwarter W. Too much of a good thing. Nature. 2014;127:159–166.

5. Dohnert K, Kostic T, McDowell R, Eudes F, Singh BK, Sarkar S, et al. Microbiome innovations for a sustainable future. Nat Microbiol. 2016;1:138–42.

6. Bakken LR, Frostegård Å. Emerging options for mitigating N2O emissions from animal production to help mitigate nitrous oxide emissions. Curr Opin Environ Sustain. 2012;25:138–42.

7. Butterbach-Bahl K, Baggs EM, Dannenmann M, Kiese R, Zechmeister-Boltenstern S. Nitrous oxide emissions from soils: how well do we understand the processes and their controls? Philos Trans R Soc B Biol Sci. 2013;368:13.

8. Tian H, Xu R, Canadell JG, Thompson RL, Winiwarter W, Suntharalingam P, et al. A comprehensive quantification of global nitrous oxide sources and sinks. Nature. 2020;586:248–56.

9. Zhang X, Liu M, Ju X, Gao B, Su F, Chen X, et al. Nitrous oxide emissions increase under the Chinese plain. Environ Sci Technol. 2018;52:12504–12513.

10. Song X, Liu M, Ju X, Gao B, Su F, Chen X, et al. Nitrous oxide emissions increase under the Chinese plain. Environ Sci Technol. 2018;52:12504–12513.

11. Zumft WG. Cell biology and molecular basis of denitrification. Microbiol Mol Biol Rev. 1997;61:533–616.

12. Qu Z, Bakken LR, Molstad L, Frostegård Å, Bergaust LL. Transcriptional and posttranscriptional effects of suboptimal pH on nitrogen oxide reductase in paracoccus denitrificans. Appl Microbiol Biotechnol. 2016;25:8378–96.

13. Liu B, Frostegård Å, Bakken LR. Impaired reduction of N2O to N2 in acid soils is due to a posttranscriptional interference with the expression of nosZ. MBio. 2014;5:e01383–14.

14. Lu H, Chandra K, Stensel D. Microbial ecology of denitrification in biological wastewater treatment. Water Res. 2014;64:237–54.

15. Sanford RA, Wagner DD, Sanford RA, Jones CM. Genomics and ecology of novel N2O-reducing microorganisms. Trends Microbiol. 2018;26:43–50.

16. Hallin S, Philippot L, Löf K.R. Jonassen et al. RSC metallobiology series 9: metalloenzymes in denitrification. Applications and environmental impacts. Cambridge: The Royal Society of Chemistry; 2017. p. 312–31.

17. Gao Y, Mania D, Moussavi SA, Lycus P, Armstrong M, Wolly K, et al. Competition for nitrous oxide (N2O)-reducing denitrifier-inoculated organic fertilizer mitigates N2O emissions from agricultural soils. Biol Fertil Soils. 2017;53:885–98.

18. Chen C, Shen X, Xie H, Hu Z, Pavlostathis SG, Zhang J. Coupled methane and nitrous oxide biotransformation in freshwater wetland sediment microcosms. Sci Total Environ. 2019;648:916–22.

19. Richter M, Bossellö-Mora R. Shifting the genomic gold standard for the prokaryotic species definition. Proc Natl Acad Sci USA. 2009;106:19216–31.

20. Lim NYN, Frostegård Å, Bakken LR. Nitrite kinetics in denitriﬁcation: the role of autochthonous reactions versus microbial reduction. Soil Biol Biochem. 2018;119:203–9.

21. Nadeem S, Bakken LR, Frostegård Å, Gaby JC, Dörsch P. Contingent effects of liming on N2O-emissions driven by autotrophic nitrification. Front Env Sci. 2020;8:598513.

22. Gao N, Shen W, Camargo E, Shiratori Y, Nishiizawa T, Isobe K, et al. Nitrous oxide (N2O)-reducing denitrifier-inoculated organic fertilizer mitigates N2O emissions from agricultural soils. Biol Fertil Soils. 2017;53:885–98.

23. Gao N, Shen W, Kakuta H, Tanaka N, Fujwara T, Nishiizawa T, et al. Inoculation with nitrous oxide (N2O)-reducing denitrifier strains simultaneously mitigates N2O emission from pasture soil and promotes growth of pasture plants. Soil Biol Biochem. 2016;97:83–91.

24. Baral KR, Laboriau R, Olesen JE, Petersen SO. Nitrous oxide emissions and nitrogen use efﬁciency of manure and digestates applied to spring barley. Agric Ecosyst Environ. 2017;239:166–177.

25. Herrero M, Henderson B, Havlik P, Thornton PK, Conant RT, Smith P, et al. Greenhouse gas mitigation potentials in the livestock sector. Nat Clim Chang. 2016;6:452–61.

26. Spio S. Regulation of denitrification, (Chapter 13). In: Ismail M, José JMG, Sofia RP, Luisa BM (editors). RSC metallobiology series 9: metalloenzymes in denitrification: applications and environmental impacts. Cambridge: The Royal Society of Chemistry; 2017. p. 312–31.

27. Gao Y, Mania D, Moussavi SA, Lycus P, Armstrong M, Wolly K, et al. Competition for nitrous oxide (N2O)-reducing denitrifier-inoculated organic fertilizer mitigates N2O emissions from agricultural soils. Biol Fertil Soils. 2017;53:885–98.

28. Peng W, Piavola A. Sustainable management of digesterage from the organic fraction of municipal solid waste and food waste under the concepts of back to earth alternatives and circular economy. Waste Biomass Valoriz. 2019;10:465–81.

29. Sun S, Siddhu V, Rong Y, Zheng Y. Pesticide pollution in agricultural soils and sustainable remediation. Methods: a Rev Curr Pollut Rep. 2018;4:240–50.

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DATA AVAILABILITY

The sequencing data for this study have been deposited in the European Nucleotide Archive (ENA) at EMBL-EBI under accession number PRJEB341816 (isolates AN, A5 and P5) and PRJEB41816 (metagenome) (https://www.ebi.ac.uk/ena/browser/view/PRJEBxxxx). Functionally annotated MAGs and metagenomic assembly are available in FigShare (https://doi.org/10.6084/m9.figshare.13012451 and https://doi.org/10.6084/m9.figshare.13012493). The proteomics data has been deposited to the ProteomeXchange Consortium (http://proteomexchange.org) via the PRIDE partner repository [49] with the dataset identifier PXD022030* and PXD023233** for the metaproteome and proteome of Azonexus sp. AN, respectively.

* Reviewer access: Username: reviewer_pxd022030@ebi.ac.uk. Password: GdTR3b3
** Reviewers access: Username: reviewer_pxd023233@ebi.ac.uk. Password: nMz52S80
47. Backer R, Rokem JS, Ilangumaran G, Lamont J, Praslickova D, Ricci E, et al. Plant growth-promoting rhizobacteria: context, mechanisms of action, and roadmap to commercialization of biostimulants for sustainable agriculture. Front Plant Sci. 2018;871:1473.

48. Poole P, Ramachandran V, Terpolilli J. Rhizobia: from saprophytes to endosymbionts. Nat Rev Microbiol. 2018;16:291–303.

49. Vizcaíno JA, Côté RG, Córcoles A, Dianes JA, Fabregat A, Foster JM, et al. The Proteomics Identifications (PRIDE) database and associated tools: status in 2013. Nucleic Acids Res. 2013;41:1063–9.

50. Foged R, Lyngsø H, Flotats X, August B, Blasi J, Palati A, et al. Inventory of manure processing activities in Europe. Technical report No. I concerning “Manure Processing Activities in Europe” to the European Commission, Directorate-General Environment. 2011;138. Publications Office of the European Union, Brussels.

51. Holm-Nielsen JB, Al Seadi T, Oleskowicz-Popiel P. The future of anaerobic digestion and biogas utilization. Bioresour Technol. 2009;100:5478–84.

52. Stenmarck. Estimates of European food waste levels, Report of the project FUSIONS (contract number: 311972) granted by the European Commission (FP7). https://doi.org/10.13140/RG.2.1.4658.4721.

53. Meyer AKP, Ehimen EA, Holm-Nielsen JB. Future European biogas: animal manure, straw and grass potentials for a sustainable European biogas production. Biomass Bioenergy. 2009;111:154–64.

54. Eurostat (2017) Agri-environmental indicator – greenhouse gas emissions. ISSN 2443-8219, https://ec.europa.eu/eurostat/statistics-explained/pdfscache/16817.pdf.

55. Vaccaro BJ, Thorgersen MP, Lancaster WA, Price MN, Wetmore KM, Poole FL, et al. Determining roles of accessory genes in denitrification by mutant fitness analyses. Appl Environ Microbiol. 2016;82:51–61.

56. Russenes AL, Korsaeth A, Bakken LR, Dörsch P. Spatial variation in soil pH controls off-season N$_2$O emission in an agricultural soil. Soil Biol Biochem. 2016;99:36–46.

**AUTHOR CONTRIBUTIONS**

KJR conducted the research, analyzed and interpreted the data, and wrote the manuscript, LHH conducted genomic and proteomic analyses of the enrichments, SHWV conducted the research and wrote the manuscript, MA contributed to analysis of the genomics and proteomics, VE and ÅF contributed to the writing of the manuscript, PL did proteomics on the isolate, LM was responsible for gas measurements and kinetics analyses, PBP contributed to the analysis of genomics and proteomics and to the writing of the manuscript, LRB designed the experiment, analyzed the data and wrote the manuscript.

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**COMPETING INTERESTS**

The authors declare no competing interests.

**ADDITIONAL INFORMATION**

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