N_2O-respiring bacteria in biogas digestates for reduced agricultural emissions

Kjell Rune Jonassen[1,3], Live H. Hagen[1], Silas H.W. Vick[1], Magnus Ø. Arntzen[1], Vincent G.H. Eijsink[1], Åsa Frostegård[1], Pawel Lycus[1], Lars Molstad[4], Phillip B. Pope[1,2], Lars R. Bakken[1*]

[1)] Faculty of Chemistry, Biotechnology and Food Science, NMBU - Norwegian University of Life Sciences, 1432 Ås, Norway

[2)] Faculty of Biosciences, NMBU - Norwegian University of Life Sciences, Norway

[3)] VEAS WWTP, Bjerkåsholmen 125, 3470 Slemmestad, Norway.

[4)] Faculty of Science and Technology, Norwegian University of Life Sciences, Norway

* corresponding author

Abstract

Inoculating agricultural soils with N_2O-respiring bacteria (NRB) can reduce N_2O-emissions, but would be impractical as a standalone operation. Here we demonstrate that digestates obtained after biogas production are suitable substrates and vectors for NRB. We show that indigenous NRB in digestates grew to high abundance during anaerobic enrichment under N_2O. Gas-kinetics and meta-omic analyses showed that these NRB's, recovered as metagenome-assembled genomes (MAGs), grew by harvesting fermentation intermediates of the methanogenic consortium. Three NRB's were isolated, one of which matched the recovered MAG of a *Dechloromonas*, deemed by proteomics to be the dominant producer of N_2O-reductase in the enrichment. While the isolates harbored genes required for a full denitrification pathway and could thus both produce and sequester N_2O, their regulatory traits predicted that they act as N_2O sinks in soil, which was confirmed experimentally. The isolates were grown by aerobic respiration in digestates, and fertilization with these NRB-enriched digestates reduced N_2O emissions from soil. Our use of digestates for low-cost and large-scale inoculation with NRB in soil can be taken as a blueprint for future applications of this powerful instrument to engineer the soil microbiome, be it for enhancing plant growth, bioremediation, or any other desirable function.

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 N_2O emissions (Tian et al 2020). Reduced emissions can be achieved by minimizing the consumption of fertilizer nitrogen through improved agronomic practice and reduction of meat consumption (Snyder et al 2014, Sutton et al, 2011), but such measures are unlikely to do more than stabilize the global consumption of fertilizer N (Erisman et al 2008). This calls for more inventive approaches to reduce N_2O emissions, targeting the microbiomes of soil (D'Hondt et al 2021), in particular the physiology and regulatory biology of the organisms involved in production and consumption of N_2O in soil (Bakken and Frostegård 2020).

N_2O turnover in soil involves several metabolic pathways, controlled by a plethora of fluctuating physical and chemical variables (Butterbach-Bahl et al 2013, Hu et al 2015).

Heterotrophic denitrification is the dominant N_2O source in most soils, while autotrophic ammonia oxidation may dominate in well drained calcareous soils (Song et al 2018 and references therein). Heterotrophic denitrifying organisms are both sources and sinks for N_2O because N_2O is a free intermediate in their stepwise reduction of nitrate to dinitrogen ($NO_3^- \rightarrow NO_2^- \rightarrow NO \rightarrow N_2O \rightarrow N_2$). 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 (Zumft 1997, Qu et al 2016). Many organisms have truncated denitrification pathways, lacking from one to three of the four reductase genes (Shapleigh 2013, Lycus et al 2017), and truncated denitrifiers can thus act as either N_2O producers (organisms without *nosZ*) or N_2O reducers (organisms with *nosZ* only). The organisms with *nosZ* only, coined non-denitrifying N_2O-reducers (Sanford et al 2013), have attracted much interest as N_2O sinks in the environment (Hallin et al 2018). Of note, organisms with a full-fledged denitrification pathway may also be strong N_2O sinks depending on the relative activities and regulation of the various enzymes in the denitrification pathway (Lycus et al 2018; Mania et al 2020). Despite their promise, feasible ways to utilize N_2O-reducing organisms to reduce N_2O emissions have not yet emerged.

A soil with a strong N_2O-reducing capacity will emit less N_2O than one dominated by net N_2O producing organisms, as experimentally verified by Domeignoz-Horta et al (2016), who showed that soils emitted less N_2O if inoculated with large numbers (10^7 - 10^8 cells g^{-1} soil) of organisms expressing Nos as their sole denitrification reductase. As a standalone operation, the large-scale production and distribution of N_2O-respiring bacteria would be prohibitively expensive and impractical. However, the use of N_2O-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 (**Figure 1**), as an element of the roadmap towards a low-carbon circular economy (Scarlat et al 2018). This means that digestates from **AD** are likely to become a major organic fertilizer for agricultural soils, with a huge potential for reducing N_2O emissions if enriched with N_2O-respiring bacteria prior to application.

Here we provide the first proof of this promising concept. Firstly, we demonstrate selective enrichment and isolation of fast-growing digestate-adapted N_2O-respiring bacteria using a digestate from a wastewater treatment plant. Secondly, we demonstrate that the use of digestates enriched with such organisms as a soil amendment reduces the proportion of N leaving soil as N_2O, confirming the suitability of such digestates for this purpose. Analysis of the enrichment process with multi-omics and in-depth monitoring of gas kinetics provides valuable insights into Nos-synthesis by the various enriched taxa, and the metabolic pathways of the anaerobic consortium providing substrates for these enriched N_2O-respiring organisms.

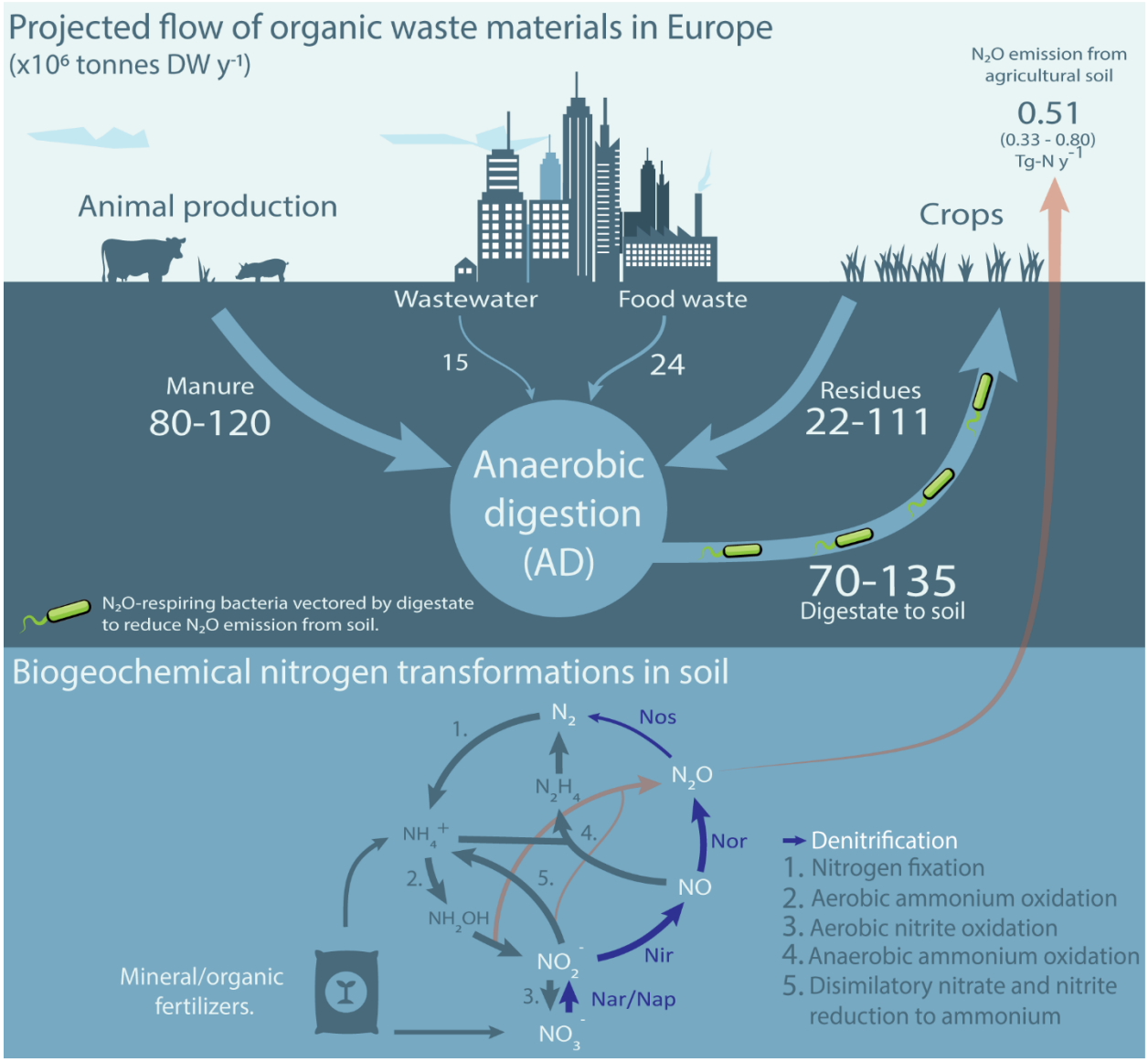

Figure 1. Possible biomass streams in a future circular economy with a central role for anaerobic digestion. Solid arrows (top section) show streams of biomass available for anaerobic digestion (AD). Numbers indicate known estimates of currently used or potentially available amounts in Europe, in million tonnes dry-weight (DW) per year (Foged et al 2011, Holm-Nielsen et al 2009, Stenmarck et al 2016, Meyer et al 2018). The arrow from anaerobic digestion to agricultural soil, indicates a credible pathway for digestate enriched with N_2O-reducing bacteria (assuming enrichment at **AD** site); fertilization with such enriched digestates strengthens the N_2O sink capacity of the soil, hence reducing N_2O emissions. N_2O emissions from agricultural soil in Europe are estimated at 0.51 tG per year (min 0.33 – max 0.80), representing some 48 % of total European N_2O emissions (Tian et al 2020), which account for approximately 3.5 % of the global warming effect from European greenhouse gas emissions and 35 % of the global warming effect from European agriculture (Eurostat 2018). The lower half of the picture shows the microbial nitrogen transformations underlying these N_2O emissions, which are fed by fertilizers. Today, **AD** is primarily used for treating urban organic wastes, which comprise only ~10 % of the biomass potentially available for **AD**. The amount of biomass treated by **AD** is expected to increase by an order of magnitude when adopted on a large scale in the agricultural sector. This would generate 70-135 Mt DW of digestate annually (assuming 50% degradation by AD), which is equivalent to 400-780 kg DW ha^{-1} y^{-1} if spread evenly on the total farmland of Europe (173 million ha).

Materials and methods

98 The digestates were taken from two anaerobic digesters, one mesophilic (37 °C) and one thermophilic
99 (52 °C), which were running in parallel, producing biogas from sludge produced by a wastewater
100 treatment. The sludge was a poly-aluminum chloride (PAX-XL61™, Kemira) and ferric chloride
101 (PIX318™, Kemira) precipitated municipal wastewater sludge, with an organic matter content of 5.6%
102 (w/w). Both digestors reduced the organic matter by approximately 60%, producing digestates
103 containing ~2.1 % organic matter, 1.8-1.9 g NH_4^+-N L^{-1}, ~16 and 32 Meq VFA L^{-1} , pH=7.6-7.8 and 8.2;
104 mesophilic and thermophilic, respectively (see Suppl Methods 1 for further details). The digestates
105 were transported to the laboratory in 1 L insulated steel-vessels and used for incubation experiments
106 3-6 hours after sampling.

107 The robotized incubation system developed by Molstad et al (2007, 2016) was used in all experiments
108 where gas kinetics was monitored. The system hosts 30 parallel stirred batches in 120 mL serum vials,
109 crimp sealed with gas tight butyl rubber septa, which are monitored for headspace concentration of
110 O_2, N_2, N_2O, NO, CO_2 and CH_4 by frequent sampling. After each sampling, the system returns an equal
111 volume of He, and elaborated routines are used to account for the gas loss by sampling to calculate
112 the production/consumption-rate of each gas for each time interval between two samplings. More
113 details are given in Suppl Methods 2.

114 Enrichment culturing of N_2O-respiring bacteria (NRB) in digestate was done as stirred (300 rmp)
115 batches of 50 mL digestate per vial. Prior to incubation, the headspace air was replaced with Helium
116 by repeated evacuation and He-filling (Molstad et al 2007), and supplemented with N_2O, and N_2O in
117 the headspace was sustained by repeated injections in response to depletion. Liquid samples (1 mL)
118 were taken by syringe, for metagenomic and metaproteomic analyses, and for quantification of
119 volatile fatty acids (VFA) and 16srDNA abundance. The samples were stored -80 °C before analyzed.
120 The growth of NRB in the enrichments was modelled based on the N_2O reduction kinetics. The
121 modelling and the analytic methods (quantification of VFA and 16srDNA abundance) are described in
122 detail in Suppl Methods 3.

123 Metagenomics and metaprotomics: Sequencing of DNA (Illumina HiSec4000), and the methods for
124 Metagenome-Assembled Gemome (MAG) binning, and the phylogenetic placement of the MAGs is
125 described in detail in Suppl Methods 4. Proteins were extracted and digested to peptides, which were
126 analyzed by nanoLC-MS/MS, and the acquired spectra were inspected, using the metagenome-
127 assembled genomes (149 MAGs) as a scaffold (Suppl Methods 5).

128 Isolation of N_2O-respiring bacteria (NRB) (Suppl Methods 6). NRB present in the enrichment cultures
129 were isolated by spreading diluted samples on agar plates with different media composition, then
130 incubated in an anaerobic atmosphere with N_2O. Visible colonies were re-streaked and subsequently
131 cultured under aerobic conditions, and 16s-sequenced. Three isolates, **AS** (*Azospira* sp), **AN** (Azonexus
132 sp) and **SP** (*Pseudomonas* sp) (names based on their 16s sequence), were selected for genome
133 sequencing, characterization of their denitrification phenotypes, and for testing their effect as N_2O
134 sinks in soil.

135 Genome sequencing and phenotyping of isolates. Three isolates were genome sequenced and
136 compared with MAG's of the enrichment culture (Suppl Methods 7). The isolates' ability to utilize
137 various organic C substrates was tested on BiOLOG Phenotype MicroArray™ microtiter plates, and
138 their characteristic regulation of denitrification was tested through a range of incubation experiments
139 as in previous investigations (Bergaust et al 2010, Liu et al 2014, Lycus et al 2018, Mania et al 2020),
140 by monitoring the kinetics of O_2, N_2, N_2O, NO and NO_2^- throughout the cultures' depletion of O_2 and
141 transition from aerobic to anaerobic respiration in stirred batch cultures with He + O_2 (+/- N_2O) in the
142 headspace (Suppl Methods 8). The kinetics of electron flow throughout the oxic and anoxic phase in

143 these experiments were used to assess if the organisms were *bet hedging*, as demonstrated for
144 *Paracoccus denitrificans* (Lycus et al 2018), i.e. that only a minority of cells express nitrate- and/or
145 nitrite-reductase, while all express Nos, in response in response to oxygen depletion. Putative *bet*
146 *hedging* was corroborated by measuring the abundance of nitrate-, nitrite- and nitrous oxide
147 reductase (Suppl Methods 9).

148 N_2O mitigation experiments (Suppl Methods 9). To assess the capacity of the isolates to reduce the
149 N_2O emission from soil, they were grown aerobically in sterilized digestate, which was then added to
150 soil in microcosms, for measuring the NO-, N_2O- and N_2- kinetics of denitrification in the soil. For
151 comparison, the experiments included soils amended with sterilized digestate, live digestate (no
152 pretreatment), and digestate in which N_2O-reducing bacteria had been enriched by anaerobic
153 incubation with N_2O (as for the initial enrichment culturing).

154 *Data availability*

155 The sequencing data for this study have been deposited in the European Nucleotide Archive (ENA) at
156 EMBL-EBI under accession number PRJEB41283 (isolates AN, AS and PS) and PRJEB41816
157 (metagenome) (https://www.ebi.ac.uk/ena/browser/view/PRJEBxxxx). Functionally annotated MAGs
158 and metagenomic assembly are available in FigShare (DOI: 10.6084/m9.figshare.13102451 and
159 10.6084/ m9.figshare.13102493). The proteomics data has been deposited to the ProteomeXchange
160 Consortium (http://proteomecentral.proteomexchange.org) via the PRIDE partner repository
161 (Vizcaino et al 2013) with the dataset identifier PXD022030* and PXD023233** for the metaproteome
162 and proteome of *Azonexus* sp. AN, respectively.

163 * Reviewer access: Username: reviewer_pxd022030@ebi.ac.uk. Password: GdTR3biE

164 ** Reviewers access: Username: reviewer_pxd023233@ebi.ac.uk Password: nMz62S8O

165

166 **Results and Discussion**

167 Enrichment of indigenous N_2O- respiring bacteria (NRB) in digestates

168 We hypothesized that suitable organisms could be found in anaerobic digesters fed with
169 sewage sludge, since such sludge contains a diverse community of denitrifying bacteria
170 stemming from prior nitrification/denitrification steps (Lu et al 2014). We further
171 hypothesized that these bacteria could be selectively enriched in digestates by anaerobic
172 incubation with N_2O. We decided to enrich at 20 ^{o}C, rather than at the temperatures of the
173 anaerobic digesters (37 and 52 ^{0}C), to avoid selecting for organisms unable to grow within the
174 normal temperature range of soils.

175 The digestates were incubated anaerobically as stirred batch cultures with N_2O in the
176 headspace (He atmosphere), and the activity and apparent growth of N_2O reducers was
177 assessed by monitoring the N_2O-reduction to N_2. **Figure 2A** shows the results for the first
178 experiment, where culture vials were liquid samplws were taken at three time points (0, 115
179 and 325 h) for metagenomics, metaproteomics, and quantification of 16S rDNA and volatile
180 fatty acids (VFAs). N_2O was periodically depleted (100-140 h) in this experiment, precluding
181 detailed analysis of the growth kinetics throughout. This was avoided in the second
182 enrichment, for which complete gas data are shown in **Figure 2BC**. Apart from the deviations
183 caused by the temporary depletion of N_2O in the first experiment, both experiments showed

very similar N_2 production rates (**Figures 2B and S1B**). The gas kinetics of the second enrichment are discussed in detail below.

Figure 2B shows declining rates of N_2-production (V_{N2}) during the first 50 h, followed by exponential increase. This was modelled as the activity of two groups of NRB, one growing exponentially from low initial abundance, and one which was more abundant initially, but whose activity declined gradually (further explained in **Figure S1**). The modelling, indicated that the cell density of the growing NRB increased exponentially (specific growth rate, $\mu = 0.1$ h^{-1}) from a very low initial density (~$2.5 \cdot 10^3$ cells mL^{-1}) to $1.6 \cdot 10^8$ cells mL^{-1} after 110 h, and continued to increase at a gradually declining rate to reach ~$3 \cdot 10^9$ cells mL^{-1} at the end of the incubation period (215 h). The modelled cell-specific electron flow rate (V_{e-}, **Figure 2C**) was sustained at around 5 fmol e^- $cell^{-1}$ h^{-1} during the exponential growth, and declined gradually thereafter, as the number of cells continued to increase, while the overall rate of N_2O-respiration remained more or less constant (V_{N2}, **Figure 2B**). Enrichment culturing as shown in **Figure 2BC** was repeated three times, demonstrating that the characteristic N_2 production kinetics was highly reproducible (**Figure S2**).

The provision of substrate for the N_2O-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 ^oC, versus 37 ^oC in the digester). In **AD**, organic polymers are degraded and converted to CO_2 and CH_4 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, H_2 and CO_2, and 4) methane production from acetate, CO_2, H_2, and methylated compounds. By providing N_2O to this (anaerobic) system, organisms that respire N_2O 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 (Stams et al 2003). Thus, the respiration and growth of the N_2O-respiring bacteria is 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_2O-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_2O confirmed the presence of an active methanogenic consortium, sustaining a methane production rate of ~0.2 µmol CH_4 mL^{-1} h^{-1} throughout (**Figure S3**). Methane production was inhibited by N_2O, and partly restored in periods when N_2O was depleted (**Figure 2A, Figures S3**&**S4**). We also conducted parallel incubations with O_2 and NO_3^- as electron acceptors. These incubations showed that methanogenesis was completely inhibited by NO_3^-, and partly inhibited by O_2 (concentration in the liquid ranged from 20 to 90 µM O_2) (**Figures S3**). The rates of O_2 and NO_3^- reduction indicated that the digestate contained a much higher number of cells able to respire O_2 and NO_3^- than cells able to respire N_2O (**Figure S5A-C**). During the enrichment culturing with NO_3^- , almost all reduced nitrogen appeared in the form of N_2O during the first 50 h (**Figure S5E**),

another piece of evidence that in the digestate (prior to enrichment culturing), the organisms reducing NO_3^- to N_2O outnumbered those able to reduce N_2O to N_2. The measured production of CH_4 and electron flows to electron acceptors deduced from measured gases (N_2, O_2 and CO_2) were used to assess the effect of the three electron acceptors (N_2O, NO_3^- and O_2) on C-mineralization. While oxygen appeared to have a marginal effect, NO_3^- and N_2O caused severe retardation of C-mineralization during the first 50 and 100 h, respectively (**Figure S5A-D**)**.** This retarded mineralization is plausibly due to the inhibition of methanogenesis, causing a transient accumulation of H_2 and VFAs until the N_2O-reducing bacteria reach a cell density that allowed them to effectively reap these compounds. This was corroborated by measurements of H_2 and VFAs (**Figure S13**).

To track the origin of the enriched N_2O-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_2O-respiring bacteria by comparing the N_2O reduction potential of wastewater sludge and the digestate. The results indicated that ≤ 1/3 of the N_2O-respiring bacteria in the sludge survived the passage (**Figure S6**). We also did enrichment culturing with a digestate from a thermophilic digester (52 ^oC) operated in parallel with the mesophilic digester (provided with the same feed), and found that it too contained N_2O reducers that could be enriched, although the estimated initial numbers were orders of magnitude lower than in the mesophilic digestate **(Figure S7)**.

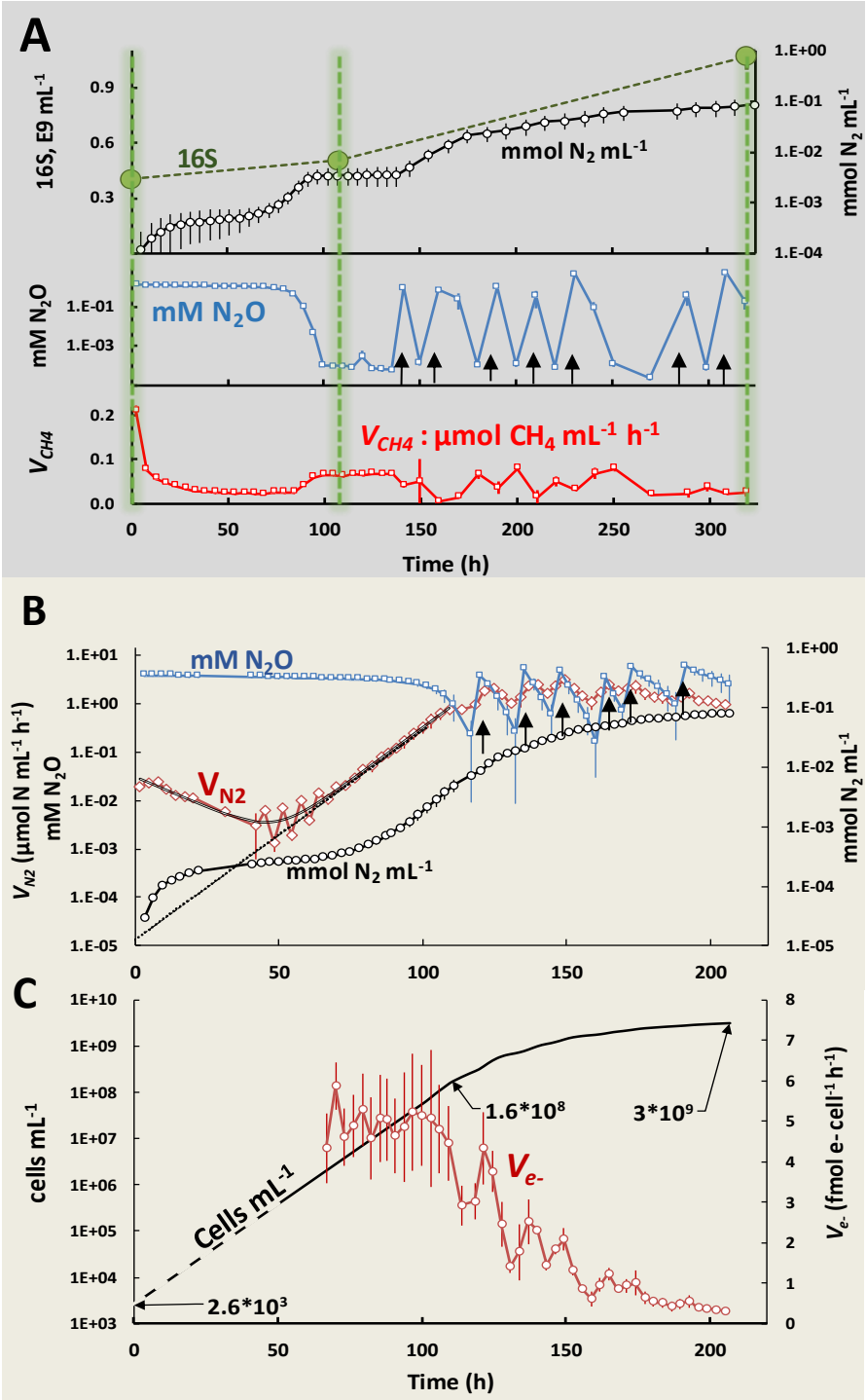

Figure 2: Gas kinetics in anaerobic enrichment cultures with digestate.

Panel A shows results for the enrichment culture (triplicate culture vials) sampled for metagenomics, metaproteomics, quantification of volatile fatty acids (VFAs) and 16S rDNA abundance (sampling times = 0, 115 and 325 hours, marked by vertical green lines). The top panel shows the amounts of N_2 produced (mmol N_2 L^{-1} digestate, log scale) and 16S rDNA copy numbers. The mid panel shows the concentration of N_2O in the digestate (log scale), which was replenished by repeated injections 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.

Panel B and C show the results of a repeated enrichment experiment where N_2O-depletion (as seen at t=100-140 h in panel A) was avoided, to allow more precise assessment and modelling of growth kinetics. Panel B: N_2O concentration in the digestate (mM N_2O), rate of N_2-production (V_{N2}) and N_2 produced (mmol N_2 mL^{-1} digestate), all log scaled. The curved black line shows the modelled V_{N2} 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 modelled density (cells mL^{-1}) of cells growing by N_2O respiration, extrapolated back to t=0 h (dashed line), and the cell specific respiratory activity (V_{e-}, fmol electrons $cell^{-1}$ h^{-1}), which declined gradually after 110 h. Standard deviations (n = 3) are shown as vertical lines. **Figure S1** provides additional data for the experiment depicted in Panel A, as well as a detailed description of the modelling procedures and their results.

MAG-centric metaproteomic analysis of the enrichment cultures

295 We analyzed the metagenome and metaproteome at three timepoints (0, 115 and 325 h,
296 **Figure 2A**), to explore the effect of the anaerobic incubation with N_2O on the entire microbial
297 consortium, and to identify the organisms growing by N_2O reduction. Metagenomic
298 sequences were assembled and resultant contigs assigned to 278 metagenome-assembled
299 genomes (MAGs), of which 149 were deemed to be of sufficient quality (completeness > 50%
300 and contamination < 20%, Supplementary Data S1) for downstream analysis. The
301 phylogenetic relationship and the relative abundance of the MAGs throughout the
302 enrichment are summarized in **Figure 3**, which also shows selected features revealed by the
303 combined metagenomic and metaproteomic analyses, including information about genes
304 and detected proteins involved in N_2O reduction, other denitrification steps, methanogenesis,
305 syntrophic acetate oxidation and methane oxidation.

306 Closer inspections of the abundance of individual MAGs, based on their coverage in the
307 metagenome and metaproteome, showed that the majority of the MAGs had a near constant
308 population density throughout the incubation, while two MAGs (260 and 268) increased
309 substantially (**Figure 4**; further analyses in **Supplementary Section B, Figures S8-S11**). The
310 stable abundance of the majority indicates that the methanogenic consortium remained
311 intact despite the downshift in temperature (20 °C versus 37 °C) and the inhibition of
312 methanogenesis by N_2O. Only 9 MAGs showed a consistent decline in abundance throughout
313 the enrichment (**Table S1**). These MAGs could theoretically correspond to microbes whose
314 metabolism is dependent on efficient H_2 scavenging by methanogens (Schink 1997), but we
315 found no genomic evidence for this, and surmise that organisms circumscribed by the
316 declining MAGs were unable to adapt to the temperature downshift from 37 °C to 20 °C.

317 Six MAGs, including the two that were clearly growing (MAG260 & MAG268) contained the
318 *nosZ* gene and thus had the genetic potential to produce N_2O-reductase (Nos) (**Figure 4**). Nos
319 proteins originating from five of these MAGs were detected in the metaproteome.
320 Importantly, while all but one of these MAGs contained genes encoding the other
321 denitrification reductases, none of these were detected in the metaproteome, suggesting
322 that the organisms can regulate the expression of their denitrification machinery to suit
323 available electron acceptors, in this case N_2O. Three of the MAGs with detectable Nos in the
324 proteome (MAG004, MAG059, MAG248) appeared to be non-growing during the enrichment.
325 The detected levels of their Nos proteins remained more or less constant, and their estimated
326 abundance in the metagenome and -proteome did not increase (**Figure 4B**). It is conceivable
327 that these three MAGs belong to the initial population of N_2O reducers whose N_2O-reduction
328 activity was present initially but gradually decreased during the early phase of the enrichment
329 (**Figure 2A**). The two growing MAGs (MAG260 and MAG268) showed increasing Nos levels
330 and increasing abundance both in terms of coverage and metaproteomic detection (**Figure**
331 **4B**), in proportion with the N_2 produced (**Figure S11**). MAG260 reached the highest
332 abundance of the two and accounted for 92% of the total detectable Nos pool at the final
333 time point. MAG260 is taxonomically most closely affiliated with the genus *Dechloromonas*
334 (GTDB, 97.9% amino acid similarity). Interestingly, Nap rather than Nar takes the role of
335 nitrate reductase in MAG260 (**Figure 4**), which makes it a promising organism for N_2O
336 mitigation since organisms with Nap only (lacking Nar) preferentially channel electrons to N_2O
337 rather than to NO_3^- (Mania et al 2020). MAG260, MAG004 and MAG088 contain a clade II

338 *nosZ*, characterized by a *sec*-dependent signal peptide, in contrast to the more common *tat*-
339 dependent clade I *nosZ*. The physiological implications of clade I versus clade II *nosZ* remains
340 unclear. Organisms with *nosZ* Clade II have high growth yield and high affinity (low k_s) for N_2O,
341 compared to thise with *nosZ* Clade II (Yoon et al 2016), suggesting a key role of *nosZ* Clade II
342 organisms for N_2O reduction in soil, but this was contested by Conthe et al (2018), who found
343 that Clade I organisms had higher "catalytic efficiency" (V_{max}/k_s) than those with Clade II.

344 The apparent inhibition of methanogenesis by N_2O seen in the present study has been
345 observed frequently (Andalib et al 2011) and is probably due to inhibition of coenzyme M
346 methyltransferase (Kengen et al 1988), which is a membrane bound enzyme essential for
347 methanogenesis and common to all methanogenic archaea (Fischer et al 1992). The gas
348 kinetics demonstrate that the inhibition of was reversible, being partly restored whenever
349 N_2O was depleted (**Figure 2**). In the enrichment culture where metagenomics and
350 metaproteomics was monitored, several such incidents of N_2O depletion occurred (**Figure 2A**)
351 and during these periods CH_4 accumulated to levels amounting to 10% of levels in control
352 vials without N_2O (**Figure S4B**). These observations suggest that methanogens would be able
353 to grow, albeit sporadically, during the enrichment, which is corroborated by the sustained
354 detection of the complete methanogenesis pathway, including the crucial coenzyme M
355 methyl-transferase, of *Methanothrix* (MAG025), *Methanoregulaceae* (MAG014) and
356 *Methanobacterium* (MAG124) at high levels in the metaproteome. In fact, both MAG
357 coverage data and 16S rDNA copy numbers assessed by ddPCR suggested that the majority of
358 the original methanogenic consortium continued to grow (**Supplementary Section B**). A
359 tentative map of the metabolic flow of the methanogenic consortium, including the reaping
360 of intermediates (monosaccharides, fatty acids, acetate and H_2) by N_2O-respiring bacteria is
361 shown in **Figure S12.** Since methane production was inhibited from the very beginning of the
362 incubation, while it took ~100 hours for the N_2O-respiring bacteria to reach high enough
363 numbers to become a significant sink for intermediates (**Figure 2**), one would expect transient
364 accumulation of volatile fatty acids and H_2, which was corroborated by measurements of
365 these metabolites (**Figure S13**).

366 Of note, we detected methane monooxygenase and methanol dehydrogenase proteins from
367 MAG087 and MAG059, respectively, in the metaproteome. This opens up the tantalizing
368 hypothesis of N_2O-driven methane oxidation, a process only recently suggested to occur
369 (Valenzuela et al 2020; Cheng et al 2019). However, a close inspection of the N_2O- and CH_4-
370 kinetics indicated that N_2O-driven methane oxidation played a minor role (**Figure S4CD**).

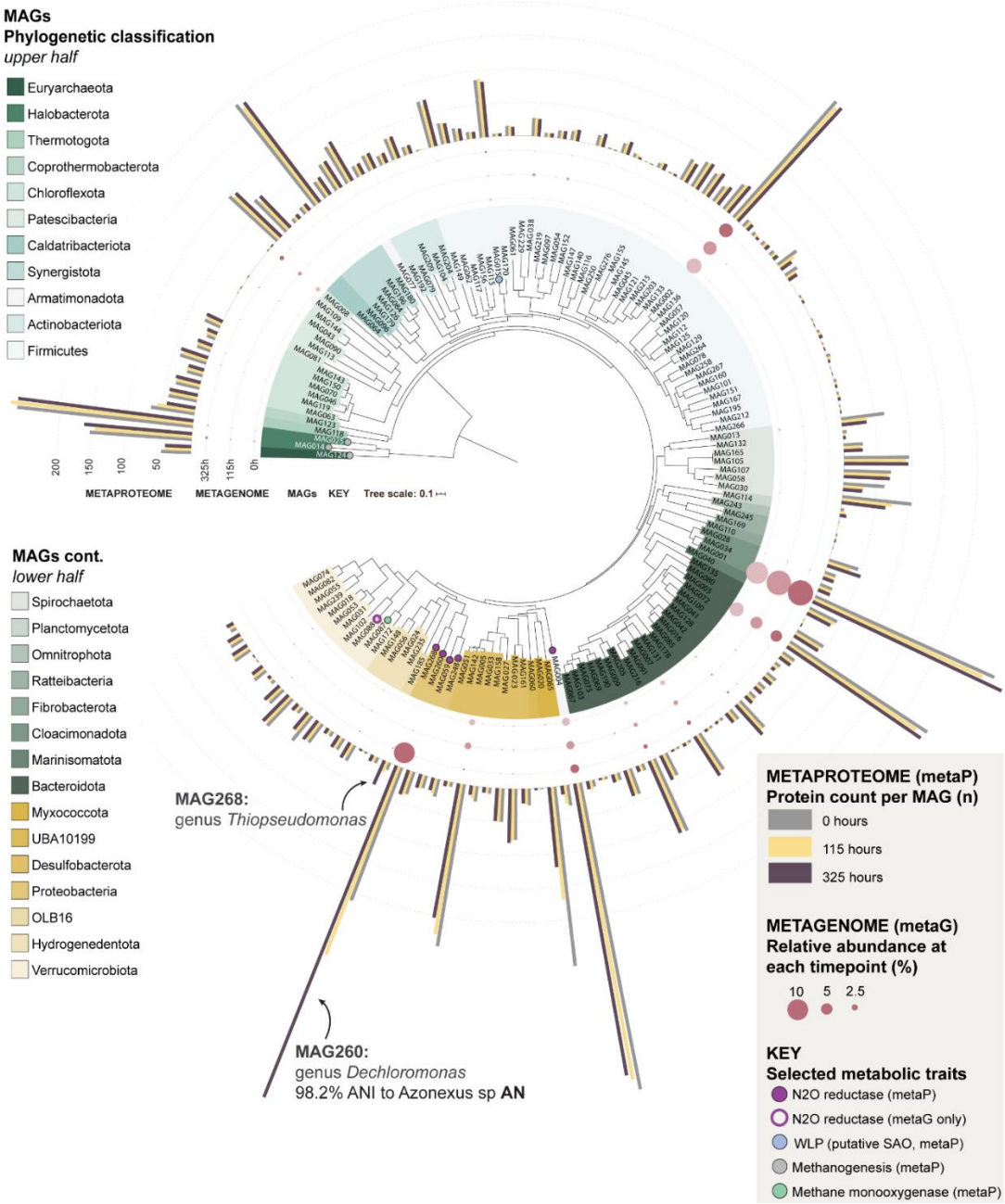

Figure 3: MAGs from the anaerobic enrichment culture with the mesophilic digestate. The figure shows a maximum likelihood tree indicating the phylogenetic placement of MAGs from the anaerobic enrichment. The tree was constructed from a concatenated set of protein sequences of single-copy genes. Taxonomic classification of the MAGs was inferred using the Genome Taxonomy Database (GTDB) and is displayed at the phylum level by label and branch coloring. Branch label decorations indicate the presence of genes involved in selected metabolic traits in the MAGs. The relative abundance of the MAG in the community as calculated from sequence coverage is indicated by bubbles at branch tips and bar charts indicate the number of detected proteins affiliated with each MAG at the three time points during incubation. Four of the 149 MAGs that met the completeness and contamination threshold for construction of the metaproteome database were lacking the universal single-copy marker genes and were omitted from the tree. Total protein counts per MAG were calculated by aggregating both secretome and cell-associated proteomes.

Isolation of N_2O-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 N_2O

408 for enrichment, a process which would be costly and require significant infrastructure. An
409 alternative approach would be to introduce an axenic or mixed culture of digestate-derived,
410 and likely digestate-adapted, N_2O-respiring bacteria to sterilized/sanitized digestates. This
411 approach has multiple benefits: 1) it would remove the need for N_2O enrichment on site as
412 isolates could be grown aerobically in the digestate material, 2) one could chose organisms
413 with favorable denitrification genotypes and regulatory phenotypes, 3) the sanitation would
414 eliminate the methanogenic consortium hence reducing the risk of methane emissions from
415 anoxic micro-niches in the amended soil, and 4) sanitation of digestates aligns with current
416 practices that require such a pretreatment prior to use for fertilization. For these reasons an
417 isolation effort was undertaken to obtain suitable digestate-adapted N_2O-respiring
418 microorganisms from the N_2O-enrichment cultures. These efforts resulted in the recovery of
419 three axenic N_2O-respiring bacterial cultures, which were subjected to subsequent genomic
420 and phenotypic characterization.

421 The isolates were phylogenetically assigned to *Pseudomonas* sp. (**PS**), *Azospira* sp. **(AS)** and
422 *Azonexus* sp. (**AN**) (working names in bold) based on full length 16S rDNA obtained from the
423 sequenced genomes (accessions ERR4842639 - 40, **Table S2**, phylogenetic trees shown in
424 **Figure S14**). All were equipped with genes for a complete denitrification pathway (**Figure 4C**).
425 **AN** and **AS** carried *napAB*, encoding the periplasmic nitrate reductase (Nap) and *nosZ* clade II,
426 whilst **PS** carried genes for the membrane bound nitrate reductase (Nar), encoded by *narG*,
427 and *nosZ* clade I. All had *nirS* and *norBC*, coding for nitrite reductase (NirS) and and nitric oxide
428 reductase (Nor), respeictivey. Pairwise comparison of average nucleotide identities (ANI) with
429 MAGs from the enrichment metagenomes showed that the isolate **AN** matched the
430 *Dechloromonas*-affiliated MAG260 with 98.2 % ANI, suggesting the isolate is circumscribed by
431 MAG260 (Richter and Resselló-Móra 2009). Given the GTDB phylogeny of **AN** and MAG260
432 and the 16S rDNA gene homology of **AN** (95.2 % sequence identity to *Azonexus hydrophilus*
433 DSM23864, **Fig S14C**), we conclude that **AN** likely represents a novel species within the
434 *Azonexus* lineage. Unfortunately, the 16S rDNA gene was not recovered in MAG260,
435 preventing direct comparison with related populations. No significant ANI matches in our
436 MAG inventory were identified for the genomes of **PS** and **AS.**

437 The carbon catabolism profiles of the isolates were assayed using Biolog^{TM} PM1 and PM2
438 microplates, to screen the range of carbon sources utilized (**Supplementary Section E)**. **PS**
439 utilized a wide spectrum of carbon sources (amino acids, nucleic acids, volatile fatty acids
440 (VFA), alcohols, sugar alcohols, monosaccharides and amino sugars), but only one polymer
441 (laminarin). **AN** and **AS** could only utilize small VFAs (*eg*. acetate, butyrate), intermediates in
442 the TCA cycle and/or the β-oxidation/methyl malonyl-CoA pathways of fatty acid degradation
443 (*eg*. malate, fumarate, succinate), and a single amino acid (glutamate). Thus, all three would
444 be able to grow in a live digestate by reaping the VFA's produced by the methanogenic
445 consortium. While the utilization of VFAs as C-substrates is one of several options for **PS**, **AN**
446 and **AS** appear to depend on the provision of VFAs. This was confirmed by attempts to grow
447 the three isolates in an autoclaved digestate: while **PS** grew well and reached high cell
448 densities without any provision of extra carbon sources, **AN** and **AS** showed early retardation
449 of growth unless provided with an extra dose of suitable carbon source (glutamate, acetate,
450 pyruvate or ethanol) (**Figure S25 and S26**). A high degree of specialization and metabolic

451 streamlining may thus explain the observed dominance of **AN** (MAG260) during enrichment
452 culturing.

453 To evaluate the potentials of these isolates to act as sinks for N_2O, we characterized their
454 denitrification phenotypes, by monitoring kinetics of oxygen depletion, subsequent
455 denitrification and transient accumulation of denitrification intermediates (NO_2^-, NO, N_2O).
456 The experiments were designed to assess properties associated with strong N_2O reduction
457 such as 1) *bet hedging*, i.e. that all cells express N_2O reductase while only a fraction of the
458 cells express nitrite- and/or nitrate-reductase, as demonstrated for *Paracoccus denitrificans*
459 (Lycus et al 2018); 2) strong metabolic preference for N_2O-reduction over NO_3^- -reduction, as
460 demonstrated for organisms with periplasmic nitrate reductase (Mania et al 2020).
461 **Supplementary section F** provides the results of all the experiments and a synopsis of the
462 findings. In short: *Azonexus* sp. (**AN**) had a clear preference for N_2O over NO_3^- reduction, but
463 not over NO_2^- reduction, ascribed to *bet hedging* with respect to the expression of nitrate
464 reductase (a few cells express Nap, while all cells express Nos), which was corroborated by
465 proteomics: the Nos/Nap abundance ratio was ~25 during the initial phase of denitrification
466 (**Figure S17**). *Azospira* sp. (**AS**) had a similar preference for N_2O over NO_3 reduction, albeit less
467 pronounced than in **AN**, and no preference for N_2O over NO_2^-. *Pseudomonas* sp. (**PS**) showed
468 a phenotype resembling that of *Paracoccus denitrificans* (Lycus et al 2018), with
469 denitrification kinetics indicating that Nir is expressed in a minority of cells in response to O_2
470 depletion, while all cells appeared to express N_2O reductase. This regulation makes **PS** a more
471 robust sink for N_2O than the two other isolates, since it kept N_2O extremely low even when
472 provided with NO_2^-.

473 In summary, **PS** appeared to be the most robust candidate as a sink for N_2O in soil for two
474 reasons; 1) it can utilize a wide range of carbon substrates, and 2) its N_2O sink strength is
475 independent of the type of nitrogen oxyanion present (NO_2^- or NO_3^-). In contrast, **AN** and **AS**
476 appear to be streamlined for harvesting intermediates produced by anaerobic consortia,
477 hence their metabolic activity in soil could be limited. In addition, they could be sources rather
478 than sinks for N_2O if provided with NO_2^-, which is likely to happen in soils, at least in soils of
479 neutral pH, during hypoxic/anoxic spells (Lim et al 2018).

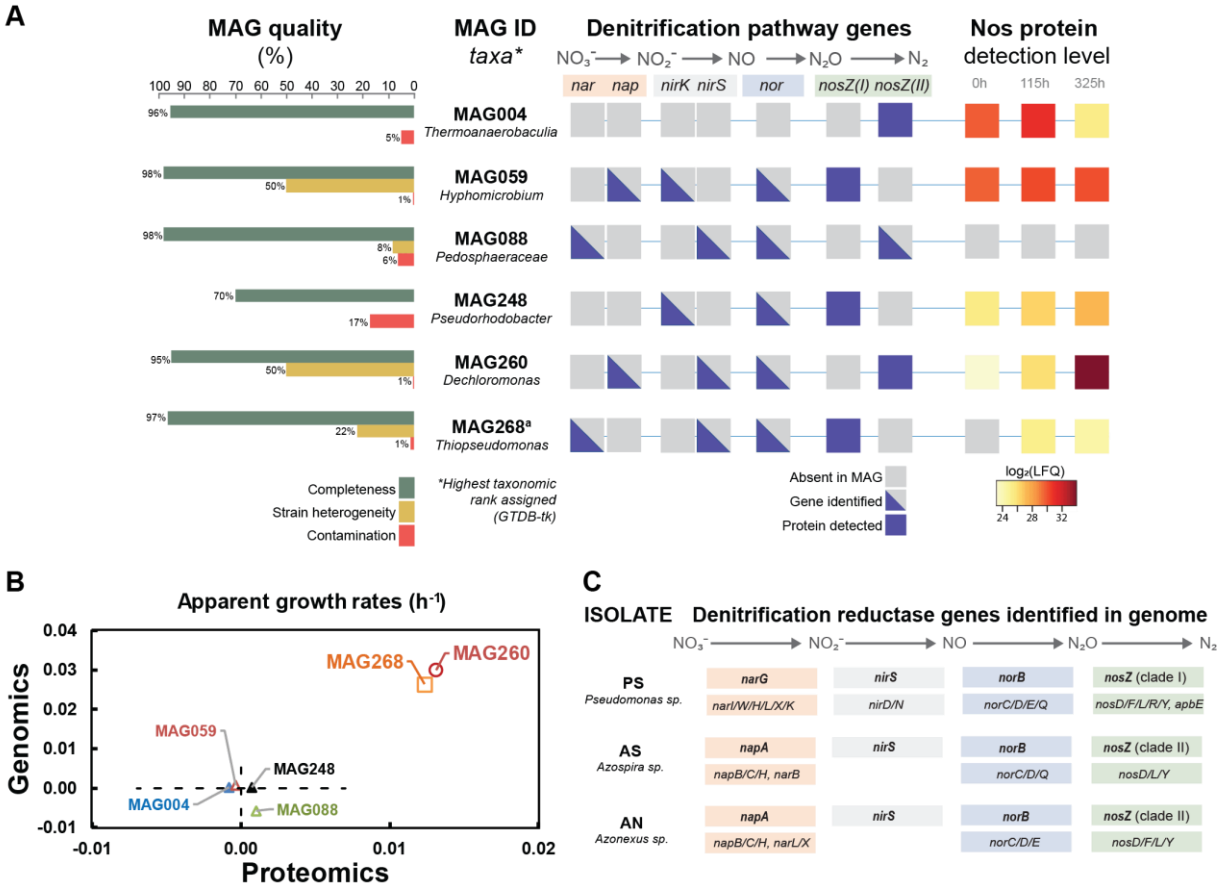

Figure 4: Overview of MAGs with *nosZ* and denitrification genes in isolated organisms. Panel A shows the quality (completeness, strain heterogeneity and contamination), taxonomic classification based on GTDB and NCBI, presence of denitrifying genes and proteins, and the detected levels of Nos (N_2O reductase, encoded by *nosZ*) throughout the enrichment culturing for the six MAGs containing the *nosZ* gene (**Supplementary Data S1** and **S2**). Nos was detected in the proteome of five MAGs, but the detection level increased significantly throughout for only MAG260 and 248, respectively. None of the MAGs produced detectable amounts of the other denitrification reductases. [a)] LFQ values for one of the two detected predicted Nos proteins for MAG268 is shown. Panel B shows the apparent growth rates of the MAGs, based on their coverage in the metagenome and metaproteome (regression of ln(N) against time; see **Figure S11** for more details). Panel C shows the taxonomic classification (16S rDNA), working names (abbreviations) and denitrification genotypes of three isolates from the enrichment culturing. The genes coding catalytic subunits of denitrification reductases are shown in bold, above the accessory genes (Vaccaro et al 2016) that were also identified. More information about accessory genes is presented in **Figure S14**. The isolate **AN** has 98.2 % ANI to MAG260.

Effects on N_2O emissions

To assess if fertilization with digestates containing N_2O-reducing bacteria could reduce N_2O emissions from denitrification in soil, we conducted a series of incubation experiments with soils fertilized with digestates with and without N_2O-reducing bacteria. The fertilized soils were incubated in closed culture vials containing He + 0.5 vol % O_2, and O_2, NO, N_2O and N_2 were monitored during oxygen depletion and anaerobic growth. The experiments included soils amended with digestates in which indigenous N_2O-reducing bacteria had been enriched by anaerobic incubation with N_2O (**Figure 2**), as well as autoclaved digestates in which the isolates from the current study had been grown by aerobic cultivation (see **Figures S25** & **S26** for cultivation details). The experiments included two types of control digestates: Live

digestate (directly from the digester), and live digestate heated to 70 °C for 2 hours (to eliminate most of the indigenous consortium). 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 N_2O reductase (Liu et al 2014), we tested the digestates with two soils from a liming experiment (Nadeem et al 2020) with different pH (pH_{CaCl2} = 5.5 and 6.6).

The transient N_2O accumulation during denitrification was generally higher in the acid than in the near-neutral soil (**Figure 5**), which was expected since the synthesis of functional Nos is hampered by low pH (Bergaust et al 2010, Liu et al 2014). Based on the kinetics of both N_2 and N_2O (see **Figure S27 and S28**), we calculated the N_2O-index ($\boldsymbol{I_{N2O}}$) which is a measure of the molar amounts of N_2O relative to N_2+N_2O in the headspace for a specific period (0-T), see equation at top of **Figure 5**). Low values of $\boldsymbol{I_{N2O}}$ indicate efficient N_2O-reduction. In this case, we calculated $\boldsymbol{I_{N2O}}$ for the incubation period until 40% of the available NO_3^- had been recovered as N_2+N_2O (=$\boldsymbol{I_{N2O\ 40}}$) and for the incubation period until 100% was recovered ($\boldsymbol{I_{N2O\ 100}}$).

Extremely low $\boldsymbol{I_{N2O}}$ values were recorded for the treatments with digestate in which N_2O-reducing bacteria were enriched by anaerobic incubation with N_2O, even in the acid soil. This is in line with the current understanding of how pH affects N_2O-reduction: low pH slows down the synthesis of functional Nos, but once synthesized, it remains functional even at low pH (Bergaust et al 2010). Functional Nos had already been expressed during the enrichment and was evidently active after amendment to the soils.

$\boldsymbol{I_{N2O}}$ values were generally high for treatment with live digestate, which probably reflects that the digestate is dominated by N_2O-producing organisms (**Figure S5E**). This interpretation is corroborated by the observed effect of heat-treating the live digestate; this lowered $\boldsymbol{I_{N2O}}$ substantially.

The presence of the isolates in the digestates had clear but variable effects on $\boldsymbol{I_{N2O}}$. Compared to the heat treated digestate ("70 C dig" Fig 5), **AN** and **AS** increased the $\boldsymbol{I_{N2O}}$-values in the soil with pH=5.5, while in the soil with pH 6.6, their effect was marginal. The high $\boldsymbol{I_{N2O}}$ for **AN** and **AS** in the acid soil plausibly reflect that the isolates were grown aerobically in the digestate, hence synthesizing their denitrification enzymes after transfer to soil, which would be hampered by low pH. In contrast to AN and AS, **PS** resulted in very low $\boldsymbol{I_{N2O}}$ values in both soils, suggesting that this organism has an exceptional capacity to synthesize functional Nos at low pH.

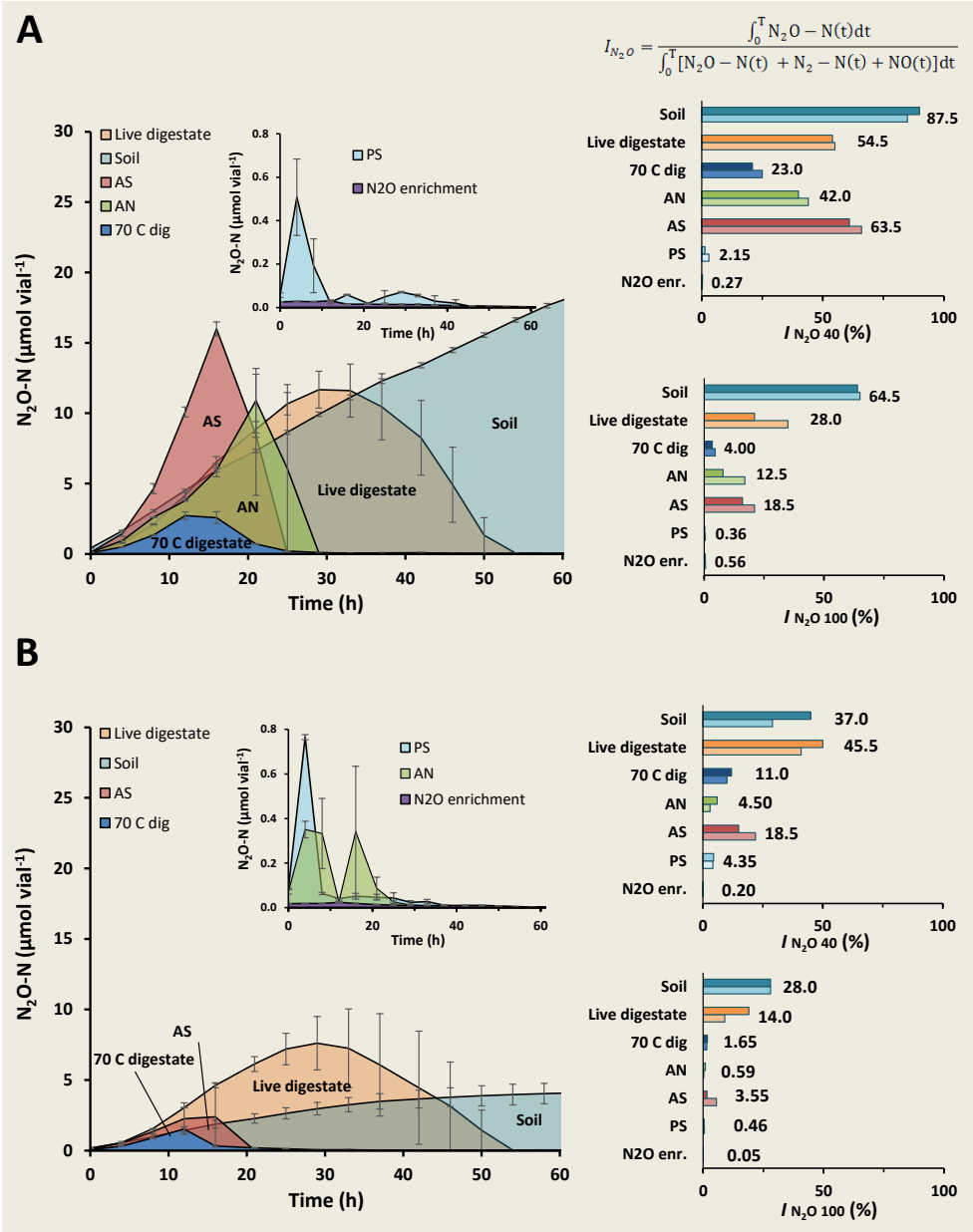

Figure 5: Soil incubations. N_2O kinetics during incubation of soils amended with six different digestates and a control sample (soil only). Panel A shows results for the pH 5.5 soil, while panel B the pH 6.6 soil. The digestates treatments are: "Live digestate", digestate directly from the anaerobic digester; "70 C dig", live digestate heat treated to 70 °C for two hours; AN, AS and PS: autoclaved digestate on which isolates AN, AS and PS had been grown aerobically (see Figure **S25&S26** for details on the cultivation); "N_2O enr"= digestate enriched with N_2O-respiring bacteria (as in **Fig 2**). The left panels show the N_2O levels observed during each treatment; the insets, with altered scaling, show N_2O levels for treatments that resulted in very low N_2O levels (the PS and N_2O enr. treatments). The bar graphs to the right show the N_2O indexes (***I_{N2O}***, bar height = single culture vial values, numerical value = average of duplicate culture vials), which are calculated by dividing the area under the N_2O-curve by the sum of the areas under the N_2O and N_2-curve, expressed as % (see equation in the figure and Liu et al 2014; the N_2 curves are provided in **Figures S27&S28**). ***I_{N2O}*** have proven to be a robust proxy for potential N_2O emission from soil (Russenes et al 2016). Two ***I_{N2O}*** values are shown: one for the timespan until 40% of the NO_3^--N was recovered as N_2+N_2O+NO ($I_{N2O\ 40\%}$), and one for 100% recovery ($I_{N2O\ 100\%}$). More details (including N_2 and NO kinetics) are shown in **Figure S27 and S28.**

These results show that the emission of N_2O from soil fertilized with digestates can be manipulated by tailoring the digestate microbiome. Interestingly, measurements of methane

553 in these soil incubations showed that the methanogenic consortia in digestates that had not
554 been heat treated (i.e. the live digestate and the N_2O enrichment) remained metabolically
555 intact in the soil, and started producing methane as soon as N_2O and nitrogen oxyanions had
556 been depleted, while no methane was produced in the soils amended with autoclaved
557 digestate, and that heated to 70 ^oC (**Figure S29**).

558 In an effort to determine the survival of the N_2O-scavenging capacity of a digestate enriched
559 with N_2O reducers, we also tested its effect on soil N_2O emissions after a 70-hour aerobic
560 storage period (in soil or as enrichment culture, at 20 °C). These experiments demonstrated
561 a sustained beneficial effect on $\boldsymbol{I_{N2O}}$ after 70 hours of aerobic storage (**Figure S30**). This result
562 indicates that the enrichment strategies discussed here are robust, although long-lasting
563 storage experiments as well as field trials are needed.

564 **Concluding remarks**

565 This feasibility study identifies an avenue for large scale cultivation of N_2O reducers for soil
566 application, which could be low cost if implemented as an add-on to biogas production
567 systems. Further efforts should be directed towards selecting organisms that are both strong
568 sinks for N_2O and able to survive and compete in soil, to secure long-lasting effects on N_2O
569 emissions. A tantalizing added value would be provided by selecting organisms (or consortia
570 of organisms) that are not only strong N_2O-sinks, but also promote plant growth and disease
571 resistance (Gao et al 2016, 2017).

572 Gas kinetics, metagenomics and metaproteomics revealed that the methanogenic consortium
573 of the digestate remains active during anaerobic incubation with N_2O, and that bacteria with
574 an anaerobic respiratory metabolism grew by harvesting fermentation intermediates. The
575 inhibition of methanogenesis by N_2O implies that the respiring organisms would have
576 immediate access to the electron donors that would otherwise be used by the methanogens,
577 i.e. acetate and H_2, while they would have to compete with fermentative organisms for the
578 "earlier" intermediates such as alcohols and VFA. The importance of fermentation
579 intermediates as a carbon source for the N_2O-respiring bacteria would predict a selective
580 advantage for organisms with a streamlined (narrow) catabolic capacity, i.e. limited to short
581 fatty acids, and our results lend some support to this: the catabolic capacity of the organism
582 that became dominant (MAG260, isolate **AN**) was indeed limited, as was also the case for
583 isolate **AS**. Such organisms are probably not ideal N_2O-sinks in soil because their ability to
584 survive in this environment would be limited. Organisms with a wider catabolic capacity, such
585 as the isolated *Pseudomonas* sp. (**PS**), are stronger candidates for long term survival and N_2O-
586 reducing activity in soil. The ideal organisms are probably yet to be found, however, and
587 refinements of the enrichment culturing process are clearly needed.

588 The digestate used in this study contained N_2O-respiring bacteria, most likely survivors from
589 the raw sludge, which however, were clearly outnumbered by bacteria that are net producers
590 of N_2O. We surmise that the relative amounts of N_2O-producers and N_2O-reducers in
591 digestates may vary, depending on the feeding material and configuration for the anaerobic
592 digestion. This could explain the observed large variation of digestates on N_2O emission from
593 soils (Baral et al 2017, Herrero et al 2016). The high abundance of both NO_3^- - and 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 NO_3^- - and O_2-respiring organisms (**Figure 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 N_2O was also experienced by Conthe et al (2018). The organisms that did grow by respiring N_2O 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 NO_3^-, NO_2^- or NO (Spiro 2016), which were all absent during the enrichment.

Two of the reconstructed MAGs had periplasmic nitrate reductase (*nap*), as was the case for two of the three isolates (AN and AS). This in itself would predict preference for N_2O- over NO_3^- reduction at a metabolic level (Mania et al 2020), but otherwise their potential for being N_2O sinks cannot be predicted by their genomes. The phenotyping of the isolates revealed conspicuous patterns of *bet hedging* as demonstrated for *Paracoccus denitricans* (Lycus et al 2018). 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 N_2O. 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 N_2O-sinks: while all isolates were strong N_2O sinks when provided with NO_3^- only, **AN** and **AS** accumulated large amounts of N_2O if provided with NO_2^-.

The N_2O 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 induced by digestates in soil (Kuzyakov and Blagodatskaya 2015). 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 (Liu et al 2014). The results for isolates **AS** and **AN** lend support to this (high $\boldsymbol{I_{N2O}}$ 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 N_2O 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 N_2O 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

636 hence is scalable. If we manage to treat a major part of waste materials in agroecosystems by
637 AD, the resulting digestates would suffice to treat a large share of total farmland, as illustrated
638 by **Figure 1**. Estimation of the potential N_2O-mitigation effect is premature, but the
639 documented feasibility and the scalability of the approach warrant further refinement as well
640 as rigorous testing under field condition. Our approach suggests one avenue for a much
641 needed valorization of organic wastes (Peng and Pivato 2019) via anaerobic digestion. Future
642 developments of this approach could extend beyond the scope of climate change mitigation
643 and include the enrichment of microbes for pesticide- and other organic pollutant
644 degradation (Sun et al 2018), plant growth promotion (Backer et al 2018) and inoculation of
645 other plant symbiotic bacteria (Poole et al 2018).

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