Bacteria in biogas digestates for reduced climate forcing

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Abstract

Mitigation of N_2O-emissions from soils is needed to reduce climate forcing by food production. Inoculating soils with N_2O-reducing bacteria would be effective, but costly and impractical as a standalone operation. Here we demonstrate that digestates obtained after biogas production may provide a low-cost and widely applicable solution. Firstly, we show that indigenous N_2O-reducing bacteria in digestates grow to high levels during anaerobic enrichment under N_2O. Gas kinetics and meta-omic analysis show that the N_2O-respiring organisms, recovered as metagenome-assembled genomes (MAGs), grow by harvesting fermentation intermediates of the methanogenic consortium. Three digestate-derived denitrifying, N_2O-reducing bacteria were obtained through isolation, one of which matched the recovered MAG of a dominant *Dechloromonas*-affiliated N_2O reducer. While the identified N_2O-reducers encoded 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. Secondly, moving towards practical application, we show that these isolates grow by aerobic respiration in digestates, and that fertilization with these enriched digestates reduces N_2O emissions. This shows that the ongoing implementation of biogas production in agriculture opens a new avenue for cheap and effective reduction of N_2O emissions from food production.

Introduction

Nitrous oxide, N_2O, is a central intermediate in the nitrogen cycle and a powerful greenhouse gas that is emitted in large volumes from agricultural soils, accounting for ~1/3 of total anthropogenic N_2O emissions (Tian et al 2020). N_2O turnover in soil involves several metabolic pathways, controlled by a plethora of fluctuating physical and chemical variables (Butterbach-Bahl 2013, Hu et al 2015). Heterotrophic denitrification, i.e. the process of reducing nitrate to N_2, is the dominant N_2O source in most soils, while autotrophic ammonia oxidation may be the dominating

source in well drained calcareous soils (Song et al 2018 and references therein). Ammonia oxidizing organisms produce N_2O aerobically as a byproduct of their oxidation of hydroxylamine to nitrite (Stein 2019). In contrast, heterotrophic denitrifying organisms are both sources and sinks for N_2O because it is a free intermediate in the stepwise reduction of nitrate to dinitrogen ($NO_3^- \rightarrow NO_2^- \rightarrow NO \rightarrow N_2O \rightarrow N_2$). Denitrification involves four enzymes; 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*. 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 one to three of the four genes (Shapleigh 2013, Lycus et al 2017), and 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). Despite their promise, feasible ways to utilize such organisms to reduce N_2O emissions have not yet emerged. Of note, organisms with a full-fledged denitrification pathway may also be strong N_2O sinks depending on regulatory traits that affect the relative activities of the various enzymes in the denitrification pathway (Lycus et al 2018; Mania et al 2020).

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) showing that the majority of tested soils emitted less N_2O if inoculated with large numbers (10^7 - 10^8 cells g^{-1} soil) of organisms possessing *nosZ* as the sole denitrification reductase. As a standalone operation, the large-scale production and distribution of N_2O-reducing bacteria needed for such heavy inoculation of soils would be prohibitively expensive and impractical. However, the operation could become feasible if adapted to an existing fertilization pipeline, such as fertilization with the nitrogen and phosphate-rich organic waste (digestates) 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 amounts of wastes produced in 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 will become a major organic fertilizer for agricultural soils, with a huge potential for reducing N_2O emissions if enriched with N_2O reducing bacteria prior to fertilization.

Here we provide the first proof of this concept by selective enrichment and isolation of N_2O reducing bacteria in a digestate from a wastewater treatment plant, and by demonstrating that digestates enriched with such organisms reduce the proportion of N leaving soil as N_2O when used as a soil amendment. Analysis of the enrichment process with multi-omics and in-depth monitoring of gas kinetics provided insights into why this strategy was successful.

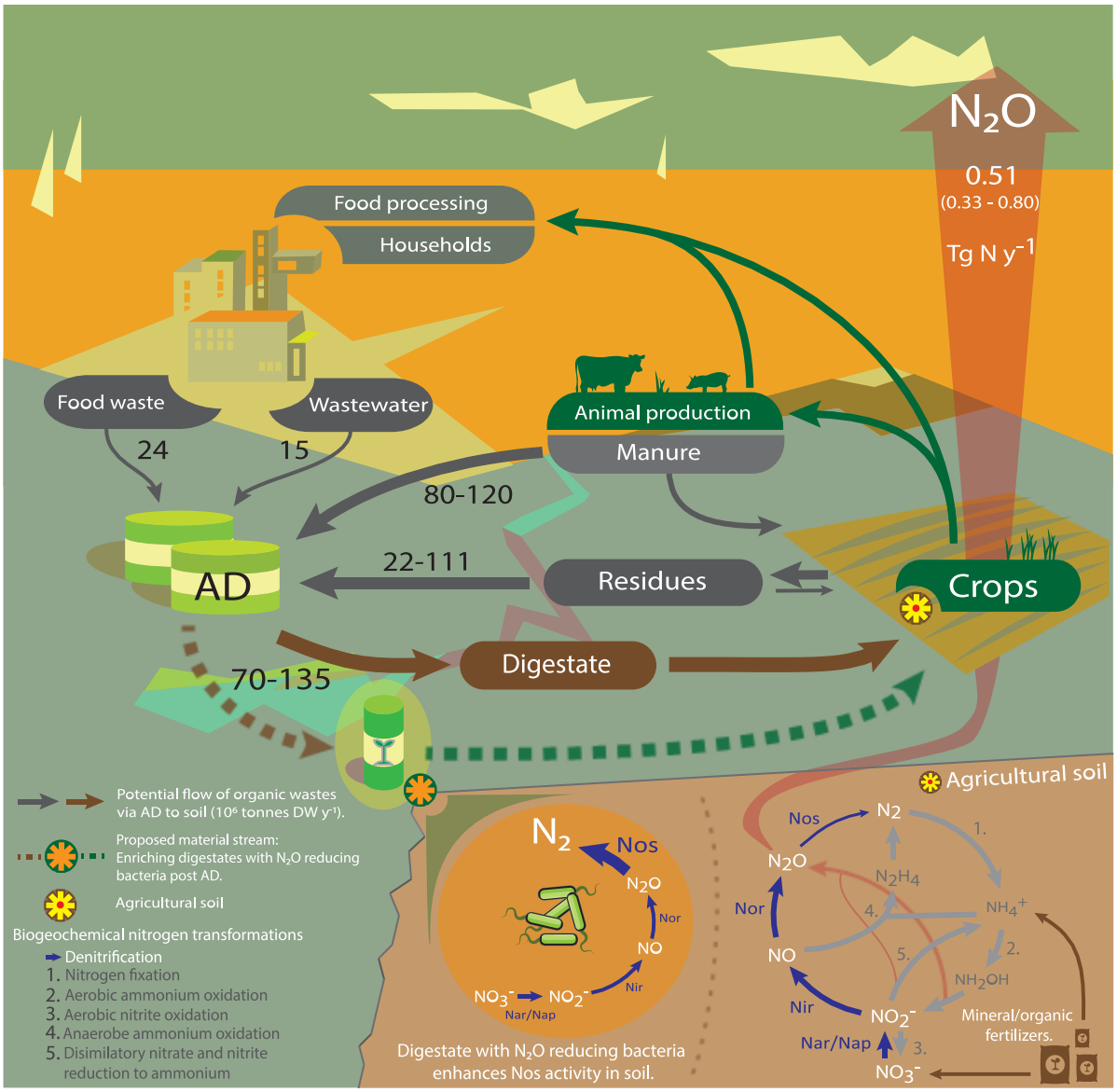

Figure 1. Material streams via biogas production in a future circular economy provides a route for effective and low-cost mitigation of N_2O emission. Waste streams in Europe currently used or available for anaerobic digestion under a circular economy model (million tonnes dry-weight (DW) year^{-1}, based on Foged et al 2011, Holm-Nielsen et al 2009, Stenmarck et al 2016, Meyer et al 2018). Lower right figure illustrates nitrogen red/ox transformations and the role of denitrification as both source and a sink for N_2O, and that the sink capacity can be strengthened by fertilizing with digestates enriched with N_2O-reducing bacteria (lower center), hence reducing the N_2O emissions. Today, AD primarily treats urban organic wastes, which is only ~1/10 of the total waste material. The amounts treated by AD will increase by an order of magnitude when adopted on a large scale by the agricultural sector, producing 70-135 Mt digestate DW 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).

Results and Discussion

Enrichment of indigenous N_2O reducers in digestates

Our aim was to identify and isolate organisms that combine two traits: fast growth to high cell densities in anaerobically digested waste material, and strong capacity to reduce N_2O. 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 (Lu et al 2014). We further hypothesized that these bacteria could be selectively enriched in digestates by anaerobic incubation with N_2O in the headspace. We decided to enrich at 20 ^oC, rather than at temperatures commonly used in the anaerobic digesters, typically 35 - 40 ^oC in "mesophilic" and 52 - 55 ^oC in "thermophilic" reactors, to avoid selecting for organisms unable to grow within the normal temperature range of soils.

To enrich these indigenous N_2O reducers, digestates were incubated anaerobically as stirred batch cultures with N_2O in the headspace (He atmosphere), and the activity and apparent growth of N_2O reducers was assessed by monitoring the kinetics of N_2O reduction to N_2 **(Figure 2**). The incubation robot used for these experiments measures O_2, CO_2, CH_4, NO, N_2O and N_2 in a single gas sample from the headspace. **Figure 2A** shows the results for the first experiment, where vials were sampled every ~4h for gasses and at three time points (0, 115 and 325 hours) for metagenomics, metaproteomics, and quantification of 16S rDNA and volatile fatty acids (VFAs). N_2O was depleted for a long period (100-140 hours) in this experiment, precluding analysis of the growth kinetics throughout. This was avoided in the second enrichment, which is shown in **Figure 2BC**. The N_2 production rate (V_{N2}) declined during the first 50 h, followed by an exponential increase. This phenomenon was modelled as the activity of two groups of N_2O-respiring organisms: one growing exponentially from low initial abundance, and one which was more abundant initially, but whose activity died out gradually (further explained in **Figure S1**). The estimated cell density of the growing population increased exponentially (μ=0.1 h^{-1}) from a very low initial density (~$2.5\cdot10^3$ cells mL^{-1}) to $1.6\cdot10^8$ cells mL^{-1} after 110 h, and continued to increase at a gradually declining rate to reach $3\cdot10^9$ cells mL^{-1} at the end of the incubation (215 h). The modelled cell specific electron flow rate (V_{e-}, **Figure 2C**) was sustained at around 5 fmol e^- cell h^{-1} during the exponential growth, and declined gradually thereafter, as the number of cells continued to increase, while the rate of substrate provision for N_2O respiration apparently remained more or less constant (V_{N2}, **Figure 2B**). The enrichment culturing was repeated three times, demonstrating that the characteristic N_2 production kinetics were highly reproducible (**Figure S2**).

The provision of substrate for respiring bacteria can be understood by considering the enrichment culture as a continuation of the AD process in the anaerobic digester, 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, each by a separate fraction of the methanogenic consortium: 1) hydrolysis of polysaccharides to monomers by organisms with carbohydrate-active enzymes, 2) primary fermentation to volatile fatty acids (VFA), 3) secondary

fermentation to acetic acid, H_2 and CO_2, and 4) methane production from acetate, CO_2+H_2, and/or from methylated compounds. By adding N_2O to this system (in the enrichment culture), 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). This means that 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. This would explain the more or less constant cell specific electron flow rate (*Ve*-, **Figure 2C**) up to ~100 h, which could be the time point when the population of N_2O respiring organisms had reached high enough cell densities to reap most of the intermediates produced by the consortium. As the N_2O reducers continued to grow, they experienced an increasing scarcity of substrates, reflected in the gradually declining V_{e-}.

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 reduce O_2 and NO_3^- than cells able to reduce 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**) which is another piece of evidence that in the digestate (prior to enrichment culturing), the organisms reducing NO_3^- to N_2O outnumbered those reducing 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 VFA until the N_2O reducers reach a cell density that allows them to effectively deplete these compounds. This was corroborated by measurements of H_2 and VFAs (**Figure S13**).

We assumed that N_2O respiring bacteria in the digestate are indigenous wastewater-sludge bacteria that survive the passage through the anaerobic digester, which had a retention time of 20-24 days. We assessed this by N_2O reduction kinetics in wastewater sludge *versus* digestate, and the results indicate that ≤1/3 of the N_2O respiring organisms in the sludge survived the passage (**Figure S6**). We also did enrichment culturing with digestate from a thermophilic digester (52 ^oC) that was operated in parallel with the mesophilic digester (with the same feed), and found that it 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)**.

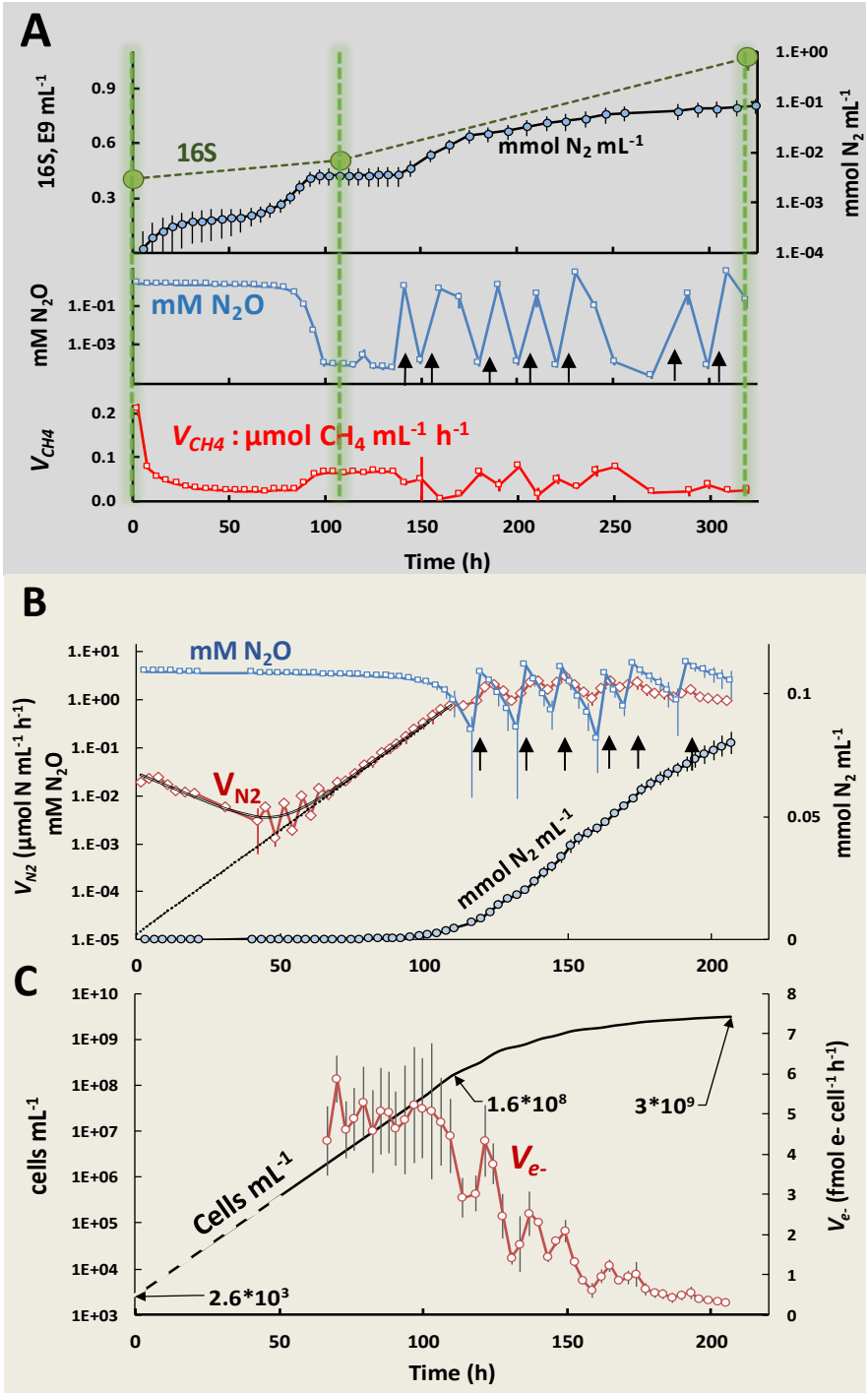

Figure 2: **Gas kinetics in anaerobic enrichment cultures with digestate.**

Panel A shows results for the enrichment cultures sampled for metagenomics, metaproteomics and quantification of volatile fatty acids (VFA) and 16S rDNA abundance (sampling times= 0, 115 and 325 hours, marked by vertical lines). The top panel shows the amounts of N_2 produced (log scale) and 16S rDNA copy numbers. The mid panel shows the concentration of N_2O in the liquid (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 deviation shown as vertical lines (n=3) in all panels.

Panel B and C show the results of a repeated experiment where complete N_2O-depletion (t=100-140 h, panel A) was avoided, to allow a better assessment of growth kinetics. Panel B: N_2O concentration in the liquid (mM N_2O), rate of N_2-production (V_{N2}) (both log-scaled), and N_2 produced (mmol N_2 mL^{-1}, linear scale). The curved black line is 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-respiraton, 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 (modelling is explained in **Figure S1**). Standard deviation is shown as vertical lines (n=3).

MAG-centric metaproteo-omic analysis of the enrichment cultures

We analyzed the metagenome and metaproteome at three timepoints (0, 115 and 325 h, **Figure 2A**) in order to explore the effect of the anaerobic incubation with N_2O on the entire microbial consortium, and to identify the organisms growing by N_2O 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%) for downstream analysis. The phylogenetic relationship and the relative abundance of the MAGs throughout the enrichment are summarized in **Figure 3**, which also shows selected features revealed by the combined metagenomic and metaproteomic analyses, including information about the presence of genes and detected proteins involved in denitrification, N_2O reduction, methanogenesis (coenzyme M methyl-transferase), syntrophic acetate oxidation and methane oxidation.

A closer inspection of the abundance of individual MAGs, based on their coverage in the metagenome and metaproteome, showed that the majority of the MAG's had a near constant population density throughout the incubation (**Supplementary Section B**), while two MAGs (260 and 268) increased substantially (**Figures 4**). The stable abundance of the majority of the MAGs indicates that the methanogenic consortium remained intact despite the downshift in temperature (20 °C versus 37 °C) and the inhibition of methanogenesis by N_2O. Only 9 MAGs showed a consistent decline in abundance throughout the enrichment (**Table S1**). These MAGs could theoretically correspond to microbes whose metabolism is dependent on efficient H_2 scavenging by methanogens (Schink 1997). However, we were unable to identify any such metabolic traits that could explain their fadeout during the enrichment, and surmise that the primary reason is failure to adapt to the temperature downshift from 37 ^{o}C to 20 ^{o}C.

Six MAGs, including the two that were clearly growing, MAG260 & MAG268, contained the *nosZ* gene and thus had the genetic potential to produce Nos for N_2O reduction (**Figure 4**). Nos proteins originating from five of these MAGs were detected in the metaproteome, while, importantly, none of the other denitrification enzymes were detected, suggesting that these organisms can regulate the expression of their denitrification machinery to suit available electron acceptors (in this case N_2O). Three of the MAGs with detectable Nos appeared to be non-growing during the enrichment: The detection level of their Nos proteins remained more or less constant, and their estimated abundance in the metagenome and -proteome did not increase (**Figure 4B**). It is conceivable that these three MAGs belong to the initial population of N_2O reducers whose N_2O-reduction activity was present initially but gradually decreased during the early phase of the enrichment (**Figure 2A**). The two growing MAGs (260 and 268) showed increasing Nos levels and increasing abundance both in terms of MAG coverage and metaproteomic detection (**Figure 4B**), in proportion with the N_2 produced (**Figure S11**). MAG260 reached the highest abundance of the two, accounting for 92% of the total detectable Nos pool at the final time point. MAG260 is taxonomically most closely affiliated with the genus *Dechloromonas* (GTDB, 97.9% AA similarity). Interestingly, Nap rather than Nar takes the role of nitrate reductase in MAG260 (**Figure 4**), which

makes it a promising organism for N_2O-mitigation since organisms with Nap prefer N_2O over NO_3^- reduction (Mania et al 2020). MAG260, MAG004 and MAG088 contain a clade II *nosZ*, characterized by a *sec* dependent signal peptide, in contrast to the more common *tat* dependent clade I *nosZ*. The physiological implications of clade I versus clade II *nosZ* genes remains unclear, early studies suggested an association with higher N_2O reduction potential, but more recent papers have called this into question (Conthe et al 2019; Hein et al 2017).

The community was originally structured as a methanogenic consortium digesting wastewater sludge at a temperature of 37 ^oC and continued to produce methane during our incubations at 20 ^oC, unless inhibited by adding N_2O, NO_3^- or O_2, as described above. Inhibition of methanogenesis by N_2O has been observed frequently (Andalib et al. 2011), and is probably due to inhibition of coenzyme M methyl transferase (Kengen et al 1988), which is a membrane bound enzyme that is essential in methanogenesis and common for all methanogenic archaea (Fischer et al 1992). The gas kinetics demonstrate that the inhibition of methanogenesis by N_2O was reversible, being partly restored whenever N_2O was depleted (**Figure 2**). In the enrichment culture used for metagenomics and metaproteomics there were several such incidents of N_2O depletion (**Figure 2A**), and indeed CH_4 accumulated to levels amounting to 10% of levels in control vials without N_2O (**Figure S4 Panel B**). 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 of *Methanothrix* (MAG025), *Methanoregulaceae* (MAG014) and *Methanobacterium* (MAG124) in the metaproteome. In fact, the measurements of MAG coverage and 16S rRNA copy number assessed by ddPCR suggested that the majority of members in the original methanogenic consortium continued to grow (**Supplementary Section B**), presumably supported via bacterial mixed acid fermentation fed by depolymerization of polymers. A tentative draft of the metabolic flow of the methanogenic consortium, including the reaping of intermediates (monosaccharides, fatty acids, acetate and H_2) by N_2O-respiring bacteria is shown in **Figure S12.** Since methane-production was inhibited from the very beginning of the incubation, while it took ~100 hours for the N_2O-respiring organisms to reach high enough numbers to become a significant sink for intermediates (**Figure 2**), one would expect transient accumulation of volatile fatty acids and H_2, which was corroborated by measurements of these metabolites (**Figure S13**).

Finally, we detected predicted methane monooxygenase and methanol dehydrogenase proteins from MAG087 and MAG059, respectively, in the metaproteome. This opens up the tantalizing hypothesis of N_2O driven methanotrophy within the enrichments which has only recently been suggested (Valenzuela et al 2020; Cheng et al 2019). However, a close inspection of the N_2O- and CH_4-kinetics indicated that N_2O-driven methanotrophy played a minor role for the carbon flow in our system (**Figure S4CD**).

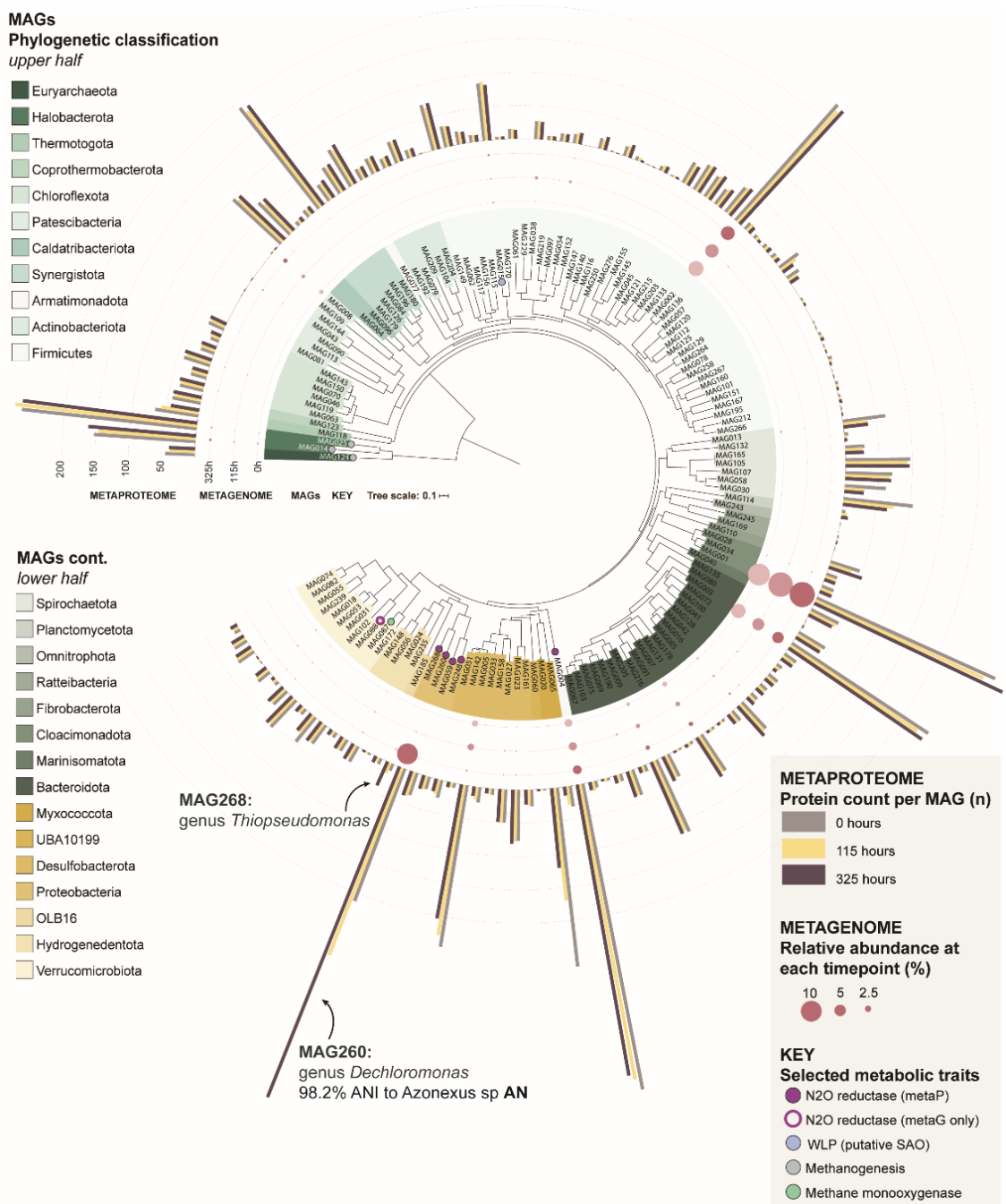

Figure 3: MAGs from the anaerobic enrichment culture with the mesophilic digestate. The figure shows a maximum likelihood tree indicating the phylogenetic placement of MAGs from the anaerobic enrichment. The tree was constructed from a concatenated set of protein sequences of single-copy genes. Taxonomic 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.

Isolation of N_2O reducing organisms and their geno- and phenotyping

Above we have described enrichment culturing of an N_2O-reducing digestate community. Whilst this enrichment culture could be used directly as a soil amendment, this approach is likely to have several limitations. First amongst these is that it would require the use of large volumes of N_2O 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 N_2O reducing organisms to sterilized/sanitized digestates. This approach has multiple benefits: 1) it would remove the need for N_2O 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 pre-treatment to be performed prior to use for fertilization. For these reasons an isolation effort was undertaken to obtain suitable digestate-derived N_2O-reducing microorganisms from the N_2O enrichment cultures. These efforts resulted in three axenic N_2O-reducing 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 rDNA obtained from the sequenced genomes (accession nr. xxx, **Table S2**, phylogenetic trees shown in **Figure S14**). All three were equipped with genes for a complete denitrification pathway; **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. *nirS* and *norBC*, coding Nir and Nor, were found in all three genomes (**Figure 4C**). 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, strongly suggesting the isolate is a strain circumscribed by MAG260 (Richter and Resselló-Móra 2009). Given GTDB phylogeny of **AN** and MAG260 and the 16S rDNA gene homology of **AN** (95.2 % sequence identity to *Azonexus hydrophilus* DSM23864, **Fig S14C**), we conclude that **AN** likely represents a novel species within the *Azonexus* lineage. Unfortunately, the 16S rDNA 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.**

The carbon catabolism profiles of these isolate strains were assayed using the formazan assay on Biolog™ PM1 and PM2 microplates, to detect the range of carbon sources utilized by the isolates (**Supplementary Section E)**. **PS** was able to utilize 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** showed carbon metabolisms limited to small VFAs (*eg*. acetate, butyrate), intermediates in the TCA cycle and/or β-oxidation/methyl malonyl-CoA pathways of fatty acid degradation (*eg*. malate, fumarate, succinate) and a single

amino acid (glutamate). Thus, all three would be able to grow in a live digestate by reaping the VFA's produced by the methanogenic consortium. While the utilization of VFA's as C-substrates is one of several options for **PS**, **AN** and **AS** appear to depend on the provision of VFA. This was corroborated by the attempts to grow the three strains in an autoclaved digestate: while **PS** grew well and reached high cell densities without any provision of extra C sources, **AN** and **AS** showed early retardation of growth unless provided with an extra dose of carbon sources (glutamate, acetate, pyruvate and ethanol) (**Figure S25 and S26**). A high degree of specialization and metabolic streamlining may explain the observed dominance of **AN** (MAG260) during enrichment culturing.

To evaluate the potentials of these isolates to act as sinks for N_2O, we characterized their denitrification phenotypes when growing in Sistrom's medium, by monitoring kinetics of oxygen depletion and subsequent denitrification and transient accumulation of intermediates (NO_2^-, NO, N_2O). The experiments were designed to unravel phenomena which could secure strong N_2O-reduction such as 1) *bet hedging*, i.e. that all cells express N_2O-reductase while only a fraction of the cells express nitrite- and/or nitrate-reductase, as demonstrated for *Paracoccus denitrificans* (Lycus et al. 2018); 2) strong metabolic preference for N_2O-reduction over NO_3^- -reduction, as demonstrated for organisms with periplasmic nitrate reductase (Mania et al 2020). **Supplementary section F** provides the results of all the experiments, and a synopsis of the findings. In short: *Azonexus sp.* (**AN**) had a clear preference for N_2O over NO_3^- reduction, but not over NO_2^- reduction, ascribed to *bet hedging* with respect to expression of nitrate reductase (few cells express Nap, while all cells express Nir and Nos), which was confirmed by proteomics (**Figure S17**). *Azospira sp.* (**AS**) had a similar preference for N_2O over NO_3 reduction, albeit less pronounced than in **AN**, and no preference for N_2O over NO_2^- . *Pseudomonas sp.* (**PS**) showed a phenotype resembling that of *Paracoccus denitrificans* (Lycus et al 2018), with denitrification kinetics indicating that Nir is expressed in a minority of cells in response to O_2-depletion, while all cells appeared to express N_2O reductase. This regulation makes **PS** a more robust sink for N_2O than the two other strains, since it kept N_2O extremely low even when provided with NO_2^-.

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

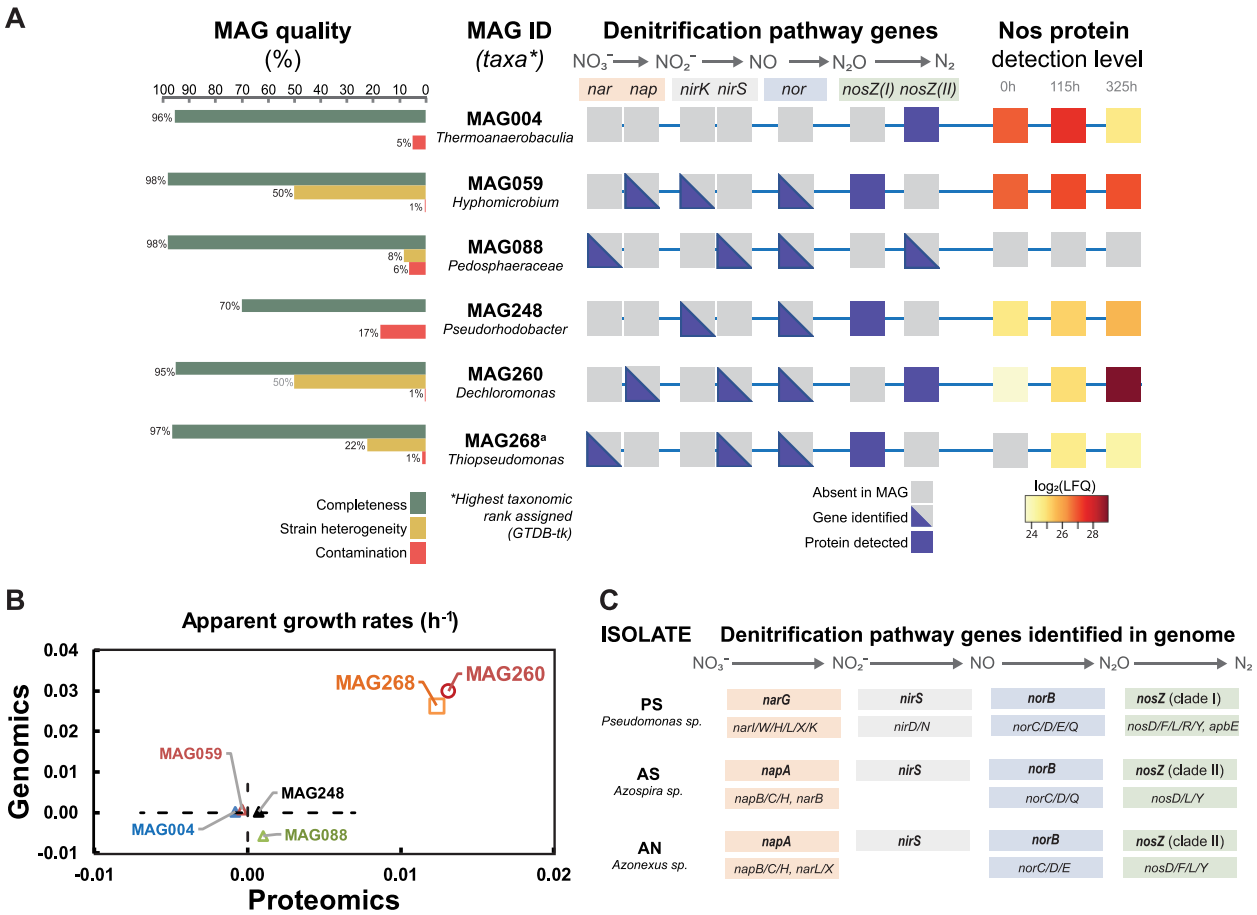

Figure 4: Abundance of MAGs with *nosZ* and overview of denitrification genes in isolated organisms. Panel A shows the quality (completeness, strain heterogeneity and contamination), taxonomic classification based on GTDB and NCBI, presence of denitrifying genes and proteins, and the detected levels of Nos (N_2O reductase, encoded by *nosZ*) throughout the enrichment culturing for the six MAGs containing the *nosZ* gene. Nos was detected in the proteome of five MAGs but the detection level increased significantly throughout for only two MAGs (260 and 268). None of the strains produced detectable amounts of the other denitrification proteins. 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, N= mean coverage in the metagenome, and summed label-free quantification (LFQ) values in the metaproteome). 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 are shown in bold, above the accessory genes (Vaccaro et al 2016) that were identified. More information about peripheral genes is presented in **Figure S14**. The strain **AN** has 98.2 % ANI to MAG260.

Effects on N_2O emissions

To assess if fertilization using digestates enriched with N_2O-reducing isolates could reduce N_2O emissions from soil, a series of incubations were conducted in which digestates with and without N_2O-reducing bacteria were added to soils. Soil samples were then incubated in closed vials with He + 0.5 vol % O_2, which were monitored O_2, NO, N_2O and N_2 during and after oxygen depletion. These experiments included soils amended with enrichment cultures, i.e. digestates in which

indigenous N_2O reducing bacteria were 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 (**Figures S25** & **S26**). The experiments included two types of control digestates: Live digestate (directly from the digester), and digestated heated to 70 ^oC for 2 hours to eliminate most of the indigenous consortium. Since soil pH 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 (limed in 2014, described by Nadeem et al, 2020) with different pH (pH_{CaCl2} = 5.5 and 6.6).

As expected, the transient N_2O accumulation during denitrification was higher in the acid than in the near-neutral soil for all treatments (**Figure 5**), 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 (***I_{N2O}*** = right hand panels in **Figure 5**) which is a measure of the molar amounts of N_2O relative to N_2+N_2O for a specific period (0-T, see equation at top of **Figure 5**). In this case, we calculated ***I_{N2O}*** for the period until 40% of the available NO_3^--N had been recovered as N_2+N_2O (=***$I_{N2O\ 40}$***) and for the period until 100% had been recovered (***$I_{N2O\ 100}$***).

The treatments with digestate in which N_2O-reducing bacteria were enriched by anaerobic incubation with N_2O had extremely low ***I_{N2O}*** values both in acid soil and in neutral soil. This corroborates 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.

The untreated digestate resulted in relatively high ***I_{N2O}*** compared to the digestate heated to 70 ^oC. This probably reflects that the population of denitrifying organisms in the live digestate was dominated by organisms that are net producers of N_2O (**Fig S5E**), which were killed by the heat treatment.

The three isolated strains were grown in the digestate by aerobic growth; hence their denitrification enzymes would have to be synthesized after being added to the soils, and therefore influenced by soil pH. Accordingly, the ***I_{N2O}*** values for **AN** and **AS** were significantly higher in the pH 5.5 soil than in the pH 6.6 soil, and the values were comparable to those for the heat treated digestate. The presence of strain **PS**, on the other hand resulted in very low ***I_{N2O}*** values throughout, surprisingly unaffected by soil pH.

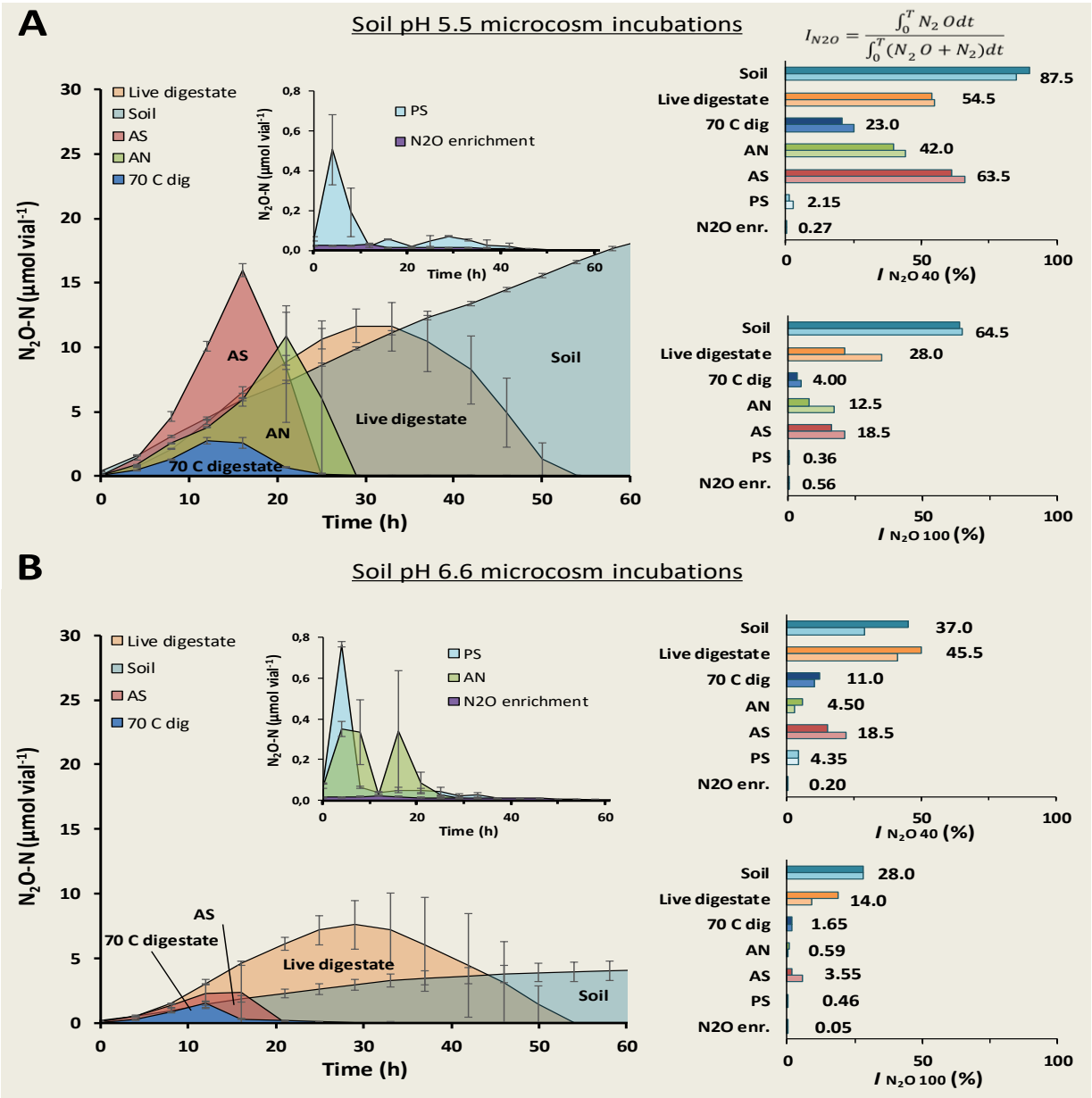

Figure 5: Soil incubations. Panels summarize the gas kinetics during incubation of soils (Panel A: soil pH=5.5, Panel B: soil pH=6.6) amended with various pretreated digestates: "Live digestate" = digestate directly from the anaerobic digester, "70 C dig" = live digestate heat treated to 70 °C for two hours, **AN**, **AS** and **PS**: autoclaved digestate with strains **AN**, **AS** and **PS** (grown aerobically), "N_2O enr"= digestate enriched with N_2O reducing bacteria (as in **Fig 2**). The main graph (panel A&B) shows the N_2O kinetics for each treatment, inset graph: altered scaling to visualize the N_2O kinetics in the treatment with low N_2O (**PS** and N_2O enrichments). The two bar graphs to the right show the N_2O indexes (***I_{N2O}***, single vial values), which is the area under the N_2O-curve divided by the area under the N_2O+N_2-curve, expressed as % (see equation in chart; Liu et al 2014), 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 is recovered as N_2+N_2O+NO-N (**$I_{N2O\ 40\%}$**), 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 is pervasively affected by the population of denitrifying bacteria in a digestate, be it the indigenous microbiota, or bacteria enriched by anaerobic or aerobic growth, and that manipulation of this microbiome can greatly affect the emission of N_2O. Interestingly, measurements of methane in these soil incubations showed that the methanogenic consortia remained metabolically intact in the soil, producing methane as soon as N_2O and oxyanions had been depleted, while no methane was produced in the soils amended with digestate heated to 70 ^oC (**Figure S29**).

In an effort to determine the survival of the N_2O-scavenging capacity of a digestate enriched with N_2O reducers, we tested its effect on soil N_2O emission after 70-hour aerobic storage (in soil or as enrichment culture), demonstrating a sustained effect on I_{N2O} over this 70-hour period of aerobic storage (**Figure S30**). Further long-lasting storage experiments as well as field trials will be made, to assess prolonged effects of N_2O-reducing digestate enrichments on soils.

Conclusive remarks

This is a feasibility study, unravelling some crucial aspects of how N_2O reducing organisms can be grown in digestates, and their effects on N_2O emissions from soils, depending on their regulation of denitrification. The study identifies an avenue for large scale cultivation of such organisms for soil application, at marginal costs if implemented as an add-on to biogas production systems. Further efforts should be on selecting organisms that are both strong sinks for N_2O, and able to survive in soil, in order to secure a long-lasting effect on the soils propensity to emit N_2O. A tantalizing option which may offset some of the economic costs associated with such a process is to select organisms (or consortia of organisms) that are not only strong N_2O sinks, but also promote plant growth and disease resistance (Gao et al 2016, 2017).

Gas kinetics, metagenomics and metaproteomics revealed that the methanogenic consortium of digestate can remain active during anaerobic incubation with N_2O, and that bacteria with an anaerobic respiratory metabolism can grow by harvesting fermentation intermediates. The inhibition of methanogenesis by N_2O 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 H_2, while they would have to compete with fermentative organisms for the "earlier" intermediates such as alcohols and VFA. But even these would become increasingly available for respiring organisms as acetate and H_2 accumulates (which was observed), since this would render the fermentation of these compounds thermodynamically unfavorable (Stams et al 2003). The importance of fermentation intermediates as a carbon source for the N_2O-respiring organisms 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 N_2O-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 in soil providing efficient reduction of N_2O emissions. The ideal organisms are probably yet to be found, however, and refinements to the enrichment culturing is clearly needed. Although the culturing in live digestate unraveled fascinating interactions between fermentative and respiratory metabolism, sanitized/sterilized digestates are likely to be a better choice for future efforts to enrich ideal organisms to combat N_2O emission from soil.

The digestate contained N_2O reducing bacteria, most likely survivors from the raw sludge. However, they were clearly outnumbered by bacteria that produce N_2O from NO_3, hence the digestate accumulated N_2O from NO_3^- under anoxia, both when providing NO_3^- to the digestate, and when incorporated into soil. We surmise that the relative amounts of N_2O producers and N_2O-reducers in digestates vary grossly, depending on the feeding material and process configuration, and have found some empirical evidence for this (unpublished data). This could be one reason for the highly variable effects of digestates on N_2O emission from soils reported (Baral et al 2017, Herrero et al 2016). Our results also show that N_2O reducing bacteria were vastly outnumbered by organisms respiring O_2. This relatively high number of both NO_3^- - and O_2-respiring organisms in the digestate has implications for the practical implementation of the culturing of inoculated N_2O reducing strains in digestates. Since their growth should be sustained by NO_3^- or O_2, rather than N_2O, for practical reasons, any inoculant would probably be outnumbered by the indigenous NO_3^- - or O_2-respiring organisms. Hence, we foresee that future implementation of the technique will require a brief heat treatment or other sanitizing procedure prior to inoculating the digestates with the desired N_2O reducing bacteria. A bonus of such sanitation is that it eliminates methane production by the digestate in soil (**Figure S29**). This underscores the need to search for bacteria that are not streamlined for feeding by the intermediates produced by an anaerobic consortium, as was the case for two or the isolated strains (**AN** and **AS**), since the heat treated digestate would not provide the necessary intermediates.

Non-denitrifying N_2O reducers (organisms lacking all other denitrification genes than *nosZ*) did not reach dominance during the enrichment, and 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 genomes had periplasmic nitrate reductase (*nap*), as was the case for two of the three isolates. 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 revealed conspicuous patterns of *bet hedging* as demonstrated for *Paracoccus denitricans*, first by modelling the denitrification kinetics (Hassan et al 2016) and subsequently confirmed experimentally (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*, suggesting that it is indeed hedging its bets when experiencing oxygen depletion, by expressing Nos in all cells, but Nir only in a few. 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. The differences had obvious implications for the strains' robust function as N_2O-sinks: while all strains 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 strains 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 strain **AS** and **AN** lend support to this: their effect on the transient N_2O accumulation was much weaker in the soil with pH=5.5 than in the soil with pH 6.6. **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 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, hence scalable to treat a major part of waste materials in agroecosystems, which would suffice to treat a large share of total farmland, as illustrated by waste material flow for Europe (**Figure 1**). Estimation of the potential N_2O-mitigation is premature, but the documented feasibility and the scalability of the approach opens an avenue for substantial reductions of N_2O emissions, much beyond that achieved by "good agronomic practice".

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