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Growth yield and selection of *nosZ* clade II types in a continuous enrichment culture of N_2O respiring bacteria

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Summary

Nitrous oxide (N_2O) reducing microorganisms may be key in the mitigation of N_2O emissions from managed ecosystems. However, there is still no clear understanding of the physiological and bioenergetic implications of microorganisms possessing either of the two N_2O reductase genes (*nosZ*), clade I and the more recently described clade II type *nosZ*. It has been suggested that organisms with *nosZ* clade II have higher growth yields and a lower affinity constant (K_s) for N_2O. We compared N_2O reducing communities with different *nosZ*I/*nosZ*II ratios selected in chemostat enrichment cultures, inoculated with activated sludge, fed with N_2O as a sole electron acceptor and growth limiting factor and acetate as electron donor. From the sequencing of the 16S rRNA gene, FISH and quantitative PCR of *nosZ* and *nir* genes, we concluded that betaproteobacterial denitrifying organisms dominated the enrichments with members within the family *Rhodocyclaceae* being highly abundant. When comparing cultures with different *nosZ*I/*nosZ*II ratios, we did not find support for (i) a more energy conserving N_2O respiration pathway in *nosZ* clade II systems, as reflected in the growth yield per mole of substrate, or (ii) a higher affinity for N_2O, defined by μ_{max}/K_s, in organisms with *nosZ* clade II.

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Introduction

Nitrous oxide (N_2O) reducing microorganisms, both denitrifying and non-denitrifying, can contribute to the N_2O sink capacity of ecosystems and may be key in reducing emissions of this potent greenhouse gas (Hallin *et al.*, 2018). The phylogeny of the nitrous oxide reductase (NosZ), encoded by the *nosZ* gene, has two major clades, clade I and II (Jones *et al.*, 2013). A high abundance and diversity of N_2O reducing bacteria harboring *nosZ* clade II, in particular, has been linked to an increased N_2O reduction potential in soils as well as lower *in situ* N_2O emissions (Jones *et al.*, 2014; Domeignoz-Horta *et al.*, 2017), but a mechanistic explanation for this is lacking. *nosZ* clade I and clade II differ in (i) the co-occurrence with other denitrification genes, with *nosZ* clade II being more often associated to non-denitrifiers (Graf *et al.*, 2014) and (ii) the accessory proteins associated to the *nos* operon. For example, *nosR* and *nosB* genes encode proteins likely to be involved in electron transport to the NosZ of clade I and clade II respectively (Sanford *et al.*, 2012). It is not understood if these differences between the two types of NosZ, apparent on the genome level, result in a differentiation in the ecophysiology of N_2O reducers harboring either *nosZ* clade.

Physiological studies with clade II-type N_2O reducers are scarce, but Yoon and colleagues (2016) recently compared five N_2O reducing bacterial species and reported lower whole-cell half-saturation constants (K_s) for N_2O and up to 1.5 times higher biomass yields per mole of N_2O for the *nosZ* clade II N_2O reducers compared to those harboring *nosZ* clade I. A lower K_s would confer *nosZ* clade II N_2O reducers a selective advantage during competition for limiting amounts of N_2O, whereas a higher biomass yield implies a greater efficiency of energy conservation in the *nosZ* clade II-associated electron transport chain (ETC). Extra charge separations during N_2O reduction could hypothetically be mediated by the predicted transmembrane protein encoded by *nosB* present in *nosZ* clade II organisms. It is an attractive hypothesis that *nosZ*II-associated ETCs generate a greater proton motive force per electron accepted than the *nosZ*I equivalent, which would explain niche

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Table 1. Conversion rates in the chemostats (negative numbers = consumption, positive = production) under N_2O limitation and carbon (C) and electron (e⁻) balances over the conversions (mean ± SD, *n* = 5).

	Dilution rate (h^{-1})	Compound conversion rates (mmol h^{-1})						C-bal (%)	e^--bal (%)
		CH_3COO^-	N_2O	N_2	NH_4^+	$CH_{1.8}O_{0.5}N_{0.2}$	CO_2		
Conthe and colleagues (2018)[a]	0.027 ± 0.001	−1.78 ± 0.20	−5.09 ± 0.90	3.88 ± 0.81	−0.30 ± 0.01	1.14 ± 0.08	2.25 ± 0.13	93	97
This study	0.026 ± 0.001	−1.94 ± 0.10	−4.40 ± 0.09	4.34 ± 0.05	−0.39 ± 0.04	1.39 ± 0.15	2.27 ± 0.07	106 ± 12	104 ± 12

a. During period IV of operation.
b. After day 21.

differentiation between the two clades. To test the competition between *nosZ* clade I and clade II N_2O reducers, we recently analyzed the performance of an enrichment culture growing for a large number of generations with N_2O as the sole electron acceptor under different dilution rates and with either the electron donor (acetate) or N_2O as the limiting factor (Conthe *et al.*, 2018). Continuous systems with enrichment cultures are optimal to study the potential dichotomy in N_2O reducer ecophysiology, as it allows competition experiments based on the affinity for a limiting substrate within a fairly complex community and provides prolonged steady state conditions to obtain reliable biomass yields. Nevertheless, irrespective of whether N_2O or acetate was the growth limiting substrate in the culture, *nosZ* clade I N_2O reducers dominated the enrichment. This led us to reject the hypothesis that *nosZ* clade II-harboring organisms have a higher overall affinity for N_2O than organisms with *nosZ* clade I, with affinity being determined by the ratio of μ_{max} over K_s. Since we did not enrich for a significant community of *nosZ* clade II N_2O reducers under the different operational conditions, we were unable to compare growth yields amongst N_2O reducers of both clades (Conthe *et al.*, 2018). However, we did observe an increase in *nosZ* clade II when the dilution rate switched from high to low, which suggest that the μ_{max} was important in the selection of N_2O reducers.

The aim of the present study was to compare the results from the period with low dilution rate and N_2O limitation from our previous experiment with an independently enriched N_2O-fed chemostat culture subject to the same conditions. Even though a functional steady state had been achieved in the previous study, a steady state in terms of microbial community composition and *nosZ*II/*nosZ*I ratio had not. Additionally, the history of reactor operation likely affects the selection of community members, and in the present study, we directly started off with continuous operation under conditions of N_2O limitation and low dilution rate without a preceding period of higher dilution rate or acetate limiting conditions. With the new enrichment approach, the abundance of *nosZ* clade II bacteria was significantly increased, which allowed us to compare the thermodynamic efficiency of *nosZ* clade II- versus clade I-associated ETCs and to gain further insight into the role of the NosZ type in the microbial competition for N_2O. The abundance of N_2O reducers was determined using quantitative real-time PCR (qPCR) of *nosZ*I and *nosZ*II along with the nitrite reductase genes *nirS* and *nirK* characteristic of denitrifying organisms. Additionally, the 16S rRNA genes were sequenced to obtain the composition of the enriched community, and fluorescent *in situ* hybridization (FISH) with probes targeting Bacteria and

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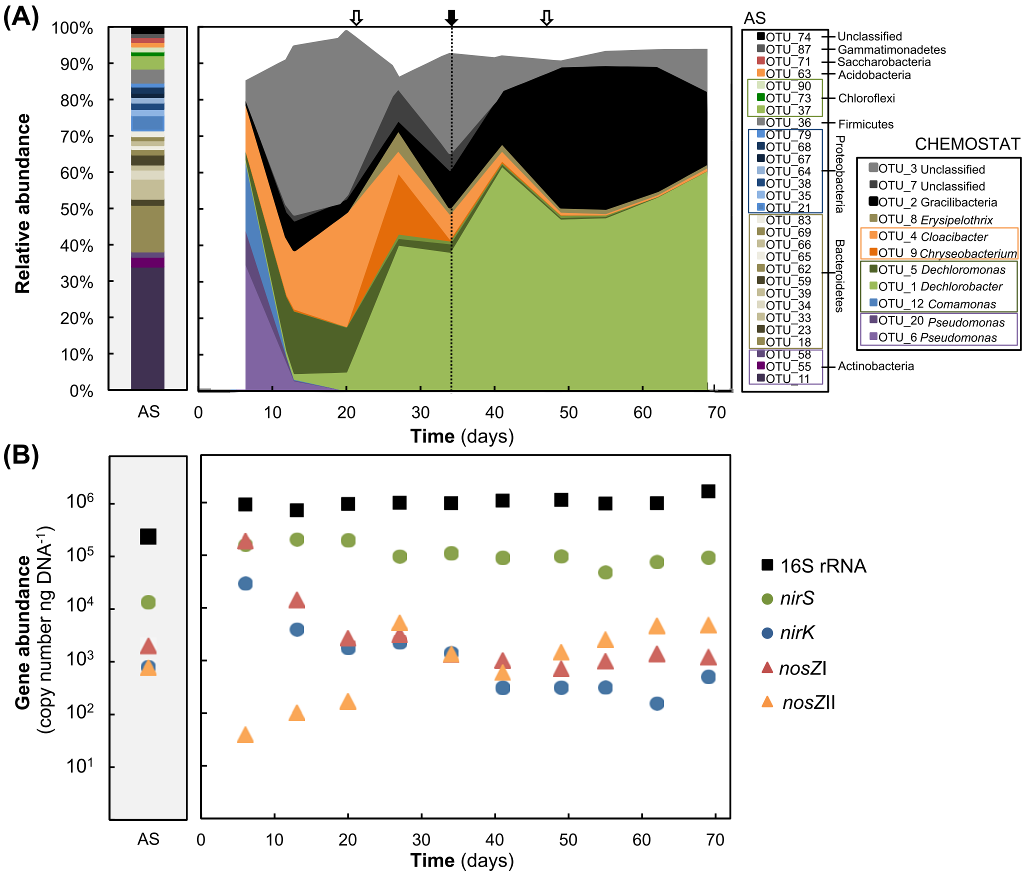

Fig. 1. Relative abundances of the 16S rRNA gene OTUs with > 5% of the sequences (A) and abundances of 16S rRNA and denitrification genes (B) in the activated sludge sample used as inoculum (AS) and in the enrichment culture throughout the operation of the chemostat. The white arrow on day 21 indicates when the influent pump tubing was changed, leading to a decrease in the dilution rate from 0.028 ± 0.001 to 0.026 ± 0.001, while the white arrow on day 47 indicates the switch from N_2 to Argon and recirculation of gas (200 ml min^{-1} of in-gas composed of Argon and N_2O with 700 ml min^{-1} of recycling, keeping the flow of N_2O constant). The black arrow indicates the time point corresponding to the FISH image (Fig. 2). Sequences are available at NCBI under BioProject accession number PRJNA430066. The procedures for DNA extraction, Illumina sequencing and bioinformatic analyses of 16S rRNA gene sequences as well as the qPCR of *nosZ* and *nir* genes can be found in Conthe and colleagues (2018). qPCR efficiencies were 97% for 16S rRNA, 80% for *nosZ*I, 87% for *nosZ*II, 93% for *nirK* and 75% for *nirS*.

Beta- and Gammaproteobacteria was performed to independently quantify the relative abundance of these taxa.

Results and discussion

Prolonged heterotrophic growth sustained by N_2O respiration

Activated sludge from the wastewater treatment plant of Harnaschpolder (the Netherlands) was used as the inoculum to enrich a microbial community growing with N_2O as the sole electron acceptor and using acetate as an electron donor at pH 7 and 20°C. After an initial batch start-up phase of 48 h, the culture was operated in continuous mode under N_2O limiting conditions during 72 days at a dilution rate of 0.027 h^{-1} (specifically at 0.028 ± 0.001 h^{-1} days 0–20 and 0.026 ± 0.001 h^{-1} days 21–72; Supporting Information Fig. S1). Nitrous oxide was supplied to the reactor at a constant rate (Supporting Information Fig. S1) and the reactor set-up, medium composition, operation and sampling are described in detail in Conthe and colleagues (2018). The microbial community was growing by N_2O reduction to N_2 at the expense of acetate oxidation, as confirmed by the elemental and electron balances (Table 1), with acetate present in excess throughout the operation (Supporting Information Fig. S1). The compound conversion rates were comparable to those obtained in our previous experiment, showing that the community functioning was similar in the two, independent enrichments (Table 1). To confirm that N_2O was growth limiting in the system, the N_2O sparging rate was increased, which resulted in an immediate increase in the biomass specific N_2O conversion rates (data not shown).

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The N_2O reducing community was dominated by betaproteobacterial denitrifiers

The composition of the enrichment culture, sampled on 10 different days during chemostat operation, and of the activated sludge used as inoculum was determined by Illumina sequencing of the 16S rRNA gene (Fig. 1A and Supporting Information Tables S1 and S2). Bacteria belonging to the family *Rhodocyclaceae*, despite representing only a small percentage of sequences in the activated sludge inoculum, made up a significant part of the enrichment with a single OTU (1) covering 40 to 60% of the reads after day 30. However, FISH performed on day 34 suggests that the relative abundance of the dominant OTU, as reflected in the abundance of bacteria hybridizing with the betaproteobacterial probe, was even much higher than estimated by sequencing (70–90% of the biovolume *vs*. 40% of sequences; Fig. 2). As far as we could see, the cells stained with the betaproteobacterial probe had the same morphology. The initial decrease of *Pseudomonas* sp. and *Comamonas* sp. that dominated at the startup of the reactor operation was followed by an increase in *Cloacibacterium* sp., *Chryseobacterium* sp. and *Dechloromonas* sp. This shift in community composition coincided with a decrease in *nosZ* clade I abundance and an increase in *nosZ* clade II (Fig. 1B). In agreement, sequenced genomes of the genera *Pseudomonas* and *Comamonas* harbor clade I *nosZ*, whereas *Dechloromonas* sp. and N_2O reducers within *Flavobacteriaceae* harbor *nosZ* clade II. After day 20, *Rhodocyclaceae* (*Dechlorobacter* sp.) dominated the enrichment. Different species within the *Rhodocyclaceae* have been shown to harbor either *nosZ* clade I or II (Jones *et al*., 2014). The only sequenced genome of *Dechlorobacter* so far has a *nosZ* sequence similar to the *nosZ* clade I from *Rhodoferax ferrireducens* and *Ralstonia pickettii* (Conthe *et al*., 2018). However, while OTU 1 was assigned to *Dechlorobacter* when using the Silva taxonomy, it was assigned to the genus *Azonexus* when using the rdp classifier, and sequenced genomes of *Azonexus* harbor *nosZ* clade II rather than clade I. This makes it difficult to speculate about the type of *nosZ* associated to this OTU. Instead, the similar abundance of both *nosZ* types suggests that OTU 1 could be a mix of closely related species within the *Rhodocyclaceae* family. Interestingly, reads related to *nosZ* clade II from *Azonexus* dominated the *nosZ* clade II community in the previous experiment under the same conditions used in the present study, although the corresponding 16S rRNA gene sequences could only be assigned at the family level (Conthe *et al*., 2018). Bacteria of the genus *Pseudomonas*, *Comamonas* and *Dechloromonas*, as well as many *Rhodocyclaceae* also possess genetic potential for denitrification (Graf *et al*., 2014), but for the *Cloacibacterium* sp. and *Chryseobacterium* sp. knowledge is limited. The *nir* genes, characteristic of denitrifying organisms, were highly abundant in the culture (Fig. 1B), indicating that the N_2O reducers dominating the enrichment were likely denitrifiers rather than non-denitrifying N_2O reducers. This shows that the availability of N_2O, even under N_2O limiting conditions, is not a selective driver for non-denitrifying N_2O reducers and highlights the strong competitive advantage of proteobacterial *nirS*-type denitrifiers under these conditions.

(A)

(B)

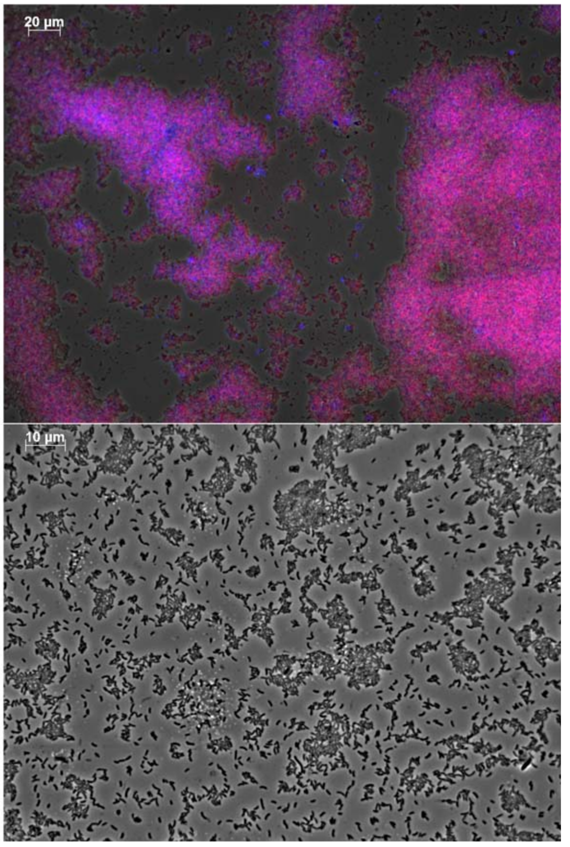

Fig. 2. FISH microscopic photographs of the enrichment.
A. FISH image (40×) of the culture on day 34 stained with a Cy5-labelled probe targeting Bacteria (EUB338, blue), a Cy3-labelled probe targeting Betaproteobacteria (Beta42a, red) and a FLUOS-labelled probe for Gammaproteobacteria (Gamma42a, green). Cells in blue only hybridized with EUB332, while cells in pink hybridized with both EUB338 and Beta42a. Gammaproteobacteria were absent from the culture. Details about the probes and protocol used for FISH can be found in Conthe and colleagues (2018).
B. 100× microscopic image of the cells.

The vast majority of the community members were presumed to harbor the *nosZ* gene required for sustained growth on N_2O respiration, translated in similar abundances of *nosZ* and 16S rRNA genes. However, the total *nosZ* gene copy numbers were two to three orders of magnitude lower than that of the 16S rRNA genes and two orders lower than the abundance of *nir*

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Table 2. Biomass yields and gene copy number ratios of the enrichment cultures.

	Dilution rate (h^{-1})	Y_{XAc} CmolX/CmolAc	Gene copy number ratios[a] *nosZ*II/*nosZ*I	*nosZ*/*nir*[a]	*nosZ*/16S rRNA[b]
Conthe and colleagues (2018)[c]	0.027 ± 0.001	0.32 ± 0.04	0.005 ± 0.002	3.253 ± 2.220	0.542 ± 0.272
This study	0.026 ± 0.001	0.36 ± 0.04	3.025 ± 0.896	0.060 ± 0.026	0.004 ± 0.002

X = biomass; Y_{XAc} = biomass yield on acetate in carbon mole biomass produced (CmolX) per carbon mole of substrate consumed (CmolS).

a. From qPCR values averaged over relevant periods (days 49–69 in this study *vs.* days 163–195 in Conthe and colleagues 2018).

b. *nosZ* includes the sum of *nosZ*I and *nosZ*II gene copy number. *nir* includes the sum on *nirS* and *nirK* gene copy number.

c. During period IV of operation.

genes after the community shift on day 21 (Fig. 1B and Table 2). This is potentially due to an underestimation of *nosZ* genes or the presence of a population incapable of N_2O reduction that was not captured when sequencing the 16S rRNA gene. We also detected a relatively high abundance of the phylum Gracilibacteria and unclassified bacteria (Fig. 1A). The only genomes of Gracilibacteria available so far were obtained from single-cell sequencing of cells from the vicinity of hydrothermal vents of the East Pacific rise. Both of the two retrieved genomes are closely related, have low G + C content and are characterized as fermentative bacteria (Rinke *et al.*, 2013). They do not have any *nos* genes that would indicate capacity for N_2O reduction, although they have a nitric oxide reductase. They may have co-existed in the chemostat by living off products of cell lysis or cross-feeding with N_2O reducers. The Gracilibacteria were also present in the enrichment in Conthe and colleagues (2018).

nosZ *clade type is not a selective driver in the competition for N_2O*

The *nosZ*II/*nosZ*I abundance ratio in the present enrichment culture was higher compared to that reported by Conthe *et al.* despite similar operating conditions (Table 2). Differences in the bacterial community composition of the inoculum or in reactor operation history, as well as a certain degree of stochasticity to be expected during colonization of any ecosystem (Roeselers *et al.*, 2006), could explain the difference in community composition between the two enrichment cultures. However, the small difference in dilution rate between the studies (0.026 ± 0.001 in this study *vs.* 0.027 ± 0.001 in Conthe *et al.*, 2018) could be an explanation considering that the minor change in dilution rate on day 21 coincided with a dramatic shift in the composition of the bacterial community (Fig. 1). Changes in community composition, either due to minor operational differences or due to potential interactions among community members, suggest that the competitive differences between *nosZ* clade I and II are small during N_2O limiting conditions.

The fact that the relative abundance of the two clades differed substantially between the two independent enrichment cultures, while conversion rates and biomass yields were very similar (Tables 1 and 2), suggests that competition among community members was not driven by the type of NosZ and that the overall energy conservation was similar in *nosZ* clade I- and *nosZ* clade II-associated ETCs present in our system. Our finding that N_2O reduction kinetics and stoichiometric yields do not distinguish bacteria harboring NosZ clade I from those with NosZ clade II contradicts the study reporting lower whole-cell K_s values and 50–80% higher growth yields in *nosZ* clade II N_2O reducers compared to organisms with *nosZ* clade I during growth on N_2O as the sole electron acceptor (Yoon *et al.*, 2016). The species that were studied might not be representative for the extant diversity known for the two clades of NosZ and furthermore, the difference in apparent K_s among the clade II species was as large as the differences among the clade I species, suggesting that differences in affinity might be taxa dependent rather than between *nosZ* clade I and II organisms. We conclude that there is no simple answer explaining the divergence and ecological differences of the two clades of NosZ observed in several studies of soils, sediments and rhizosphere (e.g., Tsiknia *et al.*, 2015; Wittorf *et al.*, 2016; Graf *et al.*, 2016; Dini-Andreote *et al.*, 2016; Juhanson *et al.*, 2017).

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References

Conthe, M., Wittorf, L., Kuenen, J.G., Kleerebezem, R., van Loosdrecht, M.C.M., and Hallin, S. (2018) Life on N_2O:

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deciphering the ecophysiology of N_2O respiring bacterial communities in a continuous culture. *ISME J* 1.
Dini-Andreote, F., Brossi, MJ. D L., van Elsas, J.D., and Salles, J.F. (2016) Reconstructing the genetic potential of the microbially-mediated nitrogen cycle in a salt marsh ecosystem. *Front Microbiol* **7**: 9023389–9023902.
Domeignoz-Horta, L.A., Philippot, L., Peyrard, C., Bru, D., Breuil, M.-C., Bizouard, F., *et al*. (2017) Peaks of in situ N_2O emissions are influenced by N_2O-producing and reducing microbial communities across arable soils. *Glob Chang Biol* **24**: 360–370.
Graf, D.R.H., Jones, C.M., and Hallin, S. (2014) Intergenomic comparisons highlight modularity of the denitrification pathway and underpin the importance of community structure for N_2O emissions. *PLoS One* **9**: e114118.
Graf, D.R.H., Zhao, M., Jones, C.M., and Hallin, S. (2016) Soil type overrides plant effect on genetic and enzymatic N_2O production potential in arable soils. *Soil Biol Biochem* **100**: 125–128.
Hallin, S., Philippot, L., Löffler, F.E., Sanford, R.A., and Jones, C.M. (2018) Genomics and ecology of novel N_2O-reducing microorganisms. *Trends Microbiol* **26**: 43–55.
Jones, C.M., Graf, D.R.H., Bru, D., Philippot, L., and Hallin, S. (2013) The unaccounted yet abundant nitrous oxide-reducing microbial community: a potential nitrous oxide sink. *ISME J* **7**: 417–426.
Jones, C.M., Spor, A., Brennan, F.P., Breuil, M.-C., Bru, D., Lemanceau, P., *et al.* (2014) Recently identified microbial guild mediates soil N_2O sink capacity. *Nat Clim Chang* **4**: 801–805.
Juhanson, J., Hallin, S., Söderström, M., Stenberg, M., and Jones, C.M. (2017) Spatial and phyloecological analyses of nosZ genes underscore niche differentiation amongst terrestrial N_2O reducing communities. *Soil Biol Biochem* **115**: 82–91.
Rinke, C., Schwientek, P., Sczyrba, A., Ivanova, N.N., Anderson, I.J., Cheng, J.-F., *et al*. (2013) Insights into the phylogeny and coding potential of microbial dark matter. *Nature* **499**: 431–437.
Roeselers, G., Zippel, B., Staal, M., Van Loosdrecht, M., and Muyzer, G. (2006) On the reproducibility of microcosm experiments – different community composition in parallel phototrophic biofilm microcosms. *FEMS Microbiol Ecol* **58**: 169–178.
Sanford, R.A., Wagner, D.D., Wu, Q., Chee-Sanford, J.C., Thomas, S.H., Cruz-García, C., *et al*. (2012) Unexpected nondenitrifier nitrous oxide reductase gene diversity and abundance in soils. *Proc Natl Acad Sci USA* **109**: 19709–19714.
Tsiknia, M., Paranychianakis, N.V., Varouchakis, E.A., and Nikolaidis, N.P. (2015) Environmental drivers of the distribution of nitrogen functional genes at a watershed scale. *FEMS Microbiol Ecol* **91**. doi:10.1093/femsec/fiv052.
Vishniac, W., and Santer, M. (1957) The Thiobacilli. *Microbiol Mol Biol Rev* **21**: 195–213.
Wittorf, L., Bonilla-Rosso, G., Jones, C.M., Bäckman, O., Hulth, S., and Hallin, S. (2016) Habitat partitioning of marine benthic denitrifier communities in response to oxygen availability. *Environ Microbiol Rep* **8**: 486–492.
Yoon, S., Nissen, S., Park, D., Sanford, R.A., and Löffler, F.E. (2016) Nitrous oxide reduction kinetics distinguish bacteria harboring clade I NosZ from those harboring clade II NosZ. *Appl Environ Microbiol* **82**: 3793–3800.

Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Fig. S1. Chemostat operation over 72 days showing (a) the liquid medium and gas flow rates (the total gas flow consisting of pure N_2O diluted in N_2 or Argon) going into the reactor, (b) the incoming and outgoing acetate and NH_4^+ concentrations in the medium and effluent and (c) the biomass concentration and optical density of the culture. Day 0 corresponds to the start of continuous operation. Medium A contained 90.6 mmol acetate ($NaCH_3COO{\cdot}3H_2O$) per liter, and medium B contained 26.6 mmol NH_4Cl, 14.8 mmol KH_2PO_4, 4.2 mmol $MgSO_4{\cdot}7H_2O$, 1 mmol NaOH, 4 mg yeast extract and 5 ml trace element solution (Vishniac and Santer, 1957) per liter. Both media were fed to the chemostat by means of one peristaltic pump with two pump heads. Even though the biomass concentration increased after day 21, growth yields remained the same. This is because the HRT decreased after replacing the influent pump tubing feeding mediums A and B to the reactor while the growth limiting substrate – N_2O – was supplied to the reactor at a constant gas flow rate. Recirculation was implemented on day 47 with the intention of reducing the amount of Argon gas used and to increase the mass transfer of gaseous N_2O to the liquid phase. However, the resulting increase in N_2O availability in the liquid was too small to be detected in the biomass yield of the culture.

Table S1. Assigned taxonomy for the main 16S rRNA-based OTUs (those with $>$ 10% sequences) of the activated sludge inoculum using the Silva database.

Table S2. Assigned taxonomy for the main 16S rRNA-based OTUs in the enrichment using the Silva database. The main OTUs were considered to be those with $>$ 5% sequences on any given sampling date, also see Fig. 1.

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