Growth yield and selection of nosZ clade II types in a continuous enrichment culture of N₂O respiring bacteria

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Summary
Nitrous oxide (N₂O) reducing microorganisms may be key in the mitigation of N₂O emissions from managed ecosystems. However, there is still no clear understanding of the physiological and bioenergetic implications of microorganisms possessing either of the two N₂O 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 (Kₛ) for N₂O. We compared N₂O reducing communities with different nosZ/nosZI ratios selected in chemostat enrichment cultures, inoculated with activated sludge, fed with N₂O 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/nosZI ratios, we did not find support for (i) a more energy conserving N₂O respiration pathway in nosZ clade II systems, as reflected in the growth yield per mole of substrate, or (ii) a higher affinity for N₂O, defined by jmax/Kₛ, in organisms with nosZ clade II.

Introduction
Nitrous oxide (N₂O) reducing microorganisms, both denitrifying and non-denitrifying, can contribute to the N₂O 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 N₂O reducing bacteria harboring nosZ clade II, in particular, has been linked to an increased N₂O reduction potential in soils as well as lower in situ N₂O 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 N₂O reducers harboring either nosZ clade.

Physiological studies with clade II-type N₂O reducers are scarce, but Yoon and colleagues (2016) recently compared five N₂O reducing bacterial species and reported lower whole-cell half-saturation constants (Kₛ) for N₂O and up to 1.5 times higher biomass yields per mole of N₂O for the nosZ clade II N₂O reducers compared to those harboring nosZ clade I. A lower Kₛ would confer nosZ clade II N₂O reducers a selective advantage during competition for limiting amounts of N₂O, 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 N₂O 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 nosZI-associated ETCs generate a greater proton motive force per electron accepted than the nosZI equivalent, which would explain niche
differentiation between the two clades. To test the competition between nosZ clade I and clade II N₂O reducers, we recently analyzed the performance of an enrichment culture growing for a large number of generations with N₂O as the sole electron acceptor under different dilution rates and with either the electron donor (acetate) or N₂O as the limiting factor (Conthe et al., 2018). Continuous systems with enrichment cultures are optimal to study the potential dichotomy in N₂O 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 N₂O or acetate was the growth limiting substrate in the culture, nosZ clade I N₂O reducers dominated the enrichment. This led us to reject the hypothesis that nosZ clade II-harboring organisms have a higher overall affinity for N₂O than organisms with nosZ clade I, with affinity being determined by the ratio of $\mu_{\text{max}}$ over $K_s$. Since we did not enrich for a significant community of nosZ clade II N₂O reducers under the different operational conditions, we were unable to compare growth yields amongst N₂O 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 $\mu_{\text{max}}$ was important in the selection of N₂O reducers.

The aim of the present study was to compare the results from the period with low dilution rate and N₂O limitation from our previous experiment with an independently enriched N₂O-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 nosZII/nosZI 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 N₂O 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 N₂O.

The abundance of N₂O reducers was determined using quantitative real-time PCR (qPCR) of nosZI and nosZII 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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<th>Compound conversion rates (mmol h⁻¹)</th>
<th>C-bal (%)</th>
<th>e–-bal (%)</th>
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<tr>
<td>CH₃COO⁻</td>
<td>N₂O</td>
<td>CH₄</td>
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<td>1.80 ± 0.01</td>
<td>0.50 ± 0.01</td>
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<th>Dilution rate (h⁻¹)</th>
<th>Compound conversion rates (mmol h⁻¹)</th>
<th>C-bal (%)</th>
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<td>0.027 ± 0.001</td>
<td>1.80 ± 0.01</td>
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Table 1. Conversion rates in the chemostats (negative numbers = consumption, positive = production) under N₂O limitation and carbon (C) and electron (e–) balances over the conversions

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Results and discussion

Prolonged heterotrophic growth sustained by N₂O respiration

Activated sludge from the wastewater treatment plant of Harnaschpolder (the Netherlands) was used as the inoculum to enrich a microbial community growing with N₂O 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 N₂O 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 N₂O reduction to N₂ 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 N₂O was growth limiting in the system, the N₂O sparging rate was increased, which resulted in an immediate increase in the biomass specific N₂O conversion rate (data not shown).

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OTU. Instead, the similar abundance of both nosZ sequence similar to the sequenced genome of Dechlorobacter species within the suggests that OTU 1 could be a mix of closely related reads related to nosZ speculate about the type of nosZ clade II rather than clade I. This makes it difficult to Azonexus to the genus bacter 2018). However, while OTU 1 was assigned to Dechloromonas sp. knowledge is limited. The nir genes, characteristic of denitrifying organisms, were highly abundant in the culture (Fig. 1B), indicating that the N2O reducers dominating the enrichment were likely denitrifiers rather than non-denitrifying N2O reducers. This shows that the availability of N2O, even under N2O limiting conditions, is not a selective driver for non-denitrifying N2O reducers and highlights the strong competitive advantage of proteobacterial nirS-type denitrifiers under these conditions.

The vast majority of the community members were presumed to harbor the nosZ gene required for sustained growth on N2O 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
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$_2$O 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$_2$O 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$_2$O reducers. The Gracilibacteria were also present in the enrichment in Conthe et al. (2018).

**nosZ clade type is not a selective driver in the competition for N$_2$O**

The nosZI/nosZI 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 (Roesselers 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$_2$O 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 nosZI clade I- and nosZI clade II-associated ETCs present in our system. Our finding that N$_2$O 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 nosZI clade II N$_2$O reducers compared to organisms with nosZ clade I during growth on N$_2$O 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 nosZI 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).

**Acknowledgements**

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**References**


**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 $N_2$ 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$_3$COO·3H$_2$O) per liter, and medium B contained 26.6 mmol NH$_4$Cl, 14.8 mmol KH$_2$PO$_4$, 4.2 mmol MgSO$_4$·7H$_2$O, 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.