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Carbon substrate selects for different lineages of N_2O reducing communities in soils under anoxic conditions

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ABSTRACT

Agricultural soils are a main source of nitrous oxide (N_2O), a potent greenhouse gas and the dominant ozone-depleting substance emitted to the atmosphere. The only known sink of N_2O in soil is the microbial reduction of N_2O to N_2. Carbon (C) availability is a key factor in determining microbial community composition in soil. However, its role in shaping the structure of N_2O reducing communities in soil is unexplored. In this study, a microcosm experiment was set up in which two arable soils with contrasting edaphic properties were incubated anaerobically for 83 days with four different C substrates: glucose, acetate, hydroxyethylcellulose (HEC) and mixture of the three. We show that the effect of C addition on the abundance and diversity of clade I and clade II *nosZ* genes, encoding different variants of the N_2O reductase, varies across the different C substrates differently in contrasting soil types, yet still plays an important role in selecting specific taxa of N_2O reducers under denitrifying conditions. We observed an increase of betaproteobacterial clade I and II N_2O reducing species with addition of HEC, whereas alphaproteobacterial clade I species and clade II species within other Proteobacteria and Bacteriodetes were associated with glucose and acetate. These results show that specific C-substrates select for certain lineages of nitrous oxide reducers and influence patterns of niche partitioning within clades of N_2O reducers, whereas other soil factors drive differences between clade I and II *nosZ* communities.

1. Introduction

Nitrous oxide (N_2O) is a major greenhouse and ozone layer-depleting gas, with almost 300 times more warming potential than carbon dioxide over 100 years due to its long residence time in the atmosphere (Shine et al., 1990; Ravishankara et al., 2009). Atmospheric N_2O concentrations have increased by more than 20% since pre-industrial times, and agricultural soils contribute more than 50% of total anthropogenic N_2O emissions at the global scale (Tian et al., 2020). Nitrous oxide is mainly produced by two microbial nitrogen (N) cycle processes, nitrification and denitrification, with denitrification being the main source of N_2O (Firestone et al., 1980; Inatomi et al., 2019; Scheer et al., 2020). Denitrification is a facultative anaerobic respiratory process in which nitrate (NO_3^-) or nitrite (NO_2^-) are reduced to nitrous oxide (N_2O) and dinitrogen gas (N_2). In agricultural soils, addition of N fertilizers stimulates the activity of denitrifying microorganisms, resulting in losses of 10–40% of applied N (Galloway et al., 2004) as well as enhanced emissions of N_2O (Bouwman et al., 2002; Shcherbak et al., 2014).

The only known process that consumes N_2O in soil is the microbial reduction of N_2O to N_2, catalysed by the N_2O reductase encoded by the *nosZ* gene found in denitrifying and non-denitrifying microorganisms (Conrad, 1996; Hallin et al., 2018). The overall diversity of N_2O reducers is divided into two groups termed *nosZ* clades I and II, based on the phylogeny of the *nosZ* gene (Sanford et al., 2012; Jones et al., 2013). Several studies indicate that organisms with *nosZ* clade II are important for mitigating N_2O emissions in arable soils (Jones et al., 2014; Domeignoz-Horta et al., 2016; Xu et al., 2020), although organisms with clade I *nosZ* associated with the roots of leguminous plants have also been shown to be potentially important N_2O sinks (Sameshima-Saito et al., 2006; Gao et al., 2021). Comparisons of the abundance and diversity of both *nosZ* groups over gradients of various edaphic factors, as well as between rhizosphere and bulk soil environments suggest niche partitioning between organisms with *nosZ* clade I and II, which may have a significant effect on net N_2O emissions (Jones et al., 2014; Tsiknia et al., 2015; Juhanson et al., 2017; Graf et al., 2019; Assémien et al., 2019; Shi et al., 2021). Although the overall availability of carbon (C) substrates is one of the key factors regulating denitrification rates as well as affecting the composition of denitrifying communities in soil

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(Wallenstein et al., 2006), the effect of different C substrates on the community composition, diversity and abundance of N_2O reducing microorganisms in soil is poorly understood. This is particularly relevant since addition of different C substrates has been shown to alter the denitrification end-product ratio, defined as the proportion of denitrification terminating with N_2O to that of N_2, in soil (Lorrain et al., 2004; Morley and Baggs, 2010). Previous work has shown that the capacity to utilize different carbon compounds is not randomly distributed across different microbial lineages (Martiny et al., 2013). Since affiliation to *nosZ* clades is highly taxa dependent, with *nosZ* clade II harbouring a diverse range of denitrifying and non-denitrifying bacterial and archaeal taxa and clade I mainly including proteobacterial denitrifiers (Jones et al., 2013; Graf et al., 2014), different C substrates could select for particular taxa within each clade of N_2O reducers. A better understanding of niche differentiation among N_2O reducers in relation to C substrates could support N_2O mitigation strategies in managed soils.

The objective of this study was to examine the effect of different C substrates on N_2O consuming microbial communities in soils that have been previously shown to have contrasting ability to consume exogenous N_2O (Philippot et al., 2011). Specifically, we investigate niche partitioning of genetically distinct N_2O reducers in soil and, by linking specific N_2O reducing phylotypes to particular C-substrates, determine if C preferences are associated with specific lineages in the *nosZ* phylogeny. For that purpose, we set up a microcosm experiment under anoxic conditions to investigate the effects of four different C substrates– acetate, glucose, hydroxyethylcellulose (HEC) and a mixture of the three – on the changes in abundances of nitrate respiring and denitrifying microbial communities over time, as well as the abundance, community structure and diversity of N_2O reducers in soil. Glucose and acetate represent low molecular weight sugars and organic acids that are typically dominant components of rhizodeposition (Kraffcyzk et al., 1984; Jones and Darrah,1996; Jones 1998) and HEC being a soluble form of cellulose i.e. a component of plant structural carbon, and utilized by a wide range of bacterial species (Eichorst and Kuske, 2012).

2. Materials and methods

2.1. Soil sampling and soil properties

Soils were collected from two agricultural fields, Ekhaga and Ulleråker, in the region surrounding Uppsala, Sweden and have been described in previous studies (Stenberg et al., 1998; Philippot et al., 2011). Briefly, both fields are mineral arable soils under cereal/ley rotation with conventional mouldboard ploughing and tillage. The Ekhaga soil is a silty clay loam that has previously demonstrated to have a poor ability to reduce exogenously supplied N_2O, whereas the Ulleråker soil is a silty clay with a higher capacity for reduction of N_2O (Philippot et al., 2011). Soil was sampled in August 2013 at a depth of 0–10 cm and homogenized by sieving through a 4 mm mesh, then stored at – 20 °C until further use (Stenberg et al., 1998). Soil properties for each site (Table 1) were determined by Agrilab (Uppsala, Sweden), an accredited laboratory using standardized protocols for soil pH (H_2O; ISO 10390:2005), total carbon (ISO 10694:1995), total N (ISO 13878:1998) and water content (KLK 1965:1). Soil texture was determined as the fraction of sand, silt and clay content (ISO 11277:2009) as well as the fraction of soil organic matter (SOM) based on loss on ignition (LOI) determination of volatile solids followed by correction for clay content (KLK 1965:1).

2.2. Microcosm setup and sampling

Soils were thawed by incubating under oxic conditions at room temperature one day before establishment of microcosms. For the experiment, soils were incubated under anoxic conditions for 83 days with KNO_3 and either high or low levels of one of the four carbon substrates: acetate, glucose, HEC, or an equimolar mixture of the three. For each microcosm, 12.5 g fresh weight soil, corresponding to 10.4 g and 11 g of soil dry weight (DW) for Ekhaga and Ulleråker soils, respectively, was weighed into sterile 100 ml Schott bottles with screw caps and airtight butyl rubber stoppers. To maintain an anoxic environment within the microcosm, a soil slurry was prepared by adding 12.5 ml of sterile water to soil and exchanging the headspace to N_2 gas after closing the microcosm. Carbon substrates were added at two levels, 1660 mg C and 166 mg C per kg DW of soil, with addition of KNO_3 solution adjusted for the different water content of each soil to reach a final concentration of 1 mM KNO_3. A control treatment was established with no carbon addition, but the same KNO_3 concentration. We used nitrate rather than N_2O as the electron acceptor since the expression of either clade of N_2O reductase is not regulated by N_2O but rather other forms of NOx as well as oxygen availability (Torres et al., 2016; Sanchez et al., 2017; Hallin et al., 2018). Thus, addition of nitrate and the subsequent production of different NOx species by denitrification under anaerobic conditions is necessary to select for a broad range of N_2O reducing organisms in complex communities.

In total, 165 microcosms were established to allow for destructive sampling of three replicate microcosms at time points 0, 14, 28, 42, 56, 70 and 83 days of incubation for treatments under both C substrate levels as well as the control for each of the two soils. Samples at time zero reflect the soils prior to addition of substrates. The microcosms were incubated on a shaker (140 rpm) at 25 °C, and a regular supply of 0.04 mg NO_3^--N and either 0, 0.33 or 3.32 mg C per g soil DW per week was provided to each microcosm by adding 0.2 ml of substrate twice a week throughout the incubation. Destructive sampling was done every two weeks for pH measurement and every four weeks for abundance of N cycle genes, whereas sequencing of N_2O reducing communities was performed only for samples taken when the experiment was terminated at day 83. The pH of the soil slurries was measured using a pH meter (Mettler Toledo SevenCompact™ pH meter S210, Urdorf, Switzerland).

2.3. DNA extraction and quantitative PCR of N-cycling genes

DNA was extracted from soil slurry equivalent to 0.3 g dry weight soil using DNeasy® PowerLyzer® PowerSoil® kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions from all replicates at time points 0, 28, 56 and 83 days of incubation. The extracted DNA was quantified using the Broad Range double stranded DNA kit with a Qubit® fluorometer (Thermo Fisher Scientific, Waltham. Massachusetts, USA) and DNA quality was inspected by agarose gel (1%) electrophoresis.

Quantitative real-time PCR was used to determine the abundances of nitrate, nitrite and nitrous oxide reducing microbial communities. The genes *narG* and *napA*, encoding the cytoplasmic and periplasmic nitrate reducases, respectively, were used as markers for different nitrate reducing communities (Bru et al., 2007), whereas *nirK* and *nirS* genes were used as markers for denitrfying coommunities that posses either the copper- or cytochrome based nitrite reductases, respecitvely. (Hallin and Lindgren, 1999; Throbäck et al., 2004). The two variants of the *nosZ*

Table 1
Properties of the soils used in the experiment.

Soil	pH (H_2O)	Total-C (%)	Total-N (%)	Soil Moisture (%)	SOM[a] (%)	Clay (%)	Silt (%)	Sand (%)
Ekhaga	6.6	4.3	0.33	17	9.5	31.0	50.5	9.0
Ulleråker	6.9	2.1	0.25	12	4.2	43.0	46.5	6.3

[a] Soil organic matter.

gene, clades I and II, were markers the two different N_2O reducing communities (Henry et al., 2006; Jones et al., 2013), and the *nrfA* gene encoding the formate-dependent nitrite reductase for organisms capable of dissimilatory nitrate reduction to ammonia (DNRA; Welsh et al., 2014) Primers and thermal cycling conditions for each gene are shown in Table S1. Each reaction consisted of 5–10 ng template DNA, 1x iQ SYBR Green Supermix (BioRad, Hercules, CA, USA), 0.1% bovine serum albumin (BSA; New England Biolabs, Ipswich, MA, USA) and 0.5–0.8 μM primer concentrations in a total volume of 15 μl. Serial dilutions of linearized plasmids harbouring a fragment of the respective genes were used to generate standard curves. Inhibition of PCR due to contaminants co-extracted with DNA were tested for all samples prior to gene quantification by amplifying a fragment of pGEM-T plasmid (Promega, Madison, Wisconsin, USA) in presence of 5–10 ng extracted DNA or water with 0.25 μM plasmid specific T7 and SP6 primers. There was no significant difference in the threshold values for quantification of controls from those with soil DNA, indicating no inhibition.

2.4. nosZ clade I and clade II amplicon sequencing

The composition and diversity of N_2O reducing communities were determined by amplicon sequencing of *nosZ* clades I and II using a two-step PCR protocol. Forward and reverse primers for the first PCR step consisted of clade-specific primer sequences as well as adaptors for the addition of barcodes in the second PCR step (Table S1). The first PCR step reactions consisted of 1 × DreamTaq buffer (Thermo-Fisher scientific, Waltham. Massachusetts, USA), 0.2 mM dNTP mix, 0.1% BSA, 25 mU/μl Dream Taq DNA Polymerase (Thermo-Fisher scientific, Waltham. Massachusetts, USA), 10 ng of template DNA and 0.8 μM primer of *nosZ* clade I or 2 μM primer of *nosZ* clade II with a final volume of 25 μl in triplicate reactions. The thermal cycling conditions were as follows: 5 min at 95 °C, 30 cycles of 95 °C for 45s, 54 °C for 45s, 72 °C for 1 min and an extension step for 10 min at 72 °C. In the second PCR step, unique barcodes (Chaudhary et al., 2020) were added to each sample using primer constructs consisting of the barcode and adaptor sequences. Each reaction of the second PCR consisted of 1 × DreamTaq buffer, 0.2 mM dNTP mix, 0.1% BSA, 25 mU/μl Dream Taq DNA Polymerase, 10 μl of pooled PCR product from first PCR step and 0.2 μM barcoded universal primer with final volume of 25 μl in quadruplicate reactions. The thermal cycling conditions were as follows: 1 min at 95 °C, 7 cycles of 95 °C for 30s, 50 °C for 30s, 72 °C for 30s and an extension step for 5 min at 72 °C. Pooled amplicons were purified using AMPure XP bead purification (Beckman Coulter, Brea, CA, USA) after each amplification step. For samples with low concentration of pooled PCR products, the number of replicate PCR reactions in the final pool was increased. After the final AMPure purification, amplicons were inspected by agarose gel (2%) electrophoresis and quantified using High Sensitivity double stranded DNA kit (Thermo Fisher Scientific) on a Qubit® fluorometer (Thermo Fisher Scientific). For each *nosZ* variant 29–30 uniquely barcoded amplicon libraries were combined into an equimolar pool, which was then purified using E.Z.N.A.® Cycle-Pure Kit, Omega Bio-Tech, Inc, Norcross, GA, USA. Quality control of the purified libraries was done using a BioAnalyzer (Agilent, Santa Clara, CA, US) and NanoDrop™ 1000 spectrophotometer (Thermo Fisher Scientific). The libraries were then sequenced on the PacBio Sequel® System (PacBio, Menlo Park, CA, USA) with one SMRT cell per pool at SciLifeLab, Uppsala, Sweden. All sequence data is available in the Short Read Archive of NCBI under BioProject accession number PRJNA832917.

2.5. Bioinformatic analysis

Prior to analysis of amplicon sequences, a reference database of full length *nosZ* sequences was created from available microbial genome assemblies obtained from NCBI (June 2019), which included both single organism and metagenome assembled genomes. Assemblies were searched using HMMer (Eddy,1998) with a *nosZ* hidden markov model (HMM) that included both clades I and II. The resulting hits were then dereplicated at 100% amino acid identity using cd-hit, and aligned to the *nosZ* HMM using HMMer. The alignment was manually inspected, and incomplete sequences or those from poor quality genomes (based on checkM scans of assemblies; Parks et al., 2014) were removed. The final alignment was manually refined using the ARB program (Ludwig et al., 2004), and a reference phylogeny was generated using IQ-Tree 2 (Minh et al., 2020) with automatic model selection and ultra-fast bootstrapping ($n = 1000$) to determine node support. The final phylogeny was based on the Le-Gascuel model (Le and Gascuel, 2008) with 10 rate-heterogeneity categories (LG + R10) and rooted at a group of potential *nosZ* homologues found in *Nitrospina* and Proteobacterial genomes obtained from assembled metagenomes. The final reference database consisted of 1684 unique *nosZ* sequences and 9 outgroup sequences, and an ARB database with sequences and the final phylogeny is available for download from the Dryad repository (https://doi.org/10.5061/dryad.pnvx0k6qz).

Processing of *nosZ* clade I and II amplicons was then performed using a combination of the reference database generated above, as well as the SCATA pipeline (https://scata.mykopat.slu.se/). Demultiplexing, quality filtering and clustering was performed using SCATA and reads shorter than 200 bp and containing bases with quality scores less than 10 were discarded. Single linkage clustering of OTUs was performed at 98.5% similarity using USEARCH (Edgar, 2010) with default parameter settings within the SCATA pipeline. Singleton OTUs were excluded from the datasets, and representative sequences for the resulting OTUs were then translated and screened against the reference database using HMMer with an HMM based on full-length *nosZ* amino acid sequences, allowing for the identification and removal of contaminating sequences. The final set of representative sequences were then aligned by amino acid to the reference *nosZ* database using HMMer, then placed into the reference phylogeny using the next-generation evolutionary placement algorithm (NG-EPA; Barbera et al., 2019) with the LG + R10 likelihood model as determined by IQ-Tree 2. Taxonomic classification of OTUs was then performed using the 'gappa' software for analysis of phylogenetic placements (Czech and Stamatakis, 2019). Phylogenetic analysis of translated representative OTU sequences was performed using FastTree2 (Price et al., 2010) using the LG + R10 substitution model.

2.6. Statistical analysis and identification of differentially abundant nosZ OTUs

All statistical analyses and plotting were done using R (R Core Team, 2013). Changes in pH over time were assessed within each combination of C substrate and level using either one-way ANOVA analysis or Kruskal-Wallis tests when assumptions of ANOVA were not met.

The effect of C substrate on functional gene abundances throughout the incubation period was determined using generalized linear models, in which carbon treatment and days of incubation were treated as fixed effects in the model whereas the pH of the microcosms was added as a covariate to control for potential confounding effects of pH. The significance of the pH term in the model was examined by *t*-tests of the resulting model coefficients as well as likelihood ratio tests between the initial model and a reduced model without pH and was excluded when it was not a significant term in the initial model ($Pr(t) > 0.05$) and no significant difference observed between the full and reduced models. A gamma distribution with a log link function was used estimate model errors for each gene abundance, as determined by inspection of diagnostic plots. The final models were then used to for subsequent ANOVA analyses, as well as determining pairwise differences between means using the '*emmeans*' package in R (Lenth, 2022). Overall changes in functional gene abundances throughout the incubation period were assessed using the AC-PCA algorithm ('Adjustment for Confounding variation Principal Components Analysis'), which removes variation associated with one or more confounding variables from the resulting principal components (Lin et al., 2016). As with the linear modelling, pH was specified as a potential confounding variable for both low and high

C substrate levels, and resulting ordinations reflect variation in gene abundances associated with C substrate and incubation time only.

Prior to analyses of *nosZ* community diversity and composition, samples with fewer than 1000 reads were discarded. The diversity of *nosZ* clade I and II communities was then calculated as Shannon's index based on rarefied OTU tables for each clade within each soil type using the 'vegan' package. The rarefied tables consisted of 1937 and 1217 reads per sample for clade I and II communities in Ekhaga soils, respectively, and 2912 and 1150 reads per sample for clades I and II communities in the Ulleråker soils, respectively. The effect of carbon substrate type was then determined using a similar linear modelling approach as described for gene abundances, in which pH was included in the models as a covariate when found to be a significant term in the initial model.

Compositional changes in the N_2O reducing communities were then examined based on Robust Aitcheson distances (Martino et al., 2019) calculated using the 'vegan' package in R (Oksanen et al., 2013). Robust Aitcheson distances are based on the centred log-ratio transformation to account for compositionality of microbial community sequence data, while at the same time dealing with the high number of zero-counts typically observed in OTU tables. Significant differences in community composition were tested using permutational multivariate analysis of variance (PERMANOVA), using the *adonis* function in 'vegan' with 999 permutations. We then used AC-PCoA, a variant of the AC-PCA method in which the effect of confounding variables is removed from Principal Coordinates Analysis (Wang et al., 2022), to examine the structure of *nosZ* communities in response to C substrate and level while controlling for differences in pH. Correlations between differences in community structure and gene abundances were determined using the *envfit* function in 'vegan'.

Detection of *nosZ* clade I and II OTUs that were significantly enriched by addition of different C amendments was performed using generalized linear modelling of centred log-ratio transformed abundances for each clade within each soil type. Prior to transformation, zero-counts within each OTU table were replaced by imputation of pseudo-counts using the 'zCompositions' package in R (Palarea-Albaladejo and Marin-Fernandez, 2015). Tables were transformed to centred log-ratios of proportional abundances, and generalized linear models were constructed for each OTU in which the effect of C treatment was adjusted for differences in pH across the microcosms. Log_2-fold increases in abundance were calculated using treatment vs. control contrasts of each C amendment (high or low) to the nitrate only controls, and the resulting p-values corrected for false discovery rate. Plotting of indicator OTUs within the reference phylogeny was performed using the 'ggtree' package in R (Yu et al., 2017).

3. Results

3.1. pH

The change in pH over the duration of the incubation period differed depending on C-substrate and soil type (Table 2). Shifts in pH larger than one unit were observed only with addition of high glucose levels in both soils, resulting in a more acidic pH. In contrast, addition of acetate resulted in a significant increase in pH, with final pH levels ranging from 7.07 to 7.49 across both soil types. The effect of other substrates on pH was less consistent, as an addition of HEC, resulted in a moderate, yet significant increase in pH only in the Ekhaga soil when added in low concentrations, whereas addition of the combination of substrates increased pH in Ekhaga soils yet decreased pH Ulleråker soil (see Table 2).

Table 2
Effect of different carbon amendments on pH in microcosms incubated for 83 days. Values show pH in microcosms at the end of the incubation period ($pH_{t=83}$), as well as the change in pH from the start of the experiment ($\Delta pH_{t(83\text{-}0)}$; mean ± standard deviation, n = 3). Values in bold indicate significant changes based on one-way ANOVA or Kruskal-Wallis tests over the incubation period within combination of substrate level and substrate.

Soil	Substrate level	Substrate	$pH_{t=83}$	$\Delta pH_{t(83\text{-}0)}$
Ekhaga	Control	Nitrate only	**7.02 ± 0.03**	**0.62 ± 0.06*****
	Low	Acetate	**7.18 ± 0.19**	**0.78 ± 0.20***
		Glucose†	6.54 ± 0.17	0.13 ± 0.19
		HEC†	**6.66 ± 0.57**	**0.26 ± 0.58***
		Combined	**6.95 ± 0.05**	**0.55 ± 0.06***
	High	Acetate†	**7.07 ± 0.04**	**0.67 ± 0.06****
		Glucose†	**4.65 ± 0.25**	**-1.75 ± 0.27****
		HEC†	6.94 ± 0.12	0.54 ± 0.14
		Combined†	**6.66 ± 0.04**	**0.26 ± 0.05****
Ulleråker	Control	Nitrate only	7.16 ± 0.14	-0.09 ± 0.15
	Low	Acetate†	**7.41 ± 0.1**	**0.16 ± 0.10***
		Glucose	**6.44 ± 0.29**	**-0.81 ± 0.29*****
		HEC†	7.25 ± 0.05	<0.01 ± 0.05
		Combined†	**6.93 ± 0.09**	**-0.32 ± 0.10***
	High	Acetate	**7.49 ± 0.04**	**0.24 ± 0.05*****
		Glucose†	**4.37 ± 0.0**	**-2.88 ± 0.02****
		HEC	7.32 ± 0.04	0.07 ± 0.05
		Combined†	**6.40 ± 0.11**	**-0.84 ± 0.11***

†Kruskal-Wallis test.
***$P < 0.001$.
**$0.001 < P < 0.01$.
*$0.01 P < 0.05$.

3.2. Abundance of functional genes

When statistically controlling for potential variation caused by differences in pH, we observed that abundances of all N cycling genes in both soils still differed significantly depending on C-substrate, with the exception of abundance of *nirK*, one of the marker genes for denitrifiers, in Ekhaga soil at low levels of C substrate addition (Table 3; Tables S2 and S3). At high substrate addition levels, the significant interaction observed between C-substrate and days under incubation also indicated that the effect of C substrate differed over time. This is likely due to the increased abundance observed for most genes in the nitrate only controls in both soils, whereas abundance decreased over time in most of the microcosms with added C, with the exception of HEC in which abundances were typically highest at 56 days (Tables S2 and S3).

Comparison of the abundance of all N cycling genes using principal components analysis adjusted for confounding variables (AC-PCA), in which variation associated with pH was removed, further indicated that assemblages of N-cycling guilds involved in inorganic N transformations varied in response to different C substrates. This was most pronounced in microcosms with high C amendment levels, as samples clearly differentiated by C amendment for both soil types (Fig. 1). While increased *narG* in soils amended with nitrate only was observed for both soil types (Fig. 1b; Table S3), the covariation of gene abundances in response to C amendment differed between soils, indicating that the composition of N-cycling guild assemblages is soil specific. In Ekhaga soil, decreasing abundance of *nirK* and *nosZ* clade I in the nitrate only microcosms were strongly correlated with increased *nirS* abundance (Fig. 1a; Table S3), whereas the increased abundance of *nirS* and *nosZ* clade I in Ulleråker soil amended with HEC was concomitant with decreased *nirK* abundance. At low substrate level, changes in N-cycling community assemblage were mostly associated with incubation time, with overall decreased abundances after 83 days (Fig. S1; Table S2). While samples were not as clearly clustered according to C substrate compared to high

Table 3
Effect of substrate type and days under incubation at high and low substrate levels on abundances of N-cycling genes based on generalized linear models, with pH as a covariate. Significant effects are shown in bold (α = 0.05, n = 3).

Soil	Substrate level	Gene	Substrate		Days		Substrate × Days		pH[a]	
			$F_{(4,30)}$	P	$F_{(2,30)}$	P	$F_{(8,30)}$	P	$F_{(1,30)}$	P
Ekhaga	Low	*narG*	**6.24**	**< 0.001**	**12.34**	**< 0.001**	0.8	0.6		NS
		napA	**14.71**	**< 0.001**	**9.5**	**< 0.001**	**3.86**	**0.003**	**5.04**	**0.03**
		nrfA	**6.78**	**< 0.001**	3.12	0.06	1.55	0.18		NS
		nirK	1.36	0.27	**22.04**	**< 0.001**	1.11	0.39		NS
		nirS	**20.29**	**< 0.001**	**8.3**	**0.001**	**9.38**	**< 0.001**	**22.59**	**< 0.001**
		nosZI	**3.99**	**0.01**	**19.35**	**< 0.001**	1.31	0.28		NS
		nosZII	**4.50**	**0.005**	**22.3**	**< 0.001**	1.2	0.33		NS
	High	*narG*	**86.88**	**< 0.001**	**26.31**	**< 0.001**	**9.12**	**< 0.001**		NS
		napA	**7.72**	**< 0.001**	**13.82**	**< 0.001**	**3.19**	**0.01**	**6.94**	**0.01**
		nrfA	**26.29**	**< 0.001**	**22.68**	**< 0.001**	**3.93**	**0.002**		NS
		nirK	**96.84**	**< 0.001**	**45.34**	**< 0.001**	**7.96**	**< 0.001**		NS
		nirS	**58.21**	**< 0.001**	**12.0**	**< 0.001**	**12.31**	**< 0.001**	**34.13**	**< 0.001**
		nosZI	**69.3**	**< 0.001**	**34.49**	**< 0.001**	**5.96**	**< 0.001**		NS
		nosZII	**115.44**	**< 0.001**	**34.41**	**< 0.001**	**6.41**	**< 0.001**		NS
Ulleråker	Low	*narG*	**6.71**	**< 0.001**	1.54	0.23	**2.67**	**0.02**	**7.71**	**0.009**
		napA	**6.81**	**< 0.001**	**4.24**	**0.02**	1.85	0.11	**7.54**	**0.01**
		nrfA	**7.53**	**< 0.001**	0.74	0.49	0.99	0.46	**5.24**	**0.03**
		nirK	**10.47**	**< 0.001**	1.66	0.21	2.33	0.051	**4.28**	**0.05**
		nirS	**5.81**	**0.001**	2.49	0.1	1.32	0.27	**8.52**	**0.006**
		nosZI	**5.26**	**0.003**	**4.95**	**0.01**	1.32	0.27	**6.58**	**0.02**
		nosZII	**7.9**	**< 0.001**	**3.49**	**0.04**	1.07	0.41	4.09	0.052
	High	*narG*	**54.34**	**< 0.001**	**11.56**	**< 0.001**	**2.51**	**0.03**		NS
		napA	**49.14**	**< 0.001**	**11.25**	**< 0.001**	**5.67**	**< 0.001**		NS
		nrfA	**114.44**	**< 0.001**	**18.22**	**< 0.001**	**6.41**	**< 0.001**		NS
		nirK	**67.68**	**< 0.001**	**18.99**	**< 0.001**	**3.92**	**0.003**		NS
		nirS	**61.45**	**< 0.001**	**8.62**	**0.001**	**2.57**	**0.03**		NS
		nosZI	**94.25**	**< 0.001**	**8.72**	**0.001**	**8.37**	**< 0.001**		NS
		nosZII	**113.63**	**< 0.001**	**35.98**	**< 0.001**	**2.98**	**0.01**		NS

[a] 'NS' indicates models in which pH was not significant, and thus excluded from the final model.

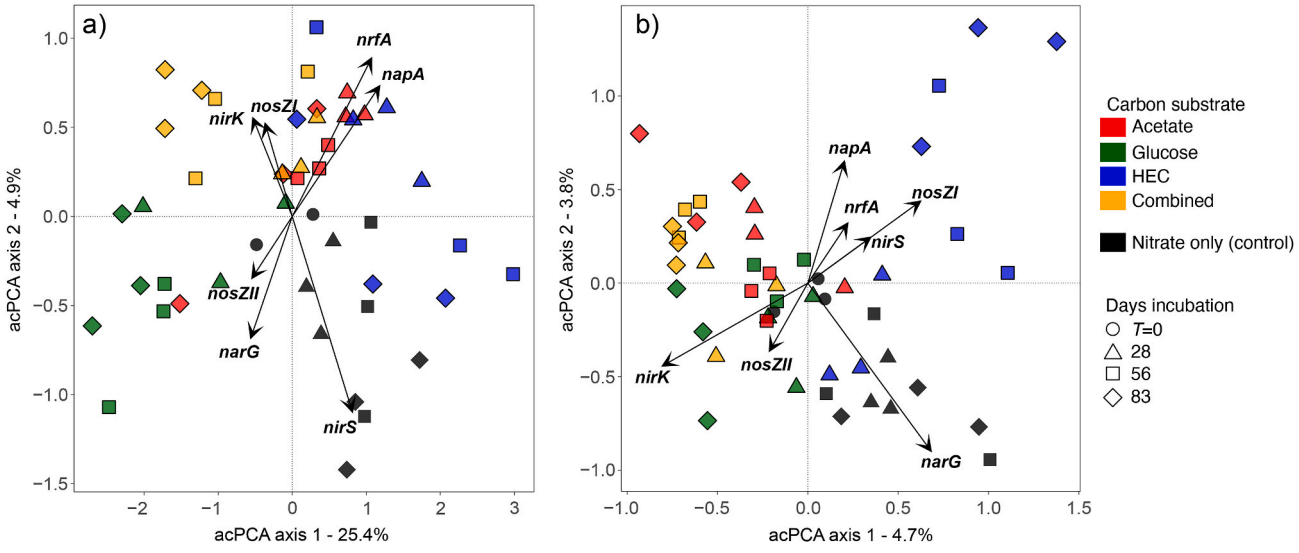

Fig. 1. Principal component analysis Adjusted for Confounding variable (AC-PCA) of anaerobic inorganic nitrogen cycling communities in soil microcosms amended with different carbon substrates and nitrate in a) Ekhaga and b) Ulleråker soils at high substrate level over 83 days. The ordination is based on $\log_{10}$ transformed abundances of marker genes representing nitrate-reducing (*napA*, *narG*), denitrifying (*nirS*, *nirK*), nitrous oxide reducing (*nosZ*I and *nosZ*II) and DNRA (*nrfA*) communities. Eigenvectors and loading factor scores are adjusted to remove variation associated with changes in pH across samples. Symbol color indicates amendments of carbon substrates and nitrate only, and symbol shape represents samples taken at different days of incubation. Variance explained by each principal component is shown in the axes labels. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

substrate levels, abundances were generally lower in acetate amended microcosms for both soils. Moreover, abundances of *nirS*, *narG, nirK* and *nosZ*II exhibited little change or even increased in nitrate-only controls in Ulleråker soil (Fig. S1b). The effect of addition of low levels of HEC was variable over time across both soil types, as *nirK* gene abundances in HEC-amended Ulleråker soil nearly doubled after 56 days then

decreased substantially at day 83 (Fig. S1b; Table S3), whereas gene abundances of *nosZ*II, *nirS, nrfA* and *napA* increased after 56 days in Ekhaga soil (Fig. S1a; Table S3).

3.3. nosZ clade I diversity and community composition

At the end of the incubation period, the diversity of *nosZ* clade I communities differed significantly across Ulleråker soils amended with different C substrates at both high and low substrate addition levels, with the lowest diversity observed in the HEC treatment (Table S4). By contrast, C-substrate addition had no effect on *nosZ* clade I diversity in the Ekhaga soil (Table S4). The composition of both *nosZ* communities differed in response to substrate source and level (Fig. 2a–d), which was confirmed by PERMANOVA in both the soils (Tables S5 and S6). Differences in *nosZ* clade I community structure associated with pH were also detected for communities in Ulleråker soils (Table S5). However, the variation explained by pH was far lower than that explained by C source, and communities were clearly differentiated by C source and level in ordinations in which variation associated with pH was removed (Fig. 2a and b), with C source have a greater effect on structure than amendment level (Table S6). *nosZ* clade I communities in HEC-amended microcosms showed the greatest degree of differentiation from communities in soils amended with other C sources. However, abundances of different genes were associated with *nosZ* clade I compositional shifts in either soil. *nirS* abundances were correlated with the community structure of Ekhaga soils amended with nitrate only or HEC (Fig. 2a) whereas *narG*, *nrfA* and *nosZ* clade I abundances were associated with the composition in Ulleråker soil with low levels of different C sources or nitrate only (Fig. 2b).

Analysis of differential abundance of OTUs in C-amended soils compared to the nitrate-only controls revealed several alphaproteobacterial *nosZ* clade I lineages that were enriched by amendment with low or high levels of acetate as well as high levels of the combined substrate in both soils (Fig. 3a and b). The majority of these were classified as *nosZ* clade I from genera within the families Bradyrhizobiaceae (*Bradyrhizobium*, *Afipia*), while others were associated with *Paracoccus* sp. within the order Rhodobacterales in Ekhaga soils (Fig. 3a), or *Skermanella* sp. within the family Rhodospirillaceae in Ulleråker soils (Fig. 3b). Other OTUs within these lineages were also enriched in microcosms with low glucose addition, whereas no *nosZ* clade I OTUs were found to be significantly enriched in microcosm with high levels of glucose added. While several OTUs classified as betaproteobacterial *nosZ* clade I increased with addition of HEC in both soils, most were observed only in the Ulleråker soil and were classified within the families Oxalobacteriaceae (*Novaherbaspirillum* sp.) or unclassified groups within the Burkholderiales.

3.4. nosZ clade II diversity and community composition

The diversity of *nosZ* clade II communities differed significantly across C-substrate amendments in both soils after controlling for pH, however this effect was dependent on the level of substrate addition for

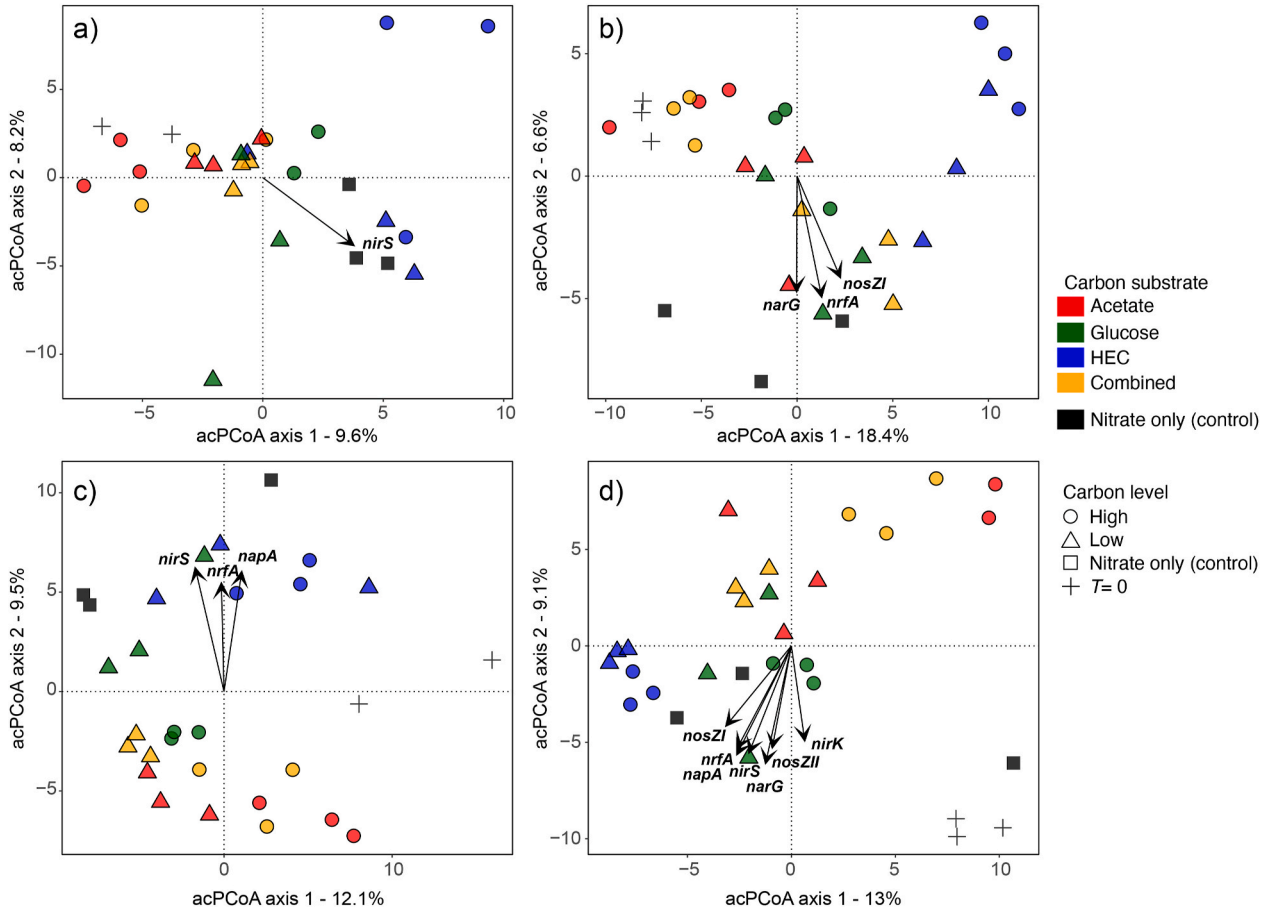

Fig. 2. Principal Coordinate analysis Adjusted for Confounding variables (AC-PCoA) of nitrous oxide reducing communities with *nosZ* clade I in a) Ekhaga and b) Ulleråker soil and *nosZ* clade II in c) Ekhaga and d) Ulleråker soil after 83 days of incubation with different carbon substrates and nitrate. Ordination scores are adjusted to remove variation associated with changes in pH across samples. Symbol color indicates amendments of carbon substrates and nitrate only, and symbol shape represents substrate levels. The initial community at the start of the experiment is indicated by crosses. Functional gene abundances that are significantly correlated ($P < 0.05$) with the community structure are shown as arrows indicating direction and strength of correlation. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

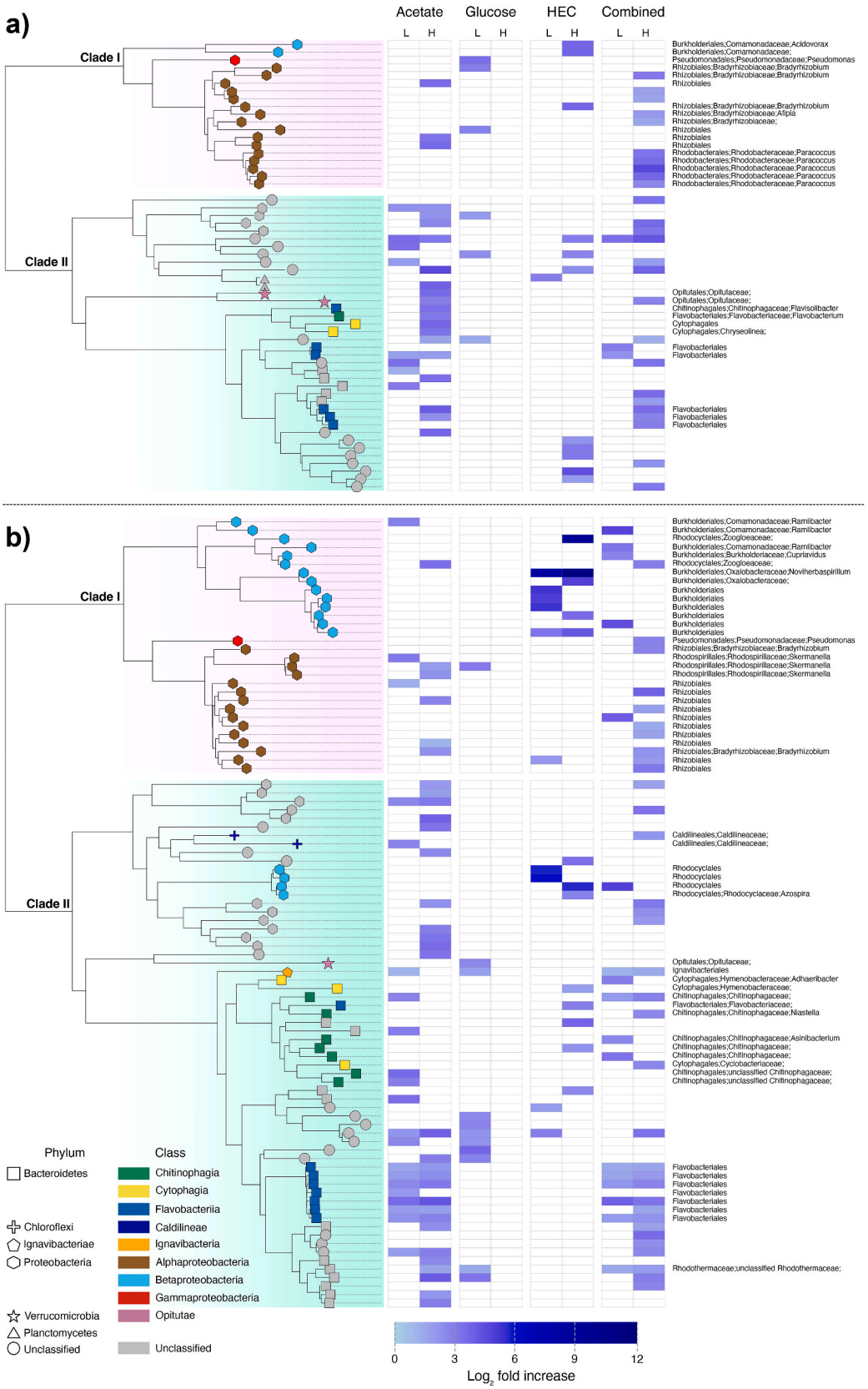

Fig. 3. Enrichment of *nosZ* clade I and clade II OTUs by addition of different carbon substrates in a) Ekhaga and b) Ulleråker soils. The heatmap shows the $\log_2$-fold increase in abundance of OTUs that were significantly higher ($P < 0.1$, FDR corrected) in microcosms amended with either low ('L') or high ('H') levels of different carbon substrates when compared to the nitrate-only controls, while controlling for the effect of pH in generalized linear models. The phylogeny at left is based on maximum likelihood analysis (LG model + R10; FastTree2) of translated OTU sequences, with shading indicating *nosZ* clade. Shape and color of tip symbols denote taxonomic classification of OTUs at the phylum and class level, respectively, with further classification to the genus level shown at right. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

each soil type. Similar to *nosZ* clade I, addition of high levels of HEC lowered the diversity of *nosZ* clade II in the Ulleråker soil, yet acetate addition at low substrate level reduced *nosZ* clade II diversity compared to the other substrates in Ekhaga (Table S4). While pH was also significantly associated with different in *nosZ* clade II community structure in both soils (Table S5), the explained variation was again lower relative to the effect of carbon amendment, and a distinct segregation of *nosZ* clade II communities according to the type and level of C-substrate was observed in ordinations in which pH-associated variation was removed (Fig. 2c–d). As with clade I, the greatest variation in structure was explained by substrate type rather than level. Increased abundances of *nirS, nrfA* and *napA* were associated with *nosZ* clade II community structure in HEC and nitrite only treatments in Ekhaga soil (Fig. 2c), whereas all N cycling genes were positively related to *nosZ* clade II communities in nitrate control, and negatively correlated to high levels of other C sources, in Ulleråker soil (Fig. 2d).

The majority of significantly enriched *nosZ* clade II OTUs were observed in soils amended with acetate or the combined C substrate, many of which were identified as *nosZ* from various sub-groups within the Bacteroidetes or Proteobacteria phyla (Fig. 3a–b). However, we also observed a greater range of taxonomic diversity amongst enriched OTUs in the Ulleråker soil, which included OTUs classified within the phyla Chloroflexi and Ignavibacteria. In both soils, abundances of OTUs classified as Chitinophagales, Flavobacteriales and Cytophagales increased with both high and low acetate amendments as well as high levels of the combined C substrate. We also observed several OTUs, from the phylum Ignavibacteriae and the rest unclassified, that increased in response to several different C-amendments, whereas several indicator OTUs that grouped closely with *Azospira* sp. within the *nosZ* clade II betaproteobacterial lineage increased with low or high HEC amendment in the Ulleråker soil only (Fig. 3b).

4. Discussion

Amendment with different C-substrates, rather than their concentration, generally played a more important role in niche differentiation within each clade of N_2O reducing microorganisms, as indicated by the greater variation in community structure explained by C-substrate compared to level in both soils. Thus, each C substrate selected for specific N_2O reducing communities, which was further confirmed by differential abundance analysis. Changes in soil pH are known to have strong effects on microbial community structure (Fierer et al., 2012), and the observed differences in pH resulting from amendment with different C sources also influenced community structure at the end of the incubation. However, with the exception of glucose, changes in pH were less than one pH unit over the duration of the incubation across the different C substrates and the variation explained by pH was much lower than that explained by C source. Thus, pH was less important than C-substrate in determining the structure of N_2O reducing communities. Despite the effects of C source on community composition of both *nosZ* clades, we found no evidence that the C-substrate or their concentration were consistent drivers of niche differentiation between clade I and II N_2O reducing communities, as the effect of C-source on the abundance and diversity of each community was largely dependent on soil type. This result was unexpected given previous work showing that N_2O reducing communities at the plant-root interface were dominated by organisms with clade I *nosZ* (Graf et al., 2016, 2022; Ai et al., 2020). Thus, amendment with more labile carbon compounds typically found in rhizodeposits, such as glucose and acetate, should result in a niche space similar to that found in the rhizosphere. However, Graf et al. (2016) also demonstrated that soil type was an overriding factor in determining the *nosZ* gene abundances in the rhizosphere. The different C amendments used in this study also likely caused differences in NO_3^- immobilization, thereby adding an indirect control on N_2O reducing microorganisms. Hemicellulose, which is not readily available to a majority of the soil microorganisms, can result in low NO_3^- immobilization in soil. For example, Wang et al. (2021) showed a significantly lower microbial NO_3^- immobilization rate upon addition of wheat and peanut straw to soil when compared with glucose. This could explain why addition of HEC resulted in a higher abundance of denitrification genes compared with the other C-treatments and similar to abundances in the control with only NO_3^-, as N addition is known to promote denitrifiers in soil (e.g. Jones et al., 2022).

Many of the alphaproteobacterial species with *nosZ* clade I are plant-associated, including *Bradyrhizobium* spp. (Tiedje, 1988; Sameshima-Saito et al., 2006) and *Paracoccus* spp. (Dias et al., 2013; Abdelfattah et al., 2021), suggesting that N_2O-reducing organisms within these lineages are adapted to utilize a range of different C sources in root exudates, including low molecular weight sugars and organic acids. *Paracoccus* spp. in particular are known for their diverse catabolic capacity, as they can possess both the glyoxalate and ethylmalonyl-CoA pathways for carbon assimilation and coordinate the expression of either pathway depending on the presence of different C-sources as a means to maximize C assimilation efficiency (Kremer et al., 2019). This may explain why the combined substrate favoured this group of N_2O reducers in the Ekhaga soil, although this effect was soil specific in this study. The majority of *nosZ* clade II OTUs that increased with addition of different carbon sources in both soils were classified as being within the phyla Proteobacteria and Bacteriodetes, the latter of which is known to include a large proportion of non-denitrifying N_2O reducing organisms (Graf et al., 2014). Similar to the alphaproteobacterial *nosZ* clade I, abundances of these lineages also increased with addition of acetate and combined C substrates, as well as HEC in the Ulleråker soil. The enrichment of species within the orders Cytophagales and Chitinophagales of Bacteriodetes by HEC is not surprising as members of these groups are known for degrading and utilizing cellulose and other complex organic C sources under oxic and anoxic conditions (Reichenbach, 2006; Rosenberg 2014; Wörner and Pester, 2019). Addition of HEC also selected for of various betaproteobacterial species within both *nosZ* clades in the two soils, which again aligns with previous work showing that many species within the family Oxalobactereacea, particularly *Herbaspirillum* and *Noviherbaspirillum*, are known to be endophytic bacteria that produce and degrade cellulose (Straub et al., 2013; Hameed et al., 2015), whereas species within the families Comamonadaceae and Zoogloeaceae, such as *Acidovorax* and *Azoarcus* sp., are capable of utilizing a variety of recalcitrant carbon compounds (Uchida et al., 2000; Liu et al., 2006).

The low diversity of *nos*Z clade I community in combination with high abundances of *nirS*, *nosZ*I and *napA* in HEC amended Ulleråker soil indicate selection of betaproteobacterial N_2O reducing lineages that are likely to be complete denitrifiers. Alternatively, there is complete denitrification capacity at the community level (Hallin et al., 2018), in which different organisms perform different steps in the denitrification pathway. The more frequent association of HEC with betaproteobacterial indicator species in Ulleråker (~13%) than Ekhaga soil (~4%) could be due to the higher soil organic matter and carbon content in Ekhaga soil. Low organic matter content in soil has been shown to favour a microbial community with complete denitrification (Surey et al., 2020), whereas metabolic specialization across the denitrification pathway has been observed in eutrophied sandy sediments (Marchant et al., 2018). Thus, resource availability likely determines the potential for incomplete over complete denitrification. *nosZ* clade II OTUs associated with betaproteobacterial species also responded positively to HEC in the Ulleråker soil, specifically those within the order Rhodocyclales that includes genera such as *Dechloromonas* and *Azospira* which are known decomposers of complex C substrates in different environments (Achenbach et al., 2001; Lu et al., 2007; Salinero et al., 2009). For example, in a groundwater system designed for nitrate removal (Xie et al., 2017), cellulose favoured denitrification by *Azospira* sp, while addition of cellulosic material in simulated constructed wetlands resulted in enhanced denitrification rates with a concomitant increase enrichment of *Dechloromonas* sp. (Si et al., 2018). However, in a pure

culture study comparing two *Azospira* sp. isolates subject to acetate, succinate, glycerol, methanol, and ethanol when using N_2O as an electron acceptor demonstrate that they readily use all these compounds although the organic acids were preferred over the alcohols and the biokinetics for each compound vary between the two isolates (Qi et al., 2022). Thus, although our results indicate selection of betaproteobacterial N_2O reducers with HEC as a C- substrate, variation within specific lineages are likely substantial.

5. Conclusion

Our study provides evidence that N_2O reducing community composition is controlled directly and indirectly by addition of different C-substrates, indicating niche partitioning within the two major clades of N_2O reducers. Although representatives of both *nosZ* clades of N_2O reducing communities were in general more favoured by acetate and the combination of C-substrates over HEC, betaproteobacterial species within both clades were positively responding to HEC. Our findings demonstrate that C substrate amendments cause substantial shifts in N_2O reducing bacterial community composition. These observations could potentially explain why the type of C substrate can influence N_2O production and reduction rates in soil (Morley et al., 2014) and alter the denitrification end-product ratio (Lorrain et al., 2004; Morley and Baggs, 2010).

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

An ARB database of nosZ reference sequences and phylogeny is available in the Dryad data repository

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.soilbio.2022.108909.

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