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Research Article

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RESEARCH ARTICLE

Habitat diversity and type govern potential nitrogen loss by denitrification in coastal sediments and differences in ecosystem-level diversities of disparate N_2O reducing communities

Lea Wittorf[1], Fabian Roger[2,†], Christian Alsterberg[2,‡], Lars Gamfeldt[2], Stefan Hulth[2], Kristina Sundbäck[2], Christopher M. Jones[1] and Sara Hallin[1,*,§]

[1]Swedish University of Agricultural Sciences, Department of Forest Mycology and Plant Pathology, Uppsala, Sweden and [2]University of Gothenburg, Department of Marine Sciences, Gothenburg, Sweden

*Corresponding author: Box 7025, 750 07 Uppsala, Sweden. Tel: +46(0)18 67 32 09; E-mail: Sara.Hallin@slu.se

One sentence summary: Within illuminated shallow-water sediments, habitat diversity was shown to promote ecosystem-level diversity of nitrous oxide reducing communities, whereas habitat type determined the denitrification potential of the benthic microbial communities.

†*Current address:* Lund University, Centre for Environmental and Climate Research, Lund, Sweden
‡*Current address:* Lund University, Department of Biology, Lund, Sweden

Editor: Julie Olson

§Sara Hallin, http://orcid.org/0000-0002-9069-9024

ABSTRACT

In coastal sediments, excess nitrogen is removed primarily by denitrification. However, losses in habitat diversity may reduce the functional diversity of microbial communities that drive this important filter function. We examined how habitat type and habitat diversity affects denitrification and the abundance and diversity of denitrifying and N_2O reducing communities in illuminated shallow-water sediments. In a mesocosm experiment, cores from four habitats were incubated in different combinations, representing ecosystems with different habitat diversities. We hypothesized that habitat diversity promotes the diversity of N_2O reducing communities and genetic potential for denitrification, thereby influencing denitrification rates. We also hypothesized that this will depend on the identity of the habitats. Habitat diversity positively affected ecosystem-level diversity of clade II N_2O reducing communities, however neither clade I *nosZ* communities nor denitrification activity were affected. The composition of N_2O reducing communities was determined by habitat type, and functional gene abundances indicated that silty mud and sandy sediments had higher genetic potentials for denitrification and N_2O reduction than cyanobacterial mat and *Ruppia maritima* meadow sediments. These results indicate that loss of habitat diversity and specific habitats could have negative impacts on denitrification and N_2O reduction, which underpin the capacity for nitrogen removal in coastal ecosystems.

Keywords: habitat loss; illuminated shallow-water sediments; benthic communities; nitrogen removal; *nosZ*; niche differentiation

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INTRODUCTION

Worldwide, homogenization of natural habitats driven by human activities (McKinney and Lockwood 1999; Western 2001) results in loss of biodiversity that may negatively affect important ecosystem functions (Elahi *et al.* 2015; Lefcheck *et al.* 2015; Newbold *et al.* 2015; Alexander, Vonlanthen and Seehausen 2017). In coastal marine sediments, habitat homogenization is an effect of eutrophication, terrestrial sediment run-off, and other natural and anthropogenic stressors (Karlson, Rosenberg and Bonsdorff 2002; Thrush *et al.* 2004; Mineur *et al.* 2015). Recently, habitat diversity has been shown to have positive effects on both multifunctionality and bacterial species diversity in illuminated shallow-water sediments (Alsterberg *et al.* 2017). An important function of these systems is the removal of fixed nitrogen (N) (Codispoti 2007; Gruber 2008). Denitrification, a microbial process in which nitrate (NO_3^-) is reduced to gaseous N compounds, is often the main pathway for N removal (Seitzinger 1988; Middelburg *et al.* 1996; Deutsch *et al.* 2010; Gao *et al.* 2012). Illuminated shallow-water sediments are thus important nutrient filters that retain, transform and remove N transported from land to sea (McGlathery, Sundbäck and Anderson 2007; Asmala *et al.* 2017). Despite this, the importance of habitat diversity for maintaining diverse denitrifying communities in benthic environments is not well known.

The capacity to denitrify is present among a diverse range of microbial taxa (Graf, Jones and Hallin 2014), suggesting high functional redundancy within this guild (Graham *et al.* 2016). However, the denitrification pathway is modular, as organisms may have the genetic capacity to perform all or only a subset of the different steps within the pathway (Zumft 1997; Graf, Jones and Hallin 2014). The reduction of nitrite (NO_2^-) to nitric oxide (NO) is performed by two structurally distinct nitrite reductases, containing either cytochrome cd_1 or copper in their catalytic center, that are encoded by the genes *nirS* and *nirK*, respectively (Zumft 1997). The cytotoxic NO is reduced to nitrous oxide (N_2O), a potent greenhouse gas and ozone depleting substance. Nitrous oxide can be further reduced to dinitrogen gas (N_2) by organisms harboring the *nosZ* gene, encoding the nitrous oxide reductase, and communities of these organisms are the only known biological sink for N_2O. Organisms with the capacity to reduce N_2O are split into two major groups, designated clades I and II based on the *nosZ* phylogeny (Sanford *et al.* 2012; Jones *et al.* 2013). Most organisms within clade I are complete denitrifiers, while the majority of those in clade II do not have genes involved in N_2O production (Graf, Jones and Hallin 2014). An increased abundance of clade II *nosZ* in soils has been associated with a higher capacity to reduce N_2O (Jones *et al.* 2014; Domeignoz-Horta *et al.* 2016), whereas efficient N_2O reduction in bioreactors was shown to be accomplished by denitrifying communities dominated by clade I *nosZ* populations (Conthe *et al.* 2018). Only a few studies have been published so far on the ecology of clade I and II type N_2O reducers in coastal marine sediments. Overall, clade I has been shown to dominate in non-illuminated and deeper sediments, while clade II may have an important role in shallow-water permeable sand sediments (Wittorf *et al.* 2016; Marchant *et al.* 2018). However, differences among sediment types and how these microbial communities may be affected by losses in habitat diversity is not known, despite the importance of these shallow-water coastal environments as nutrient filters.

The aim of this study was to determine how habitat diversity regulates the diversity of N_2O reducing communities at the ecosystem scale (gamma diversity) in illuminated shallow-water sediments. We hypothesized that increasing habitat diversity will result in a concomitant increase in the gamma diversity of the N_2O reducing communities. If there is a link between *nosZ* diversity and community functioning an increased diversity would result in increased denitrification activity and genetic potential for N_2O reduction and N removal through denitrification from these coastal sediments. This could either be due to complementarity and/or selection effects, i.e. the increased probability of diverse communities including high-performing genotypes. The genetic potential was defined as the size of the denitrifying and N_2O reducing communities, as determined by the abundances of *nirS* and *nirK* genes and *nosZ* clade I and II genes, respectively. In addition to the hypothesized effect of habitat diversity on the gamma diversity of N_2O reducers, specific habitats can be more influential by having higher activities and genetic potentials for denitrification and N_2O reduction, making them potentially more beneficial for N-removal in these ecosystems.

MATERIALS AND METHODS

Sampling and experimental set-up

To investigate the effects of habitat diversity and habitat type on denitrification rates and denitrifying and N_2O reducing communities in illuminated shallow-water sediments, we analysed samples and data collected in a previous study (Alsterberg *et al.* 2017). Briefly, a total of 112 sediment cores (inner diameter = 8 cm, height = 11 cm) were collected from four naturally occurring habitats on the west coast of Sweden in the Skagerrak area in summer 2013: sandy beach, silty mud, cyanobacterial mat and *Ruppia maritima* meadow sediments. For each core, pore water concentrations of ammonium and nitrate + nitrite (as a measure of dissolved inorganic nitrogen (DIN)) were determined according to standard colorimetric procedures (Strickland and Parson 1972). To extract sediment pore water, 50 ml of sediment was centrifuged (32 000 x g) for 30 min (Eppendorf Centrifuge 5810 R), and the pore water was filtered (0.45-μm surfactant-free cellulose acetate filters) and then immediately frozen at −80°C before analyses using a TRAACS autoanalyzer (Technicon, SEAL Analytical Inc.).

Sediment cores were assembled into mesocosms that represented ecosystems with different levels of habitat diversity, ranging from 1 (presence of only one habitat type) to 4 (all four habitat types were present). This was achieved by varying the presence and abundance of sediment cores from each habitat type (Fig. 1). Each mesocosm consisted of four sediment cores of either the same or a combination of different habitat types that were placed together in a larger cylinder (inner diameter = 25 cm, hight = 25 cm). All habitat levels were replicated four times, but for levels 2 and 3 the four combinations were randomly drawn from the pool of habitat types and therefore all possible combinations were not represented (Fig. S1, see online supplementary material). The mesocosms were placed in a greenhouse and supplied with a continuous flow of water (~20 L h^{-1}) coming directly from an adjacent bay for 2 weeks. This time span was chosen as it constitutes enough time for the sediment cores to interact with the overlying water and recover from sampling, but minimizes changes in the microbial community as an effect of incubation (Alsterberg *et al.* 2017). When

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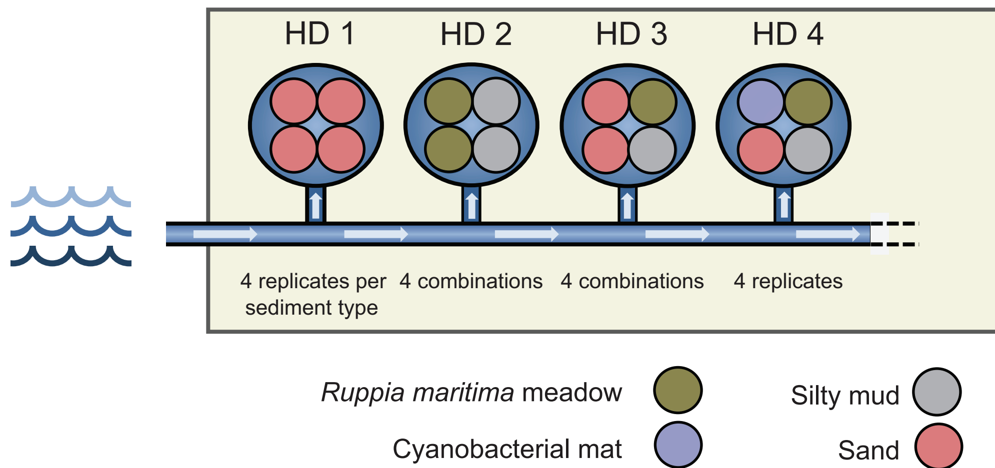

Figure 1. Experimental set-up (adapted from Alsterberg *et al.* 2017). Colors indicate sediment cores from four naturally occurring habitats on the west coast of Sweden: *Ruppia maritima* meadow, cyanobacterial mat, sandy and silty mud sediment. Mesocosms with different levels of habitat diversity levels (HD), ranging from HD 1 (presence of only one habitat type) to HD 4 (all four habitat types were present) are indicated, as well as the supply of sea water from an adjacent bay. Mesocosms were placed in a greenhouse, as indicated by the grey box. All habitat diversity levels were replicated four times, but all possible combinations were not represented for levels 2 and 3 (see Fig. S1).

terminating the experiment, the top layer of 0.5 cm of the cores were sliced off. After sampling for variables related to primary producers from the undisturbed top 5 mm, the slice was homogenized. Top slices of the same habitat type within each mesocosm were pooled to a single sample resulting in 52 samples in total, of which 13 were not considered in this study due to sample loss (Fig. S1).

Denitrification activity

The potential denitrification activity of each sediment sample was assessed by stable isotope amendments of $^{15}NO_3^-$ and subsequent measurements of $^{29/30}N_2$ concentrations above their natural abundances. The method and results were described previously by Alsterberg *et al.* (2017). Briefly, 2 mL of homogenized sediment was pre-incubated for 24 h in glass Exetainers (Labco Limited) flushed with helium and finally incubated with $^{15}NO_3^-$ ($Na^{15}NO_3$, Europa Science Ltd.) at a final concentration of 50 μM in the sediment pore water for 2.5 h in the dark. The samples were continuously shaken and the reaction was stopped by addition of 6.1 M $ZnCl_2$. The samples were analysed for $^{29/30}N_2$ concentrations at the University of California and potential denitrification activity was calculated as described by Thamdrup and Dalsgaard (2002).

DNA extraction and quantification of 16S rRNA, *nirK*, *nirS* and clade I and II *nosZ* genes

At the end of the experiment, ~5 g of homogenized sediment was taken from each sample and DNA was extracted from ~0.3 g of sediment using a FastDNA Spin Kit (MP Biomedical). The concentration of DNA was quantified using a Qubit Fluorometer (Life Technologies Corporation) and quality was verified by gel electrophoresis. Abundances of total bacteria were determined by quantifying the genes for 16S rRNA by quantitative real-time PCR (qPCR) and using the primers described by López-Gutiérrez *et al.* (2004). For denitrifying and N_2O reducing communities, nitrite reductase genes *nirS* and *nirK*, and the nitrous oxide reductase clade I and II *nosZ* genes were quantified using the primers described by Throbäck *et al.* (2004) and Jones et *al.* (2013). Each reaction contained 10 ng of template DNA, iQ SYBR Green Supermix (BioRad), 0.1% bovine serum albumin (BSA) and primer concentrations between 0.25 and 0.8 μM in a total reaction volume of 15 μl. Independent, duplicate runs were performed for each gene. Efficiencies, primer sequences and thermal cycling conditions are listed in Supplementary Table S1, see online supplementary material. For the standard curve, we used a linearized plasmid containing fragments of the respective genes. To exclude the possibility that the quantifications were affected by PCR inhibition, a plasmid specific qPCR assay was performed containing the pGEM-T plasmid (Promega) as template and 10 ng of extracted sample DNA. There was no inhibition of the PCR reaction in any of the samples.

Community analysis of N_2O reducers

The structure, composition and diversity of the N_2O reducing communities were determined by 454 pyrosequencing of both clades of the *nosZ* gene. A two-step PCR was performed (Berry *et al.* 2011) and for the first step duplicate PCR reactions were done for each sample consisting of 20 ng of DNA, DreamTaq Green PCR Mastermix (Fermentas), 0.1% BSA, 3 mM $MgCl_2$ and 0.8 μM *nosZ* clade I or clade II specific primers (Table S1). From the pooled product, 2.5 μl was added as template for the second PCR step and gene-specific primers were used that included the barcode, sequencing key and adapter sequences. The second PCR was performed in triplicate, and the final products were pooled and purified using Agencourt AMPure beads (Beckman Coulter Life Sciences). Sequencing was performed on a 454 FLX Genome Sequencer (Roche) using Titanium FLX+ chemistry by Microsynth (Microsynth AG, Switzerland). The nucleotide sequences obtained in this study were submitted to the NCBI short read archive and are available under Bioproject accession number PRJNA398484.

Raw sequences of *nosZ* clade I and II were demultiplexed, screened and filtered in QIIME (Caporaso *et al.* 2010) with the default parameters. All subsequent sequence processing steps and analyses were done with the help of an updated version of the *nosZ* reference alignment in Jones *et al.* (2014) consisting of 1139 full-length *nosZ* sequences obtained from genomes and assembled metagenomes. Removal of contaminating sequences

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and frameshift correction was performed with HMM-FRAME (Zhang and Sun 2011) and the HMM profiles were built separately for each clade from the reference alignment. The sequences were screened for chimeras (*de novo* and reference-based with *nosZ* sequences retrieved from FunGene; Fish *et al.* 2013) and clustered into OTUs at a nucleotide similarity threshold of 97% with QIIME ('pick_otus.py' using the 'usearch' option). This resulted in 250509 high-quality sequences clustered into 1669 OTUs for *nosZ* clade I and 193514 sequences clustered into 1984 OTUs for *nosZ* clade II.

To determine the structure and diversity of the N_2O reducing communities, representative sequences of all 3653 OTUs were aligned to the reference alignment using HMMER (Eddy 1998), and the final alignment was inspected and edited manually in ARB (Ludwig *et al.* 2004). The phylogeny of *nosZ* OTUs was based on the final amino acid alignment and calculated using FastTree2 (Price, Dehal and Arkin 2010) with the WAG+CAT substitution model (Whelan and Goldman 2001).

The community structure of the N_2O reducing communities was assessed by non-metric multidimensional scaling of generalized UniFrac distances calculated from the OTU-based phylogeny for both *nosZ* clades I and II using the 'vegan' and 'GUniFrac' packages in the R environment (Chen *et al.* 2012; Oksanen *et al.* 2019). Phylogenetic placement of the full set of 444023 high-quality *nosZ* sequences from both clades was performed by aligning all sequences to the reference *nosZ* amino acid alignment with HMMER, then mapping reads into the reference phylogeny (Jones *et al.* 2014) using pplacer (Matsen, Kodner and Armbrust 2010). The placement of reads was summarized using the *-fat* command within the 'guppy' suite and Archeaopteryx was used for tree visualization (Han and Zmasek 2009).

Alpha diversity was calculated as effective number of species of order $q = 1$ (Jost 2006), taking OTUs as 'species' and the relative sequence abundance as weights. We used the bias correction proposed by Chao *et al.* (2015) to account for uneven sampling depth without rarefaction. The effective number of species of order $q = 1$ is equivalent to the exponential of the Shannon index and can be interpreted as the number of species in an equally diverse community with evenness = 1. For the gamma diversity of each mesocosm we used the multiplicative diversity framework for true diversities (Jost 2007). Thus, data from each of the samples in a mesocosm were pooled by calculating the average abundances for each OTU. In the mesocosm from habitat diversity level 3, the average was weighed by the proportion of each sample (habitat type) among the four samples in each mesocosm. Estimated gamma diversity was calculated based on all possible combinations of the replicated sediment communities from habitat diversity level 1. As two sediments had one missing replicate, only three replicates from each sediment were used for the calculations of estimated gamma diversity.

Statistical analysis

Differences in denitrification activity, gene abundance and alpha diversity of N_2O reducing communities between habitat types were analysed using one-way analysis of variance (ANOVA) and least significant difference *post hoc* test (LSD), including Tukey's adjustment for multiple comparisons (Tukey's HSD) using the 'stats' package in R (R Core Team 2018). When assumptions about heteroscedasticity and normality were violated, the data was transformed either by log, square-root or rank transformation. The effect of habitat diversity on the gamma diversity was assessed by a linear model fitted to the observed gamma diversity values only. Correlations between alpha diversity and the initial DIN in the pore water were calculated by a bootstrap Pearson's correlation method with 10000 permutations. The effect of habitat type on the community structure of *nosZ* clade I and II communities was tested by permutational multivariate analysis of variance (PERMANOVA) using generalized UniFrac distances, and pairwise comparisons were performed with the multi-response permutation procedure (MRPP), a non-parametric method that tests the hypothesis of no difference between groups. The resulting *P*-values were adjusted for multiple comparisons using false discovery rate (FDR) procedures. The variation in community composition amongst replicates within each habitat type was analysed, based on generalized UniFrac distances. Pairwise comparisons of group dispersion between the different habitat types were done using Tukey's HSD. All multivariate analyses were performed with the 'vegan' package within the R environment (Oksanen *et al.* 2013).

RESULTS AND DISCUSSION

Effects of habitat diversity

As hypothesized, gamma diversity, defined as the diversity at the ecosystem level (i.e. mesocosm level), increased significantly with increasing habitat diversity in the mesocosms, although only for the *nosZ* clade II communities (Fig. 2A; *nosZ* clade I $R^2 = 0.06$, $P = 0.14$; *nosZ* clade II $R^2 = 0.54$, $P < 0.001$). Since this analysis was based on rather few data points in the higher habitat diversity levels, we also calculated the estimated gamma diversity, based on *in silico* combinations of the microbial communities from different replicate mesocosms with habitat diversity level 1. We did this to ascertain theoretical upper and lower bounds on the gamma diversity at these levels. The estimated gamma diversity was similar to the observed data, which always lay within the estimated values (Fig. 2A). The decrease in gamma diversity of *nosZ* clade II with decreasing habitat diversity underscores the negative impact of habitat homogenization on the diversity of N_2O reducers. The increase in gamma diversity of clade II but not clade I *nosZ* communities with increasing habitat diversity may reflect differences in trait diversity between the two N_2O reducing communities. Clade I communities are more likely to be taxonomically and functionally constrained than clade II. Clade II *nosZ* is present among a diverse set of taxa (Hallin *et al.* 2018) and trait diversity among clade II microorganisms in benthic environments is therefore likely to be higher if they are represented by more distantly related taxa. Indeed, phylogenetic placement of *nosZ* reads obtained from the sediments showed that *nosZ* clade II sequences were related to a more diverse range of taxa compared to clade I (Figs S2 and S3, see online supplementary material). Further, in clade I, *nosZ* is mainly involved in the denitrification pathway, whereas in clade II, *nosZ* can be involved in the denitrification pathway as well as in N_2O reduction in non-denitrifying N_2O reducers, or be linked to respiratory ammonification (Sanford *et al.* 2012; Jones *et al.* 2013). Thereby clade I organisms occupy a smaller trait space compared to clade II with respect to N_2O reduction. Altogether, if these differences between the clades reflect differences in trait space, increases or decreases in landscape diversity may have a less pronounced effect on the ecosystem-level diversity of clade I *nosZ*.

Although habitat diversity has been shown to affect both individual functions and multifunctionality in the sediments used in this study (Alsterberg *et al.* 2017), there was no effect of habitat diversity on the denitrification rates (Fig. 2B). This

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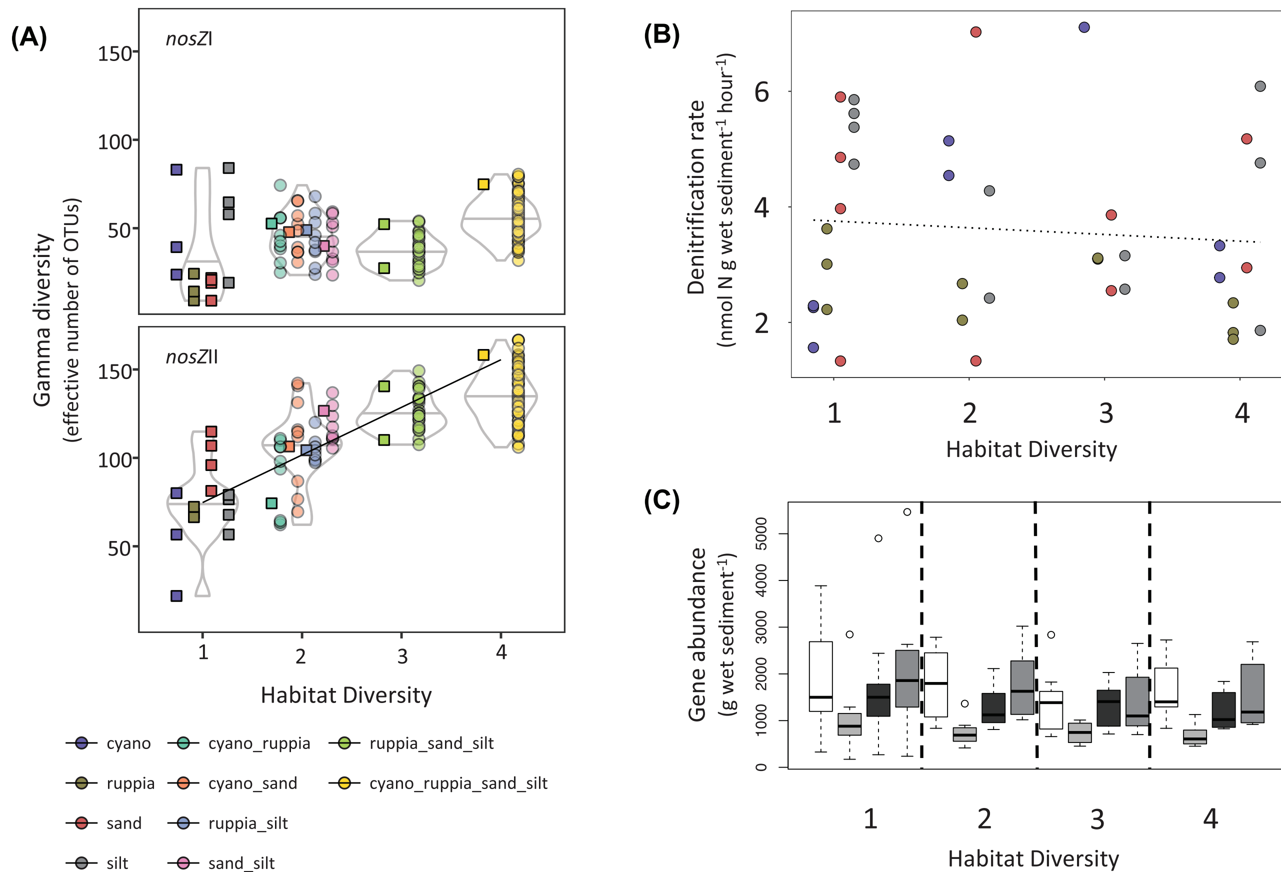

Figure 2. Effects of habitat diversity on the gamma diversity of N_2O reducing communities, denitrification activity, and abundances of nitrite and nitrous oxide reductase genes in illuminated shallow-water sediments. (**A**) Gamma diversity of *nosZ* clade I (upper panel) and clade II (lower panel) in ecosystems (mesocosm-scale) with different levels of habitat diversity, calculated as effective number of species. The observed (squares) and estimated (circles) gamma diversity for the combinations of 1 to 4 habitats are indicated. Linear models were applied to the observed values only (*nosZ* clade I: $R^2 = 0.06$, $P = 0.14$, *nosZ* clade II: $R^2 = 0.54$, $P < 0.001$) and only the significant relationship is shown. The habitat combination is indicated by the color key below the graph. (**B**) Denitrification activity (nmol N g wet sediment^{-1} h^{-1}, data from Alsterberg *et al.* (2017)). The dotted line shows the general trend of activity across habitat diversity level, however it is not significant ($P = 0.5$). (**C**) Gene abundances of *nirS* (white), *nirK* (lighter grey), *nosZ* clade I (darker grey) and clade II (dark grey). Gene abundances are presented as square root transformed gene copy numbers g wet sediment^{-1}. Whiskers indicate 1.5 × interquartile range and circles denote outliers. There was no significant relationship between gene abundances and habitat diversity ($P > 0.05$).

was also reflected in the genetic potential for denitrification and N_2O reduction, as shown by the lack of response in the abundances of *nir* and *nosZ* genes (Fig. 2C). The saturation in gamma diversity observed already at diversity level 2 for clade I N_2O reducers, mainly representing denitrifiers according to genome information (Graf, Jones and Hallin 2014), corroborate the results showing minimal influence of habitat diversity on denitrification activity in these sediments. This is not conclusive because not all denitrifiers have the capacity to reduce N_2O, and we may therefore have underestimated the effect of habitat diversity on dentrifier diversity since we did not specifically look into the gamma diversity of *nir* gene communities. Nevertheless, ecosystem-level diversity of N_2O reducers and denitrifiers could be important to increase the chance of maintaining this function under fluctuating or changing conditions (Yachi and Loreau 1999). A previous study showed that higher diversity in denitrifier communities results in a broader operating range for denitrification activity in soils under short-term temperature and salinity gradients (Hallin *et al.* 2012). Consequently, more diverse denitrifier communities in coastal shallow-water systems may respond efficiently to changes in environmental conditions. Further work is needed to determine temporal differences and effects of habitat diversity under fluctuating conditions.

Effects of habitat type

The differences in gamma diversity for both *nosZ* clades I and II between habitats at habitat diversity level 1 and the lack of effects of habitat diversity on denitrification rates and genetic potential (Fig. 2) suggest that certain habitat types are more influential than others. In fact, communities of both clades I and II *nosZ* were significantly different between the four habitat types (Fig. 3; PERMANOVA test statistics for *nosZ*I: $R^2 = 0.464$ and $P = 0.001$, *nosZ*II: $R^2 = 0.446$ and $P = 0.001$). Pairwise comparisons of community structure amongst habitat types showed significant differences between all habitat types ($P = 0.001$ for all comparisons). The largest differences were observed between the *nosZ* communities in cyanobacterial mat and sandy sediments (corrected within-group agreement $A = 0.22$ and $A = 0.21$ for clade I and II, respectively). *Ruppia* and sandy sediment *nosZ* communities were more similar in composition (*nosZ*I $A = 0.12$, *nosZ*II $A = 0.11$), yet still significantly different. This confirms the importance of habitat type for maintaining different N_2O reduc-

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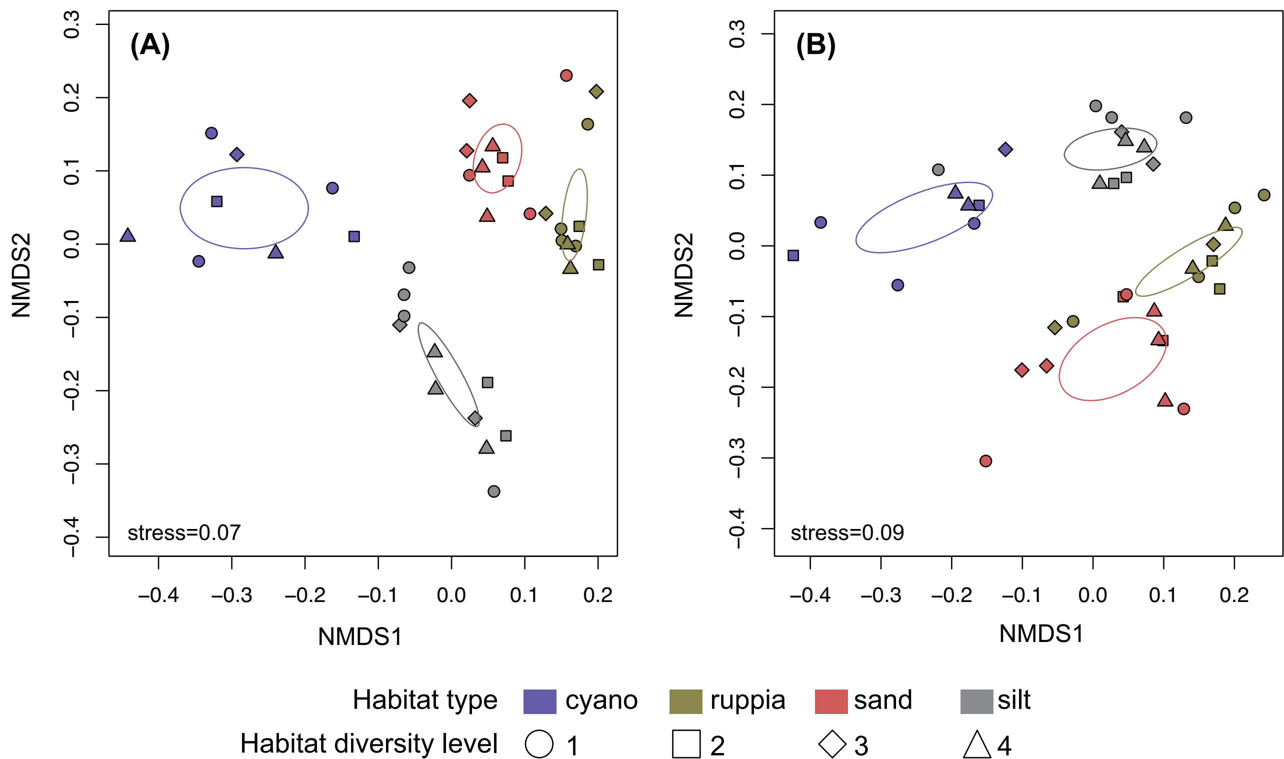

Figure 3. Community structure of N_2O reducing communities in illuminated shallow-water sediments from four habitat types: cyanobacterial mat, *Ruppia maritima* meadow, sandy and silty mud sediment. Non-metric multidimensional scaling of generalized phylogenetic UniFrac distances of (**A**) *nosZ* clade I and (**B**) *nosZ* clade II communities in cores from different habitat types incubated under habitat diversity levels 1, 2, 3 and 4 as indicated by the shape. The ellipses show the standard error within a confidence limit of 0.95 around the centroid.

ing communities, of which many, especially within clade I, are denitrifiers (Graf, Jones and Hallin 2014). Betadispersal, which measures the degree of compositional variation in communities from a single habitat, was significantly affected by habitat type in clade I communities (Fig. S4a, see online supplementary material; ANOVA $F = 4.34$, $P(F) = 0.01$). Highest variation was observed in cyanobacterial mat sediments, in accordance with data showing that this habitat type had the highest degree of heterogeneity in terms of its sediment characteristics (Alsterberg *et al.* 2017). By contrast, no significant effect of habitat type was detected for betadispersal of *nosZ* clade II (Fig. S4b; $F = 1.06$, $P(F) = 0.38$), emphasizing that the differences observed for these two clades are likely connected to their trait space.

The genetic potential for N removal through denitrification and N_2O reduction, defined as the abundance of *nir* and *nosZ* genes, was also affected by sediment type (Fig. 4). We detected significantly higher abundances of all nitrite (*nirS* and *nirK*) and nitrous oxide reductase genes (*nosZ* clades I and II) in sandy and silty mud sediments compared to cyanobacterial mat and *Ruppia* sediments. These differences were unrelated to differences in the abundance of the total bacterial community, even though the abundance of bacterial 16S rRNA genes also differed significantly among the four habitat types ($F_{(3,35)} = 16.04$, $P(F) < 0.001$), with higher abundances in silty mud and cyanobacterial mat sediments (Table S3, see online supplementary material). Overall, a significantly higher abundance of *nirS*-type nitrite reductase genes was observed compared to the abundances of the *nirK*-type, with a *nirK* to *nirS* ratio of 0.27 (standard deviation (SD) $\pm$ 0.16) across all habitat diversity levels and habitat types (Fig. S5a, see online supplementary material). Similarly, *nirS* type denitrifiers typically dominate over *nirK* types in marine environments (Abell *et al.* 2010; Jones and Hallin 2010; Mosier and Francis 2010; Marchant *et al.* 2018). Regarding the potential for N_2O reduction, clade II *nosZ* genes were more abundant than those of clade I, with a *nosZ* clade I to clade II ratio of 0.77 (SD $\pm$ 0.41; Fig. S5b). This is in contrast to observations in deeper sediments on the Swedish west coast, which were clearly dominated by *nosZ* clade I (Wittorf *et al.* 2016). This further emphasizes niche differentiation between *nosZ* clade I and II N_2O reducers (Hallin *et al.* 2018) and highlights that non-denitrifying N_2O reducers found within *nosZ* clade II could play an important role in illuminated shallow-water sediments.

Similar to the genetic potential, estimates of alpha diversity for clade I and clade II *nosZ* communities differed significantly between the habitat types (Fig. 5A). The two clades of *nosZ* showed contrasting patterns, as the alpha diversity of *nosZ* clade I was highest in silty mud and cyanobacterial mat sediments and lowest in sandy and *Ruppia* sediments. By contrast, *nosZ* clade II showed the highest level of diversity in sandy sediments, intermediate levels in silty mud and *Ruppia* sediments and lowest levels in cyanobacterial mat sediments. Qualitatively, similar trends were observed for the number of OTUs (Table S2, see online supplementary material). The contrasting patterns of diversity for each *nosZ* clade indicate niche differentiation between the two clades, in accordance with previous studies in coastal sediments (Graves *et al.* 2016; Wittorf *et al.* 2016). The availability of N could be an important factor determining the niches of the two clades, as suggested by the fact that DIN measured in the pore water (data from Alsterberg *et al.* 2017) was positively correlated to the effective number of OTUs of *nosZ* clade I across the habitats (mean Pearson's $r = 0.728$, $P = 0.047$, 95% quantile is 0.009 and 0.995), whereas no relationship was

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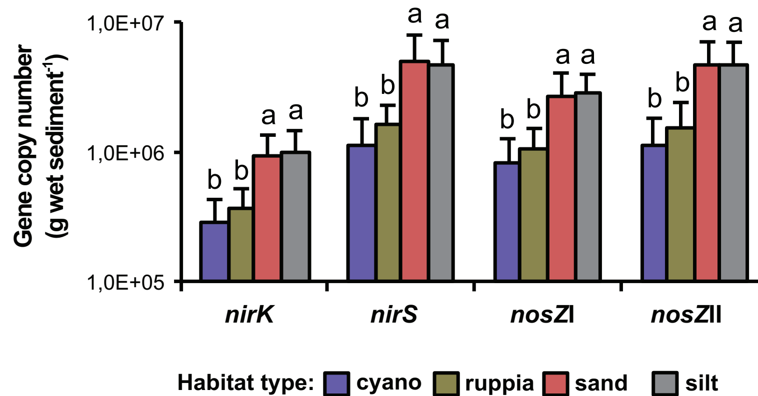

Figure 4. Abundances of *nirK*, *nirS and nosZ* clade I and II genes in the four habitats: cyanobacterial mat, *Ruppia maritima* meadow, sandy and silty mud sediment. Significant differences (ANOVA followed by Tukey's HSD) in gene abundances between habitat types are indicated by different letters (*nirK*: $F_{(3,35)} = 11.2$, *nirS*: $F_{(3,35)} = 9.1$, *nosZ* clade I: $F_{(3,35)} = 14.9$, *nosZ* clade II: $F_{(3,35)} = 8.9$; $P < 0.001$ for all comparisons). Error bars indicate standard deviation.

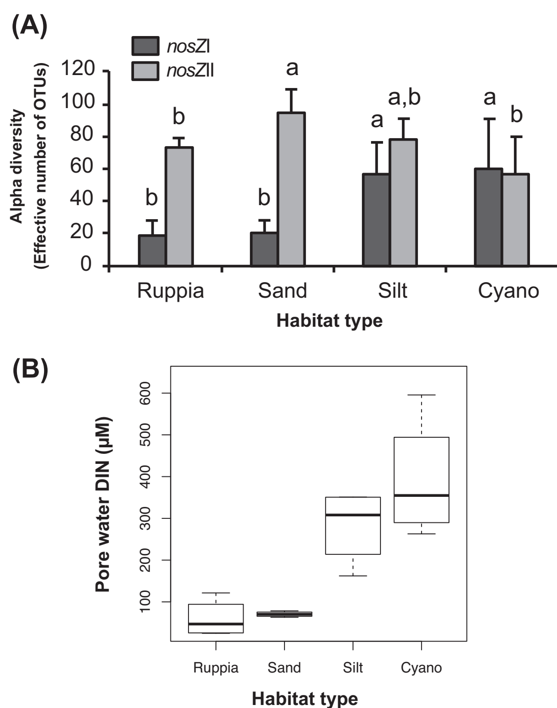

Figure 5. Diversity of N_2O reducing communities compared to dissolved inorganic nitrogen (DIN) content in illuminated shallow-water sediments from four habitats. (**A**) Alpha diversity of N_2O reducers by habitat type. Bars show the mean effective number of OTUs across cores of each habitat type for *nosZ* clade I and clade II communities, and error bars indicate standard deviation. Letters indicate significant differences (ANOVA followed by Tukey's HSD) between the habitat types for *nosZ* clade I and clade II (*nosZ* clade I: $F_{(3,35)} = 16.4$, *nosZ* clade II: $F_{(3,35)} = 8.5$, $P < 0.001$ for both comparisons). (**B**) DIN in the pore water of the sediment prior to incubation in mesocosms (data from Alsterberg *et al.* 2017). Differences in pore water DIN were significant between habitat types (ANOVA: $F_{(3,12)} = 14.1$, $P < 0.001$).

observed for *nosZ* clade II (Fig. 5). As organisms with clade I *nosZ* are more likely to be complete denitrifiers (Graf, Jones and Hallin 2014), this result suggests that higher concentrations of DIN in cyanobacterial mats and silty mud sediments select for more diverse communities of complete denitrifiers. The diversity of clade II communities was also higher in silty mud sediments compared to cyanobacterial mats and *Ruppia* sediments, however the highest values were observed in sandy sediments.

This pattern corresponded with the abundances of denitrification genes measured across the different habitat types, and suggests that silty mud and sandy sediments could be particularly important for N removal and mitigation of N_2O emissions in illuminated shallow-water sediments like those in the Skagerrak and Kattegat coastal areas. This is further supported by the observed trend of higher denitrification activities in silty mud and sandy sediments, although these differences were not statistically significant (Fig. S6, see online supplementary material). Other areas with sandy sediments, especially permeable and eutrophied sediments, also show high N removal rates by denitrification (e.g. Rao *et al.* 2007; Gao *et al.* 2012). However, studies from sandy sediments in the Wadden Sea show that 1–2% of the nitrate ends up as N_2O instead of N_2 in the overlying water due to incomplete denitrification (Marchant *et al.* 2018). Whether this is the case in the present study is not known and further investigations on the end-product ratios of denitrification in illuminated coastal sediments are warranted.

Genetic potential for denitrification and N_2O reduction in illuminated shallow-water sediments

Phylogenetic placement of *nosZ* reads into a reference phylogeny showed that the majority of *nosZ* clade I sequences were closely related to Alpha- and Gamma-proteobacteria (Fig. S4). Within the Alphaproteobacteria, most reads mapped with *nosZ* from Rhodobacterales, such as Sedimentitalea and a lineage of Rhodobacteraceae. Alphaproteobacteria have been previously described to be abundant in communities from marine sediments (e.g. Hunter, Mills and Kostka 2006; Wittorf *et al.* 2016). Rhodobacterales in particular have been shown to mainly harbor genes for a complete denitrification pathway (Graf, Jones and Hallin 2014). Many of the *nosZ* clade II sequences mapped with Bacteroidetes, especially with sequences from *Rhodothermus*, and with the gammaproteobacterial endosymbiont of *Olavius algarvensis* (Fig. S5). Bacteroidetes with *nosZ* clade II often lack genes encoding dissimilatory nitrite reductases and could therefore act as an N_2O sink and positively affect the N_2O reduction capacity of microbial communities (Domeignoz-Horta *et al.* 2016). Thus, the community composition suggests that these illuminated shallow-water sediments have the capacity to be efficient in removing N from the system without producing high levels of N_2O emitted to the atmosphere.

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The results on gene abundances further corroborate the hypothesis that these sediments have a high genetic potential for N_2O reduction compared to its production. The sediments from all habitats had a *nosZ* to *nir* ratio of 1.5 (SD ± 0.54; Fig. S5c), which agrees with reported ratios between N_2O:N_2 fluxes within the range 0.1–6% from estuarine environments and sandy sediments (Seitzinger and Kroeze 1998; Dong and Nedwell 2006; Murray, Erler and Eyre 2015; Marchant *et al.* 2018). The relative proportion of organisms capable of N_2O reduction in relation to those with the capacity to produce N_2O could have been overestimated due to lower coverage of the primers for nitrite reductase genes *nirS* and *nirK* (Bonilla-Rosso *et al.* 2016). However, in soil systems the *nosZ* to *nir* gene abundance ratios obtained with these primers usually indicate the opposite to what we found in these sediments (e.g. Juhanson *et al.* 2017; Krause *et al.* 2017; Domeignoz-Horta *et al.* 2017). The higher levels of *nirS* compared to *nirK* and clade II *nosZ* compared to clade I in these sediments are also indicative of low genetic potential for N_2O emissions, as organisms carrying *nirS* are likely capable of denitrification terminating with N_2, while organisms with clade II *nosZ* typically lack genes involved in N_2O formation (Graf, Jones and Hallin 2014).

Overall, abundance and sequencing data point towards a community with the genetic potential to perform complete denitrification, either by organisms carrying the genes for a complete dentification pathway or by coupled N_2O producing and reducing reactions performed by different organisms within the community. Coupled reactions have previously been reported in permeable sand sediments and salt marshes (Graves *et al.* 2016; Marchant *et al.* 2018), and tightly linked reactions in time and space within a network of N_2O producing and reducing organisms is needed to control N_2O emissions. Even though our results indicate a high potential for complete conversion of nitrate to N_2, there were differences in capacity among sediment types. The loss of certain habitats could therefore negatively impact efficient removal of N as well as N_2O emissions from coastal sediment environments. Further understanding of differences in denitrification and N_2O production and reduction among habitat types of coastal sediments is needed since this is relevant to consider when mapping habitats to obtain bay-wide or global estimates of N removal capacity and N_2O emissions.

SUPPLEMENTARY DATA

Supplementary data are available at FEMSEC online.

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