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Chironomus plumosus larvae increase fluxes of denitrification products and diversity of nitrate-reducing bacteria in freshwater sediment

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ABSTRACT

Benthic invertebrates affect microbial processes and communities in freshwater sediment by enhancing sediment-water solute fluxes and by grazing on bacteria. Using microcosms, the effects of larvae of the widespread midge *Chironomus plumosus* on the efflux of denitrification products (N_2O and $\text{N}_2 + \text{N}_2\text{O}$) and the diversity and abundance of nitrate- and nitrous-oxide-reducing bacteria were investigated. Additionally, the diversity of actively nitrate- and nitrous-oxide-reducing bacteria was analyzed in the larval gut. The presence of larvae increased the total effluxes of N_2O and $\text{N}_2 + \text{N}_2\text{O}$ up to 8.6- and 4.2-fold, respectively, which was mostly due to stimulation of sedimentary denitrification; incomplete denitrification in the guts accounted for up to 20% of the N_2O efflux. Phylotype richness of the nitrate reductase gene *narG* was significantly higher in sediment with than without larvae. In the gut, 47 *narG* phylotypes were found expressed, which may contribute to higher phylotype richness in colonized sediment. In contrast, phylotype richness of the nitrous oxide reductase gene *nosZ* was unaffected by the presence of larvae and very few *nosZ* phylotypes were expressed in the gut. Gene abundance of neither *narG*, nor *nosZ* was different in sediments with and without larvae. Hence, *C. plumosus* increases activity and diversity, but not overall abundance of nitrate-reducing bacteria, probably by providing additional ecological niches in its burrow and gut.

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Introduction

Benthic invertebrates influence the reaction rates of microbial and geochemical processes and the abundance and distribution of microbes in sediments [1,26,32]. Particle reworking through bioturbation activity and ventilation of tubular burrows by the invertebrates strongly enhance the solute exchange between sediment and water compared to molecular diffusion across the bare sediment-water interface [1,26,39]. Consequently, transport of substrates and products of microbial and geochemical processes is more efficient, which increases reaction rates in the sediment. Additionally, benthic invertebrates excrete spinning silk, mucus, feces, and ammonium and thereby enrich the burrows with organic matter and nutrients [15,28,48], which makes them preferred colonization sites for bacteria [29,33].

Benthic invertebrates also directly influence bacterial abundance in sediments by their grazing activity. Deposit-feeding chironomid larvae significantly decrease bacterial abundance at the

sediment surface [2,20] and change the bacterial community structure by selective grazing [58]. Not all of the ingested bacteria are digested in the invertebrate gut [20,36], but the effect of differential survival on microbial community structure in the sediment is not generally known; it might be minor because recolonization of feces occurs through immigration of bacteria from ambient sediment rather than through regrowth of survivors [37].

An inverse effect of grazing on microbial communities in sediments occurs when ingested bacteria do not only survive the gut passage, but also remain or even become metabolically active in the gut. Certain metabolic pathways may be induced by the shift in microenvironmental conditions from water or sediment surface to invertebrate gut. In the gut of *Chironomus plumosus* larvae (Insecta, Diptera), for instance, low O_2 and high NO_3^- concentrations prevail [47,50]. As a consequence, denitrification activity is induced in some of the ingested bacteria that are normally exposed to O_2 in the water column or at the sediment surface [49]. Denitrification is the dissimilatory reduction of nitrate (NO_3^-) either completely to dinitrogen (N_2) or to nitrous oxide (N_2O) only, when the last reduction step is absent, repressed or running at a lower rate than the preceding reduction steps [59]. Strikingly, the N_2O yield of gut denitrification is much higher than that of sedimentary denitrification [49], probably because of delayed induction of the nitrous

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oxide reductase gene after the oxic-anoxic shift [4,12,19]. The feces of *C. plumosus* larvae might therefore be rich in surviving denitrifiers with all reductase genes expressed, except for maybe the nitrous oxide reductase gene. In addition to denitrifiers, bacteria capable of dissimilatory nitrate reduction to ammonium (DNRA) may find suitable conditions in the invertebrate gut and their genes may also be expressed during the gut passage. The same accounts for bacteria that reduce NO_3^- to nitrite (NO_2^-) and not further (e.g., many fermenters, enteric bacteria, or *Bacillus* species). Since the feces excreted by *C. plumosus* may serve as microbial inocula to the ambient sediment, the larval grazing activity may eventually promote bacteria mediating dissimilatory nitrate reduction pathways and increase their abundance in the sediment.

The present study focused on the direct and indirect effects of *C. plumosus* larvae on nitrate reduction, incomplete denitrification to N_2O , and complete denitrification to N_2 in freshwater sediment. *C. plumosus* larvae are common and abundant in lake sediments where they construct U-shaped burrows which they periodically ventilate [40]. The diet of the larvae consists of organic particles and the attached bacteria and microalgae that are acquired by deposit- and filter-feeding [57]. Two hypotheses were tested: (1) *C. plumosus* larvae increase the sediment-water flux of denitrification products (N_2O and $\text{N}_2 + \text{N}_2\text{O}$), and (2) *C. plumosus* larvae increase abundance and diversity of nitrate- and nitrous-oxide-reducing bacteria in the sediment. Our objectives were to (1) quantify the direct larval emissions and the indirect larval stimulation of sedimentary emissions at different larval densities, and (2) to quantify abundance and diversity of the genes encoding the nitrate reductase (*narG*) and the nitrous oxide reductase (*nosZ*) in sediments with and without larvae. To test for direct effects of *C. plumosus* larvae on nitrate- and nitrous-oxide-reducing bacteria, the diversity of *narG* and *nosZ* transcripts was analyzed in the larval gut content. The differential appearance of individual *narG* and *nosZ* phylotypes in three gene libraries (i.e., sediment with and without larvae, gut content) was used to identify the mechanisms behind the *C. plumosus* effects on nitrate- and nitrous-oxide-reducing bacteria.

Materials and methods

Sedimentary and larval emission of denitrification products

Sediment was collected in Lake Großer Binnensee (Northern Germany, 54°19'40"N, 10°37'30"E). After defaunation by sieving, sediment was filled into 24 120-mL glass bottles that were left open and submersed in four 10-L tanks containing NO_3^- -enriched tap water ($500 \mu\text{mol L}^{-1}$). Each bottle was flushed with air through a hypodermic needle positioned in the bottleneck. The air bubbles escaping from the bottles also provided for a continuous exchange of water between the tank and the bottles. After a two-week equilibration period, the sediments were spiked with 0, 2, 4, or 6 fourth-instar *C. plumosus* larvae from Lake Großer Binnensee per bottle (six replicates each), which corresponded to 0, 1150, 2300, or 3450 individuals per m^2 , respectively. After an incubation period of one week, the bottles were retrieved from the tanks and the total efflux of denitrification products (N_2O and $\text{N}_2 + \text{N}_2\text{O}$) was determined in each bottle according to [51]. Briefly, the bottles were sealed with a rubber septum leaving a 5-mL air-filled headspace in the bottleneck. The accumulation of N_2O in the headspace was monitored for 3–5 h by gas chromatography and used to calculate the total N_2O efflux (i.e., the total N_2O flux out of the sediment into the overlying water) as a measure of incomplete denitrification. Additional incubations without NO_3^- added to the water column revealed that the N_2O efflux due to nitrification, the sequential oxidation of NH_4^+ via NO_2^- to NO_3^- , was negligible (data not shown). The bottles were incubated a second time with the overlying water

adjusted to 10% saturation of acetylene to inhibit the last step of denitrification [44]. In this case, the accumulation of N_2O in the headspace was used to calculate the total $\text{N}_2 + \text{N}_2\text{O}$ efflux as a measure of complete denitrification.

The direct emission of N_2O and $\text{N}_2 + \text{N}_2\text{O}$ by *C. plumosus* larvae was quantified according to [49]. Briefly, larvae were collected from the sediment and individually incubated in air-filled, gas-tight vials (3 mL). The accumulation of N_2O in the vials was monitored for 4–5 h by gas chromatography and used to calculate the individual N_2O emission rate of the larvae. A second batch of larvae was incubated in an atmosphere of 10% acetylene and 90% air. In this case, the accumulation of N_2O in the vials was used to calculate the individual $\text{N}_2 + \text{N}_2\text{O}$ emission rate of the larvae.

Sampling for analysis of nitrate- and nitrous-oxide-reducing bacteria

Defaunated lake sediment was homogenized and filled into six 300-mL glass beakers that were submersed into two 10-L tanks containing NO_3^- -enriched tap water ($500 \mu\text{mol L}^{-1}$). Mixing of water within the tanks was achieved by aeration using an air pump and a diffusor. After a two-week equilibration period, three beakers were each spiked with six fourth-instar *C. plumosus* larvae (which corresponded to 1333 individuals per m^2), while the other three beakers served as controls. After an incubation period of two weeks, the beakers were retrieved from the tanks and in each beaker the upper 8 mm of the sediment, which covered both the oxic sediment layer and the anoxic nitrate-reducing layer, was sampled with a sterile core liner. The remaining sediment was searched through to retrieve the living larvae. Both sediment samples and larvae were immediately frozen at -80°C .

DNA and RNA extraction

DNA was extracted in triplicates from the sediment samples by combined enzymatic and mechanical lysis as described previously [13]. DNA concentrations were determined using Picogreen (Invitrogen, USA) on a Nanodrop fluorometer (Thermo-Scientific, USA) [25]. RNA was extracted from the gut contents of dissected *C. plumosus* using the FastRNA[®] Pro Soil-Direct Kit (Qbiogene Inc., California, USA). RNA extracts tested negatively for residual DNA by 16S rRNA gene-targeted PCR [19]. cDNA was generated from RNA extracts with random hexamer primers using the iScript cDNA Synthesis Kit (Biorad, California, USA).

Diversity of *narG* and *nosZ*

Gene fragments of *narG* (690 bp) and *nosZ* (700 bp) were amplified, cloned, sequenced, and phylogenetically analyzed as previously described [49]. To broaden primer coverage for *nosZ*, a second fragment (1100 bp) was amplified following the protocol of [41], but using four annealing temperatures in parallel (51.2, 53, 55.2, and 57°C). The resulting PCR products were pooled, cloned, sequenced, and added to the phylogenetic analysis described above. Nine clone libraries were analyzed at nucleic acid level: 3 samples (S_0 : sediment without larvae, S_1 : sediment with larvae, G: gut contents) $\times$ 3 gene fragments ($1 \times \text{narG}$, $2 \times \text{nosZ}$). Sequences were assigned to phylotypes in DOTUR [42] using 97, 90, and 80% sequence identity cut-off values. Phylotype richness of each clone library was estimated using the software package compiled by [23]. One sequence of each phylotype was deposited in GenBank (accession no. EU052803 to EU053075).

The appearance of individual *narG* and *nosZ* phylotypes in the libraries for the three samples was listed in a presence/absence matrix. Individual phylotypes could appear either in one, two, or three libraries. Thus, seven different categories of phylotype

distribution between the samples were possible: S_0 , S_1 , G , $S_0 + S_1$, $S_0 + G$, $S_1 + G$, and $S_0 + S_1 + G$. The relative frequency of each category was determined for each gene analyzed. To this end, all *nosZ* phylotypes were combined because the two primer pairs retrieved non-overlapping sets of phylotypes. The evaluation was repeated at 97, 90, and 80% sequence identity cut-off values.

Abundance of *narG* and *nosZ* genes

Gene copy numbers of *narG* were quantified by qPCR with primers *narG*1960F and *narG*2008R as previously described [25]. For qPCR targeting *nosZ*, primers *nosZ*1181F.b (5'-GNT GTT CMT CGA CAG YCA R-3') and *nosZ*1382R (5'-ARC GRT CYT TSG AGA AYT TG-3') were designed based on *nosZ* sequences obtained in this study using the ARB software package [30]. A 20 μ L reaction mix contained 10 μ L LC-FastStart DNA Master SYBR® Green I Master mix (Roche), 1 μ L template DNA (diluted 1:10), 0.5 mg mL⁻¹ bovine serum albumin, and 0.5 μ mol L⁻¹ of each primer. Three independent serial dilutions (10^8 to 10^3 gene copies μ L⁻¹) of a *nosZ*-containing plasmid (EU053038) served as standards. Thermal cycling on a LC480 Lightcycler (Roche) was as follows: 300 s enzyme activation at 95 °C; then cycles of denaturation (95 °C), 30 s; annealing (59 °C), 30 s; extension (72 °C), 30 s; and data acquisition (80 °C). Amplification efficiency of the new qPCR assay was 66.2% ($r^2 = 0.988$), specificity was confirmed by detection of the desired 201 bp-qPCR product by agarose gel electrophoresis and by cloning and sequencing of the PCR product, which revealed exclusively *nosZ* among 20 randomly picked clones.

Gene copy numbers of *narG* and *nosZ* were quantified in triplicate in three independent DNA extracts from sediment with and without *C. plumosus*, normalized for DNA concentrations, and differences in gene abundance between sediment with and without *C. plumosus* were evaluated using Student's *t*-test.

Results and discussion

Sedimentary and larval emission of denitrification products

The total N_2O efflux from the sediment microcosms increased linearly with *C. plumosus* density (Fig. 1(A)). Direct larval N_2O emission due to incomplete denitrification in the gut [49] contributed 15.4, 19.2, and 19.4% to this increase at larval densities of 1150, 2300, and 3450 individuals per m², respectively (Fig. 1(A)). Thus, while most of the increase in total N_2O efflux was due to stimulation of sedimentary N_2O emission, the direct contribution by the larvae was still substantial. The total $N_2 + N_2O$ efflux also increased with larval density in the sediment, but leveled off at a density higher than 2300 individuals per m² (Fig. 1(B)). The contribution of direct $N_2 + N_2O$ emission by *C. plumosus* larvae to the total $N_2 + N_2O$ efflux was negligible (Fig. 1(B), inset). Maximally, the total effluxes of N_2O and $N_2 + N_2O$ increased 8.6- and 4.2-fold, respectively, relative to the background fluxes in the absence of larvae.

The stimulation of sedimentary N_2O and $N_2 + N_2O$ emission probably takes effect directly in the larval burrows from which the gases are efficiently transported into the water column due to the periodic ventilation activity of the larvae [35,53]. Chironomid burrows are distinct microsites in the sediment enriched with particulate organic matter [27,48] and show pronounced fluctuations of O_2 and NO_3^- concentrations [38,46,49]. Hence, denitrification activity and concomitant N_2O production may prevail during the anoxic phase [50], whereas nitrification activity with N_2O as a byproduct of ammonia oxidation [7] may prevail during the oxic

Fig. 1. Total efflux of (A) N_2O and (B) $N_2 + N_2O$ from lake sediment measured at different densities of *C. plumosus* larvae (●) and extrapolated from rates of direct larval emissions (○). Direct larval N_2O emission contributed approximately 15–20% to the total N_2O efflux, while direct larval $N_2 + N_2O$ emission (highlighted in inset) did not significantly contribute to the total $N_2 + N_2O$ efflux. Error bars indicate standard error of the mean ($n = 6$).

phase. In fact, the stimulation of nitrification and total efflux of N_2O by *C. plumosus* were correlated in a lake sediment studied by [54]. In the lake sediment used here, however, denitrification was likely the main source of N_2O , since in control microcosms without NO_3^- addition, N_2O emission was negligible (data not shown).

The N_2O yield of both nitrification and denitrification generally reaches high values under hypoxic [10] and fluctuating O_2 conditions [24,43]. Accordingly, the ratio of total N_2O to total $N_2 + N_2O$ emissions increased from 0.5% in microcosms without burrows to 1.2% in microcosms with the highest density of periodically ventilated burrows (calculated from data in Fig. 1). The larvae also contribute directly to this increase by the extremely high N_2O yield of denitrification in their guts (15–68%) [49]. Unlike in the burrows, O_2 is largely absent from the gut contents of *C. plumosus* larvae [47]. The high N_2O yield is therefore not explained by hypoxic or fluctuating O_2 conditions in the gut, but rather by delayed induction of the nitrous oxide reductase in the gut contents [19,49]. Additionally, the high ratio of total N_2O to total $N_2 + N_2O$ emissions at the highest larval density is explained by the absence of a further increase in the total $N_2 + N_2O$ emission (Fig. 1(B)). At very high animal density, the sediment between burrows is increasingly oxygenated, which eventually inhibits denitrification in the burrow layer [14].

Density-dependent increases in the total efflux of denitrification products ($N_2 + N_2O$) have been observed for a number of marine and freshwater invertebrates [3,11,34,51,52,55]. The main mechanism of increased sedimentary denitrification is the efficient transport of NO_3^- from the water column to deep, anoxic sediment layers by the periodic ventilation activity of the animals. Little is known about the concomitant increase in total efflux of N_2O from bioturbated sediments. Aside from *C. plumosus* [49,50,54], only two more sediment-dwelling freshwater species have been studied, the mayfly *Ephemera danica* and the alderfly *Sialis lutaris* [51]. *C. plumosus* and *E. danica* larvae contribute to the total N_2O efflux by

Table 1
Abundance of the *narG* and *nosZ* genes in sediment samples.

	Sediment without <i>C. plumosus</i>	Sediment with <i>C. plumosus</i>	<i>P</i> -value
<i>narG</i> copies per ng DNA	1.9 × 10 ⁶ (± 0.8 × 10 ⁶)	2.4 × 10 ⁶ (± 1.1 × 10 ⁶)	0.726
<i>nosZ</i> copies per ng DNA	4.1 × 10 ⁵ (± 1.6 × 10 ⁵)	4.1 × 10 ⁵ (± 1.9 × 10 ⁵)	0.498

The differences in gene abundance between sediment with and without *C. plumosus* larvae were evaluated with Student's *t*-test; *P*-values >0.05 indicate no significant difference between the two samples. Mean values (standard deviation) of three replicates are given.

Table 2
Clone libraries of the *narG* and *nosZ* genes in sediment and gut samples.

Gene fragment	Sample	No. of clones screened	No. of phylotypes observed	Coverage (%)*	Estimated no. of phylotypes by Chao1**
<i>narG</i>	Sediment without <i>C. plumosus</i>	79	40	71	68
	Sediment with <i>C. plumosus</i>	102	68	49	170
	Gut content of <i>C. plumosus</i>	109	25	86	47
<i>nosZ</i> (1100 bp)	Sediment without <i>C. plumosus</i>	53	10	98	10
	Sediment with <i>C. plumosus</i>	53	7	98	7
	Gut content of <i>C. plumosus</i>	33	1	100	n.a.
<i>nosZ</i> (700 bp)	Sediment without <i>C. plumosus</i>	99	14	92	21
	Sediment with <i>C. plumosus</i>	82	16	90	22
	Gut content of <i>C. plumosus</i>	16	7	75	10

*Coverage (%): Coverage = 1 – (N_{singletons}/N_{total}) [31]; **Chao1: Bias-corrected phylotype richness estimator [8]. Analysis was done at nucleic acid sequence identity cut-off values of 97, 90, and 80% (results not shown for 90 and 80%). The results were essentially the same at all three sequence identity cut-off values. The abundance-based coverage estimator (ACE) by [9] showed similar results as the Chao1-model (results not shown).

gut denitrification and by stimulating sedimentary denitrification, while *S. lutaria* only stimulates sedimentary denitrification. More recently, also mussels and snails living on top of freshwater and marine sediments were found to emit N₂O [16,17,52]. The invasive freshwater mussel *Dreissena polymorpha* emits N₂O produced by nitrification in the biofilm growing on its shell and by denitrification in its gut. Apparently, this type of dual N₂O emission is also widespread in coastal marine mussels and snails [16,17].

Abundance and diversity of nitrate- and nitrous-oxide-reducing bacteria

The higher N₂O and N₂ + N₂O fluxes in sediments inhabited by *C. plumosus* may be explained by an increase in abundance and/or cell-specific activity of nitrate-reducing and denitrifying bacteria through bioturbation, ventilation, and grazing activities of the larvae. Gene copy numbers of *narG* and *nosZ* were not

Fig. 2. Pie charts: Relative frequency of appearance of *narG* and *nosZ* phylotypes in clone libraries constructed for sediment with and without *C. plumosus* (DNA) and the gut content of *C. plumosus* (RNA). The total number (i.e., 100%) of *narG* and *nosZ* phylotypes in the three clone libraries was 114 and 42, respectively. The three libraries are represented as vertical columns; the seven possible categories of phylotype appearance in the libraries are represented as rows of one or more colored boxes. Each category represents one or more putative mechanisms of *C. plumosus* effects on the nitrate reducer and/or denitrifying community in the sediment. For example, *narG* phylotypes, exclusively detected in sediment with larvae are represented by the color orange, were likely promoted through niche construction by *C. plumosus*, and made up 45% of all *narG* phylotypes observed. This evaluation gave identical results at sequence identity cut-off values of 97%, 90%, and 80% (results not shown for 90% and 80%).

The community composition of nitrate- and nitrous-oxide-reducing bacteria was indeed affected by the presence of *C. plumosus* larvae: estimated phylotype richness of the *narG* gene was higher in sediment with larvae (170 phylotypes) compared to sediment without larvae (68 phylotypes) (Table 2). The additional *narG* phylotypes in sediment with larvae covered a broad

taxonomic spectrum (Fig. 3(A)). In contrast, the estimated phylotype richness of the *nosZ* gene was highly similar in sediment with larvae (29 phylotypes) and without larvae (31 phylotypes) (Table 2). Nevertheless, there was little overlap between phylotypes of the two communities, indicating a change in community composition also for *nosZ*-containing denitrifiers (Fig. 3(B)). The *nosZ* phylotypes in sediment with and without larvae covered a relatively broad taxonomic spectrum but more narrow than for *narG* (Fig. 3(B)). The generally lower *nosZ* abundance and phylotype richness compared to *narG* can be explained by the fact that nitrate reducers usually outnumber denitrifiers in terrestrial and aquatic environments [18,19,45,56], and that even of true denitrifiers about 30% do not carry a nitrous oxide reductase [21,59]. It should also be noted that the primer sets used for *nosZ* here (as in most other studies) do not target Gram-positive nitrous-oxide

reducers [22]; their importance for freshwater sediment is as yet unknown. Additionally, the estimated phylotype richness of *narG* and *nosZ* transcripts in the gut was 47 and 10, respectively (Table 2); the observed *narG* and *nosZ* phylotypes covered a broad taxonomic spectrum (Fig. 3(A) and (B)).

Clone-based sequencing approaches may bias the analysis of microbial community composition due to limited sampling depth. Next-generation sequencing allows to produce 2–3 orders of magnitude more sequences from any given sample than clone-based sequencing and thereby to overcome bias due to undersampling. In the *narG* and *nosZ* clone libraries, significant undersampling was evident for *narG* in sediment with and without *C. plumosus* larvae (49 and 71% coverage, respectively) and for *nosZ* transcripts in the gut of the larvae (75% coverage) (Table 2). It can thus be expected that a larger sequencing effort would have revealed an even greater

Fig. 3. (Continued)

B

Fig. 3. (Continued)

difference in *narG* phylotype richness between sediment with and without *C. plumosus* larvae and also a higher phylotype richness of *nosZ* transcripts in the gut of the larvae. In contrast, a major difference in *nosZ* phylotype richness between sediment with and without *C. plumosus* larvae would not be expected because of the similarly high coverage of the respective clone libraries (Table 2).

Putative mechanisms behind *C. plumosus*' effects on the nitrate reducer and denitrifier community

The observed shift in the community composition of nitrate reducers and denitrifiers may be explained in three ways: (1) habitat structuring, i.e., addition or removal of ecological niches by the larvae (e.g., by adding the distinct microenvironments of burrow

and gut); (2) metabolic induction of bacteria in the gut followed by inoculation of the sediment with active bacteria; and (3) elimination of certain bacterial species via digestion in the gut. For a first indication of the mechanisms possibly at play, the diversity of *narG* and *nosZ* phylotypes actively expressed in the gut of *C. plumosus* larvae was compared to the diversity of *narG* and *nosZ* phylotypes in sediments with and without larvae (Fig. 2). The total number of *narG* and *nosZ* phylotypes observed in the gut (G), in sediment without larvae (S_0), and sediment with larvae (S_1) was 114 and 42, respectively. Individual phylotypes could appear in either one, two, or three of the G, S_0 , and S_1 libraries; the relative frequency of each possible phylotype distribution was expressed as a fraction of the total number of *narG* or *nosZ* phylotypes (Fig. 2).

For nitrate-reducing bacteria, the quantitative assessment of putative mechanisms in Fig. 2 suggests that niche construction by the larvae was of greatest importance for the observed increase in phylotype richness (Table 2), since 45% of all *narG* phylotypes were only detected in sediment with larvae, not in sediment without larvae and not as transcripts in the gut either (Fig. 2, orange). Apparently, induction in the gut or elimination through digestion did not occur for these additional phylotypes. Only a small fraction of all *narG* phylotypes (5%) contributed to the observed increase in phylotype richness through gut induction followed by sediment inoculation (Fig. 2, light blue). In a considerably larger fraction of all *narG* phylotypes, however, gut induction was not sufficient to inoculate the sediment with metabolically active nitrate reducers (Fig. 2, yellow and white). Elimination occurred for one fourth of all *narG* phylotypes (Fig. 2, white and dark blue). Higher coverages of the S_0 and S_1 libraries would probably not have rendered niche construction by the larvae a less important factor for increased phylotype richness. The role of gut induction, however, may have been underestimated due to the particularly low coverage of the S_1 library.

For nitrous-oxide-reducing bacteria, no difference in overall species richness was observed between sediments with and without *C. plumosus* (Table 2). Nevertheless, niche construction by the larvae was important, since 33% of all *nosZ* phylotypes were exclusively detected in sediment with larvae (Fig. 2, orange). In contrast, gut induction followed by sediment inoculation was not observed at all (Fig. 2, light blue). In fact, for a significant proportion of all *nosZ* phylotypes, gut induction occurred, but did not lead to sediment inoculation (Fig. 2, yellow and white). However, the extent of gut induction followed by sediment inoculation may have been underestimated due to the low coverage of the G library. Elimination of *nosZ* phylotypes was of greater relative importance than observed for *narG* phylotypes and obviously prohibited an overall increase in *nosZ* phylotype richness (Fig. 2, white and dark blue).

Conclusion and perspective

Our results indicate that the most important factor for increased denitrification, N_2O emission, and nitrate reducer diversity, and for the change in denitrifier community composition is niche construction by *C. plumosus* larvae. Although a local increase in nitrate reducer and denitrifier abundance at the burrow walls (below the sensitivity of the qPCR assay) cannot be excluded, overall abundances do not change; therefore the increased rates are best explained by a higher sediment volume ideal for denitrification and hence a higher fraction of active denitrifiers, possibly together with higher cell-specific denitrification rates of the altered community. Finally it can be speculated that nitrate reducers can cope better with the dynamic conditions inside the *C. plumosus* burrow than complete denitrifiers because they only have to express one rather than four reductases during the recurring oxic-anoxic shifts inside the burrow. Hence, the burrow microenvironment may select for non-denitrifying nitrate reducers and against denitrifying nitrous-oxide reducers.

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