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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 $N_2 + N_2O$) 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 $N_2 + N_2O$ 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.

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 preceeding 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 $N_2 + N_2O$), 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 μmol 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 $N_2 + N_2O$) 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 $N_2 + N_2O$ efflux as a measure of complete denitrification.

The direct emission of N_2O and $N_2 + N_2O$ 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 $N_2 + N_2O$ 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 μmol 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) × 3 gene fragments (1 × *narG*, 2 × *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

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