Nitrous oxide emission by aquatic macrofauna

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A large variety of aquatic animals was found to emit the potent greenhouse gas nitrous oxide when nitrate was present in the environment. The emission was ascribed to denitrification by ingested bacteria in the anoxic animal gut, and the exceptionally high N_2O-to-N_2 production ratio suggested delayed induction of the last step of denitrification. Filter- and deposit-feeding animal species showed the highest rates of nitrous oxide emission and predators the lowest, probably reflecting the different amounts of denitrifying bacteria in the diet. We estimate that nitrous oxide emission by aquatic animals is quantitatively important in nitrate-rich aquatic environments like freshwater, coastal marine, and deep-sea ecosystems. The contribution of this source to overall nitrous oxide emission from aquatic environments might further increase because of the projected increase of nitrate availability in tropical regions and the numeric dominance of filter- and deposit-feeders in eutrophic ecosystems.

aquatic animals | eutrophication | sediment | gut microbiology | denitrification

The global increase of the atmospheric nitrous oxide concentration correlates with enhanced nitrogen fertilization, biomass burning, and industrial processing (1). However, magnitude and regulation of the biogenic production of nitrous oxide in the world's oceans, fresh waters, and soils is poorly understood (1, 2), and present estimates of global emissions and atmospheric sinks are still under debate (3, 4). Microorganisms mediating biologic nitrogen conversions, such as nitrification and denitrification, are the main producers of nitrous oxide in sediments and soils (2, 5). Recently plants have also been shown to emit nitrous oxide at very low rates (6), and plants can act as conduits between soil and atmosphere for nitrous oxide produced by microbes (7). Whereas soil-living earthworms are known to be globally important emitters of nitrous oxide (8), aquatic animals have never been shown to emit this greenhouse gas. We therefore initiated an extensive field survey in which we observed that benthic invertebrates emitted nitrous oxide depending on the feeding guild to which they belonged. Laboratory experiments with *Chironomus plumosus* (Insecta, Diptera) and *Ephemera danica* (Insecta, Ephemeroptera), 2 abundant filter- and deposit-feeders in freshwater ecosystems, revealed that nitrous oxide was produced in the anoxic animal gut because of incomplete denitrification by ingested bacteria.

Results and Discussion

The field survey covered 21 different invertebrate taxa sampled at 7 field sites that included several creeks and streams, a lake, and the seashore. Rates of nitrous oxide emission differed markedly between taxa; emission rates were, however, not correlated with taxonomic affiliation or habitat type of the invertebrates but rather with the feeding guild to which the species belonged (Fig. 1). Filter- and deposit-feeders were grouped into 1 feeding guild (FD-feeders) because many of their representatives are able to switch between the 2 feeding modes (9). FD-feeders emitted the highest amounts of nitrous oxide per individual, shredders and grazers took an intermediate position, and predators played an insignificant role (Fig. 1). This overall pattern was also evident when the rate of nitrous oxide emission was expressed per milligram dry weight of the specimens (Table S1). Because FD-feeding species ingest greater numbers of microbes than predators do (10), this observation suggests microbial nitrogen conversions in the gut of the invertebrates as the source of nitrous oxide. Given anoxic conditions and the availability of easily degradable organic carbon in the gut of terrestrial and aquatic invertebrates (8, 11), we hypothesized that nitrous oxide production was due to the activity of denitrifying bacteria in the animal gut.

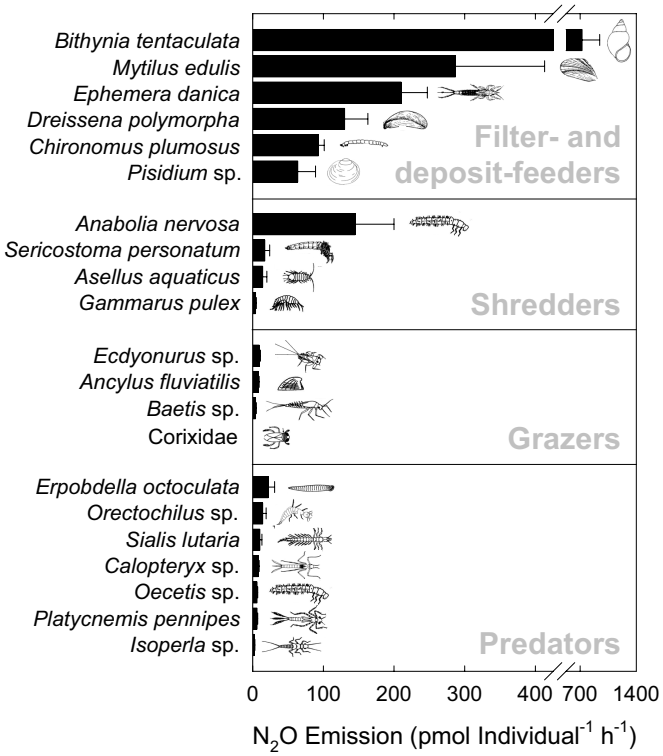

Fig. 1. Nitrous oxide emission by various aquatic invertebrate taxa. Animals are grouped into 4 different feeding guilds and sorted according to emission rate. Error bars indicate SEM (n = 3–23). Line drawings of animal taxa are stylized and not to scale. See Table S1 for details on sampling locations and taxonomy.

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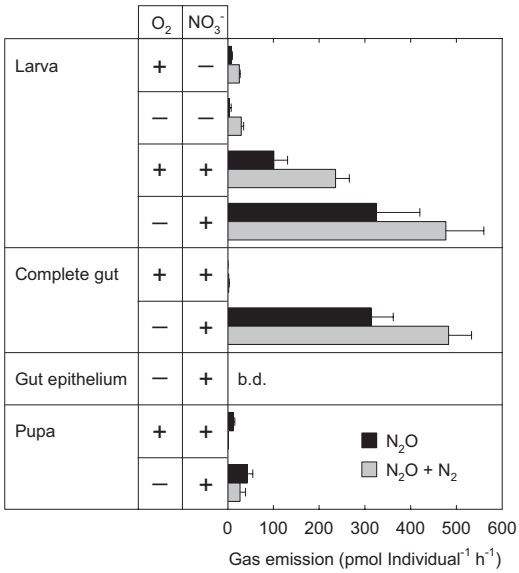

Fig. 2. Nitrogen gas emissions from *C. plumosus*. Nitrous oxide emission and total denitrification (i.e., nitrous oxide plus dinitrogen emission) measured in live larvae, dissected complete guts, gut epithelia, and pupae with or without nitrate in the sediment–water microcosms and with or without oxygen in the incubation vials. Error bars indicate SEM (n = 4–18). b.d., below detection limit.

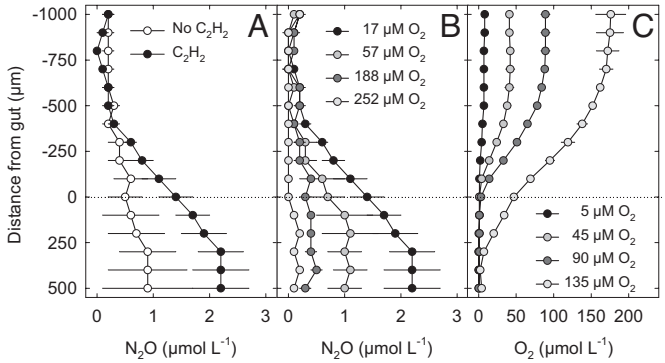

Fig. 3. Distribution of nitrous oxide (*A* and *B*) and oxygen (*C*) in dissected guts of *E. danica* larvae as measured with microsensors. Guts were incubated in a flow cell with physiologic salt solution adjusted to 50 μmol L^{-1} NO_3^- and various O_2 concentrations and with or without 10% acetylene to inhibit the last step of denitrification. Microsensor profiles were measured perpendicular to the gut and are plotted relative to the surface of the gut content (dotted line). In detail, the conditions were (*A*) 17 μmol L^{-1} O_2, no and 10% acetylene, (*B*) 17–252 μmol L^{-1} O_2, 10% acetylene, and (*C*) 5–135 μmol L^{-1} O_2, no acetylene. Error bars indicate SEM (n = 4–9, *A*) and range (n = 2, *B* and *C*).

To test this hypothesis, we carried out detailed experiments with the midge larvae *C. plumosus*. These worm-shaped larvae often dominate benthic invertebrate communities of freshwater ecosystems and can reach levels of abundance from several hundred to thousands of individuals per square meter (12). The larvae live in U-shaped burrows in the sediment and feed on suspended and settled particulate organic matter, thereby ingesting large numbers of bacteria (13). Our investigation of larvae, dissected guts, dissected gut epithelia, and pupae of *C. plumosus* unambiguously demonstrated nitrate-dependent denitrification activity and nitrous oxide production associated with bacteria coingested with particulate organic matter (Fig. 2). In the absence of oxygen, larvae and dissected guts showed identical emission rates, and thus denitrification and the production of nitrous oxide by *C. plumosus* larvae were exclusively associated with the gut (Fig. 2). More specifically, they were associated with anaerobic processes in the gut contents, given that only complete guts but not isolated gut epithelia denitrified and emitted nitrous oxide (Fig. 2). Pupae, possessing a nonfunctional gut, showed very low rates of nitrous oxide emission and denitrification (Fig. 2). Further evidence for a dietary origin of denitrifying bacteria active in the larval gut was provided by the analysis of expressed denitrification genes in the gut content of *C. plumosus*. Transcripts of nitrate and nitrous oxide reductase genes (*narG* and *nosZ*), coding for the first and last step in denitrification, had high sequence identities (76–99%) with diverse genes retrieved from freshwater sediments and soils [supporting information (SI) Fig. S1], suggesting that the denitrifiers had indeed been coingested with the food of the larvae. These collective results demonstrate that the nitrous oxide emission of *C. plumosus* is not due to specific symbionts in the animal gut; although we cannot fully exclude symbiotic nitrous oxide production for the other invertebrate species tested, the strict dependence of the nitrous oxide emission on the feeding guild and the very low emission rates of animals with empty guts are strong arguments against such a scenario.

The gut of FD-feeding invertebrates is obviously the distinct microsite of denitrification and production of nitrous oxide, as confirmed with microsensors on dissected guts of the mayfly larvae *E. danica* (Fig. 3). When incubated under close-to-anoxic conditions in an experimental flow cell (11), the concentration of nitrous oxide increased toward the center of the gut (Fig. 3*A*). In the presence of acetylene, an inhibitor of the last step of denitrification (14), nitrous oxide concentrations were higher than in the absence of acetylene, indicating complete denitrification activity in the gut (Fig. 3*A*). Oxygen inhibited denitrification activity in the gut in proportion to the concentration that was maintained in the medium surrounding the dissected gut (Fig. 3*B*). The *in vivo* concentration of oxygen in the hemolymph of *E. danica* larvae is not known, but our microsensor measurements indicate that at a concentration of 90 μmol L^{-1} or lower the gut will be completely anoxic and thus a favorable microsite for denitrification (Fig. 3*C*). From the concentration profiles in Fig. 3*A* (without acetylene), an average rate of nitrous oxide production of 189 pmol gut^{-1} h^{-1} was calculated using a diffusion-reaction model for radial geometry (11). This was very close to the rate that was measured for living larvae (Fig. 1). Hence, just as with *C. plumosus* larvae, the gut of *E. danica* seems to be the exclusive production site of nitrous oxide in these FD-feeding animals.

A striking feature of gas emissions by larvae of *C. plumosus* and *E. danica* was the incomplete denitrification with nitrous oxide accounting for 43–68% and 15–29% of the nitrogen gas flux, respectively (Figs. 2 and 3*A*), as compared with only 1% normally observed in aquatic sediments (15, 16). However, similarly high N_2O-to-N_2 ratios have been reported from pure cultures (17) and soils (18) after sudden shifts from oxic to anoxic conditions, and from earthworms ingesting oxic soil into their anoxic guts (19). Aquatic FD-feeders ingest bacteria-colonized particles that normally are exposed to oxic, and thus non-denitrifying, conditions at the sediment surface and in the water column. The transfer of bacteria into the anoxic animal gut apparently leads to a transient imbalance of gene expression during the onset of denitrification (20, 21), with accumulation of intermediates and emission of nitrous oxide as the unavoidable consequence (Fig. 4*C*). The detection of only 7 actively expressed phylotypes of *nosZ* in the gut contents compared with 26 of *narG* (Table 1) may indicate that the gut residence time in *C. plumosus* larvae of 2 to 3 h at 15 °C is for some denitrifiers too short to induce the full set of denitrification genes. Other phylotypes of *narG* might originate from nondenitrifying, dis-

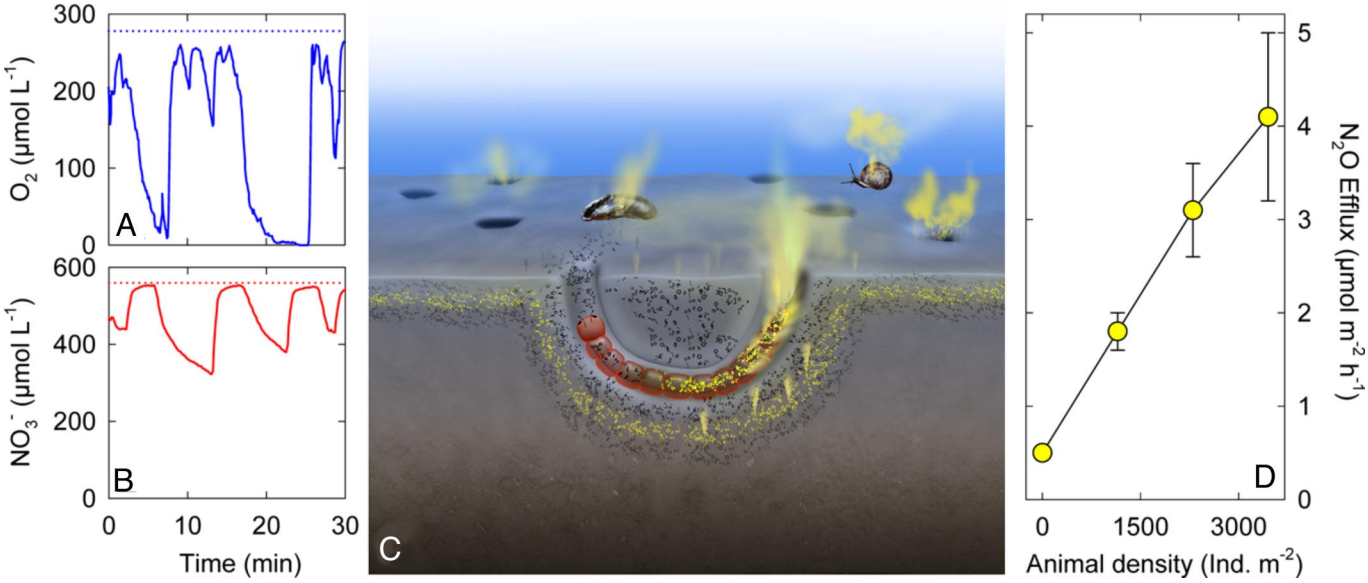

Fig. 4. Effect of *C. plumosus* larvae on sediment environment and nitrous oxide emission from the lake bottom. The larvae ventilate their U-shaped burrows, resulting in oscillating concentrations of oxygen (*A*) and nitrate (*B*) inside the burrows. Dotted lines indicate concentrations in the water column. (*C*) Conceptual model of nitrous oxide emission. The larvae feed on particles with attached bacteria (black), which inside the anoxic gut turn on nitrate reduction and denitrification (yellow), resulting in nitrous oxide emissions (yellow plumes). Also illustrated are point emissions from a filter-feeding bivalve and a deposit-feeding snail and the diffusive emission from anoxic, nitrate-containing sediment layers. (*D*) Effect of density of *C. plumosus* larvae on nitrous oxide emission from the sediment. Error bars indicate SEM ($n = 6$).

similatory nitrate-reducing bacteria, which only convert nitrate to nitrite and not further to gaseous products. These bacteria might be indirectly involved in nitrous oxide production via the accumulation of nitrite in the animal gut. Nitrite has been shown to stimulate nitrous oxide production in estuarine sediment (22) and the earthworm gut (23) more effectively compared with nitrate.

Rates of gut denitrification and nitrous oxide production increased in the presence of nitrate and decreased in the presence of oxygen (Fig. 2). By adding ^{15}N-nitrate to sediment–water microcosms, it was proven that nitrate from the water column was the precursor of nitrous oxide produced in the gut of *C. plumosus* (data not shown). Favorable conditions for gut production of nitrous oxide in the form of high nitrate availability and periodic anoxia were measured directly within the burrows with microsensors (Fig. 4 *A* and *B*). Measurements in sediment–water microcosms also showed a profound effect of *C. plumosus* larvae on the emission of nitrous oxide from lake sediment, reaching an 8-fold enhancement at the highest abundance (Fig. 4*D*). This enhancement, however, was 5 times higher than computed from larval abundance and the average emission rate of separated larvae in anoxic and oxic incubations (Figs. 2 and 4*D*). The discrepancy might be due to additional nitrous oxide emission from stimulated denitrification in the burrow walls and in fecal pellets, or suboptimal conditions in the incubations of isolated larvae. Notably, the emission of dinitrogen due to denitrification also increased with animal density, but to a slightly lower degree than that of nitrous oxide (data not shown). Thereby, the N_2O-to-N_2 ratio of the sedimentary effluxes of gaseous nitrogen compounds more than doubled from 0.51% to 1.25% with 0 and 3,450 *C. plumosus* larvae per square meter, respectively.

In a previous study of *C. plumosus*, an increase of sedimentary nitrous oxide flux was also observed but was attributed to nitrification in the sediment and not to denitrification in the gut of the larvae (24). For burrowing animals, the surrounding sediment acts as an additional sink or source of nitrous oxide, which may weaken the relative impact of gut denitrification (Fig. 4*C*). This is not the case for animals colonizing hard substrates or the sediment surface (e.g., snails and bivalves on rocky shores), and we suggest that the very high nitrous oxide fractions and emissions measured at intertidal hard substrate sites (25) could be due to gut denitrification of the epifauna. The rate of nitrous oxide emission from benthic invertebrates may thus not only depend on the rate of nitrous oxide production in their gut but also on the site of the animal habitat relative to the sediment–water interface (5).

Our findings prove valuable for predicting the effect of local management of nitrate pollution and eutrophication on nitrous

Table 1. Phylotype richness of *narG* and *nosZ* transcripts in the gut of *C. plumosus* larvae

Gene fragment	No. of clones screened	No. of phylotypes observed	Coverage* (%)	Estimated phylotype richness†	
				Chao1	ACE
narG	109	26	86	47	51
nosZ	16	7	75	10	12

*Coverage of each clone library was calculated from the number of phylotypes observed only once (i.e., $N_{singletons}$) and the total number of phylotypes observed (i.e., N_{total}) according to the equation: coverage = $[1 - (N_{singletons}/N_{total})] \times 100\%$.

†Phylotype richness was calculated using the bias-corrected estimators Chao1 and ACE (references in *SI Text*) with a sequence dissimilarity cutoff set at 3%. The results were essentially the same at sequence dissimilarity cutoff values of 10% and 20%.

oxide emission. The main conclusion is that animal-enhanced nitrous oxide emission is both nitrate dependent and feeding guild dependent. Thus, the contribution of the invertebrate community to the total emission of nitrous oxide of a given, nitrate-polluted aquatic habitat can be estimated using the rates typical of certain species or feeding guilds. In the hypertrophic lake Großer Binnensee, representative of a human-impacted aquatic ecosystem, *C. plumosus* reaches an abundance of 1,600 larvae per square meter and is the dominant benthic invertebrate species (26). During summer, nitrate concentration and temperature can exceed 100 μmol L^{-1} and 20 °C, respectively (27). In this setting, gut denitrification of *C. plumosus* increases the sedimentary nitrous oxide emission by 32–104% (according to data presented in Figs. 2 and 4). Restoration measures that reduce the nitrate concentration will decrease the overall emission of nitrous oxide. Additionally, restoration measures that remove the numeric dominance of FD-feeders (28), which according to our findings are the principal nitrous oxide emitters, will decrease the animal-associated emission of nitrous oxide.

Nitrous oxide emission by aquatic invertebrates will be quantitatively important in environments with extensive nitrate inputs from agriculture, like lakes, streams, and coastal marine ecosystems (4, 29, 30) or from nutrient-rich subsurface water in coastal upwelling zones (3, 31, 32). In such eutrophic systems, FD-feeders are often the numerically dominant feeding guild of the benthic invertebrate community (28), which may further enhance animal-associated nitrous oxide emission. In deep-sea environments with naturally high nitrate concentrations, such as hydrothermal vents (33), nitrous oxide might be produced by symbiotic microbes of the hot-vent fauna. Some tropical aquatic ecosystems will receive much more nitrate over the next few decades because of agricultural intensification (34), and nitrous oxide emission is already several times higher for tropical mangroves than for temperate estuaries and intertidal flats (35). Thus, the rates of nitrous oxide emission associated with tropical aquatic invertebrate species could also be particularly high, a hypothesis that needs to be confirmed in future studies.

Materials and Methods

Nitrous Oxide Emission. Aquatic invertebrates were collected at 7 freshwater and marine sites in Denmark and Germany (Table S1). Individual specimens were placed in 3-mL gas-tight vials that contained 200 μL of 0.2-μm-filtered water from the sampling site (i.e., at *in situ* nitrate concentration) to maintain a moist atmosphere. The 2 bivalve species were incubated in 10-mL vials with 5 mL of 0.2-μm-filtered water to allow the specimens to ''filter-feed'' during the incubation and thereby exchange gases with the incubation vial. For standardization, all species were incubated under oxic conditions at 21 °C. Gas samples were taken hourly for 4 to 5 h and analyzed with a gas chromatograph with a ^{63}Ni electron capture detector (Shimadzu GC-8A). The linear increase of nitrous oxide concentration in the incubation vial was used to calculate the nitrous oxide emission rate of the animal, also taking into account the fraction of nitrous oxide that was dissolved in the water phase.

C. plumosus larvae were kept in sieved sediment overlaid with aerated fresh water that contained either no or 500 μmol L^{-1} nitrate. Sediments and larvae were incubated at 15 °C for 1 week, during which some of the larvae metamorphosed into pupae. For rate measurements, larvae, pupae, and dissected guts were incubated individually in gas-tight vials. Guts were incubated as a whole (*complete gut*) or with the gut contents removed (*gut epithelium*) (11). Rates of nitrous oxide emission were obtained under oxic and anoxic conditions as described above. Denitrification rates were determined in separate vials with the acetylene inhibition technique (14). Oscillation of oxygen and nitrate concentrations inside the larval burrows was measured with microsensors (36). The efflux of nitrous oxide from lake sediment inhabited by different densities of *C. plumosus* larvae was measured in glass bottles (120 mL) filled with sieved sediment and nitrate-containing, aerated water. After a 1-week equilibration period (37), the efflux of nitrous oxide from the sediment was determined from the short-term increase of nitrous oxide concentration in the headspace after sealing the bottles.

E. danica larvae were collected in Klosterkanal (Jutland, Denmark) and incubated in stream sediment overlaid with nitrate-containing and aerated water. After 2 days, the guts of these larvae were dissected and incubated in a flow cell as described by Stief and Eller (11). The physiologic salt solution passing through the flow cell was adjusted to 50 μmol L^{-1} nitrate and various oxygen concentrations at 21 °C. Microsensors for oxygen (38) and nitrous oxide (39) were used as described previously (11). Gut denitrification was measured with nitrous oxide microsensors in the presence of 10% acetylene (14). For extrapolation of concentration gradients of nitrous oxide to *per capita* production rates, we used a diffusion-reaction model for radial geometry, a diffusion coefficient of nitrous oxide of 2.1×10^{-5} cm^2 s^{-1}, and assumed a food bolus of 10 mm in length and 0.8 mm in diameter.

Expression of Denitrification Genes. RNA was extracted from the gut content of *C. plumosus* larvae reared in lake sediment as described above and reversely transcribed into cDNA using random hexamers. Partial sequences of *narG* and *nosZ*, encoding for the dissimilatory nitrate and nitrous oxide reductase, respectively, were amplified, cloned, and sequenced using published primers and protocols. Sequence analysis was performed with the software packages ARB (Lehrstuhl für Mikrobiologie and Lehrstuhl für Rechnertechnik und Rechnerorganisation/Parallelrechnerarchitektur, Technische Universität München) and DOTUR (Department of Microbiology, College of Natural Resources and the Environment, University of Massachusetts). Detailed descriptions of materials and methods and references are given in *SI Materials and Methods*.

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