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Shell Biofilm Nitrification and Gut Denitrification Contribute to Emission of Nitrous Oxide by the Invasive Freshwater Mussel *Dreissena polymorpha* (Zebra Mussel)

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Nitrification in shell biofilms and denitrification in the gut of the animal accounted for N_2O emission by *Dreissena polymorpha* (Bivalvia), as shown by gas chromatography and gene expression analysis. The mussel's ammonium excretion was sufficient to sustain N_2O production and thus potentially uncouples invertebrate N_2O production from environmental N concentrations.

Nitrous oxide (N_2O) is a powerful greenhouse gas that contributes to stratospheric ozone destruction (3, 4). In natural systems, the production of N_2O is primarily associated with the turnover of inorganic nitrogen compounds by nitrifying and denitrifying microorganisms, often in oxic/anoxic transition zones in soil and sediment (34). Nitrifiers (both ammonia-oxidizing bacteria and archaea) produce N_2O as a by-product of ammonia oxidation (6, 28), especially under oxygen limitation, while for denitrifiers, N_2O is an intermediate in anaerobic respiration (40). Besides soils and aquatic systems, invertebrates are sites of a globally significant N_2O production, first discovered for earthworms (15, 19) and subsequently found for diverse freshwater and marine invertebrates (11, 36). This animal-associated N_2O production has been attributed to incomplete denitrification by ingested microorganisms in the anoxic invertebrate gut (13, 14, 35). In addition, biofilms covering shells and exoskeletons of marine invertebrates have been identified as sites of N_2O emission (11). Their relative contribution to animal-associated N_2O production, the pathways involved, and their distribution among marine and freshwater invertebrates are still unknown. The objective of the present study was therefore to quantify the biofilm-derived N_2O production and its mechanism(s) using the N_2O-emitting (36) freshwater bivalve *Dreissena polymorpha* (zebra mussel) as a model organism. This species is considered invasive in North America and Europe and can occur at extremely high abundance. Local populations in the Gudenå River system (Denmark) occasionally form large reefs at the sediment surface with more than 100,000 individuals per m^2 (1).

Site of N_2O production in *D. polymorpha*. Mussels were sampled in April 2010 in the river Remstrup, which is part of the Gudenå system. Living animals or shells dissected from living animals were pooled in sets of 7 to 15 individuals for replicate incubations (n = 5 to 6) at 21°C in gas-tight bags (10) filled with air-saturated artificial freshwater (33) containing NH_4^+ and NO_3^- (50 μM each) and a headspace of atmospheric air. Shells incubated with 50% $ZnCl_2$ to kill biological activity served as negative controls. N_2O emission rates were determined from linear increase of N_2O concentrations in 3-h incubations as previously described (36); in short, water samples were hourly withdrawn from the bags, transferred to N_2-flushed, $ZnCl_2$-containing Exetainers, and N_2O was measured by gas chromatography (36). Bags were still oxic (>50%) after the 3-h incubation, as confirmed with an O_2 microelectrode (26).

N_2O emission was approximately linear over time both in incubations of whole mussels and in biofilm-covered dissected shells; for whole mussels, the rates were similar to those for *D. polymorpha* collected in August 2006 in the river Rhine (36). The shell biofilm contributed approximately 25% to the total N_2O emission from *D. polymorpha* specimens (Fig. 1). N_2O production was an exclusively biological process, indicated by the linearity of the emissions and confirmed by the absence of N_2O emissions in the killed control.

Pathways of N_2O production. Additional whole animals and dissected shells were incubated with allylthiourea (ATU; 100 μM) to inhibit NH_3 oxidation (8). N_2O emission from ATU-incubated shells was almost completely eliminated, pointing to nitrification as the dominant N_2O-producing pathway in the shell biofilm of *D. polymorpha* (Fig. 1). In contrast, N_2O emission from the animal itself was not reduced by ATU, which indicates that denitrification was responsible for N_2O production inside the animal, in agreement with gut-associated N_2O production via denitrification in other freshwater invertebrates (36).

These results were supported by the detection of transcripts for bacterial ammonia monooxygenase (*amoA*), the key enzyme of ammonia oxidation, and for nitrite reductase (*nirK* and *nirS*), a key enzyme of denitrification. RNA was extracted from dissected whole guts and from biofilm material (sampled in June 2010) with the FastRNA Pro Soil-Direct kit (MP Biomedicals) and DNase treated (Ambion) for 30 min to remove DNA, as confirmed by (lack of) 16S rRNA gene-specific PCR amplification. Reverse transcription-PCR (RT-PCR) (35 cycles) was performed with the OneStep RT-PCR kit (Qiagen). Published protocols and primers specific for bacterial *amoA*, amoA1F-amoAR-TC (24, 27), and for *nirK* and *nirS*, F1aCu-R3Cu (9) and Cd3aF-R3cd (21, 39), respectively, were used. Bacterial *amoA* mRNA was only detected in biofilm samples, while mRNAs of *nirK* and *nirS* were only de-

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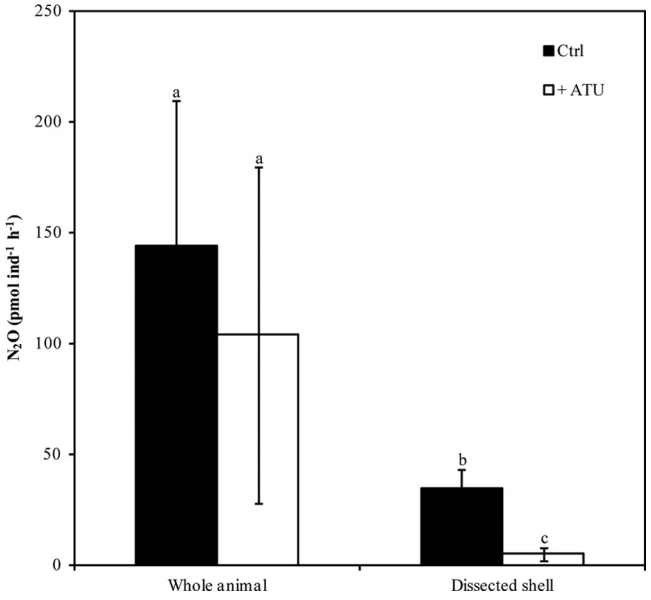

FIG 1 N_2O emission from living animals or shells dissected from living animals incubated in artificial freshwater with (+ ATU) or without inhibition of NH_3 oxidation by ATU (Ctrl). Error bars represent standard deviations (SD) of the mean ($n = 5$ to 6, and each replicate consists of 7 to 15 animals or shells). Different lowercase letters indicate significant differences between treatments ($P < 0.05$, *t* test).

tected in gut samples (Table 1). Since archaeal *amoA* genes were never detected by PCR (12) in any of the samples (see below), detection of archaeal *amoA* transcripts was not attempted.

Additional animals were collected in December 2010 and analyzed by reverse transcription-quantitative PCR (RT-qPCR). Mussels were incubated for 4 h under similar conditions to those used during N_2O rate measurements. Then total nucleic acids were extracted in triplicate by a phenol-chloroform protocol (7, 25), and one aliquot of the nucleic acid extract was DNase treated as described above. cDNA synthesis with the Omniscript reverse transcription kit (Qiagen) was primed by random hexamers, and cDNA copy numbers of bacterial *amoA*, *nirK*, and *nirS* were quantified in a LightCycler 480 (Roche) as described previously (12). Annealing temperatures were adjusted to 55°C for *nirS* and to 57°C for bacterial *amoA* and *nirK*; detection limit (10 to 13 cDNA copies) was defined as 3× the standard deviation (SD) of the nontemplate control, while the limit of quantification was defined by the lower limit of the linear range of the standard curves (85 to 100 cDNA copies).

Copy numbers of all cDNAs were low (always below the limit of quantification, for *nirS* always below the detection limit), but confirmed the results of the qualitative RT-PCR assay for bacterial *amoA* and *nirK*: bacterial *amoA* cDNA was only detected in biofilm samples, while *nirK* cDNA was only detected in gut samples (Table 1).

To test for the metabolic potential of the biofilm and gut microbial community, gene copy numbers of bacterial *amoA*, *nirK*, and *nirS* were quantified in the nucleic acid extracts from December 2010. Amplification of archaeal *amoA* (12) was attempted several times, but the result was always negative, indicating that archaeal ammonia oxidizers were not relevant in these samples. qPCRs were performed as described above, and functional gene copy numbers were normalized against 16S rRNA gene copy numbers amplified with primer pair 341F-907R (22, 23), with annealing at 57°C. 16S rRNA gene copy numbers (per mg wet weight) were $4.83 \times 10^6 \pm 6.2 \times 10^5$ in the gut and $3.48 \times 10^8 \pm 1.7 \times 10^7$ in the shell samples. Copy numbers of all functional genes were above the limit of quantification. Relative abundance ± SD was low in gut samples: i.e., $1.6 \times 10^{-3} \pm 1.5 \times 10^{-3}$ for bacterial *amoA*, $2.7 \times 10^{-1} \pm 6.5 \times 10^{-2}$ for *nirK*, and $2.5 \times 10^{-1} \pm 3.8 \times 10^{-2}$ for *nirS*. Biofilm samples showed higher relative abundances: i.e., $2.0 \times 10^{-2} \pm 2.4 \times 10^{-3}$ for bacterial *amoA*, $1.6 \times 10^{1} \pm 9.6 \times 10^{-1}$ for *nirK*, and $1.6 \times 10^{0} \pm 1.2 \times 10^{-1}$ for *nirS*. These data indicate a potential for ammonia oxidation and denitrification in both gut and biofilm, if environmental conditions allow. Expression of bacterial *amoA*, *nirK*, and *nirS* is affected by a variety of environmental factors, including O_2 partial pressure and availability of N substrates (29, 40). Inside the mussel gut, O_2 will most likely be depleted (35). In accordance with the data presented here, denitrification will therefore be induced and ammonia oxidation repressed when denitrifiers and ammonia oxidizers, respectively, enter the gut. Mussel biofilms analyzed in this study, on the other hand, were relatively thin and presumably fully oxic, as indicated by preliminary O_2 microsensor measurements (data not shown). N_2O is therefore mainly produced by nitrification, while denitrification is repressed. However, high *nir* gene abundance indicates that denitrification may contribute to N_2O production, if anoxic microsites develop within the biofilm (30).

Diversity of expressed *amoA*, *nirK*, and *nirS*. To assess the diversity of the active ammonia oxidizers and denitrifiers, clone libraries were constructed from cDNA of bacterial *amoA* (biofilm samples) and *nirK* or *nirS* (gut samples) of animals collected in June and December 2010. RT-PCR products were cloned using the pGEM-T cloning kit (Promega), with approximately 30 randomly picked clones per sample, the genes were sequenced (GATC Biotech; Macrogene), and the cDNA clone sequences were deposited in GenBank. Sequences were aligned by the integrated aligner tool in the ARB software (18) together with sequences of their closest relatives found by nucleotide BLAST, translated into amino acid sequences and used for phylogenetic tree construction in ARB using neighbor-joining and maximum likelihood analysis with 1,000 bootstrap replications. Both methods resulted in identical tree topologies.

Sequences of expressed bacterial *amoA* were in June 2010 affiliated with the *Nitrosomonas europaea* and *Nitrosomonas oligotropha* lineage and a lineage without cultured relatives, while in December 2010, they were affiliated with the *Nitrosospira* and *N. oligotropha* lineage (Fig. 2a). Since both clone libraries were well

TABLE 1 Expression of genes encoding ammonia monooxygenase (*amoA*) and nitrite reductase (*nirK* and *nirS*) in animals collected in June and December 2010

Material	Expression of[a]: *amoA* June	*amoA* December (cDNA copies/mg wet wt)	*nirK* June	*nirK* December (cDNA copies/mg wet wt)	*nirS* June	*nirS* December (cDNA copies/mg wet wt)
Gut	−	−	+	205–1,585[b]	+	<240[c]
Shell biofilm	+	200–2,000[b]	−	−	−	−

[a] −, not detected by RT-PCR or RT-qPCR; +, detected by RT-PCR.
[b] Above the limit of detection but below the limit of quantification for RT-qPCR.
[c] Below the limit of detection for RT-qPCR but detected and cloned after RT-PCR.

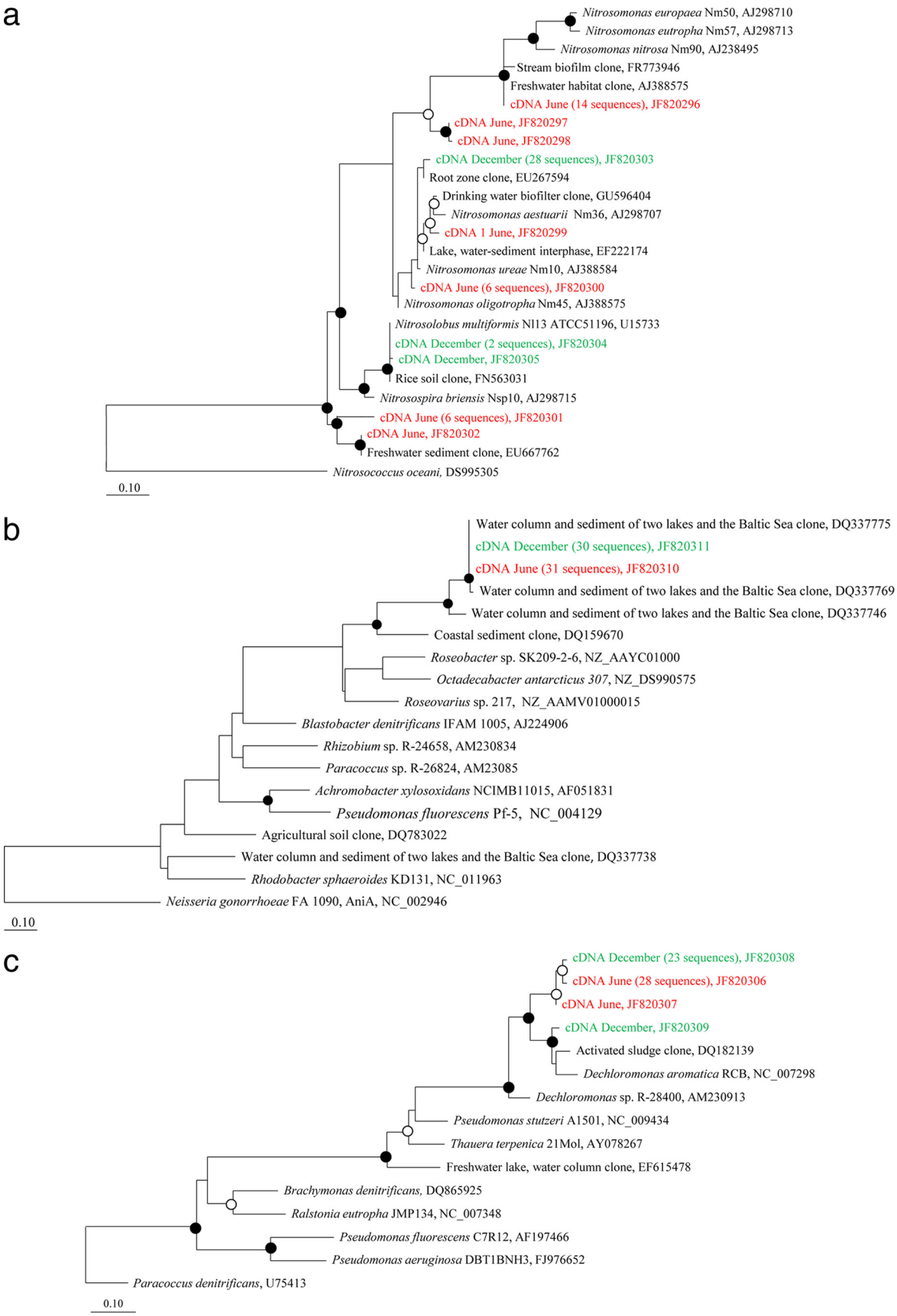

FIG 2 Neighbor-joining tree of amino acid sequences deduced from cDNA obtained in June 2010 (red) or December 2010 (green). Sequences of bacterial *amoA* (a) are from shell biofilms, while sequences of *nirK* (b) and *nirS* (c) are from the gut of *D. polymorpha*. The number of identical sequences (at least 97% nucleotide identity) is shown in parentheses. Scale bar, 10% amino acid sequence divergence. Node symbols indicate bootstrap support by maximum likelihood analysis: closed circles, >75%; open circles, >50%.

covered (Good's coverage of >98% based on a 97% nucleotide similarity threshold), the most probable explanation is differential activity of ammonia oxidizers at the different sampling times, possibly related to their different substrate affinities (16).

In contrast, diversity of expressed *nirK* and *nirS* was very low, and sequences retrieved from animals collected in June and December were highly similar or identical. *nirK* was affiliated with *Dechloromonas aromatica* (87% DNA sequence similarity), while *nirS* genes were only distantly related (70% DNA sequence similarity) to various members of the *Alphaproteobacteria*: e.g., *Rhodopseudomonas palustris* or *Rhodobacter sphaeroides* (Fig. 2b and c). This limited diversity of active denitrifiers in the gut may be explained by the fact that mussels are capable of feeding on a diet of bacteria due to the high lysozyme content in their digestive organs (20, 32). Consequently, only a minor part of the ingested denitrifiers may survive and induce their denitrification genes during the gut passage in *D. polymorpha*.

Ammonium excretion by *D. polymorpha*. The availability of NH_3 (as a substrate for ammonia oxidation) is usually low in natural freshwater systems but can be high in environments infested with *D. polymorpha* (5, 17). Ammonium excretion rates of *D. polymorpha* were measured by incubating groups of 1 to 8 living mussels ($n = 6$) in artificial freshwater without amendment of any N sources. NH_4^+ concentrations were quantified spectrophotometrically (2) every half hour for a total of 3 h. The average excretion rate ± SD was 0.128 ± 0.063 μmol NH_4^+ individual (ind)$^{-1}$ h^{-1}, which is >1,000 times the N needed to explain the N_2O production by nitrification in shell biofilms. Therefore, a significant part of the mussels' N_2O emission is sustained by the animals' N excretion.

Environmental implications. The results presented here are important on three accounts. First, they provide quantitative data for the contribution of shell biofilms to the overall N_2O emission by a benthic freshwater invertebrate, hence extending earlier qualitative observations on marine invertebrates (11). Second, with a substantial part of N_2O produced via nitrification, which can be entirely fueled by the mussels' own ammonia excretion, the data suggest that invertebrate-associated N_2O emissions can be decoupled from environmental nitrate concentrations, one of the main drivers of gut denitrification (19, 37, 38); in addition, biofilm nitrification may not only directly produce N_2O but also provide nitrate for denitrification-derived N_2O production inside the mussel.

The data also show a considerable potential of the invasive *D. polymorpha* to contribute to overall N_2O emissions from zebra mussel-infested ecosystems. Maximum densities of up to 100,000 individuals per m^2 in the river Gudenå and a potential emission rate of 144 pmol N_2O ind^{-1} h^{-1} amount to an emission potential of 28 μmol N_2O-N m^{-2} h^{-1} for *D. polymorpha*, or up to 400 times the areal N_2O fluxes reported for (noninfested) freshwater environments (31). Finally, shell biofilms, ammonium excretion, and coupled nitrification-denitrification are likely to combine also for other freshwater and marine invertebrates into significant N_2O emission potentials (I. M. Heisterkamp, A. Schramm, L. H. Larsen, N. B. Svenningsen, G. Lavik, D. de Beer, and P. Stief, unpublished data). It should however be noted that for assessment of their true environmental impact, *in situ* studies will be necessary, combining activity measurements and molecular analyses throughout the seasonal cycle.

Nucleotide sequence accession numbers. cDNA clone sequences obtained in this study have been deposited in GenBank under accession no. JF820296 to JF820311.

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