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FEATURE ARTICLE

Nitrous oxide production associated with coastal marine invertebrates

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ABSTRACT: Several freshwater and terrestrial invertebrate species emit the greenhouse gas nitrous oxide (N_2O). The N_2O production associated with these animals was ascribed to incomplete denitrification by ingested sediment or soil bacteria. The present study shows that many marine invertebrates also emit N_2O at substantial rates. A total of 19 invertebrate species collected in the German Wadden Sea and in Aarhus Bay, Denmark, and 1 aquacultured shrimp species were tested for N_2O emission. Potential N_2O emission rates ranged from 0 to 1.354 nmol ind.$^{-1}$ h^{-1}, with an average rate of 0.320 nmol ind.$^{-1}$ h^{-1}, excluding the aquacultured shrimp *Litopenaeus vannamei*, which showed the highest rate of N_2O emission measured so far for any marine species (3.569 nmol ind.$^{-1}$ h^{-1}), probably due to very high nitrate concentrations in the rearing tanks. The N_2O emitted by *L. vannamei* was almost exclusively produced in its gut by incomplete denitrification. Statistical analysis revealed that body weight, habitat, and exoskeletal biofilms were important determinants of animal-associated N_2O production. The snail *Hinia reticulata* emitted about 3.5 times more N_2O with an intact exoskeletal biofilm on its shell than with an experimentally cleaned shell. Thus, the N_2O production associated with marine invertebrates is apparently not due to gut denitrification in every species, but may also result from microbial activity on the external surfaces of animals. The high abundance and potential N_2O emission rates of many marine invertebrate species suggest significant contributions to overall N_2O emissions from coastal marine environments and aquaculture facilities.

KEY WORDS: Marine invertebrate · Animal–microbe interaction · Gut microbiology · Exoskeletal biofilm · Coastal marine ecosystem · Aquaculture

N_2O production associated with the snail *Hinia reticulata* partly results from microbial activity in exoskeletal biofilms covering the shell.

Photo: I. M. Heisterkamp

INTRODUCTION

Nitrous oxide (N_2O) is the third most important greenhouse gas after carbon dioxide and methane. Its atmospheric concentration is rapidly increasing, and it contributes significantly to global warming (IPCC 2007) and to the depletion of the stratospheric ozone layer (Ravishankara et al. 2009). Biogenic N_2O emission originates primarily from soils and oceans, where microbial nitrification and denitrification are the major N_2O-producing processes (Mosier et al. 1998, Stein & Yung 2003). During nitrification (the 2-stage oxidation of ammonium to nitrate) N_2O is produced as a by-product in the first oxidation step (Goreau et al. 1980), whereas in denitrification (the respiratory reduction of nitrate or nitrite to nitrogenous gases) N_2O is produced

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as a true intermediate (Zumft 1997). The complete denitrification pathway involves 4 enzymes that reduce nitrate to dinitrogen stepwise via the intermediates nitrite, nitric oxide, and N_2O. The 4 reductases are induced sequentially under anoxic conditions when oxidized inorganic nitrogen compounds and appropriate electron donors are available (Tiedje 1988, Zumft 1997). Whether denitrification acts as a source or sink of N_2O depends on the presence and activity of nitrous oxide reductase, which shows a higher sensitivity towards oxygen, lower carbon-to-nitrate ratios, and lower pH than the other 3 enzymes (Tiedje 1988, Bonin & Raymond 1990).

Important sites of N_2O emission are environments that are characterized by high input and turnover rates of inorganic nitrogen, such as fertilized soils and coastal areas (Mosier et al. 1998, Seitzinger & Kroeze 1998, Bange 2006). Microbial nitrogen conversions and concomitant N_2O production are especially stimulated in coastal sediments and in rock biofilms, due to high riverine input of nitrogen (Seitzinger & Nixon 1985, Law et al. 1992, Middelburg et al. 1995, Robinson et al. 1998, Magalhaes et al. 2005). Nitrification activity prevails at the oxic sediment surface and is fuelled by ammonium from organic matter degradation. Denitrification activity prevails in the anoxic subsurface layer and is driven by nitrate from nitrification (i.e. coupled nitrification–denitrification) or the water column (Jenkins & Kemp 1984). Sedimentary denitrification is commonly assumed to be the major source of N_2O to the water column, with benthic N_2O fluxes making up approximately 1% of the dinitrogen fluxes (Seitzinger 1988, Magalhaes et al. 2007, Ferrón et al. 2009). Sedimentary nitrification can, despite lower N_2O production rates, significantly contribute to benthic N_2O fluxes, due to its proximity to the sediment surface (Meyer et al. 2008). Oversaturation of N_2O in the water column occurs in many coastal areas (Kieskamp et al. 1991, Middelburg et al. 1995, Robinson et al. 1998, Dong et al. 2002).

Besides the microbial N_2O production in soils, sediments, and water bodies, N_2O is also emitted by earthworms and freshwater invertebrates (Karsten & Drake 1997, Drake & Horn 2007, Stief et al. 2009). This animal-associated N_2O production is due to the denitrification activity of ingested bacteria in the anoxic gut. The specific *in situ* conditions of the earthworm gut, including anoxia and high concentrations of easily degradable organic carbon, as well as nitrate or nitrite, stimulate the activity of ingested N_2O-producing soil bacteria (Drake et al. 2006). A similar mechanism has been suggested for freshwater invertebrates, whose N_2O emission is largely explained by their preferred diet: filter- and deposit-feeders show high, shredders and grazers intermediate, and predators very low N_2O emission rates (Stief et al. 2009). This suggests that N_2O emission is caused by bacteria that are co-ingested with the food taken up by freshwater invertebrates. N_2O emission rates of both terrestrial and freshwater invertebrates increase with nitrate and temperature and decrease with oxygen availability, indicating the important role of these environmental factors for gut denitrification (Karsten & Drake 1997, Matthies et al. 1999, Stief et al. 2009, 2010, Stief & Schramm 2010).

The N_2O emission potential of marine invertebrates has so far been neglected, although coastal marine sediments are presumably hot spots of N_2O emission, since they are densely inhabited by filter- and deposit-feeding invertebrates (Williams et al. 2004, Philippart et al. 2007) and exposed to high nitrate concentrations (Kieskamp et al. 1991, Van Beusekom et al. 2008). High N_2O emission can also be expected from aquaculture facilities in which animals are typically reared at high densities and high nitrate concentrations. The present study, therefore, investigated the N_2O emission potential of different marine invertebrate species from coastal sediments of the North Sea and Baltic Sea and of the aquacultured shrimp *Litopenaeus vannamei.* To understand how the N_2O emission potential of marine invertebrates is controlled by abiotic and biotic factors, correlations between potential N_2O emission rates and species-specific traits were investigated by statistical analysis.

MATERIALS AND METHODS

Sampling of animals. We tested the N_2O emission potential of 19 benthic invertebrate species from the German Wadden Sea and Aarhus Bay in Denmark, and of the aquacultured shrimp *Litopenaeus vannamei* (provided by Ecomaris Marifarm Kiel, Germany). Sampling was carried out between March and June 2008 at the mixed sediment intertidal flat near Dorum-Neufeld (53° 45′ N, 8° 21′ E) and at 3 different sites in Aarhus Bay (56° 9.75′ N, 10° 16.80′ E; 56° 9.29′ N, 10° 19.15′ E; 56° 6.44′ N, 10° 27.96′ E). Animals from the Wadden Sea were sampled at low tide. Epifaunal species were collected by forceps or hand, and infaunal species by digging up the sediment with a spade to a depth of approximately 25 cm and searching it by hand. Animals were placed in beakers filled with a layer of wet sediment from the sampling site until further processing in the laboratory. Sampling in Aarhus Bay was carried out from a research vessel by dredging the sediment with a triangle net. Some animals such as shore crabs and ascidians were sampled from rocks or pontoons in the harbor area of Aarhus. Sampled animals were kept in buckets filled with seawater from

the upper water column (15°C) until incubation in the laboratory was started. The temperature of the water was measured at each sampling site, and water samples were filtered (0.2 µm) and stored at –20°C until nitrate concentration was measured using the VCl_3 reduction method (Braman & Hendrix 1989) with a chemiluminescence detector (CLD 66 S NO/NO_x-Analyser, Eco Physics).

Classification of species. The screening included Crustacea, Mollusca, Echinodermata, Polychaeta, and Ascidia (Table 1). For each species, the affiliation to a feeding type and to a benthic habitat was determined (Table 1). Species that feed by several feeding modes were assigned to their dominant feeding mode. The description 'infaunal + epifaunal' refers to infaunal species that feed at the sediment surface or in the water column. Species were further characterized by their wet weight and by the presence/absence of a visible microbial biofilm on exoskeletal surfaces such as molluscan shells, crustacean exoskeletons and shell plates of polychaetes (Table 1). Most species with sturdy external surfaces carried such exoskeletal biofilms, but some of the crustacean and molluscan species (i.e. *Corophium volutator*, *Pagurus bernhardus* and *Litopenaeus vannamei*, *Macoma balthica*, *Scrobicularia plana*, *Cerastoderma edule*) did not.

Rate of N_2O emission. N_2O emission of the specimens was determined by incubating freshly collected, living animals (exception: *Litopenaeus vannamei*) in gas-tight vials with septa that allowed repeated sampling of the headspace for N_2O. The incubations were standardized regarding temperature (21°C) and oxygen (initially oxic headspace), since the main goal of the screening was to search for species-specific rather than environmental controls of N_2O emission. In many cases, the standardized conditions in the incubation vial were different from those in the natural habitat of the animals. Therefore, the N_2O emission rates measured with this approach represent potential rather than actual or *in situ* rates.

Incubation of animals was started after sampling, transport, and preparation of incubation vials, which

Table 1. List of taxa tested for N_2O emission with sampling details (temperature and nitrate concentration in the overlaying water column at the sampling site). Taxa are sorted by descending weight within each taxonomic group. Sampling sites—AB: Aarhus Bay; WS: Wadden Sea; AQ: aquaculture; Feeding types—C: carnivore; DF: deposit-feeder; FF: filter-feeder; G: grazer. Habitat—E: epifaunal; I: infaunal; EI: epifaunal + infaunal

Species	Site	Temp. (°C)	Nitrate (µM)	Wet weight (g)	Feeding type	Habitat	Exoskeletal biofilm
Ascidia							
Ascidia sp.	AB	16	0–4	7.18	FF	E	Yes
Crustacea							
Carcinus maenas	AB	15	0–4	2.95	C	E	Yes
Pagurus bernhardus	AB	7	0–4	2.81	C	E	No
Corophium volutator	WS	8	20	0.01	DF	EI	No
Echinodermata							
Echinocyamus pusillus	AB	7	0–4	0.71	DF	I	No
Echinocardium cordatum	AB	7	0–4	0.27	DF	I	No
Mollusca							
Scrobicularia plana	WS	15	20	4.63	DF	EI	No
Cerastoderma edule	WS	15	20	2.07	FF	EI	No
Mytilus edulis	AB	7	0–4	0.97	FF	E	Yes
Macoma balthica	WS	15	20	0.31	DF	EI	No
Polyplacophora[a]	AB	7	0–4	0.27	G	E	Yes
Littorina littorea	WS	22	20	2.22	G	E	Yes
Hinia reticulata	AB	7	0–4	1.70	C	EI	Yes
Gibbula sp.	AB	7	0–4	0.78	G	E	Yes
Hydrobia ulvae	WS	21	20	0.01	G	E	Yes
Polychaeta							
Arenicola marina	WS	8	20	2.06	DF	I	No
Lepidonotus squamatus	AB	7	0–4	0.49	C	E	Yes
Nephtys hombergii	WS	8	20	0.33	C	I	No
Nereis diversicolor	WS	8	20	0.15	DF	EI	No
Crustacea							
Litopenaeus vannamei	AQ	28–30	1000	21.16	DF	E	No

[a]Not determined to genus level

Table 2. Potential N_2O emission rates per g wet wt (mean ± SD for N ≥ 3; mean and range for N = 2) of the 20 tested species. N: number of replicates per species (ind. per incubation vial). Initial and highest N_2O concentration in the incubation vial

Species	N_2O emission (nmol g^{-1} h^{-1})	N (range)	N_2O conc. (nM) Initial	Highest
Ascidia sp.	0.043 ± 0.024	5 (1–4)	5.9	54
Carcinus maenas	0.369 ± 0.137	3 (1–3)	12.5	311
Pagurus bernhardus	0.020 ± 0.018	5 (1–3)	8.5	167
Corophium volutator	0.955 ± 0.664	2 (6–7)	10.2	123
Echinocyamus pusillus	0.040 ± 0.027	3 (1–3)	12.7	40
Echinocardium cordatum	0.069	1 (5)	12.2	20
Scrobicularia plana	0.302 ± 0.083	3 (2–3)	9.2	263
Cerastoderma edule	0.126	1 (5)	9.5	187
Mytilus edulis	0.269 ± 0.280	7 (1)	10.2	264
Macoma balthica	1.098 ± 1.066	7 (4–30)	9.8	287
Polyplacophora[a]	0.471 ± 0.237	2 (6)	12.5	465
Littorina littorea	0.237 ± 0.208	6 (5–15)	9.7	167
Hinia reticulata	0.608 ± 0.265	7 (1–3)	13.1	542
Gibbula sp.	0.107 ± 0.037	2 (2–4)	13.1	345
Hydrobia ulvae	5.449 ± 1.822	4 (25–50)	10.7	463
Arenicola marina	0.045 ± 0.032	3 (1–2)	11.3	55
Lepidonotus squamatus	0.666	1 (3)	12.5	466
Nephtys hombergii	0.082 ± 0.053	3 (1–2)	0.1	5.6
Nereis diversicolor	0.398 ± 0.319	9 (1–2)	11.7	21
Litopenaeus vannamei	0.183 ± 0.066	6 (1)	12.5	250

[a]Not determined to genus level

took from 3 to 5 h. Species were incubated in 3, 6, 10, or 100 ml sterile gas-tight vials, depending on the size and number of individuals. Most species were found in sufficient quantity to prepare several vials with different numbers of individuals (Table 2). Bivalves and ascidians were submerged in seawater to allow the individuals to be active and thereby exchange gases with the incubation vial. To the other species, only a small volume of seawater was added (0.05 to 2 ml) to maintain a moist atmosphere in the vials. Species from Aarhus Bay were supplied with 0.2 µm filtered seawater collected while sampling the animals; species from the intertidal flat were supplied with autoclaved seawater from the same site, collected during high tide and stored in an opaque tank until used for incubations. Animals were thus exposed to *in situ* nitrate and ammonium concentrations. The ammonium concentration in the incubation vials was initially below the detection limit of 0.5 µM and may have increased due to excretion of ammonium by the animals, which was in the range from 0.1 to 1.0 µmol $ind.^{-1}$ h^{-1} (I. M. Heisterkamp unpubl. data). The shrimp *Litopenaeus vannamei* were killed in ice-water before incubating them in 100 ml bottles with 2 ml of 0.2 µm filtered aquarium water that contained 1 mM nitrate and 14 µM ammonium. Additionally, dissected guts of *L. vannamei* were incubated in 3 ml exetainers (Labco) supplied with 50 µl of 0.2 µm filtered aquarium water.

Animals were cleaned of loosely attached sediment and algal tufts by washing them in autoclaved seawater and drying them on paper tissue; the tightly attached biofilms largely remained on the external surface of the animals. To explicitly test for effects of this exoskeletal biofilm on the N_2O emission potential, the snail *Hinia reticulata* was incubated both with biofilm-covered shells and with shells that were cleaned by thoroughly brushing them with a sterile toothbrush, although cleaning still left residues of biofilm in the grooves of the shell surface.

The accumulation of N_2O in the incubation vial was followed over a period of 4 to 6 h by regularly taking gas samples and analyzing them by gas chromatography. Samples from the Wadden Sea were measured with the GC 7890 (Agilent Technologies) with a CP-PoraPLOT Q column, and samples from Aarhus Bay with the GC-8A (Shimadzu) with a Porapak Q column. Both gas chromatographs were equipped with a ^{63}Ni electron capture detector. Injection volumes were 1 ml for the samples analyzed with the GC 7890 and 0.3 ml for samples analyzed with the GC-8A. After each headspace sampling, the incubation vials were pressure-equilibrated with air by inserting a hypodermic needle through the septum for 1 s. On both GCs, calibration standards were prepared by adding known amounts of N_2O to N_2-flushed gas-tight bottles of known volume and analyzed repeatedly during the incubation. The linear part of the increase of the N_2O concentration in the incubation vials over time was used to calculate the potential N_2O emission rate per individual and per biomass. The dilution of the gas phase and the equilibrated distribution of N_2O between the gas and water phases (Weiss & Price 1980) were taken into account when calculating the potential N_2O emission rate. This rate corresponds to the net N_2O production rate (i.e. gross production less consumption) and thus also depends on N_2O levels. Since the N_2O reduction rate was not directly assessed, the initial and final N_2O concentrations in the incubation vials are reported in Table 2 so that the experiments can be reproduced.

Rate of total denitrification. To determine the potential rate of total denitrification (i.e. production of N_2 + N_2O) in the shrimp gut, freshly killed *Litopenaeus vannamei* were dissected and the guts were incubated in an atmosphere of 10% acetylene and 90% dinitrogen

Table 3. Species traits and phenotypes used for statistical analysis of N_2O emission by marine invertebrates. Phenotypes were sorted according to their hypothesized promotion of N_2O production (Hypothesis) and then numerically coded (Value)

Species trait Phenotype	Hypothesis	Value
Feeding type	Increasing number of N_2O-producing gut bacteria	
Carnivore (predator + scavenger)		0
Grazer		1
Deposit-feeder		2
Filter-feeder		3
Habitat	Increasing nitrate availability	
Infaunal		0
Infaunal + epifaunal		1
Epifaunal		2
Exoskeletal biofilm	More biofilm bacteria	
No		0
Yes		1

gas. Acetylene inhibits the last step of denitrification (Sørensen 1978), and thus the accumulation of N_2O in the incubation vials is indicative of total denitrification. The linear increase of N_2O concentration in the incubation vials over time was used to calculate the potential total denitrification rate per gut.

Statistical analysis. The potential N_2O emission rates were tested for correlation with the species traits Feeding type, Habitat, Exoskeletal biofilm, and Weight using the statistical analysis software SPSS. The categories within the species traits Feeding type, Habitat, and Exoskeletal biofilm were ranked according to their hypothesized effects on N_2O emission rates and were transformed into a numerical code for correlation analysis (Table 3). The hypotheses were that the rate of N_2O emission is positively correlated to (1) the amount of ingested bacteria, (2) the availability of nitrate, and (3) the presence of a microbial biofilm growing on the external surfaces of the animal. The ranking of the categories was based on the assumptions that (1) the amount of ingested bacteria is determined by the feeding type and increases from carnivores over grazers and deposit-feeders to filter-feeders; (2) the nitrate concentration varies with habitat, being highest in the water column and lowest in the sediment; and (3) the shell and exoskeleton provide colonization surfaces for microbial biofilms. The high rank of filter-feeders regarding the amount of ingested bacteria may be questioned because only a few bivalve species filter unattached bacteria (e.g. *Mytilus edulis*; McHenery & Birkbeck 1985). However, species that filter-feed close to the sediment surface, where the concentration of suspended detritus is particularly high, ingest large amounts of attached bacteria (Kach & Ward 2008).

RESULTS

The potential N_2O emission rates of coastal marine invertebrate species ranged from 0 to 1.354 nmol ind.$^{-1}$ h^{-1} (Fig. 1), with an average rate of 0.320 nmol ind.$^{-1}$ h^{-1}. The weight-specific emission rates ranged from 0 to 5.448 nmol g^{-1} h^{-1}, with an average rate of 0.598 nmol g^{-1} h^{-1} (Table 2). The highest potential N_2O emission rate of 3.569 nmol ind.$^{-1}$ h^{-1} was found for the aquacultured shrimp *Litopenaeus vannamei* (not included in the above rates) that is exposed to very high nitrate concentrations (≥1 mM) and to high temperatures (28 to 30°C) in the rearing tanks (Table 1). The N_2O emission rate of dissected guts of *L. vannamei* was almost as high as the N_2O emission rate of the whole animal (Fig. 2). Dissected guts showed a total denitrification rate of 12 nmol ind.$^{-1}$ h^{-1} under anoxic conditions (Fig. 2).

The nitrate concentrations at the sampling sites in the Wadden Sea and Aarhus Bay were low (0 to 20 µM), and temperature was 7 to 8°C (exception: 15 to 22°C at the Wadden Sea site in May 2008; Table 1). The capacity to emit N_2O occurred across all taxonomic groups and was not restricted to a certain feeding type (Table 1). Most species possessing a shell or exoskeleton had potential N_2O emission rates higher than the average rates (e.g. the common periwinkle *Littorina littorea* and the shore crab *Carcinus maenas*). These conclusions were also true when the rate of N_2O emission was expressed per gram body weight (Table 2). The potential N_2O emission rates per individual tended to be higher for larger species than for smaller species (e.g. the bivalves *Scrobicularia plana* vs. *Macoma balthica*), while the highest potential N_2O emission rates per gram body weight were shown by the smallest species (e.g. *Hydrobia ulvae*, *Corophium volutator*).

The correlation analysis revealed that the potential N_2O emission rate per individual was positively correlated with the body weight with a Pearson coefficient of $R = 0.506$ ($p = 0.027$) for linear correlation and with a Spearman coefficient of $R = 0.728$ ($p < 0.001$) for non-linear correlation. The species traits Habitat and Exoskeletal biofilm showed positive non-linear correlations with the potential N_2O emission rate per individual with Spearman coefficients of $R = 0.460$ ($p = 0.047$) and $R = 0.481$ ($p = 0.037$), respectively. No correlation between the potential N_2O emission rate and the feeding type was found (Spearman coefficient of $R = -0.135$, $p = 0.581$).

The importance of the species trait Exoskeletal biofilm was further highlighted by the comparison of the N_2O emission rates of the snail *Hinia reticulata*, which were measured both with the natural biofilm on the surface of the shell and with cleaned shell surfaces. The snails with an exoskeletal biofilm emitted more N_2O than the cleaned individuals during the incu-

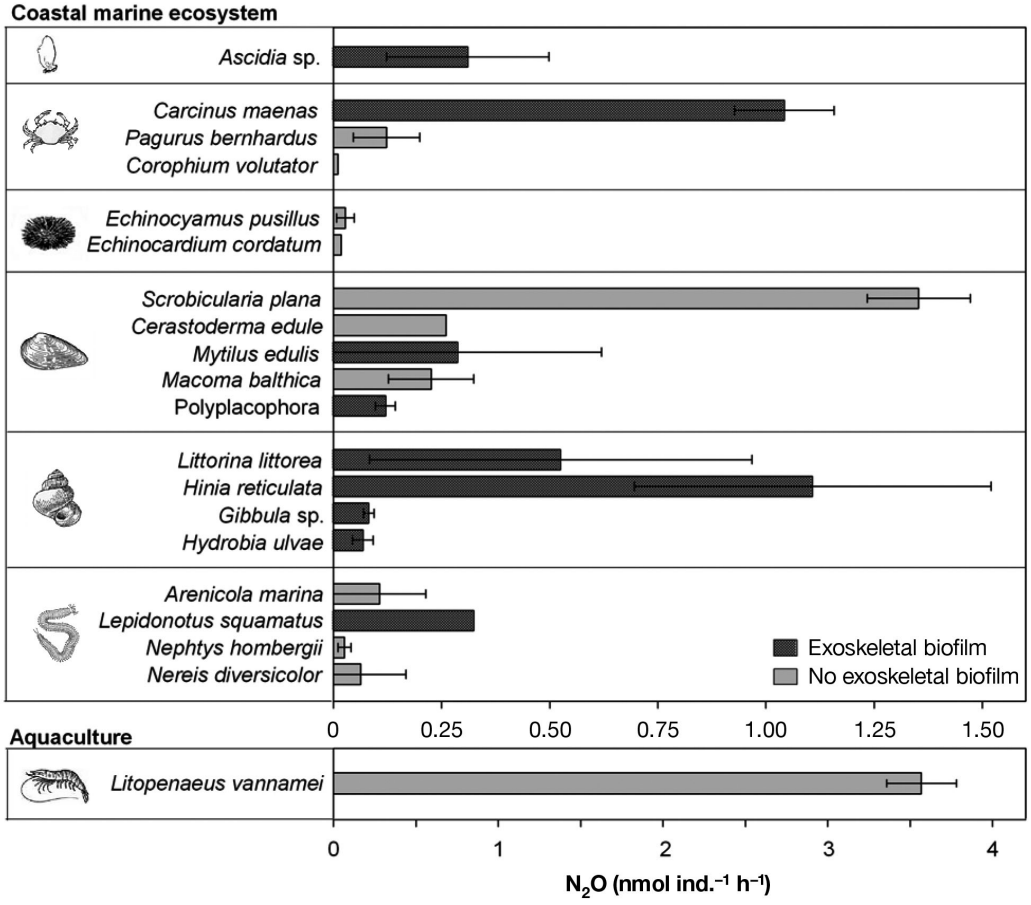

Fig. 1. Potential N_2O emission rates of various marine invertebrate taxa. Individuals were incubated in gas-tight vials under oxic conditions at 21°C, and N_2O emission was analyzed by gas chromatograph measurements over 4 to 6 h. Species are sorted by descending weight within each taxonomic group. Data are mean ± SD for species with at least 3 replicates analyzed

bation period of 4.5 h (Fig. 3). The mean potential N_2O emission rate of the biofilm-covered individuals (1.108 nmol ind.$^{-1}$ h^{-1}) was about 3.5 times higher than the rate of the cleaned individuals (0.306 nmol ind.$^{-1}$ h^{-1}). The mean potential N_2O emission rates of biofilm-covered and cleaned individuals were assessed by a *t*-test and marginally failed significance with p = 0.057 (t = –3.06; df = 2.92).

DISCUSSION

Nitrous oxide emission potential

The present study revealed that many coastal marine invertebrate species emit N_2O, representing a source that has been overlooked. The average potential N_2O emission rate of 19 marine invertebrate species was 0.320 nmol ind.$^{-1}$ h^{-1}, excluding the aquacultured shrimp *Litopenaeus vannamei*, which had an exceptionally high rate. For 20 freshwater invertebrate species, an average potential N_2O emission rate of only 0.072 nmol ind.$^{-1}$ h^{-1} was reported (Stief et al. 2009). In addition to the higher average rate, the N_2O emission potential of marine invertebrates is apparently influenced by species-specific traits (i.e. body weight, habitat, and presence of an exoskeletal biofilm) that differ from those that influence the N_2O emission potential of freshwater species (i.e. feeding type) (Stief et al. 2009).

Correlation with species traits

At a first glance, the positive correlation with body weight suggests that larger animals with presumably larger guts produce more N_2O than smaller animals because of the larger number of microbes passing through their gut. This interpretation is consistent with the hypothesis that, in marine invertebrates, N_2O production is also mediated by ingested microbes, as is the case for earthworms and freshwater invertebrates (Drake et al. 2006, Stief et al. 2009).

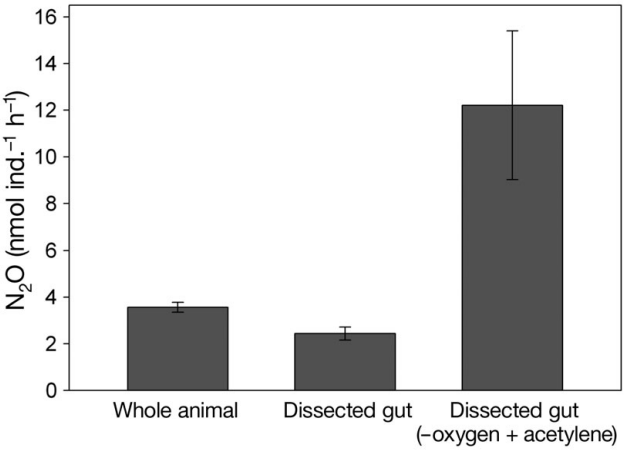

Fig. 2. *Litopenaeus vannamei*. Potential N_2O emission rates (mean ± SD; n = 3 to 6) of the shrimp *L. vannamei* and its dissected guts under oxic conditions at 21°C. Dissected guts of *L. vannamei* were also incubated under anoxic conditions with 10% acetylene, which inhibits the last step of denitrification. The resulting N_2O production indicates total denitrification

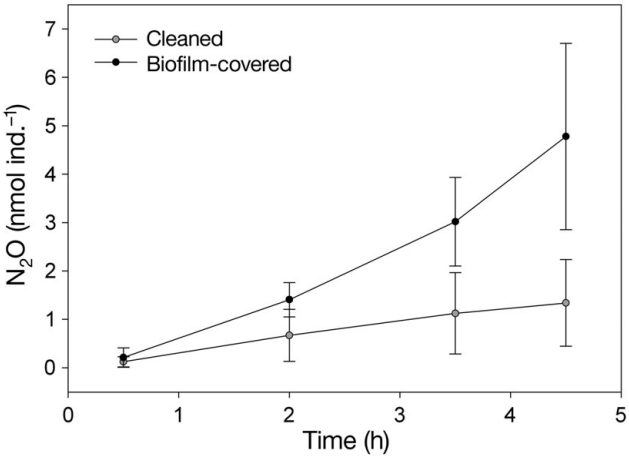

Fig. 3. *Hinia reticulata*. Potential N_2O emission (mean ± SD; n = 3) by cleaned and biofilm-covered individuals of the snail *H. reticulata* during the incubation period of 4.5 h

The correlation between potential N_2O emission rate and the presence of an exoskeletal biofilm suggests that N_2O production associated with marine invertebrates is not always due to denitrification in the gut (as proven for the shrimp *Litopenaeus vannamei*), but may also result from microbial activity on the external surfaces of the animal. Lower potential N_2O emission rates of individuals of the snail *Hinia reticulata* with an experimentally cleaned shell surface further substantiate that N_2O production is also linked to microbial activity in the exoskeletal biofilm. Furthermore, for this type of animal-associated N_2O production, the shell of larger animals is presumably colonized with larger numbers of bacteria involved in N_2O production, which is in line with the weight-dependence of N_2O emission. The microbial pathway for biofilm-associated N_2O production still needs to be identified. Depending on the oxygen availability inside the biofilm, nitrification or denitrification or both might contribute to the production of N_2O (Meyer et al. 2008). Likewise, N_2O production in the exoskeletal biofilm might be driven by ammonium from animal excretion or by nitrate from the water column, or by both. If an oxic–anoxic transition zone prevails in the biofilm, then nitrification and denitrification are probably coupled, as known for sediments in which denitrification is driven by nitrate from nitrification (Jenkins & Kemp 1984). Thick biofilms were not established on the exoskeleton of every molluscan and crustacean species tested in the present study. The exoskeleton of *Corophium volutator*, *Pagurus bernhardus*, and *L. vannamei*, for instance, may not allow the formation of a persistent biofilm due to rather short time intervals between molting events, and the shells of infaunal molluscs (i.e. *Macoma balthica*, *Cerastoderma edule*, and *Scrobicularia plana*) may not be suitable for the formation of an exoskeletal biofilm due to physical abrasion in the sediment. It remains to be investigated whether certain freshwater invertebrate species have persistent biofilms on external surfaces of their body that produce N_2O.

Habitat (a proxy for nitrate availability in the immediate environment of the animal) was also significantly correlated with the N_2O emission rate. The high potential emission rate of the epifaunal shrimp *Litopenaeus vannamei*, which is exposed to very high nitrate concentrations, agrees with this assumption. The effect of the habitat on N_2O emission could be greater during autumn and winter, when nitrate concentrations in the water column at the 2 study sites are higher than in spring and summer (Kieskamp et al. 1991, Sømod 2005) and most of the animals studied are abundant and active.

N_2O emission rate and species feeding type and diet were not correlated, which contrasts with the finding that N_2O emission of freshwater invertebrates is diet-dependent (Stief et al. 2009). Since marine species are usually larger and have longer guts and gut residence times than freshwater species (Bayne et al. 1987, Navarro et al. 1993), bacteria might be exposed long enough to anoxic conditions in the gut to express the full set of denitrification genes. In that case, complete denitrification will prevail and the main product will be dinitrogen rather than N_2O. Conversely, many of the ingested sediment bacteria might be efficiently digested in the gut of marine detritivorous species due to a high lysozyme activity (Plante & Mayer 1994,

Lucas & Bertru 1997), which inhibits microbial N_2O production. Lysozyme activity of dissected guts was approximately 5 times higher for the ragworm *Nereis diversicolor* (a marine non-emitter) than for the mayfly larva *Ephemera danica* (a freshwater emitter) (P. Stief unpubl. data).

Ecosystem perspective

Many species that tested positive for N_2O emission in the present study are very abundant in coastal soft-bottom habitats; *Macoma balthica* and *Cerastoderma edule* can reach densities of 1000 ind. m^{-2} (Fujii 2007), *Scrobicularia plana*, 250 ind. m^{-2} (Cabral & Murta 2004), and *Arenicola marina*, 100 ind. m^{-2} (Flach & Beukema 1994). The mud snail *Hydrobia ulvae* can reach densities of up to 100 000 ind. m^{-2} in intertidal sediments (Barnes 1999). This epifaunal species emits N_2O directly into the water column or the atmosphere without diffusion through the sediment, as it lives at the sediment surface where it can be exposed to high nitrate concentrations and temperatures. Taking its potential N_2O emission rate of 0.068 nmol ind.$^{-1}$ h^{-1}, this small snail could emit 6.8 µmol N_2O m^{-2} h^{-1}, which is on the same order of magnitude as the benthic N_2O fluxes reported for estuarine intertidal sediments (Middelburg et al. 1995) and intertidal rocky biofilms (Magalhaes et al. 2005).

For infaunal species, extrapolations are less robust because N_2O conversion may take place inside the burrows of animals (Stief & Schramm 2010). N_2O produced by certain infaunal species is partially consumed while diffusing towards the sediment surface (Meyer et al. 2008), whereas other infaunal species increase benthic N_2O flux more by their bioirrigation activity than by stimulating N_2O production in their gut or in exoskeletal biofilms (Stief & Schramm 2010). A second difficulty in scaling up animal-associated N_2O production to ecosystem level lies in the discrepancy between potential and *in situ* rates. The contribution of animal-associated N_2O production to overall benthic N_2O emission can be better estimated from rate measurements made at different times of the year at the prevailing environmental conditions (Stief et al. 2010, Stief & Schramm 2010). A rather constant N_2O emission rate can be expected for the aquacultured species *Litopenaeus vannamei*, since it is exposed to the same conditions throughout the year. Given its very high potential N_2O emission rate and the high growth rates of the aquaculture industry, N_2O emission by other cultured species should be investigated.

Conceptually, N_2O production associated with marine and freshwater invertebrates constitutes a link between reactive nitrogen (i.e. nitrate and ammonium) in aquatic ecosystems and N_2O in the atmosphere that has been overlooked. Aquatic invertebrates complement the known sites of N_2O production in the sediment with 3 additional microsites of N_2O production: (1) the anoxic gut, a transient microbial habitat in which denitrification prevails (Stief et al. 2009); (2) the burrow, a microbial habitat with fluctuating conditions in which nitrification and denitrification co-occur (Svensson 1998); and (3) the exoskeletal biofilm, a microbial habitat with a yet unknown microenvironment in which nitrification and/or denitrification may occur (present study). The environmental controls of sedimentary and animal-associated N_2O production may be similar (e.g. higher N_2O production rates at higher temperature and nitrate or ammonium concentrations) and require further investigation.

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