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In Vivo Emission of Dinitrogen by Earthworms via Denitrifying Bacteria in the Gut

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Earthworms emit the greenhouse gas nitrous oxide (N_2O), and ingested denitrifiers in the gut appear to be the main source of this N_2O. The primary goal of this study was to determine if earthworms also emit dinitrogen (N_2), the end product of complete denitrification. When [^{15}N]nitrate was injected into the gut, the earthworms *Aporrectodea caliginosa* and *Lumbricus terrestris* emitted labeled N_2 (and also labeled N_2O) under in vivo conditions; emission of N_2 by these two earthworms was relatively linear and approximated 1.2 and 6.6 nmol N_2 per h per g (fresh weight), respectively. Isolated gut contents also produced [^{15}N]nitrate-derived N_2 and N_2O under anoxic conditions. N_2 is formed by N_2O reductase, and acetylene, an inhibitor of this enzyme, inhibited the emission of [^{15}N]nitrate-derived N_2 by living earthworms. Standard gas chromatographic analysis demonstrated that the amount of N_2O emitted was relatively linear during initial incubation periods and increased in response to acetylene. The calculated rates for the native emissions of N_2 (i.e., without added nitrate) by *A. caliginosa* and *L. terrestris* were 1.1 and 1.5 nmol N_2 per h per g (fresh weight), respectively; these emission rates approximated that of N_2O. These collective observations indicate that (i) earthworms emit N_2 concomitant with the emission of N_2O via the in situ activity of denitrifying bacteria in the gut and (ii) N_2O is quantitatively an important denitrification-derived end product under in situ conditions.

Earthworms occur in diverse terrestrial habitats, may have densities of up to 2,000 individuals per square meter of soil, and represent the dominant soil fauna (9, 24, 25). Earthworms are divided into three feeding guilds. (i) Epigeic species (e.g., *Lumbricus rubellus*) live above the mineral soil and feed preferentially on litter. (ii) Endogeic species (e.g., *Aporrectodea caliginosa*) live in the upper zone of the mineral soil, ingest high amounts of mineral soil, and feed in the rhizosphere. (iii) Anecic species (e.g., *Lumbricus terrestris*) live in deeper zones of the mineral soil, ingest medium amounts of soil, and feed on litter that they drag into their burrows (3, 7). Earthworms contribute to the aeration, pore volume, water-holding capacity, nutrient availability, and mineralization rates of soils by virtue of their feeding and burrowing habits (9, 22, 24, 25). Casts (i.e., the fecal discharge) of diverse earthworms contain higher concentrations of nitrate, ammonium, and organic carbon than does the surrounding soil (10, 13, 31, 32, 40, 41, 49). Earthworms thus contribute to the cycling of elements in terrestrial ecosystems.

Terrestrial habitats are responsible for approximately 70% of the greenhouse gas nitrous oxide (N_2O) that is produced globally (5, 6), and the ecophysiological activities of earthworms include the emission of this gas (19, 27). This activity can be important at the local level; up to 56% of the in situ emission of N_2O from certain soils might be earthworm derived (2, 19, 27) and might yield 3×10^8 kg N_2O globally each year (8). The N_2O that is emitted by earthworms originates in the anoxic core of the earthworm gut, and emission of N_2O is likely due to the in situ activities of ingested nitrate- and nitrite-reducing bacteria that are activated during gut passage (14, 16). Denitrification appears to be the primary N_2O-producing process in the earthworm gut (14). Dinitrogen (N_2) is the terminal product of complete denitrification and is formed by *nosZ*-encoded N_2O reductase (56). However, the in vivo emission of N_2 by earthworms has thus far not been assessed. The emission of N_2 by earthworms is highly probable, based on their capacity to emit N_2O (19, 27) and the occurrence of a large number of phylogenetically diverse *nosZ* sequences in earthworm gut contents (15). In the present study, [^{15}N]nitrate and mass spectrometry were utilized to determine if earthworms emit N_2 by gut-associated microbial processes and to likewise determine if N_2 is the dominant denitrification-derived gas that is emitted.

MATERIALS AND METHODS

Collection, storage, and N_2O emission status of earthworms. Specimens of *A. caliginosa* and *L. terrestris* were collected in late summer and early autumn 2004 from the meadow Hofmanns Wiese in Bayreuth, Germany (14). Worms were identified by standard protocols (4) and stored in the dark in soil at 5°C until used; worms were utilized within 1 week of collection. As in earlier studies (14, 16, 19, 27), most of the earthworms collected emitted N_2O. In a random sampling of 50 specimens, 2 emitted <0.05 nmol N_2O per h per g (fresh weight), and the average rate of emission was 1.5 nmol N_2O per h per g (fresh weight) The maximum rate observed was 11.1 nmol N_2O per h per g (fresh weight).

^{15}N experiments with living earthworms. Earthworms were washed with water, dried slightly by being blotted with tissue paper, weighed, and then given three 50 μl injections of 20 mM sodium nitrate (10% ^{15}N) into the gut; the three injections were along the first two-thirds of the gut. Three such earthworms having a collective fresh weight of approximately 3 g were placed in a 10-ml serum vial. The vial was crimp sealed, and the gas phase was adjusted to He:O_2 (80:20) (200-kPa overpressure). Vials were incubated at room temperature (21°C) in the dark, and the gas phase was analyzed periodically. The amount of [^{15}N]nitrate injected and the number of worms per vial could theoretically yield a maximum of 450 nmol of [^{15}N]N_2 per vial; this experimental design was necessitated because of the detection limits of the mass spectrometry analysis. For experiments with nitrite, earthworms received three 10-μl injections of 20

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mM sodium nitrite (10% ^{15}N) along the first two-thirds of the gut (note: the amount of nitrite injected was one-fifth that of nitrate, to avoid the potentially toxic effects of nitrite).

^{15}N experiments with gut contents. Gut contents were pressed out of washed earthworms (14). The gut contents (approximately 0.4 g [wet weight]) of three worms having a collective fresh weight of approximately 3 g were placed in a 10-ml serum vial and supplemented with 450 μl of 20 mM sodium nitrate (10% ^{15}N). The vial was crimp sealed, and the gas phase was adjusted to 100% He (200-kPa overpressure); vials were incubated as described above.

Acetylene experiments. Serum vials containing earthworms were prepared as outlined above. The He:O_2 gas phase of the serum vials was modified to 15% (vol/vol) acetylene, and the effect of acetylene on the production of ^{15}N-labeled products was determined.

Earthworms were also evaluated for the effects of acetylene on the production of N_2O by standard gas chromatography. Washed earthworms were incubated in crimp-sealed vials (unless otherwise indicated, one worm, which was approximately 1 g [fresh weight], per 10-ml vial); the gas phase was air or air supplemented with acetylene (15% [vol/vol]) (50-kPa overpressure; the overpressure was less than that used in the ^{15}N experiments, because the amount of gas sampled was only 0.2 ml). In some cases, earthworms were given a single 10-μl injection of 10 mM sodium nitrate into the gut behind the clitellum.

Comparative emission rates for the gut contents and gut wall were obtained from microcosms prepared as previously described (16). Supplemental acetylene was 1% (vol/vol) in the headspace, and values are means of three replicates incubated for 20 h.

Analytical methods. The concentrations and the isotopic compositions ($^{15}N/^{14}N$ ratio) of gaseous N_2 and N_2O were analyzed with a GC-C-IRMS system (Thermo Electron, Bremen, Germany) consisting of a Hewlett-Packard Co. 6890 gas chromatograph (Agilent, Palo Alto, CA) and a GC/C III combustion and reduction system (Thermo Electron, Bremen, Germany) coupled via open split to a Mat 253 mass spectrometer (Thermo Electron, Bremen, Germany). Two-milliliter gaseous samples were taken with gas-tight syringes prepurged with He and injected into a split-splitless injector (1:1 ratio). Gases were separated on a Poraplot Q-FS capillary column (25 m plus 2.5 m precolumn by 0.32 mm by 10 μm; Chrompack, Darmstadt, Germany) with He (carrier gas) flow set at 1.5 ml per min. The oven was isothermic at 40°C. The system was calibrated with certified reference gases (Linde, Lübeck, Germany), and the concentrations were measured with relative standard deviations of <3.5%. The detection limits were <10 nmol for N_2O and <5 nmol for N_2.

The isotopic compositions were determined by calculating the ratios of the masses 28 and 29 with relative standard deviations of <0.5% after the instrument was calibrated with N_2 reference gas (purity was 99.995%; Linde). This reference gas had an N-isotopic composition of 0.366 ^{15}N atoms, which is nearly identical to that of air. The isotopic ratios were measured as the percentage of atoms and calculated to the number of moles [^{15}N]; the preciseness of the isotope ratio measurement was $< 2 \times 10^{-4}$ atom %. The resulting detection limits for [^{15}N]N_2 and [^{15}N]N_2O were <1 nmol ^{15}N excess, which corresponded to approximately 1/900 and 1/450, respectively, of the ^{15}N applied. Although mass 30 was not used for calculations (this was not possible, due to the small amount of this mass in the experiments), mass 30 was used for testing the consistency of the data and assessing nonstatistical distribution of the isotopomers.

[^{15}N]$NaNO_3$ and [^{15}N]$NaNO_2$ were obtained from Cambridge Isotope Laboratories (CIL International, Promochem, Wesel, Germany) with an isotope enrichment of >98% ^{15}N. A Hewlett-Packard Co. 5980 series II gas chromatograph equipped with an electron capture detector was used for the analysis of N_2O (14, 19).

RESULTS

Production of [^{15}N]N_2 from [^{15}N]nitrate by earthworms and gut contents. Specimens of *A. caliginosa* and *L. terrestris* emitted labeled N_2 when [^{15}N]nitrate was injected into the gut; the emission of labeled N_2 was concomitant to the emission of labeled N_2O and continued for several hours postinjection (Fig. 1). Although the relative amounts of [^{15}N]nitrate-derived N_2 and [^{15}N]nitrate-derived N_2O varied from one experiment to the next, the amount of [^{15}N]nitrate-derived N_2O tended to be slightly greater than that of [^{15}N]nitrate-derived N_2 (Table 1, experiments A to C). Earthworms injected with [^{15}N]nitrite, an intermediate in denitrification (56), also emitted labeled N_2 and N_2O (data not shown). The gut of the earthworm is anoxic

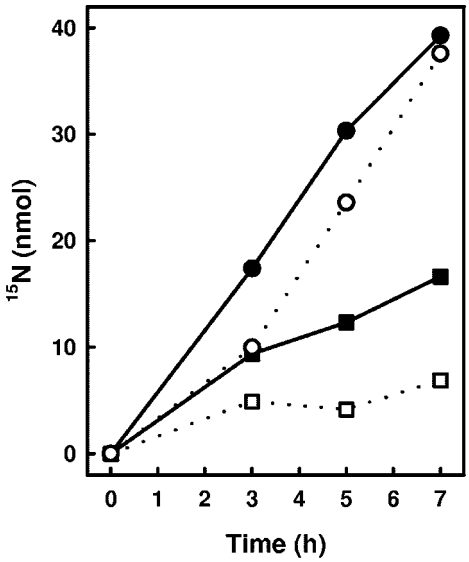

FIG. 1. In vivo emission of [^{15}N]N_2O (●, ■) and [^{15}N]N_2 (○, □) by *L. terrestris* (●, ○) and *A. caliginosa* (■, □) following the injection of [^{15}N]nitrate into the gut. Values at 0 h are from control vials that lacked earthworms. Values are means of duplicate experiments.

(14), and isolated gut contents produced [^{15}N]nitrate-derived N_2 and N_2O under anoxic conditions (Table 1, experiment D). This result indicated that the emission of [^{15}N]nitrate-derived gases by living earthworms was linked to microbial processes in the anoxic gut. Neither [^{15}N]N_2 nor [^{15}N]N_2O was detected in [^{15}N]nitrate-supplemented control vials that lacked earthworms.

Acetylene enhances the production of [^{15}N]nitrate-derived N_2O. Acetylene is an inhibitor of N_2O reductase, the N_2-forming terminal oxidoreductase in complete denitrification (54, 56). The emission of [^{15}N]nitrate-derived N_2 by living earthworms was not observed when they were incubated in the presence of acetylene. For example, in a 12-h incubation, *A. caliginosa* formed 0 and 23 nmol [^{15}N]nitrate-derived N_2 and N_2O per g (fresh weight), respectively, in the presence of acetylene. The emission of [^{15}N]nitrate-derived N_2 was also inhibited when earthworms were exposed to acetylene after several hours of emitting [^{15}N]nitrate-derived N_2 (Table 1, experiment C).

Acetylene enhances the production of N_2O. The results above demonstrated that living earthworms emit N_2. However, because these experiments necessitated the use of gut supplements (i.e., the injection of labeled nitrate into the gut), the results did not provide information on the status of natural emissions. The acetylene block technique and standard gas chromatographic analysis were used to circumvent this problem and verify the in vivo emission of N_2.

Exposing earthworms to acetylene increased the amount of N_2O they emitted (Fig. 2). Injection of 100 nmol of sodium nitrate into the gut of earthworms resulted in a slight increase in the amount of N_2O emitted (Fig. 3), a result that was consistent with the emission of [^{15}N]N_2 from injected [^{15}N]nitrate (above). The emission of N_2O was relatively linear during initial incubation periods both with and without acetylene, and the rate at which N_2O was emitted was enhanced when earth-

TABLE 1. Emission of [^{15}N]nitrate-derived [^{15}N]N_2 and [^{15}N]N_2O by living earthworms (experiments A to C) and isolated earthworm gut contents (experiment D)

Expt	Species	Time (h)	N_2[a]		N_2O[a]		$^{15}N_{N_2}$ / ($^{15}N_{N_2}$ + $^{15}N_{N_2O}$) (%)[b]
			^{15}N atom (% excess)	N mol ^{15}N g_{FW}^{-1}	^{15}N atom (% excess)	N mol ^{15}N g_{FW}^{-1}	
A	*A. caliginosa*	19	0.37	32.1	13.46	42.3	43
	L. terrestris	18	3.77	62.5	5.32	30.5	67
B	*A. caliginosa*	4	0.04	0.6	3.80	1.6	26
		6	0.06	1.1	3.86	2.8	29
		17	0.23	4.9	6.62	10.3	32
	L. terrestris	4	0.47	2.3	7.48	5.0	32
		6	1.10	8.2	7.31	9.1	47
		18	3.88	53.5	4.51	21.7	71
C	*A. caliginosa*	3	0.04	1.4	7.76	2.7	34
		5	0.03	1.2	6.22	3.6	25
		7	0.04	2.0	6.24	4.8	29
		19 (C_2H_2 at 7 h)[c]	0.01	1.4	8.38	18.7	7
	L. terrestris	3	0.32	2.3	6.60	4.0	36
		5	0.62	5.4	7.16	7.0	44
		7	0.95	8.6	7.17	9.0	49
		27 (C_2H_2 at 7 h)[c]	0.10	9.0	8.88	71.1	11
D[d]	*A. caliginosa*	4	0.08	0.8 (5)	8.10	4.0 (27)	16
	L. terrestris	4	0.29	1.0 (9)	7.32	3.8 (31)	22

[a] g_{FW}, grams (fresh weight).
[b] Values have been rounded to the nearest percentage point.
[c] Acetylene was injected into the gas phase to a concentration of 15% (vol/vol) at 7 h.
[d] Isolated gut contents. Parenthetical values are nmol ^{15}N per g (wet weight) of gut contents. Values are the means of two replicate incubations.

worms were exposed to acetylene after a period of actively emitting N_2O (Fig. 4). The relative linearity of emission suggested that denitrification in the gut was ongoing from the onset of incubation. These results provided additional evidence that earthworms emit N_2 and thus corroborated the results obtained in the ^{15}N-labeling experiments. They also extended the previous observation that earthworms bathed in nitrate solution emitted more N_2O when exposed to acetylene (27).

Localization of N_2 production in the gut. Microcosms prepared from gut contents and gut wall from *A. caliginosa* produced 66 ± 26 and 8 ± 2 nmol N_2O per g (fresh weight), respectively, under conditions that simulated the in situ conditions of the gut interior (14). Acetylene-supplemented gut contents and gut wall microcosms under such conditions yielded 92 ± 33 and 5 ± 3 nmol N_2O per g (fresh weight), respectively. Thus, gut wall-associated microbiota did not appear to be a significant origin of N_2.

N_2 emission rates. *A. caliginosa* and *L. terrestris* emitted 1.2 and 6.6 nmol nitrate-derived N_2 per h per g (fresh weight), respectively, during initial incubation periods in the ^{15}N-labeling experiments (these values are averages and are based on the amounts of N_2 formed during the first 6 and 7 h of incubation in experiments B and C [Table 1], respectively, and take into account the fact that the sodium nitrate injected into the gut was 10% ^{15}N). Based on the assumption that the acetylene-dependent increase in emitted N_2O is equal to the N_2 emitted without acetylene, the calculated rates for the native emissions of N_2 (i.e., without gut supplement) by *A. caliginosa* and *L. terrestris* approximated 1.1 and 1.5 nmol N_2 per h per g (fresh weight), respectively (Fig. 2). Earthworms that did not receive a gut injection of supplemental nitrate emitted, on average, 1.5 (±2.1) nmol N_2O per h per g (fresh weight) (data are from 50 specimens) in the absence of acetylene. Thus, the amounts of N_2 and N_2O emitted by living earthworms appeared to be similar. The acetylene method underestimates the amount of N_2 formed by denitrification because acetylene may not totally block N_2O reductase (45); thus, the amounts of N_2 estimated with this method in the present study should be regarded as minimum estimates. In contrast to the apparent coemission of similar amounts of N_2 and N_2O by living earthworms, N_2O was the dominant [^{15}N]nitrate-derived product of isolated gut contents (Table 1, experiment D), suggesting that the physical disruption of the gut decreased the relative amounts of N_2 formed by denitrifiers in gut contents.

DISCUSSION

The results of the present study demonstrate that earthworms emit N_2. The emission of [^{15}N]nitrate-derived N_2 by these invertebrates is consistent with previous results that identified denitrification as an ongoing process in the earthworm gut (14, 27). That the *nosZ* sequences detected in gut contents are phylogenetically nearly identical to those detected in soil (15) supports the hypothesis that the N_2 emitted by earthworms is primarily derived from ingested soil denitrifiers during gut passage.

The results of this study indicate that complete denitrification occurs in the earthworm gut. The expression of *nosZ*, and therefore the reduction of N_2O to N_2, is under regulatory control and is influenced by anaerobiosis and the availability of substrates (1, 28, 56). The feeding habits of earthworms might have an effect on the in situ conditions of their gut and therefore influence the amounts of N_2 that they emit. When supplemental nitrate was injected into the gut, the litter feeder *L. terrestris* tended to emit higher amounts of N_2 than did the soil-rhizosphere feeder *A. caliginosa*. However, differences in the calculated native emission of N_2 between these worm species were minor. This observation is consistent with studies that

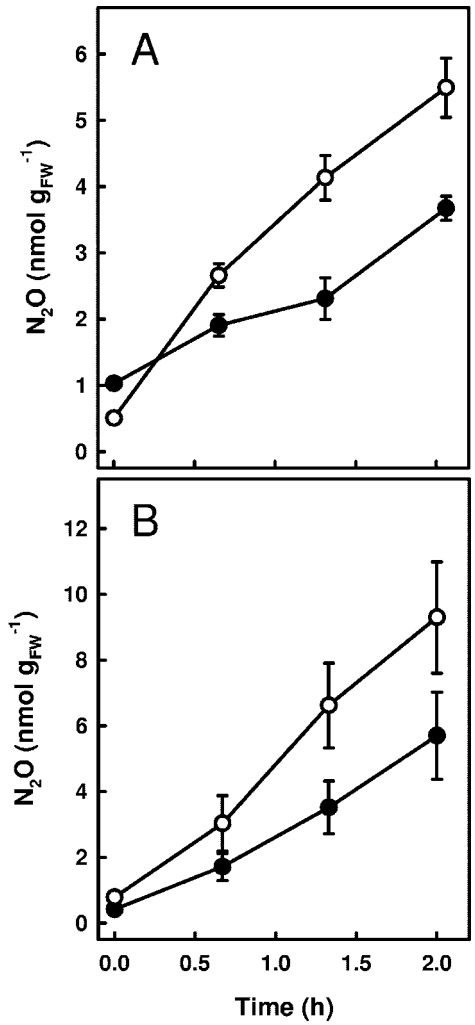

FIG. 2. In vivo emission of N_2O by *A. caliginosa* (A) and *L. terrestris* (B). Symbols: ●, vials without acetylene; ○, vials with acetylene. Bars indicate the range of values obtained from four replicate vials, each containing four earthworms. Abbreviation: g_{FW}, g (fresh weight).

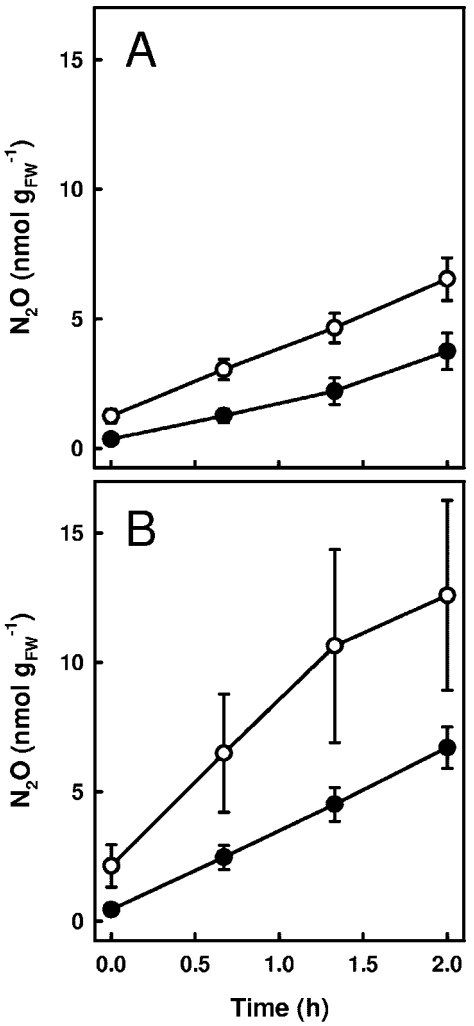

FIG. 3. In vivo emission of N_2O by *L. terrestris* without gut supplement (A) or following the injection of 100 nmol sodium nitrate into the gut (B). Symbols: ●, vials without acetylene; ○, vials with acetylene. Bars indicate the range of values obtained from four replicates. Abbreviation: g_{FW}, g (fresh weight).

indicated the feeding guilds of earthworms do not significantly affect the amounts of N_2O they emit (27).

Bathing earthworms in dilute concentrations of nitrate stimulates the emission of N_2O by earthworms (27), and the addition of nitrate to earthworm gut contents also stimulates the anaerobic production of N_2O by microbes in the gut (14). In the present study, [^{15}N]nitrate injected into the gut was likewise reduced to [^{15}N]N_2 and [^{15}N]N_2O, as shown by their emission from earthworms. These observations indicate that the in situ source of reductant required for the formation of N_2 and N_2O via denitrification is not limiting in the gut. Indeed, supplemental organic carbon does not increase the rate at which nitrate is reduced to N_2O by gut contents in anoxic microcosms (16). The aqueous phase of earthworm gut content contains readily utilizable sources of organic carbon, including up to 80 mM glucose, 40 mM amino sugars, and 10 mM maltose, as well as up to 1 mM of combined nitrate and nitrite; thus, the in situ conditions of the anoxic earthworm gut make it ideal for denitrification (14). The large amounts of readily available organic carbon in gut contents are likely derived from (i) ingested plant- and soil-derived materials that are partially degraded by digestive enzymes (e.g., proteases, chitinases, *N*-acetyl-glucosaminases, and maltases) (38, 39, 48, 51, 55) and (ii) the initial breakdown products of the intestinal mucus that is secreted by the earthworm to aid the passage of ingested material through the gut (26, 52).

Many gut ecosystems, including that of mammals, e.g., humans (37, 47), and various invertebrates, e.g., termites (12, 46, 50), harbor indigenous, autochthonous microorganisms. However, cell densities of earthworm gut wall-associated microbes are low, and the gut of the earthworm appears to have a quantitatively insignificant indigenous microbial biome (11, 15, 17, 24, 34, 35, 42, 53). Nonetheless, the gut of the earthworm constitutes a unique microenvironment to which ingested microbes are transiently subjected. The activation of certain members of the soil microbial community appears to increase their culturability (8, 16, 18, 19), indicating that gut passage

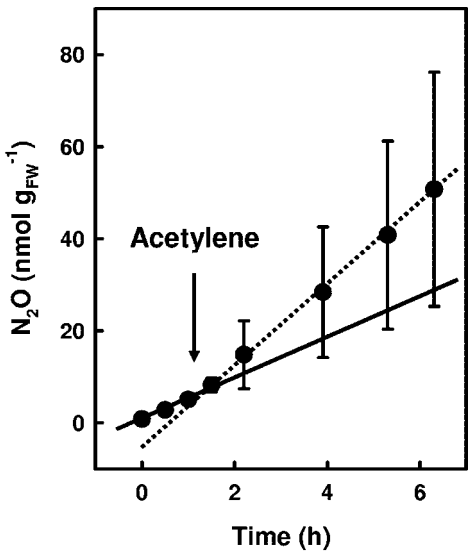

FIG. 4. In vivo emission of N_2O by *A. caliginosa* following an injection of 100 nmol sodium nitrate into the gut. Acetylene was injected into vials at 1.6 h (arrow). Bars indicate the range of values obtained from three replicates. Solid and broken lines correspond to the rates of emission prior to and after the addition of acetylene, respectively. Abbreviation: g_{FW}, g (fresh weight).

might affect the general life cycles and survival strategies of certain soil microbes. It has been postulated that the earthworm-secreted mucus in the gut might stimulate ingested soil microorganisms in a mutualistic digestive system (23).

Denitrification is catalyzed by facultative microbes under anoxic conditions and might therefore occur in many digestive tracts (which tend to have anoxic compartments or zones). However, little is known about the occurrence of this microbial process in gut ecosystems or the emission of denitrification-derived nitrogenous gases from animals. Humans exhale N_2O, and the amounts of N_2O exhaled may be (i) correlated with aging and the concentrations of nitrate and nitrite in gastric juices (21, 30) and (ii) affected by the amount of nitrate in ingested foods (29). The N_2O that is exhaled by humans might be derived from denitrifiers. However, nitrate dissimilators (i.e., anaerobes that dissimilate nitrate to ammonium) can give rise to N_2O as a side product of nitrate reductase (43, 44). Thus, nitrate dissimilators in the human gut (33) might also be a source of the N_2O exhaled by humans. Traces of N_2O are formed in the bovine rumen during the dissimilatory reduction of nitrate to ammonium (20). N_2O is not reduced further to N_2 in the bovine rumen, and denitrification appears to be insignificant in this gut ecosystem. With the exception of the earthworm, the exhalation and/or emission of microbially derived N_2 by other animals has not been reported.

Although earthworms are well recognized for their importance to the general fertility of soil (9, 22, 25), the potential importance of the earthworm gut to specific microbial processes that occur at the local level is less well understood. N_2 is classically considered the end product of denitrification at neutral pH, but the in situ production of similar amounts of denitrification-derived N_2 and N_2O by denitrifiers in the near pH neutral gut of the earthworm suggests that this process can be incomplete under in situ conditions considered favorable for complete denitrification. The loss of nitrogen from the terrestrial ecosphere via denitrification at a global level is estimated at 10^{11} kg N per year (36). The results of the present and earlier (2, 19, 27) studies illustrate the potential impact that soil fauna might have on the turnover dynamics of soil nitrogen (e.g., the ratio of N_2O/N_2 emitted) via their transient hosting of ingested soil microorganisms.

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