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Denitrifying Bacteria in the Earthworm Gastrointestinal Tract and In Vivo Emission of Nitrous Oxide (N_2O) by Earthworms

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Earthworms (*Lumbricus rubellus* and *Octolasium lacteum*) and gut homogenates did not produce CH_4, and methanogens were not readily culturable from gut material. In contrast, the numbers of culturable denitrifiers averaged 7×10^7 and 9×10^6 per g (dry weight) of gut material for *L. rubellus* and *O. lacteum*, respectively; these values were 256- and 35-fold larger than the numbers of culturable denitrifiers in the soil from which the earthworms were obtained. Anaerobically incubated earthworm gut homogenates supplemented with nitrate produced N_2O at rates exceeding that of soil homogenates. Furthermore, living earthworms emitted N_2O under aerobic conditions, and N_2O emission was stimulated by acetylene. For earthworms collected from a mildly acidic (pH 6) beech forest soil, the rates of N_2O emission for earthworms and soil averaged 884 and 2 pmol per h per g (fresh weight), respectively. In contrast, for earthworms collected from a more acidic (pH 4.6) oak-beech forest soil, N_2O emission by earthworms and soil averaged 145 and 45 pmol per h per g (fresh weight), respectively. Based on the extrapolation of this data, earthworms accounted for an estimated 16 and 0.25% of the total N_2O produced at the stand level of these beech and oak-beech forest soils, respectively.

Earthworms play an important role in the structure and fertility of soils (12, 28). The earthworm gut may be enriched with various aerobic and facultative microbes (8, 23, 24, 26, 29, 32). However, there are conflicting observations relative to the proliferation of specific bacterial groups in earthworm gut microflora; indeed, passage through the gut may selectively decrease the numbers of certain microbes, such as *Serratia marcescens* and *Escherichia coli* (11, 32). Although relatively little is known about in situ anaerobic processes in the earthworm gastrointestinal tract, various N_2-fixing species of *Clostridium* have been isolated from the gut of *Eisenia foetida* (6), and deposits of earthworm casts (feces) from the family Eudrilidae (different from those of the present study) contain denitrifying bacteria (31). In addition, the gastrointestinal tracts of *Lumbricus rubellus* Hoffmeister and *Octolasium lacteum* (Oerl.) harbor a substantially higher number of bacteria capable of anaerobic growth than does the soil from which the worms are obtained (21). These observations suggest that the earthworm gut may be enriched in microbes capable of anaerobic growth and activity. Methane (CH_4) and nitrous oxide (N_2O) are produced by microbial processes under anaerobic or low-oxygen conditions and are collectively responsible for approximately 25% of the greenhouse gases that theoretically contribute to global warming (10, 27, 38). The main objective of the present study was to determine if earthworms constitute a potential microsite for the production of CH_4 or N_2O in terrestrial ecosystems.

MATERIALS AND METHODS

Collection of earthworms and soils. Worms and A_h horizon soil were collected as previously described from a forest near Geisberg, Germany (21). The main site was dominated by beech (soil pH of approximately 6.0), while the second site had a mixed primary vegetation of oak and beech (soil pH of approximately 4.6).

Protocol for enrichment of methanogens. Gut homogenates were prepared anaerobically in a Mecaplex (Grenchen, Switzerland) anaerobic chamber (N_2 gas phase) as previously described (21), and standard anaerobic techniques for the enrichment and cultivation of methanogens were utilized (3, 41).

Enumeration of culturable denitrifiers. Dissection of earthworm guts and preparation of gut and soil homogenates were performed under aerobic conditions (21). Each gut homogenate contained the gut section (gut content plus gut wall) posterior of the gizzard from one worm. The initial homogenates contained 19 ml of glucose-free tryptic soy broth (2.88 g per liter of distilled water, pH 6.8) per g (fresh weight) of gut or soil material. Each homogenate was serially diluted (1:10) in tryptic soy broth supplemented with the following per liter: 2 mmol of glucose, 5 mmol of KNO_3, and 0.75 g of cycloheximide (to inhibit fungi). Ten replicate 0.2-ml aliquots from each dilution were transferred to microtiter plate wells. The plates were incubated in an anaerobic jar (100% Ar gas phase) at 20°C and observed weekly for growth. No further growth-positive cultures developed after 5 weeks. Most probable numbers (MPN) were calculated with a computer program (25). Aliquots of growth-positive cultures were checked for consumption of nitrate and production of nitrite and ammonia by colorimetric assays (5, 9, 18) in separate microtiter plates with a Spectra-II-photometer (SLT, Crailsheim, Germany). Cultures were considered positive for denitrification if less than 20% of the nitrate N was recovered in nitrate, nitrite, or ammonia. Additional studies confirmed that more than 90% of the cultures that met this condition were denitrifiers, i.e., reduced nitrate to N_2O or N_2 under anaerobic conditions.

Gas production by earthworm gut and soil homogenates. Gut and soil homogenates were prepared anaerobically (21). The homogenization buffer (pH 6.9) was prepared anaerobically with 100% Ar and contained, in grams per liter, the following: $Na_2HPO_4 \cdot 2H_2O$, 1.68; $NaH_2PO_4 \cdot H_2O$, 0.95; KNO_3, 2.53; KH_2PO_4, 0.5; NaCl, 0.4; NH_4Cl, 0.4; $MgCl_2 \cdot 7H_2O$, 0.05; and $CaCl_2 \cdot 2H_2O$, 0.01. Each gut homogenate contained the content of the gut section posterior to the gizzard of one worm. From each homogenate, 2 ml was transferred to gas-tight Hungate tubes (1.6 by 12.5 cm) containing a 100% Ar gas phase; the tubes were incubated at 20°C. The production of N_2O and N_2 was measured periodically. Results are indicative of duplicate experiments.

Microcosm studies with living earthworms. Microcosms contained fresh soil (A_h horizon) or one earthworm (*L. rubellus* or *O. lacteum*) with or without soil; supplemental nutrients were not provided. The microcosms were septum-sealed bottles containing sterile air; the amount of soil approximated 1 g (fresh weight) per 30 ml of gas phase. In each case, worms were provided with enough oxygen for respiration. Microcosms were incubated at 20°C in the dark, and the production of N_2O or CH_4 was measured periodically. Rates of N_2O emission by earthworms were calculated from the initial period of N_2O production and prior to defecation.

Analytical methods. For studies with gut and soil homogenates, N_2O and N_2 were quantitated with a Hewlett-Packard (Palo Alto, Calif.) 5980 series II gas chromatograph equipped with a thermal conductivity detector, a Hewlett-Packard 3396 series II integrator, and a Chromosorb 102 (Alltech, Unterhaching, Germany) column (length, 2 m; inner diameter, 3.2 mm) with 100% Ar as the carrier gas (25 ml per min); the injector temperature was 150°C, the column temperature was 40°C, and the detector temperature was 175°C. For studies with living worms, N_2O was determined with a Hewlett-Packard 5980 series II gas chromatograph equipped with an electron capture detector, a Hewlett-Packard 3396 series II integrator, and a Porapak Q-80/100 (Supelco, Bellefonte, Pa.) column (length, 4 m; inner diameter, 3.2 mm) with Ar-CH_4 (95:5) as the carrier

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TABLE 1. Comparative culturability under anaerobic conditions of denitrifying (MPN_{dnf}) and total (MPN_{total}) bacteria obtained from earthworm gut sections and soil[a]

Material (*n*)	Avg anaerobic MPN g (dry wt)$^{-1}$ (10^5)[b]		MPN_{dnf}/MPN_{total}	Gut MPN_{dnf}/soil MPN_{dnf}
	MPN_{dnf}	MPN_{total}		
L. rubellus gut (6)	682 (48.5–2,520)	6,650 (481–25,900)	0.103	256
O. lacteum gut (6)	92.8 (0–411)	3,430 (1,220–8,140)	0.027	35
Soil (3)	2.7 (2.4–2.8)	700 (374–863)	0.004	NA[c]

[a] Worms and soil (A_h horizon) were collected from the beech forest site on 21 September 1994.
[b] Ranges are given in parentheses.
[c] NA, not applicable.

gas (20 ml per min); the injector temperature was 150°C, the column temperature was 60°C, and the detector temperature was 300°C. CH_4 was determined by gas chromatography (39). The total gas pressures of tubes or bottles were measured with a Ballmoos (Horgen, Switzerland) DMG 2120 needle manometer. Soil pH, dry weights of soils and gut materials, and organic carbon were determined as previously described (21).

RESULTS AND DISCUSSION

Absence of culturable methanogens in earthworms. Terrestrial arthropods, ruminants, and other higher animals harbor culturable methanogenic bacteria in their gastrointestinal tracts and emit methane (2, 17, 20, 30). However, attempts to enrich for methanogens from the gut contents of *L. rubellus* and *O. lacteum* failed. In addition, neither of these earthworm species emitted methane; likewise, gut homogenates did not produce methane.

Enumeration of culturable denitrifiers. For gut content homogenates prepared from *L. rubellus* and *O. lacteum*, approximately 10 and 3%, respectively, of the bacteria capable of anaerobic growth in glucose-supplemented tryptic soy broth were also capable of denitrification (Table 1). In addition, the guts of *L. rubellus* and *O. lacteum* contained 256- and 35-fold more culturable denitrifiers, respectively, than did the beech forest soils from which worms were obtained (Table 1). *L. rubellus* is an epigeic worm and feeds more on litter than does the endogeic worm *O. lacteum*, which feeds more on soil (28); in this regard, the gut of *L. rubellus* tends to have higher nitrogen and organic carbon contents than does that of *O. lacteum* (21). Because feeding habits can influence the gut microbiota of animals (2, 16, 20), the differences in culturable denitrifiers between these two earthworm species might have been partly due to their different diets. All six of the *L. rubellus* guts examined contained significantly more culturable denitrifiers than did the soil. In contrast, only two of the six *O. lacteum* guts examined contained significantly more culturable denitrifiers than did the soil (a difference was considered statistically significant [$P = 0.05$] if one value was at least 3.281 times higher than the other [1]).

Production of N_2O and N_2 by earthworm gut content homogenates. Anaerobic homogenates of *L. rubellus* and *O. lacteum* gut contents that were supplemented with nitrate produced N_2O and N_2 (Fig. 1). N_2O was initially the predominant N gas detected in gut homogenates. After prolonged incubation, the N_2O produced by gut homogenates decreased and N_2 continued to accumulate in the headspace, suggesting that the microorganisms involved in N_2O production initially catalyzed an incomplete denitrification of nitrate. Sterile controls and controls lacking supplemental nitrate produced negligible N_2O and N_2, and the final amounts of N_2O and N_2 produced in nitrate-supplemented gut homogenates indicated that the supplemental nitrate was fully consumed over the course of the incubation (Fig. 1).

Soil homogenates produced substantially less N_2O and N_2 than did gut homogenates; in addition, N_2 was always produced in excess of N_2O by soil homogenates (Fig. 1). Because endogenous sources of organic carbon were utilized for reductant and energy by N_2O- and N_2-producing microflora, the lower capacities of soil to produce N_2O and N_2 may have been partly due to qualitative differences in the organic carbon of the soil compared to that of the earthworm gut. These results reinforced the concept that defecated earthworm material can enhance denitrification in soils (13, 35).

Emission of N_2O by earthworms under in vivo conditions. Living earthworms produced N_2O in sealed microcosms under aerobic conditions (Fig. 2A). After several hours of incubation and subsequent to defecation, N_2O levels decreased (Fig. 2A), suggesting that earthworms or defecated material was capable of consuming N_2O. When earthworms were combined with soil in such microcosms, N_2O was also produced (Fig. 2B), indicating that N_2O produced by earthworms was, at least in part, emitted from the soil rather than being reduced to N_2 by soil denitrifiers.

The inclusion of 10 kPa of acetylene in aerobic microcosms appeared to stimulate N_2O production by living earthworms. For individuals of *L. rubellus* collected in spring and summer of 1994 from the beech forest site, the rates of N_2O emission with and without acetylene averaged 6,520 ($\pm$2,979) and 1,980 ($\pm$608) pmol per h per g (fresh weight), respectively. This result underscored the potential involvement of denitrifiers in N_2O production.

For worms collected from the more pH-neutral beech forest soil (pH 6), the average rate of N_2O emission for earthworms and soil approximated 884 and 2 pmol per h per g (fresh weight), respectively (Table 2). In the more acidic oak-beech forest soil (pH 4.6), the average rate of N_2O emission for earthworms and soil approximated 145 and 45 pmol per h per g (fresh weight), respectively (Table 2). Not all of the earthworms examined emitted N_2O, but a larger percentage of the worms collected from the beech forest soil emitted N_2O than did worms collected from the oak-beech forest soil (Table 2, footnote *b*). However, if an earthworm emitted N_2O, the rate (on a fresh-weight basis) was always higher than the rate of N_2O production by soils. The production of methane by cockroaches and humans is correlated with the numbers of specific gut microbes (16, 20, 30); thus, the different N_2O emission patterns observed from worm to worm might be partly due to differences in the numbers of active N_2O-producing microorganisms in the worm gut. Different concentrations of N_2O precursors (e.g., nitrate) in the gut would also theoretically contribute to different N_2O emission patterns.

Denitrification takes place under anaerobic conditions but can also occur in the presence of trace levels of oxygen. Denitrifying bacteria are facultative anaerobes, and small amounts of oxygen increase the N_2O/N_2 ratio during denitrification (15, 37). For example, steady-state cultures of *Paracoccus halodeni-*

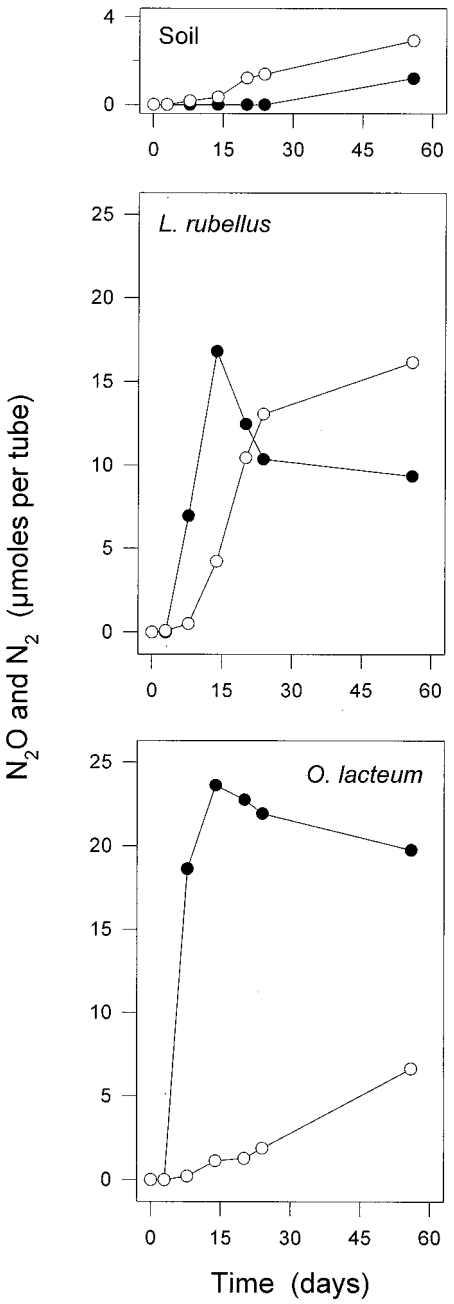

FIG. 1. Production of N_2O (●) and N_2 (○) by soil homogenates and gut content homogenates supplemented with nitrate (50 μmol per tube). The dry weights (in milligrams) and organic carbon contents (in micromoles), respectively, for homogenates were as follows: soil, 41 and 256; *L. rubellus* gut, 11 and 236; and *O. lacteum* gut, 37 and 399. Worms and soil were collected from the beech forest site on 15 April 1993.

trificans reduce nitrate mostly to N_2O when influent O_2 levels approximate 5% (19). The termite gut contains both aerobic and anaerobic microsites (4), and it is very likely that the earthworm gut is also not strictly anaerobic. Indeed, the numbers of microbes capable of aerobic growth are also larger in the guts of *L. rubellus* and *O. lacteum* than in the soil from which worms are obtained (21), thus indicating that the gut might also constitute a microsite for enriched aerobic as well as anaerobic processes.

Theoretical aspects and conclusions. Soils account for approximately 70% of the global N_2O budget (10). When N_2O emission rates were extrapolated to the stand level, N_2O emission by earthworms accounted for approximately 16 and 0.25% of the total N_2O produced by the beech and oak-beech forest soils, respectively (Table 2). Although the present study can give only a rough estimate of the quantitative contribution of earthworms to N_2O production at the ecosystem level, earthworms appear to be a mobile "hot spot" for the production of N_2O in certain terrestrial ecosystems.

Under more acidic conditions, N_2O reductase is inhibited (36), and the production of N_2O rather than N_2 can predom-

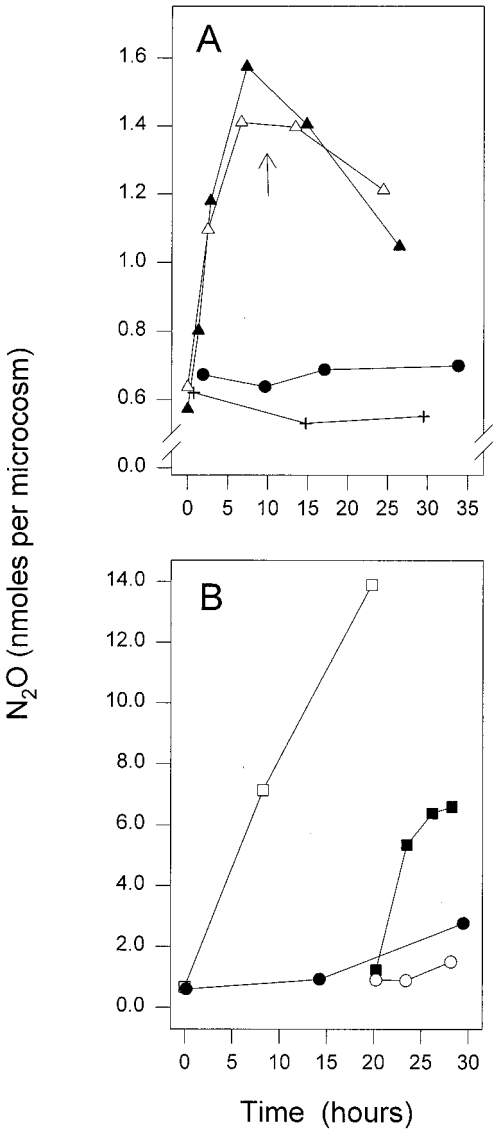

FIG. 2. Emission of N_2O by living earthworms and forest soils. (A) Microcosms contained one *L. rubellus* worm (1.64 [▲] or 1.76 [△] g [fresh weight]; worms were washed free of soil with sterile H_2O), beech forest soil (2.49 g [fresh weight]) from which worms were obtained (●), or neither worm nor soil (sterile control) (+). The arrow indicates the time of defecation by the worms. (B) Microcosms contained one *L. rubellus* worm in oak-beech forest soil (1.25 g [fresh weight] of worm and 2.29 g [fresh weight] of soil) (□) or oak-beech forest soil (2.26 g [fresh weight]) from which the worm was obtained (●); after 20 h of incubation, the worm (■) and soil (○) were transferred to separate microcosms. Worms and soils were collected on 9 May 1994.

TABLE 2. Mean N_2O emission rates in earthworm or soil microcosms[a]

Site (soil pH)	Microcosm (*n*)	N_2O emission	
		Microcosm[b] (pmol h^{-1} g [fresh wt]$^{-1}$)	Extrapolation to stand level[c] (μg of N_2O N h^{-1} m^{-2})
Beech (6.0)	Worms (8)	884 (0–2,637)	0.4
	Soil (5)	2 (0.4–8.8)	2.1
Oak-beech (4.6)	Worms (9)	145 (0–1,010)	0.2
	Soil (3)	45 (22–89)	80.0

[a] Worms and soils were collected in April, May, and September 1994. Microcosms were incubated under aerobic conditions and were not supplemented with nutrients.

[b] For worms from the beech forest, one of eight individuals did not emit N_2O; for worms from the oak-beech forest, four of nine individuals did not emit N_2O. Ranges are given in parentheses.

[c] N_2O emission rates at the stand level were estimated by using values of 49.5 kg (fresh weight) of soil (A_h horizon) per m^2 in the beech forest and 63.0 kg (fresh weight) of soil (A_h horizon) per m^2 in the oak-beech forest (40). Based on the overall samplings (reference 21 and this study), biomasses of earthworms were estimated to be 16 and 40 g (fresh weight) per m^2 in the beech and oak-beech forests, respectively.

inate during denitrification (14). The acidic oak-beech forest soil displayed a larger capacity for N_2O production than did the more pH-neutral beech forest soil. The gastrointestinal tract of the earthworm is nearly pH neutral (28), and the pH values of the gut contents of earthworms collected from the two field sites in the present study were similar and approximated 6.8. Nonetheless, N_2O was produced by both gut homogenates (prepared in buffer having a pH of 6.9) and living worms.

The collective results do not prove that the production of N_2O by living earthworms is derived solely from denitrification. Alternative N_2O-producing processes such as nitrification (10, 14, 33), assimilatory reduction of nitrate (37), and dissimilatory reduction of nitrate to ammonium (7, 34, 37) could also contribute to N_2O production under in vivo conditions. For example, the production of N_2O in the rumen appears to be associated primarily with the reduction of nitrite to ammonium rather than denitrification (22). Additional studies will be required to resolve both the specific metabolic origins of N_2O and the actual magnitude to which earthworms contribute to in situ N_2O emission in specific terrestrial ecosystems. In this regard, soils that are subjected to N deposition might be prone to enhanced N_2O emission by earthworms. Studies that address these issues are currently under way.

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