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In Situ Hydrogen and Nitrous Oxide as Indicators of Concomitant Fermentation and Denitrification in the Alimentary Canal of the Earthworm *Lumbricus terrestris*[▽]

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The earthworm gut is a unique microzone in aerated soils that has been proposed to selectively stimulate ingested soil microorganisms by its in situ conditions, which include anoxia, high water content, a near-neutral pH, and high concentrations of organic compounds. The central objective of this study was to resolve potential links between in situ conditions and anaerobic microbial activities during the gut passage of *Lumbricus terrestris*. Both H_2 and N_2O were emitted by living earthworms, and in situ microsensor analyses revealed both H_2 and N_2O in the O_2-free gut center. The highest H_2 concentrations occurred in foregut and midgut regions, whereas the highest N_2O concentrations occurred in crop/gizzard and hindgut regions. Thus, H_2-producing fermentations were more localized in the foregut and midgut, whereas denitrification was more localized in the crop/gizzard and hindgut. Moisture content, total carbon, and total nitrogen were highest in the foregut and decreased from the anterior to posterior end of the gut. Nitrite, ammonium, and iron(II) concentrations were highest in the crop/gizzard and decreased from the anterior to posterior end of the alimentary canal. Concentrations of soluble organic compounds were indicative of distinct fermentation processes along the alimentary canal, with maximal concentrations of organic acids (e.g., acetate and butyrate) occurring in the midgut. These findings suggest that earthworms (i) contribute to the terrestrial cycling of carbon and nitrogen via anaerobic microbial activities in the alimentary canal and (ii) constitute a mobile source of reductant (i.e., emitted H_2) for microbiota in aerated soils.

Earthworms constitute the dominant soil macrofauna in many soils (15, 34, 36) and emit the greenhouse gas nitrous oxide (N_2O) concomitantly with molecular nitrogen (N_2) via denitrifying bacteria in the gut (10, 11, 24, 26, 30, 39). The in situ conditions of the earthworm gut, which include anoxia, high water content, a near-neutral pH, and high concentrations of organic compounds, may activate certain ingested soil bacteria, in particular bacteria capable of anaerobiosis (10, 11, 27, 28–30, 33, 62). In this regard, the numbers of cultured anaerobes and denitrifiers are two to three orders of magnitude greater in the earthworm gut than in soil (28–30), and the amount of bacterial long-chain fatty acids is higher in the gut than in soil (46).

Gut contents of earthworms contain up to 80% intestinal mucus (i.e., up to 0.8 g mucus per g [dry weight] gut content) that consists of monosaccharides, low-molecular-weight amino acids, and glycoproteins (5, 37, 42, 62). This large amount of degradable organic carbon in the anoxic gut suggests that fermentation is very active during gut passage (27). Indeed, fermentative bacteria are abundant in the gut of earthworms (25, 28, 30). Nitrate reducers also are abundant in the earthworm gut (28) and can produce fermentation products when nitrate is not available (58). Many fermentative or facultative microorganisms also can reduce nitrite as well as nitrate (7, 63). However, fermentation processes in the alimentary canal of earthworms (Fig. 1) are not resolved, and relatively little is known about in situ conditions and microbial activities in the anterior part of the alimentary canal, i.e., in the crop and gizzard of earthworms.

Based on these collective findings, we hypothesized that fermentation is ongoing and concomitant with denitrification during gut passage. The central objective of this study was to address this hypothesis by resolving potential links between in situ conditions and anaerobic microbial activities along the alimentary canal of the anecic earthworm *Lumbricus terrestris*.

MATERIALS AND METHODS

Earthworms. Adult *L. terrestris* L. organisms were purchased from ANZO (Bayreuth, Germany) and maintained in soil at 15°C for approximately 10 days. Soil was obtained from the meadow Trafo Wiese in Bayreuth (Germany), which is described elsewhere (27).

Emission of H_2 and N_2O by living earthworms. Earthworms were washed with water, dried with tissue paper, and weighed. Two to three earthworms having a collective fresh weight of approximately 7 g were placed in a gas-tight 34-ml serum vial that then was crimp sealed under air and incubated at room temperature (approximately 20°C). The production of H_2 and N_2O was analyzed periodically; unless otherwise stated, values are the means from four replicates.

Microsensor measurements. Earthworms were anesthetized with 100% carbon dioxide to prevent defecation and subsequently were sedated with 20% ethanol for approximately 15 min. Earthworms were embedded horizontally in agar (1.5%) and overlaid with agar and double-distilled water (for the measurement of N_2O) or agar and 0.9% NaCl (for the measurement of H_2 or O_2), respectively. (Calibration tests demonstrated that these contrasting conditions were needed for microsensor stability.) This protocol was not lethal to earthworms. Microsensors for the measurement of N_2O (1), H_2 (67), and O_2 (44) were purchased from Unisense (Aarhus, Denmark) and were mounted on a micromanipulator (Märtzhäuser, Wetzlar, Germany). Radial concentration profiles were measured at room temperature for the crop/gizzard, foregut, midgut, or hindgut region of the alimentary canal (Fig. 1). Alternatively, two-point mea-

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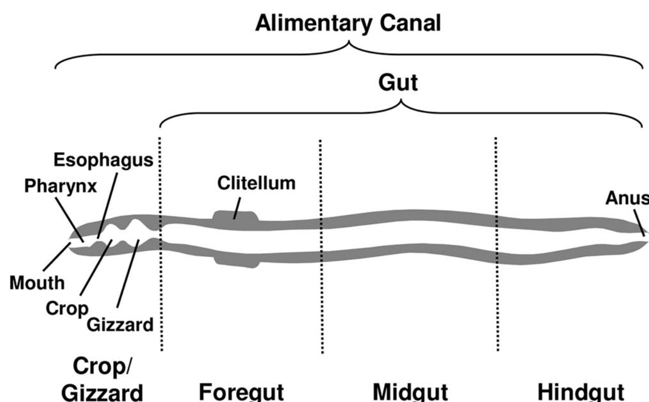

FIG. 1. Diagram of the earthworm alimentary canal. Modified from reference 27.

surements at the worm surface and the center of the alimentary canal were obtained for all four regions.

Production of H_2 by dissected guts. Earthworms were washed, sacrificed by brief immersion in 70°C water, and transferred into an O_2-free chamber (Mecaplex, Grenchen, Switzerland). Guts were dissected and placed in gas-tight 34-ml serum vials (three to four gut samples with a collective fresh weight of approximately 4.5 g per vial) that were flushed with 100% argon. Guts were incubated at room temperature in the dark, and the production of H_2 was analyzed periodically. Unless otherwise stated, values are means from four replicates.

Production of N_2O by earthworm sections. Earthworms were washed, dried with tissue paper, and sedated on ice. Earthworms were transferred into an O_2-free chamber and separated into crop/gizzard, foregut, midgut, and hindgut sections with sterile scissors. Sections were weighed and placed in gas-tight 15-ml serum vials (two sections with a collective fresh weight of approximately 2 g per vial) that were flushed with 100% argon. Earthworm sections were incubated at room temperature in the dark. The emission of N_2O was determined with and without acetylene (15%, vol/vol), an inhibitor of N_2O-reductase that reduces N_2O to N_2 (68, 70). The emission of N_2O was analyzed periodically. Unless otherwise stated, values are means from three replicates.

Extraction of soil and gut contents. Earthworms were washed and sacrificed by brief immersion in 70°C water. A total of 15 earthworms were dissected under oxic conditions (29, 30). The alimentary canal was divided into four regions (containing crop/gizzard, foregut, midgut, or hindgut). Material from the same section of five specimens was pooled to obtain approximately 0.5 g (fresh weight) per sample. Valeric acid was added as an internal standard (32). Soil or gut content was extracted with 1.5 ml double-distilled water at 60°C by being vortexed at maximum speed for approximately 1 min. Samples subsequently were cooled on ice and homogenized with an end-over-end shaker at 5°C overnight. Solid matter was separated by centrifugation (23,700 × *g*, 6 min, 4°C) and used for the determination of total nitrogen, total carbon, and organic carbon contents. Supernatant fluids were filtered (0.2 μm pore size) and stored at −20°C until analyzed for soluble organic compounds, nitrate, nitrite, iron(II), and ammonium.

Analytical techniques. The moisture content was determined by weighing the soil and gut content before and after it was dried at 60°C for 72 h. Oven-dried solid matter was ground with a ball mill (MM2; Retsch, Haan, Germany) and analyzed for total nitrogen content, and total carbon content was analyzed with an NC analyzer (Flash EA 1112; CE Instruments, Wigan, United Kingdom). Inorganic carbon was estimated as the amount of carbon lost by treatment with 8% HCl overnight followed by being at 80°C for 1 to 2 h; organic carbon was defined as the difference between total and inorganic carbon. Nitrate was analyzed with a Dionex DX-100 ion chromatograph equipped with an IonPac AS4A-SC ion-exchange column and an ED40 electrochemical detector (Sunnyvale, CA). Nitrite and iron(II) levels were determined photometrically (20, 55). Ammonium was measured by flow injection analysis (FIA-LAB; MLE, Dresden, Germany). N_2O and H_2 levels were determined by gas chromatography (30, 32); the rates of production were calculated by linear regression analysis. Organic acids were analyzed with a 1090 Series II high-performance liquid chromatograph (HPLC) (Hewlett-Packard Palo Alto, CA) equipped with a refractive index detector, a UV detector (210 nm) (both Series 1200; Agilent Technologies, Böblingen, Germany), and an Aminex Ion Exclusion HPX-87H column (300 by 7.8 mm; Bio-Rad, Richmond, CA). The column temperature was 60°C; the mobile phase was 4 mM H_3PO_4 at a flow rate of 0.8 ml min^{-1}. Chromatograms were evaluated using the ChemStation software Rev.B.02.01 (Agilent Technologies, Böblingen, Germany). Saccharides were determined with an HPLC equipped with a Dionex ED40 electrochemical detector (100-nC range), a gold electrode, and an Ag/AgCl reference electrode (Sunnyvale, CA). The hydrolysis of poly- and oligosaccharides into monosaccharides was carried out with 4 M trifluoroacetic acid (100°C, 1 h) (49). Saccharides were separated on a CarboPac PA100 column (250 by 4 mm) and eluted with stepped NaOH gradients (Table 1) (PU-1580 intelligent HPLC pump; Jasco, Großumstadt, Germany). The column temperature was 30°C (column thermostat Jet Stream Plus; Jasco, Großumstadt, Germany). Chromatograms were monitored using the software Borwin, version 1.50 (JMBS, Grenoble, France).

RESULTS

In vivo emission of H_2 by *L. terrestris* and anaerobic production of H_2 by dissected guts. H_2 was emitted by living earthworms at a rate that approximated 6.3 ± 4.8 nmol g (fresh weight)$^{-1}$ h^{-1} (n = 4). Dissected guts yielded 5.9 ± 1.8 μmol H_2 g (fresh weight)$^{-1}$ (n = 4) after 8 h under anoxic conditions. Consistently with previous findings (30, 39), N_2O also was emitted under in vivo conditions at rates that approximated 0.4 ± 0.2 nmol g (fresh weight)$^{-1}$ h^{-1} (n = 10). These initial results suggested that the alimentary canal had a high capacity for fermentative activity, and that this activity manifested itself in the in vivo emission of H_2 concomitantly with the emission of N_2O.

In situ concentrations of H_2, N_2O, and O_2. In situ H_2 and N_2O concentrations were highest in the O_2-free core of crop/gizzard radial profiles (Fig. 2). O_2 was not detected in the crop/gizzard, foregut, midgut, or hindgut (Fig. 2 and data not shown). N_2O measurements were initiated at the cuticle, since preliminary tests demonstrated that the levels of N_2O above the cuticle were below the detection limit. The steep decline of N_2O toward the cuticle, which has been documented previously (27), suggests that the cuticle is a diffusion barrier.

Maximal concentrations of H_2 occurred in the center of the foregut (5 μM) and midgut (6 μM) (Fig. 3). Despite the high variability among replicates, (i) the pattern of gases in a given worm section was similar, and (ii) H_2, an indicator of ongoing fermentation, was detected in every replicate. Maximal concentrations of N_2O occurred in the center of the crop/gizzard (2 μM) and hindgut (1 μM) (Fig. 3). The average N_2O concentration at the worm surface of the midgut region was higher than that in the worm center.

TABLE 1. Sodium hydroxide gradients used for separation of saccharides by HPLC[a]

Treatment	Time (min)	Eluent A NaOH gradient (%)[b]
Nonhydrolyzed	0	2
	15	2
	35	42
	37	70
	39	70
	40	2
Hydrolyzed	0	3
	7.5	3
	18	4
	26	70
	29	70
	30	3

[a] The flow rate was 1 ml min^{-1}.
[b] Eluent A, 0.5 M NaOH; eluent B, double-distilled H_2O.

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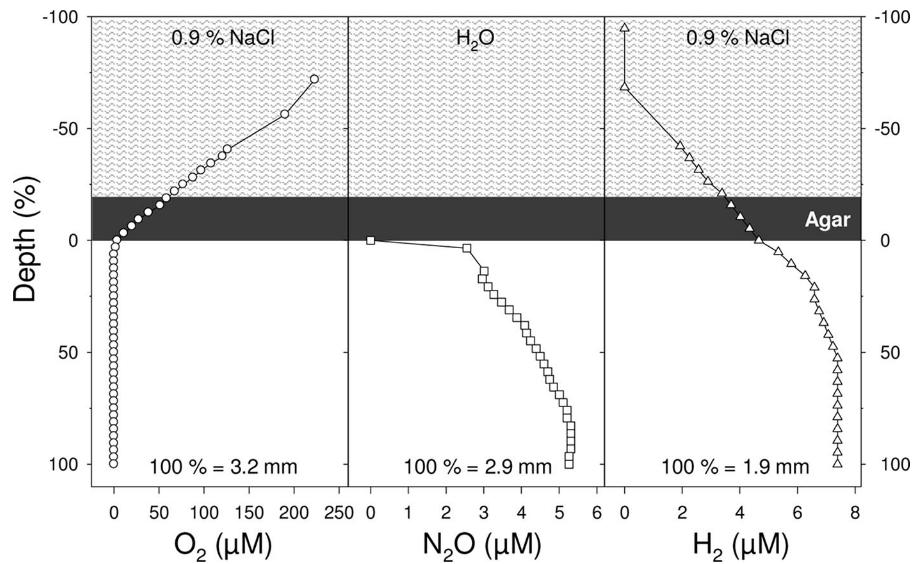

FIG. 2. Radial microsensor profiles of O_2, N_2O, and H_2 for the crop/gizzard of sedated *L. terrestris* worms. The worm radius equals 100% (as defined in the figure). Representative patterns of replicate analyses are shown.

Denitrification capacities of earthworm sections. All earthworm sections emitted N_2O without appreciable delay under anoxic conditions (Fig. 4 and data not shown). Crop/gizzard and hindgut sections emitted the largest amounts of N_2O (Fig. 4), which is consistent with the results obtained by N_2O microsensor analysis (Fig. 3). N_2O emission rates increased by factors of approximately 2, 3, 4, and 5 for crop/gizzard, foregut, midgut, and hindgut, respectively, when earthworm sections were exposed to acetylene (Fig. 4), indicating that (i) N_2O was derived from denitrification, (ii) N_2 production exceeded N_2O production, and (iii) the relative amount of N_2 increased from the anterior to the posterior end of the alimentary canal.

In situ parameters potentially linked to anaerobic activities along the alimentary canal. The results described above indicated that distinct anaerobic microbial processes occurred at different places along the alimentary canal. In situ parameters were evaluated to further resolve these microbial processes.

Moisture content along the alimentary canal was approximately twofold greater than that of the soil in which the worms were incubated (Table 2). Moisture content, total nitrogen,

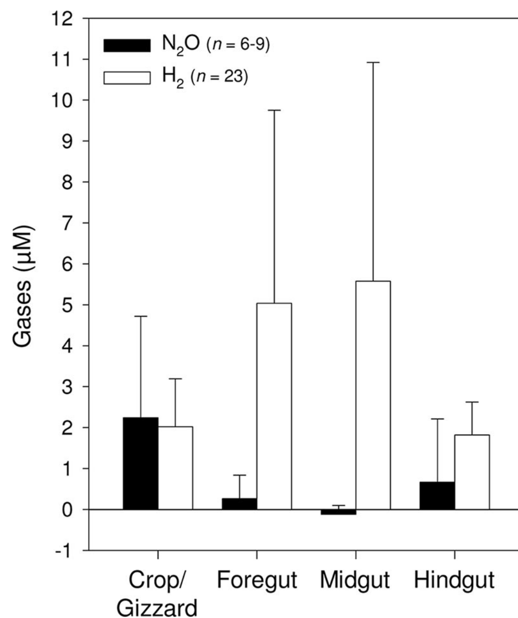

FIG. 3. In situ concentrations of N_2O and H_2 along the alimentary canal of *L. terrestris*. Values are the means from 6 to 23 replicates; error bars indicate positive standard deviations.

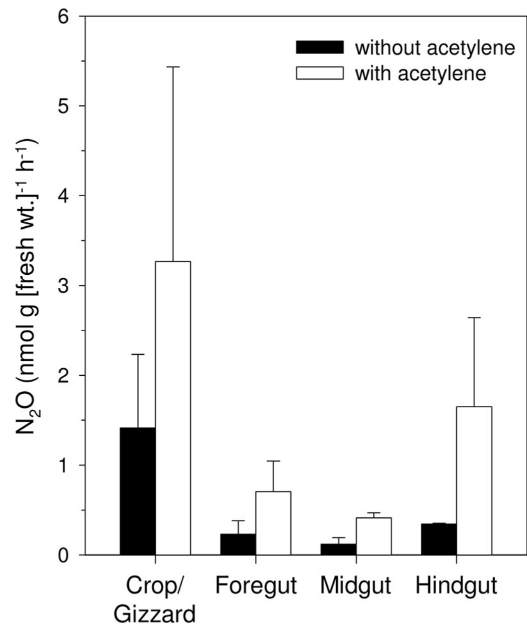

FIG. 4. Production of N_2O by earthworm sections with and without acetylene. Values are the means from triplicates; error bars indicate positive standard deviations.

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TABLE 2. In situ parameters along the alimentary canal of *L. terrestris*[a]

Alimentary canal site	Moisture content (%)	Total nitrogen (mg g [dry wt]$^{-1}$)	Total carbon (mg g [dry wt]$^{-1}$)	Organic carbon (mg g [dry wt]$^{-1}$)	Nitrate (mmol liter [water content]$^{-1}$)	Nitrite (mmol liter [water content]$^{-1}$)	Ammonium (mmol liter [water content]$^{-1}$)	Iron(II) (mmol liter [water content]$^{-1}$)
Crop/gizzard	41.8 ± 3.0	6.8 ± 0.5	54.9 ± 5.1	49.5[b]	0.3 ± 0.1	3.8 ± 0.2	17.9[c]	5.4 ± 0.5
Foregut	51.9 ± 0.2	9.0 ± 0.4	62.0 ± 3.5	58.8 ± 2.8	0.1 ± 0.0	2.5 ± 0.5	15.8[b]	2.9 ± 0.5
Midgut	49.1 ± 2.3	5.2 ± 1.2	49.6 ± 8.5	48.4 ± 8.2	0.1 ± 0.0	0.2 ± 0.1	11.0 ± 1.6	0.5 ± 0.3
Hindgut	44.0 ± 3.3	4.4 ± 0.8	42.1 ± 6.5	41.3 ± 5.9	0.3 ± 0.0	0 ± 0	11.5 ± 1.4	0.1 ± 0.1
Soil	19.9 ± 1.4	3.4 ± 0.8	38.6 ± 8.6	37.3 ± 8.2	0.9 ± 0.1	0.2 ± 0.1	0.8 ± 0.2	0.1[c]

[a] Unless otherwise stated, values are the means from triplicates (± standard deviations); each worm replicate consists of gut content derived from five worms.
[b] No standard deviation is given because three replicates had to be pooled to obtain sufficient material for analysis.
[c] No standard deviation is given because two replicates were analyzed, and the value is the average for the replicates.

and total carbon content were highest in the foregut and decreased from the anterior to the posterior end of the gut. Compared to the amounts in the soil, the amounts of total nitrogen, total carbon, and organic carbon were enriched in all portions of the alimentary canal. Approximately 90% of the total carbon was organic in crop/gizzard content, a value somewhat lower than those of other earthworm regions and soil. Excreted calcium carbonate in the esophagus followed by the release of mucus into the foregut (15) may have accounted for the higher relative amount of inorganic carbon in crop/gizzard contents. The concentrations of nitrite were greater in the aqueous phase of crop/gizzard contents and foregut contents than in the aqueous phase of soil. In contrast, nitrate concentrations were highest in soil, indicating that nitrate-reducing organisms were active in the crop/gizzard and other regions of the alimentary canal. The concentrations of iron(II) and ammonium were highest in crop/gizzard content and decreased along the alimentary canal. The concentration of iron(II) at the end of gut passage was equivalent to that of soil.

The alimentary canal contained high concentrations of soluble organic compounds. Maltose, isomaltose, and glucose were dominant nonpolymeric saccharides in the aqueous phase of the alimentary canal (Fig. 5). The numbers and concentrations of detected monosaccharides increased significantly after hydrolysis with trifluoroacetic acid, indicating that saccharides in the earthworm gut were primarily polymeric. After hydrolysis, approximately 110 mM monosaccharide equivalents were obtained from crop/gizzard contents, with mannose, glucose, galactose, arabinose, rhamnose, and fucose being dominant monosaccharides. Concentrations of saccharides decreased from the anterior to the posterior end of the alimentary canal, indicating that saccharides were consumed during gut passage.

The highest concentrations (approximately 32 mM total) and highest diversity of organic acids were detected in the aqueous phase of midgut content, indicating that fermentative organisms were very active in this region of the alimentary canal (Fig. 6). Lactate and propionate occurred primarily in crop/gizzard contents and foregut contents, whereas butyrate and acetate dominated in mid- and hindgut contents. Methylbutyrate was likewise a major product in the midgut.

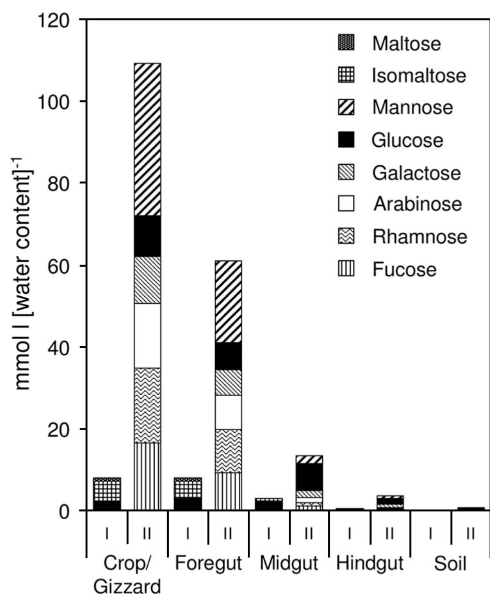

FIG. 5. Concentrations of saccharides along the alimentary canal of *L. terrestris* and in soil. I, not hydrolyzed; II, after hydrolysis with trifluoroacetic acid. Values are the means of triplicates; each replicate consists of gut content derived from five worms.

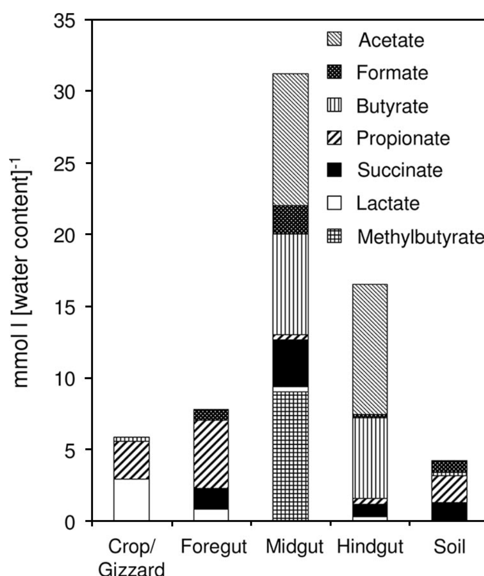

FIG. 6. Concentrations of fatty acids along the alimentary canal of *L. terrestris* and in soil. Values are the means from triplicates; each replicate consists of gut content derived from five worms.

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DISCUSSION

Impact of in situ parameters on the microbiota along the alimentary canal of *L. terrestris*. Cultured numbers of microbes capable of growth under anoxic conditions are higher in the earthworm gut than in soil (28–30), and it has been proposed that the in situ conditions of the earthworm gut activate ingested soil bacteria (11, 27, 33). The pH varies relatively little along the alimentary canal of *L. terrestris* (22). Intestinal mucus concentrations of earthworm species decrease along the alimentary canal (5, 62), suggesting that in situ conditions change during gut passage. The moisture content, total nitrogen, and total carbon of gut contents were greater than those of surrounding soil and decreased from the anterior to the posterior end of the gut (Table 2), findings that are consistent with previous studies (5, 27). The levels of iron(II), ammonium, and nitrite decreased along the alimentary canal (Table 2), underscoring the likelihood that ingested microorganisms are subjected to changing in situ conditions during gut passage. Free O_2 was not detected in the crop/gizzard (Fig. 2), suggesting that anaerobic activities are favored immediately after soil enters the alimentary canal. The absence of free O_2 in the alimentary canal and worm tissues suggests that it was rapidly bound by hemoglobin (15) and subject to respiration.

Total levels of organic carbon are higher in the earthworm alimentary canal than in soil (5, 27) (Table 2). The hydrolysis of the aqueous phase of alimentary canal contents yielded mannose, glucose, galactose, arabinose, rhamnose, and fucose (Fig. 5). The hydrolysis of the plant heteropolymer hemicellulose typically yields the pentoses xylose and arabinose, the hexoses glucose, mannose, and galactose, and the disaccharide cellobiose (45), and fucose occurs in plant cell walls, bacterial extracellular polysaccharides, and animal glycoproteins (66). Thus, at least some of the monosaccharides detected in the earthworm gut might be derived from ingested biomass. However, the earthworm gut contains up to 80% intestinal mucus (37, 42, 62), and it is likely that most of the monosaccharide equivalents detected were mucus derived. Indeed, glucosamine, galactosamine, glucose, galactose, mannose, and fucose are components of mucopolysaccharides from *Lumbricus* spp. (42). Polysaccharide-degrading enzymes (e.g., amylase, cellulase, xylanase, and chitinase) occur in the earthworm gut (40, 59, 61, 64, 65). The highest amylase and cellulase activities in the anterior part of the alimentary canal (59, 61) coincide with the highest organic carbon and saccharide concentrations in the crop/gizzard and foregut (Table 2 and Fig. 5). Monosaccharide-releasing enzymes (e.g., mannosidase, galactosidase, and glucosidase) are active in the earthworm gut (35) and might contribute to the occurrence of monosaccharides in the alimentary canal. These collective findings reinforce the hypothesis that the in situ conditions in the earthworm alimentary canal are ideally suited for heterotrophs capable of anaerobiosis.

Concomitant fermentation and denitrification in the alimentary canal. H_2 was emitted in vivo and produced in situ (Fig. 2 and 3). The decomposition of organic matter via fermentation can result in the formation of H_2 (53). That the decrease in the amount of saccharides along the alimentary canal (Fig. 5) was concomitant to the production of H_2 and other fermentation products (Fig. 3 and 6) supports the conclusion that fermentation is ongoing in the earthworm gut. Fungal hyphae are either disrupted mechanically in the anterior part of the alimentary canal or digested during gut passage (50, 51), suggesting that bacteria play a dominant role in gut fermentation (27, 28). Propionate was detected primarily in soil, crop/gizzard, and foregut (Fig. 6). Propionate is a fermentation product of propionibacteria that belong to the actinobacteria (9), and 16S rRNA gene sequences indicative of actinobacteria have been detected in casts and in the gut of *Lumbricus rubellus* (54). Propionibacteria ferment monosaccharides but also can utilize lactate, which was detected in the crop/gizzard and foregut. Lactic acid bacteria form a large fraction of the cultivable gut microbiota of wood- and soil-feeding termites (2, 52, 57). Most lactic acid bacteria are aerotolerant (8, 56) and, thus, might be poised to react quickly to anoxia after ingestion. Indeed, the detection of lactate primarily in the crop/gizzard (Fig. 6) suggests that it is formed in the anterior part of the alimentary canal and is subject to consumption in subsequent regions of the gut, which is consistent with the high capacity of gut homogenates to produce lactate and subsequently consume it under anoxic conditions (29).

High concentrations of H_2 occurred concomitantly with formate, acetate, and succinate in the foregut and midgut. Formate, acetate, succinate, and H_2 are characteristic products of facultative aerobes, including *Enterobacteriaceae* that are capable of fermenting various monosaccharides (e.g., glucose, fucose, and rhamnose (4, 19). *Enterobacteriaceae*-related species and other *Gammaproteobacteria* have been isolated from the gut of *Aporrectodea caliginosa* (28), and 16S rRNA gene sequences indicative of *Gammaproteobacteria* were retrieved from the gut of *L. rubellus* (54). The H_2-producing fermenter *Paenibacillus terrae* MH72 (of the *Firmicutes*) was isolated from *A. caliginosa* (25). Methylbutyrate was detected in the midgut. Methylbutyrate often is produced when amino acids (e.g., valine and isoleucine) are fermented (17), suggesting that amino acids that are abundant in the earthworm gut (11, 27) were utilized as carbon sources. Products indicative of butyrate fermentation (i.e., butyrate and H_2) were detected in the midgut and hindgut. Butyrate was produced in anoxic most-probable-number dilutions of gut homogenates obtained from *A. caliginosa* (28). *Clostridiaceae*-related species have been isolated from the gut of the earthworm *A. caliginosa* (28). Hydrogenases of butyrate-producing clostridia are O_2 sensitive (6), suggesting that this functional group is not active immediately upon ingestion. Thus, a broad diversity of bacteria might be linked to the anaerobic degradation of organic matter in the earthworm gut.

The proposal that denitrification and the dissimilatory reduction of nitrate are active in the earthworm gut (27, 28) is supported by (i) the near absence of nitrate in the alimentary canal (Table 2), (ii) the increased concentration of nitrite in crop/gizzard and foregut contents compared to that of soil (Table 2), (iii) the occurrence of N_2O in all regions of the anoxic alimentary canal (Fig. 3), and (iv) the acetylene-dependent enhancement of N_2O production by earthworm sections (Fig. 4). In situ concentrations of N_2O (Fig. 3) and N_2O production by worm sections (Fig. 4) suggest that denitrification is more localized in the crop/gizzard and hindgut. Complete denitrification (i.e., the reduction of nitrate or nitrite to N_2) in the crop/gizzard indicates that denitrifiers were activated without significant delay upon ingestion.

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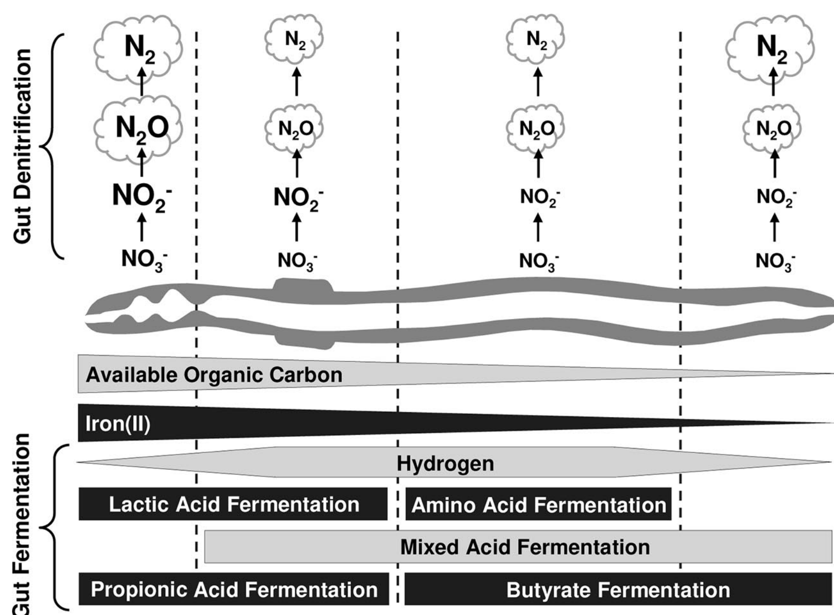

FIG. 7. Hypothetical model of links between in situ conditions and anaerobic activities during the gut passage of *L. terrestris*. Concentrations are indicated by the font size. The tapering off of a shaded element indicates that the item identified decreases in quantity in the direction of the taper. Gases in clouds indicate the in vivo emission by the worm.

The molecular analysis of *nosZ* (a structural gene for N_2O reductase [70]) in earthworm gut contents indicated that species related to *Bradyrhizobium*, *Flavobacterium*, *Dechloromonas*, *Brucella*, *Sinorhizobium*, *Pseudomonas*, *Ralstonia*, and *Paracoccus* are involved in gut denitrification (24).

Gaseous emission in the context of worm respiration. The respiration rate of *L. terrestris* approximates 3 μmol O_2 g (fresh weight)$^{-1}$ h^{-1} (34), suggesting that 12 μmol reducing equivalents g (fresh weight)$^{-1}$ h^{-1} is directed toward the reduction of O_2 by the worm. H_2 was emitted by living earthworms at a rate that approximated 6 nmol g (fresh weight)$^{-1}$ h^{-1}, a value corresponding to 12 nmol reducing equivalents g (fresh weight)$^{-1}$ h^{-1}. N_2O was emitted at rates that approximated 0.4 nmol g (fresh weight)$^{-1}$ h^{-1}. Assuming that the amount of N_2 emitted was equal to that of N_2O (26), approximately 7 nmol reducing equivalents g (fresh weight)$^{-1}$ h^{-1} was directed toward the emission of nitrogenous gases. These values indicate that the amount of reductant lost as emitted H_2 and nitrogenous gases is minimal compared to the overall flow of reductant toward the respiration of the earthworm. The millimolar amounts of organic compounds in the alimentary canal support this conclusion, i.e., that the main flow of reductant at the level of the system is not toward denitrification and H_2 production. It is obvious that a serious loss of reductant (i.e., source of energy) would be problematic for the earthworm.

Trophic links along the alimentary canal. A hypothetical model of anaerobic processes and potential trophic links along the alimentary canal of *L. terrestris* is proposed (Fig. 7). *L. terrestris* is anecic (i.e., lives in deeper soil zones, ingests moderate amounts of mineral soil, and feeds on litter dragged into its burrow) and was selected as a model earthworm in this study. However, the feeding habits of endogeic (feed in the rhizosphere, ingest substantial amounts of mineral soil, and preferentially live in upper mineral soil; e.g., *A. caliginosa*) and epigeic (feed on litter and preferentially live above the mineral soil; e.g., *L. rubellus*) earthworms may result in different processes along the alimentary canal than those shown in the model. Nonetheless, the model serves to illustrate the spatial differences among anaerobic microbial processes that might occur during gut passage.

The highest in situ H_2 concentrations occurred in foregut and midgut regions. In contrast, the highest in situ N_2O concentrations occurred in crop/gizzard and hindgut regions (crop/gizzard and hindgut sections likewise produced the largest amount of N_2O under anoxic conditions). Thus, H_2-producing fermentations were localized primarily in the foregut and midgut, whereas denitrification was localized primarily in the crop/gizzard and hindgut. Oxidizable carbon is essential to both denitrifiers and fermenters. Total organic carbon and saccharides decrease along the alimentary canal, indicating that organic compounds are utilized by microorganisms during gut passage. However, organic compounds likely are subject to assimilation by the earthworm, since (i) long-chain fatty acids derived from ingested bacteria can be assimilated into earthworm tissue (46), and (ii) the capacity to assimilate organic molecules in the gastrointestinal tract is a general trait of animals (3, 13, 16, 23, 43, 60). Thus, it is very likely that fermenters, denitrifiers, and the earthworm compete for available carbon. Indeed, the capacity of the earthworm to assimilate organic compounds derived from the breakdown of intestinal mucus and ingested biomass is essential to its survival (15, 48). The occurrence of large amounts of iron(II) in the crop/gizzard suggests that iron(III) reduction occurred during the initial ingestion phase; the decreasing amount of iron(II) in the posterior regions of the gut suggests that it is subject to assimilation by either microorganisms or the worm during gut passage.

Numerous anaerobes occur in the earthworm gut (29, 30)

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and are proposed to be the main source of organic acids in the alimentary canal (Fig. 6). Fermentation products indicative of distinct fermentations occurred spatially and consequently in temporal sequence in the alimentary canal. Total cell counts and numbers of cultured bacteria increase from foregut to hindgut (18, 31, 51). Long-chain fatty acid profiles and cell numbers for bacterial phyla (i.e., *Alpha*-, *Beta*-, *Gamma*-, and *Deltaproteobacteria*) detected by fluorescent in situ hybridization differ significantly along the earthworm alimentary canal of *L. terrestris* (47, 50). These collective findings indicate that both the microbial community structure and associated activities change during gut passage.

H_2 was a stable end product in glucose-supplemented anoxic gut homogenates (29) and was produced by gut sections and living earthworms. Methane is neither emitted by earthworms nor formed by gut homogenates (29). Thus, although H_2 forms important trophic links to methanogenesis and acetogenesis (12, 21, 69), methanogens and acetogens are not metabolically significant in the earthworm gut (29). In contrast, the specialized digestive system of termites yields large amounts of H_2 that are tightly linked to acetogenesis in the hindgut paunch (41). Although it is currently unknown if there are reductant sinks for H_2 in the earthworm alimentary canal, the occurrence of different inorganic electron acceptors (Table 2) suggests that it is subject to consumption as well as emission.

Living earthworms (i.e., *L. terrestris*) emitted approximately 6 nmol H_2 g (fresh weight)$^{-1}$ h^{-1}. Assuming worm densities of up to 2,000 individuals per square meter (14, 15) and an average worm weight of 2 g, earthworms can emit up to 600 μmol H_2 per square meter per day. This value for H_2 is sevenfold higher than the daily emission of N_2O per square meter of pasture (38). Thus, earthworms might constitute a mobile source of reductant (i.e., emitted H_2) for the microbiota in aerated soils.

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