

Carbon dioxide and nitrous oxide fluxes from soil as influenced by anecic and endogeic earthworms

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ABSTRACT

Earthworm-microbial interactions may stimulate CO2 and N2O emissions from soil. This study examined the influence of anecic and endogeic earthworms, represented by Lumbricus terrestris L. and Aporrectodea caliginosa Savigny, on CO₂ and N₂O fluxes, and on the processes (denitrification, nitrification) that lead to N₂O flux from an agricultural soil. Laboratory microcosms, with and without earthworms, were incubated at 15 °C and 40% water-filled pore space, and headspace gases were sampled after 1, 4, 7, 14, 21, and 28 days. Denitrification and nitrification processes were then evaluated in a 24 h acetylene inhibition experiment. Earthworms were responsible for 7–58% of the total CO_2 flux from soil, compared to the control (no earthworms), but did not affect the N_2O flux. The CO_2 flux was greater when more earthworms were present, and in microcosms with mixed L. terrestris and A. caliginosa populations, suggesting that microbial respiration could be stimulated by the interactions of anecic and endogeic earthworms. Denitrification was the dominant process leading to N_2O production from microcosms with L. terrestris, while nitrification was more important in microcosms with A. caliginosa. Microcosms with mixed populations produced more N₂O from denitrification than nitrification. Species-specific stimulation of nitrifiers and denitrifiers may be related to unique structures (casts, burrows) produced by L. terrestris and A. caliginosa, but this remains to be confirmed.

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1. Introduction

Soil microbial activities like decomposition, nitrification and denitrification lead to the emission of CO_2 and N_2O , important greenhouse gases linked to climate change (IPCC, 2007). The earthworm gut and associated structures (casts, burrows, middens) represent microhabitats that can support distinct microbial communities and greater microbial activity than the bulk soil (Brown et al., 2000; Drake and Horn, 2006; Marhan et al., 2007). As a result, earthworm–microbial interactions may stimulate CO_2 and N_2O emissions from soil, but this has been difficult to demonstrate in field experiments due to temporal fluctuations in soil moisture content and available substrates (Schindler Wessells et al., 1997; Bertora et al., 2007).

In a microcosm study with agricultural soil, Caravaca et al. (2005) found that 40% of the total CO_2 emission from soils with *Eisenia fetida* and composted residues was due to earthworm activity. Microcosm studies using forest (Karsten and Drake, 1997; Borken et al., 2000) and garden soils (Matthies et al., 1999) have likewise shown that earthworms may be responsible for 30–56% of the total N₂O emitted from the soils they inhabit.

Interactions between earthworms and denitrifying microbes have received special attention because in situ conditions in the earthworm gut (anoxia, availability of carbon substrates and nitrate/nitrite) stimulate the growth and activity of ingested denitrifiers, leading to N_2O and N_2 emissions from earthworms (Drake and Horn, 2006). In vivo N_2O fluxes from Lumbricus terrestris and Aporrectodea caliginosa

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may range from 0 to $11 \text{ nmol } N_2 \text{O g}^{-1}$ earthworm (fresh wt.) h^{-1} , which is about 0–0.48 µg N₂O g⁻¹ h⁻¹ (Horn et al., 2006). Earthworms mix soil and organic residues as they feed and burrow, which often stimulates aerobic respiration and may create anaerobic microsites that favor denitrifying bacteria (Burtelow et al., 1998). For example, earthworm casts and burrow linings have greater nitrification and denitrification rates than bulk soil (Svensson et al., 1986; Elliott et al., 1990; Parkin and Berry, 1994, 1999). The middens created by L. terrestris contain less NO3-N than bulk soil, possibly due to NO₃-N losses via denitrification (Subler and Kirsch, 1998). Mineralization, nitrification, and denitrification processes are probably affected by earthworm functional diversity, although the interactions between earthworm functional groups, microbial communities and N transformations are complex (Postma-Blaauw et al., 2006; Sheehan et al., 2006). How earthworm functional groups may interact with the nitrifying and denitrifying bacteria that produce N₂O has not yet been fully examined, although this could be done with a differential acetylene inhibition assay. An acetylene concentration of 5-10% (v/v) will block nitrification and nitrate reductase, providing information about N2O + N2 production, while an acetylene concentration of 0.01% (v/v) is sufficient to block nitrification, giving a measure of N₂O production (Davidson et al., 1986; Tiedje et al., 1989). We hypothesized the following: (1) soils with more earthworms will have greater CO₂ and N₂O fluxes, (2) gaseous fluxes will be affected by earthworm functional groups and soil conditions, and (3) N₂O production from earthworm-worked soils will come mainly from denitrification.

The objective of this study was to determine how earthworm functional groups influenced CO_2 and N_2O fluxes, as well as the processes that lead to N_2O production (nitrification, denitrification). This study was done in microcosms with agricultural soil and we used *L. terrestris* and *A. caliginosa* as representatives of anecic and endogeic functional groups, respectively.

2. Materials and methods

2.1. Earthworms and soil

Earthworms were collected from a field on the Macdonald Campus Research Farm, Ste. Anne de Bellevue, Quebec, Canada (45° 28′ N, 73° 45′ W) in May 2006 by handsorting and formalin extraction. The earthworms were separated by species and kept in 37 l plastic containers with field soil and several grams of dried soybean leaves placed on the surface as a food source. Containers with earthworms were stored in an incubator at 15 °C for 1 month before the experiment began in June 2006. Soil was collected from the same field in May 2006, sieved through a 6-mm screen, and stored in 37 l plastic containers in a laboratory at 20 °C. The soil was a sandy loam, mixed, frigid Typic Endoaquent of the Chicot series. It contained 580 g kg⁻¹ of sand, 300 g kg⁻¹ of silt and 120 g kg⁻¹ of clay, with 34.2 g organic C kg⁻¹, 3.6 g total N kg⁻¹ and pH 5.7.

2.2. Microcosms and experimental design

Microcosms were 1 l jars with 500 g of air-dried soil, packed to a bulk density of 1 g cm $^{-3}$ and moistened to 40% water-filled pore space (WFPS) with distilled water. A total of 115 microcosms were prepared and stored overnight at 4 °C, then microcosms were incubated at 15 °C in the dark for 7 days. Ten microcosms were removed to assess baseline soil conditions, and the rest received earthworm treatments. There were 7 earthworm treatments with 15 replicates arranged in a completely randomized design: control (C, no earthworms), A. caliginosa (A), L. terrestris (L), and both species (AL) at natural $(1\times)$ and double $(2\times)$ population levels. The number and biomass of earthworms added is provided in Table 1. Earthworms (pre-clitellate to fully clitellate adults) were added to the microcosms after voiding their guts for 24 h so as to minimize the introduction of exogenous soil microorganisms. We prepared 70 microcosms for repeated

Table 1 – Earthworms added to microcosms (Initial) and recovered after 28 days (Final), and the mean CO ₂ and N ₂ O fluxes during the 28 days study											
Treatment	Earthworm numbers (individuals per microcosm)		Earthworm biomass (g fresh wt. per microcosm)		$\begin{array}{c} \text{CO}_2 \text{ flux} \\ \text{(mg CO}_2\text{-C g}^{-1} \text{ h}^{-1} \text{)} \end{array}$	$N_2 O \mbox{ flux} \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \$					
	Initial	Final	Initial	Final							
С	0	0	0	0	$0.14\pm0.02^{\rm d}$	48 ± 23					
A1×	3	$\textbf{3.1}\pm\textbf{0.1}$	$\textbf{1.6}\pm\textbf{0.1}$	$\textbf{1.4}\pm\textbf{0.1}$	$0.23\pm0.04^{\rm bc}$	30 ± 14					
A2×	6	5 ± 0.5	$\textbf{2.8}\pm\textbf{0.2}$	$\textbf{2.4}\pm\textbf{0.2}$	0.22 ± 0.02^{bcd}	$\textbf{8.2}\pm\textbf{8.1}$					
L1×	1	1	$\textbf{3.8}\pm\textbf{0.4}$	$\textbf{3.4}\pm\textbf{0.3}$	$0.15\pm0.02^{\rm cd}$	7.4 ± 5.5					
L2×	2	2	$\textbf{7.1}\pm\textbf{0.4}$	$\textbf{6.4}\pm\textbf{0.3}$	$0.22\pm0.04^{\rm bc}$	24 ± 16					
AL1×	4	$\textbf{3.3}\pm\textbf{0.3}$	$\textbf{4.6} \pm \textbf{0.3}$	$\textbf{3.8}\pm\textbf{0.3}$	0.25 ± 0.04^{ab}	50 ± 27					
AL2×	8	$\textbf{7.5}\pm\textbf{0.2}$	$\textbf{9.7}\pm\textbf{0.9}$	8.3 ± 0.4	$0.33\pm0.03^{\texttt{a}}$	42 ± 18					
Contrast analysis											
A versus L					NS	NS					
A versus AL					P = 0.0324	NS					
L versus AL					P = 0.0021	NS					

Values are the mean \pm standard error, n = 15 (earthworms) or n = 5 (gas fluxes). Within a column, values with the same letter are not statistically different (P < 0.05, Tukey test). Treatments: C, control; A, A. caliginosa; L, L. terrestris; AL, A. caliginosa and L. terrestris combined; $1\times$, ambient population, $2\times$, twice the ambient population.

gas flux measurements and 35 microcosms as controls in the differential acetylene inhibition assay.

After the earthworms were added, microcosms were covered with a square piece of plastic mesh (1.5 mm) held tightly with a rubber band to prevent earthworms from escaping and to allow aeration. No food was added to microcosms during the study. Ten blanks (microcosms with no soil and no earthworms) were also prepared, and all microcosms were placed in an incubator (15 °C) in the dark. Soil moisture content was maintained at 40% WFPS (maximum water-holding capacity for this soil = 100% WFPS) by weighing the microcosms every 2–3 days and adding distilled water as needed. The WFPS was calculated as:

$$WFPS(\%) = Pw\left(\frac{D}{S}\right) \times 100$$
 (1)

where Pw is the gravimetric soil moisture content. We assumed a bulk density (D) of 1 g cm^{-3} and a particle density (S) of 2.65 g cm⁻³ (Elliott et al., 1999).

2.3. Gas sampling

Gas sampling occurred 1, 4, 7, 14, 21, and 28 days after earthworm addition. On the gas-sampling day, microcosms were capped using a lid with a septum. About 20-25 ml of headspace gas was collected after 0 min (from 35 experimental jars and 5 blanks) and 30 min (from another set of 35 jars and 5 blanks). We used two sets of microcosms to avoid repeated measures in the gas flux measurements. After the final gas flux measurements on day 28, we added 10 Pa (0.01%, v/v) of acetylene to 35 microcosms (0 min) and 10 kPa (10%, v/v) of acetylene to the other 35 microcosms (30 min) after removing the same amount of air from the headspace. Soils and earthworm treatments were not disturbed. The 70 microcosms that received acetylene plus 35 control microcosms without acetylene were then incubated at 15 °C for 24 h before headspace gases were sampled. All gas samples were stored in evacuated 12 ml exetainers (Labco, High Wycombe, UK) until analysed. A gas chromatograph with a thermal conductivity detector was used for CO₂ analysis, and another of the same model equipped with a 63Ni electron capture detector was used for N₂O analysis (Hewlett-Packard 5890 Series II, Hewlett-Packard Company, Avondale, PA, USA).

2.4. Gas analysis

Fluxes of CO₂ and N₂O were calculated by first converting the gas concentrations in ppm to mg l^{-1} , using the equation from Holland et al. (1999):

$$C_{\rm m} = \frac{(C_{\rm v} M P)}{R T}$$
(2)

where $C_{\rm m}$ is the mass/volume concentration in mgl⁻¹, e.g., mg CO₂–C l⁻¹; C_v is the concentration (v/v) in ppm; M is the molecular weight of the trace species, e.g., CO₂ = 12 µg C µmol⁻¹ CO₂⁻¹; P is the atmospheric pressure, 1 atm; T is room temperature, 293 °K; and R is the universal gas constant, 0.082 l atm mol⁻¹ K⁻¹. Then, the flux was calculated based on

the equations of Hutchinson and Mosier (1981) and Robertson et al. (1999):

$$f = \frac{V(C_1 - C_0)}{Wt} \tag{3}$$

where f is the gas flux in mg m⁻² h⁻¹; V is the volume of the headspace of the microcosm (0.6795 l); C₁ – C₀ is the change in concentration in mg l⁻¹; W is the dry mass of soil in the microcosm (500 g); and t is the time between the first and second gas sample collection (0.5 h). The mean CO₂ flux and mean N₂O flux were the average of the fluxes measured on the six sampling dates during this study.

Acetylene added at 10 kPa (10%, v/v) was assumed to inhibit N₂O production from nitrification and prevent N₂O reduction to N₂ (Davidson et al., 1986). The N₂O production from denitrification was the N₂O + N₂ flux, accumulated as N₂O in the headspace of microcosms treated with 10 kPa acetylene during a 24 h incubation, expressed as μ g N₂O–N g soil⁻¹ day⁻¹. The N₂O production from nitrification (N, in μ g N₂O–N g soil⁻¹ day⁻¹) was calculated as

$$N = C - n \tag{4}$$

where C is N₂O flux from control jars without acetylene and *n* is the N₂O flux from microcosms treated with 10 Pa (0.01%, v/v) acetylene. We assumed that the 10 Pa acetylene treatment inhibited nitrification, but not N₂O reduction to N₂ (Davidson et al., 1986; Tiedje et al., 1989).

2.5. Soil analysis

Soils was taken from 10 microcosms to assess baseline conditions before earthworms were added, and from 35 microcosms with earthworm treatments (control jars without acetylene) at the end of the incubation study. Soils were stored in polyethylene bags at 4 °C for 1 week before they were analyzed. The NO₃-N and NH₄-N concentrations in 0.5 M K₂SO₄ soil extracts (1:5 soil:extractant) were determined with a Lachat Quick-Chem AE flow injection autoanalyzer (Lachat Instruments, Milwaukee, WI, USA). To determine the microbial biomass N (MBN) and dissolved organic N (DON), soil samples were extracted with the chloroform fumigation-extraction procedure followed by persulfate digestion (Voroney et al., 1993). The equation to calculate MBN was: [(total extractable N after fumigation – total extractable N before fumigation)/ $k_{\rm EN}$ where k_{EN} is the extraction coefficient 0.54 (Joergensen and Mueller, 1996). The equation to calculate DON was: (NO₃⁻-N in the persulfate digested extract - mineral N in initial extract) (Cabrera and Beare, 1993). The dissolved organic C (DOC) concentration in unfumigated and fumigated soil extracts was determined with a Shimadzu TOC-V carbon analyzer (Shimadzu Corporation, Kyoto, Japan). The microbial biomass C (MBC) concentration was calculated using the equation: [(fumigated soil extract – unfumigated soil extract)/ k_{EC}], where $k_{\rm EC}$ is the extraction coefficient 0.45 (Joergensen, 1996).

2.6. Statistical analysis

Data were tested for normality using the PROC UNIVARIATE function of SAS (Version 9.1, SAS Institute Inc., Cary, NC).

Then, the effect of earthworm treatments on weekly CO_2 and N_2O fluxes were analyzed by repeated measures analysis of variance using the PROC MIXED function of SAS. The PROC MIXED function was also used to evaluate the effect of earthworm treatments on the mean CO_2 flux, the mean N_2O flux and N_2O production from denitrification and nitrification. Mean values from significant treatments were compared with a Tukey test at the 95% confidence level. Single degree of freedom orthogonal contrasts were used to compare the effect of earthworm species on the mean CO_2 and N_2O fluxes, and N_2O production. Stepwise backward regression analysis with the PROC REG function of SAS was used to evaluate the effect of earthworm numbers and soil conditions on the mean CO_2 and mean N_2O fluxes.

3. Results

3.1. Earthworm survival and growth

The earthworm mortality in microcosms was relatively low (7% of the earthworms died during the study). Three microcosms were removed from the experiment on day 7 because dead earthworms were observed on the soil surface, but there was also some mortality of earthworms below the soil surface in microcosms incubated for 28 days. Earthworms lost weight in all treatments, and biomass decreased by 10–17% during the study (Table 1).

3.2. Dynamics of CO₂ and N₂O fluxes

The repeated measures ANOVA for CO₂ fluxes showed that the earthworm treatments and the sampling day affected the CO₂ fluxes significantly (P < 0.001), but the treatment × sampling day interaction was not significant (Table 2). In contrast, N₂O fluxes were affected by both sampling day and the treatment × sampling day interaction, but not by earthworm treatments (Table 2). The largest CO₂ fluxes were measured 1 day after earthworm addition (0.34 \pm 0.02 mg CO₂–C g⁻¹ h⁻¹) and the smallest 28 d after earthworm addition (0.11 \pm 0.008 mg CO₂–C g⁻¹ h⁻¹). In contrast, N₂O production was near zero on days 1, 4 and 7 and peaked at 105 \pm 24.7 μ g N₂O–N g⁻¹ h⁻¹ on day 14 after earthworm addition (Fig. 1).

3.3. Effect of earthworms on mean CO_2 flux and mean $N_2\text{O}$ flux

The mean CO_2 flux was greater in the AL1×, AL2×, A1× and L2× treatments than the control (Table 1). Contrast analysis revealed that the AL combination produced a greater mean CO_2 flux than the single species (A, L) treatments (Table 1). The mean CO_2 flux was related to the number of earthworms initially added to microcosms, but not to soil conditions at the end of the incubation. A simple linear regression, fitted to data from Table 1, shows the relation:

Table 2 – Repeated measures analysis of variance of the effect of earthworm treatment (trt) and sampling day (day) on CO_2 and N_2O fluxes from microcosms (n = 35) during a 28 d study

Effect	$ m CO_2~flux$ (mg C g soil ⁻¹ h ⁻¹)			$N_2 O$ flux (µg N g soil $^{-1}$ h^{-1})		
	df	F-value	Pr	df	F-value	Pr
trt	6, 156	4.38	0.0004	6, 156	1.74	0.1143
day	5, 156	8.92	< 0.0001	5, 156	14.56	< 0.0001
$trt \times$	30, 156	1.08	0.3696	30, 156	2.72	< 0.0001
day						

The mean N_2O flux was not affected by earthworm treatments, the earthworm functional group or the number of earthworms added to microcosms (Table 1). Stepwise backward regression revealed no relationship between mean N_2O flux and soil conditions at the end of the incubation.

3.4. Production of N₂O via nitrification and denitrification in earthworm microcosms

Earthworms appeared unaffected by exposure to acetylene for 24 h, i.e. their bodies and movement seemed normal. The N₂O production via nitrification was greater in AL than L treatments (P = 0.008, contrast analysis) and greater in A than L treatments (P = 0.032, contrast analysis). In addition, there was more nitrification from the AL1× treatment than the control, and more denitrification from the AL1× and AL2× treatments than the control (Fig. 2). Denitrification produced more N₂O in microcosms with the AL combination than L treatments (P = 0.028, contrast analysis) or A treatments (P < 0.001, contrast analysis). There was also more denitrification in microcosms with the L treatments than the A treatments (P < 0.009, contrast analysis).

4. Discussion

4.1. Experimental constraints

All earthworms in our study lost weight and there was some mortality. Weight loss is not uncommon in microcosm



Fig. 1 – Fluctuations in CO_2 and N_2O fluxes from microcosms during the 28 days study. Data points are the mean (with standard error bars, n = 35) of the seven treatments described in Table 1.



Fig. 2 – Production of N₂O from denitrification and nitrification processes, determined after 24 h exposure to 10 kPa or 10 Pa acetylene in the headspace of microcosms with earthworm treatments (described in Table 1). Mean values (with standard error bars, n = 5) with different letters were significantly different (P < 0.05, Tukey test), with bold letters for denitrification and italicized letters for nitrification values.

studies (Whalen et al., 2004), although it was probably exacerbated by a lack of food (Curry and Bolger, 1984; Daniel et al., 1996). We did not add organic substrates to the microcosms to avoid the confounding effect of earthworminduced litter decomposition and N mineralization that could stimulate microbial activity. This may have been overly cautious, since the endogeic earthworms Octolasion lacteum and A. caliginosa stimulated the same amount of CO₂ production in microcosms without plant litter as in microcosms with litter (Potthoff et al., 2001; Vetter et al., 2004). Reproduction of A. caliginosa occurred in microcosms; we found one hatchling and two cocoons in the A treatments at the end of the study. Despite the lack of food, most earthworms survived in microcosms for 28 days, perhaps because we selected soil temperature and moisture conditions that were favorable for A. caliginosa and L. terrestris.

It is very difficult to design an experiment to compare gas fluxes amongst earthworm functional groups. The standard microcosm (11 jars with 500 g of soil and no residues) appeared to be a good habitat for A. caliginosa, but it is almost certain that L. terrestris feeding and burrowing activities were constrained. To better simulate natural earthworm behavior, larger microcosms could be built to optimize the activity of L. terrestris. However, a larger microcosm would contain more soil, which could pose problems for detecting differences in N2O concentrations among treatments and reduce the reliability of acetylene inhibition assays. The earthworm functional group in a microcosm could be based on the number of individuals or their biomass, but is it relevant to compare the gas flux from soil containing many, small endogeic earthworms versus a few large anecic earthworms? The Simplex design used by Sheehan et al. (2006) permitted interpretation of the separate effects of functional groups and their interactions on soil N transformations, and may hold promise for resolving some of these experimental constraints.

4.2. CO_2 and N_2O fluxes as influenced by earthworm numbers and species

The mean CO₂ flux increased when more earthworms were present in microcosms, probably due to earthworm respiration as well as earthworm-induced microbial respiration. Binet et al. (1998) observed a positive correlation between CO₂ emissions and the biomass of L. terrestris in microcosms. Earthworms were responsible for 7–58% of the total CO₂ flux from microcosms. Similarly, Caravaca et al. (2005) reported that 40% of the total CO_2 production was from E. fetida in microcosms with soil and composted residues. The mean CO₂ flux was greater from the AL combination than the single species treatments, suggesting a stimulation of microbial respiration resulting from interactions between endogeic and anecic functional groups. Postma-Blaauw et al. (2006) reported an increase in bacterial growth rate and soil C mineralization in microcosms with mixed populations of L. terrestris and A. caliginosa, but not for single species populations. Jégou et al. (2001) proposed that microbial stimulation can occur when A. caliginosa feeds on the burrow linings of L. terrestris, and that L. terrestris burrowing increases in the presence of A. caliginosa. Earthworms had no effect on the N₂O flux from microcosms in this study, which is in contrast to other reports (Karsten and Drake, 1997; Matthies et al., 1999; Borken et al., 2000). Finally, soil conditions in microcosms at the end of the incubation period were not related to the mean CO₂ and mean N₂O fluxes.

4.3. Denitrification and nitrification rates as influenced by earthworms

Despite the limitations of the acetylene inhibition method (Groffman et al., 2006), it remains a useful method for assessing the potential N_2O production from soils and understanding the contribution of denitrification and nitrification processes to N₂O emissions. We found that denitrification was the dominant process in the AL treatment (69-88% of total N₂O production) and L treatment (100% of total N₂O production). In a laboratory study with L. terrestris, Svensson et al. (1986) reported that casts produced 2.5 times more N₂O from denitrification than surrounding soil, likely due to anaerobic conditions in casts that favored denitrifying bacteria. We observed surface casts in all microcosms, but it may be that denitrification occurred only in casts produced by L. terrestris. Schrader and Zhang (1997) suggested that litter consumption by L. terrestris produces casts that are nutrientrich, compared to the casts generated by soil-feeding earthworms like A. caliginosa. Further study is needed to evaluate denitrification in the casts produced by these species, as well as earthworm-worked soil.

There was no nitrification in the L treatment, but it was an important process in the A treatment (58–85% of total N₂O production) and to a lesser extent in the AL treatment (12–31% of total N₂O production). Burrows have higher nitrification rates than bulk soil (Parkin and Berry, 1999) and A. *caliginosa* creates extensive horizontal burrows in microcosms (Jégou et al., 1998; Perreault and Whalen, 2006). We observed more burrows in microcosms containing A. *caliginosa* than L. *terrestris* alone and suggest that burrowing activities of A. *caliginosa* led to N₂O production via nitrification. Clearly, more

work needs to be done to assess the microbial activity in earthworm structures like casts and burrows, to better understand the contribution of earthworms in N_2O production and emissions from soils.

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