Soil Biology & Biochemistry 68 (2014) 117–124

Contents lists available at ScienceDirect

Soil Biology & Biochemistry

journal homepage: www.elsevier.com/locate/soilbio

Earthworms reduce soil nitrous oxide emissions during drying and rewetting cycles

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ARTICLE INFO

Article history:
Received 7 May 2013
Received in revised form
18 September 2013
Accepted 22 September 2013
Available online 2 October 2013

Keywords:
Earthworm
N_2O emissions
Soil moisture
Wetting–drying cycles
Denitrifier gene copies
Denitrification enzyme activity

ABSTRACT

Nitrous oxide (N_2O) is a greenhouse gas that is released from both nitrification and denitrification processes. Soil moisture content is a key controller of the biochemical pathways leading to N_2O emission, causing a switch between nitrification and denitrification processes. Earthworms are reported to increase N_2O emissions from soil under aerobic and anaerobic conditions, but how earthworm-induced N_2O emissions are affected by soil drying and rewetting cycles is unknown. The objectives of this study were to (1) evaluate earthworm-induced N_2O emissions from soils with aerobic, anaerobic, and fluctuating soil moisture conditions; and (2) determine the earthworm effects on soil denitrifiers responsible for N_2O fluxes. Soils were kept in mesocosms (polyvinyl chloride plastic tubes, 10 cm diameter, filled with soil to 15 cm depth) at constant 33% water-filled pore space (WFPS), constant 97% WFPS or underwent three wetting-drying cycles (WD). Each soil moisture treatment had 2 earthworm treatments, including (1) a mixture of endogeic *Aporrectodea turgida* and anecic *Lumbricus terrestris* and (2) no earthworm treatment. These gave a total of 6 treatments in this study, with 5 replicates for each treatment. The N_2O fluxes were quantified every one to three days, and the soil denitrifier activities were measured after 69 days, when the experiment ended. Soil moisture significantly affected N_2O emissions and the WD treatment had the highest cumulative N_2O emissions. Earthworms increased N_2O emissions by 50% in the 33% WFPS treatment but decreased N_2O emissions by 34% in the 97% WFPS treatment, probably due to more complete reduction of N_2O to N_2. Earthworms strongly reduced N_2O emission rate in WD treatment, and they significantly reduced cumulative N_2O emissions by 82%. Soil denitrification enzyme activity (DEA) increased significantly when earthworms were present. Abundance of 16S rRNA, *nirS*, and *nosZ* genes was affected significantly by the earthworm × soil moisture interaction, with the highest 16S rRNA and *nosZ* abundance in soil from the WD treatments. We conclude that the decrease in cumulative N_2O emissions from soil at 97% WFPS and the WD treatment by earthworms was due to an alteration of the denitrifying bacterial community composition.

1. Introduction

Soil moisture changes constantly as a result of rewetting events (e.g., rainfall, snowmelt, irrigation and flooding) and drying, as water drains through the profile or returns to the atmosphere via (evapo)transpiration. Soil moisture regulates redox potential and therefore influences microbially-mediated reactions in the nitrogen (N) cycle. Most nitrogenous compounds in the soil N cycle are produced under a narrow range of soil moisture conditions, but nitrous oxide (N_2O) is released from nitrification and nitrifier-denitrification under aerobic conditions (<70% water-filled pore space (WFPS)), with substantial N_2O fluxes occurring during denitrification in anaerobic soils (≥70% WFPS) (Kool et al., 2011; Linn and Doran, 1984; Wrage et al., 2005, 2001). Rapid rewetting of dry soil can trigger a pulse of N_2O, which is attributed to the following causes: (i) a number of facultative aerobic soil microorganisms can switch to anaerobic metabolism, leading to gaseous N_2 and N_2O emissions (Khahil and Baggs, 2005; Kool et al., 2011; Linn and Doran, 1984); (ii) release of the osmolytes accumulated in the drying phase, cell lysis and breakdown of aggregates supply abundant substrates to denitrifiers (Fierer et al., 2003; Gordon et al., 2008); and (iii) anaerobic microbial activity will be stimulated, especially denitrification enzyme activity (DEA) (Guo et al., 2010). Previous drying-rewetting studies showed that N_2O emissions could be affected by the frequency of the drying and rewetting cycles (Fierer and Schimel, 2002), soil compaction (Beare et al.,

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http://dx.doi.org/10.1016/j.soilbio.2013.09.020

2009), the type of crop residue present (Zhong et al., 2011) and fertilizer inputs (Ruser et al., 2006). However, most of those studies were conducted in the absence of soil macrofauna, notably earthworms, which contribute to soil N_2O emissions.

There is ample evidence that earthworm interactions with soil microorganisms increase soil N_2O emissions, with 42% more N_2O emitted from earthworm-worked soil, on average, than without earthworms (Lubbers et al., 2013). There are two sources of N_2O from earthworms – the earthworm body, which can release 0–11 nmol N_2O h^{-1} g^{-1} earthworm (Horn et al., 2006) and its biostructures (casts, middens, and burrows) (Drake and Horn, 2006, 2007). Earthworm biostructures modify the soil structure, i.e., fresh casts function like stable macroaggregates while burrows change soil water-flow dynamics and gas diffusivity (Giannopoulos et al., 2010; Lubbers et al., 2011; Shipitalo and Bayon, 2004), and are thus considered to be an indirect effect of earthworms on N_2O emissions. Earthworm-induced N_2O emissions vary depending on earthworm species (Rizhiya et al., 2007; Speratti and Whalen, 2008), food placement (residues incorporated vs. surface applied) (Giannopoulos et al., 2010) and plant N uptake (Lubbers et al., 2011) when soil water content was kept constant (from 40% to 100% WPFS in those studies). Less is known about how earthworm-induced N_2O emissions are affected by soil moisture. Bertora et al. (2007) reported that *Aporrectodea longa* enhanced N_2O production under 25% gravimetric soil water content, but not at 19% or 12.5% gravimetric soil water content, yet Rizhiya et al. (2007) found no difference in earthworm-induced N_2O production at 44% WFPS and 100% WFPS. Earthworm survival and growth are constrained in dry and flooded soils, such that about 57%–69% WFPS is optimal for earthworm activities (Eriksen-Hamel and Whalen, 2006; Moreau-Valancogne et al., 2013), and likely controls the direct and indirect effects of earthworms on soil N_2O emissions. Wetting and drying cycles are expected to cause earthworms to move vertically in the soil profile as they seek zones with favorable soil moisture conditions, although whether this affects the dynamics of earthworm-induced N_2O emissions under fluctuating soil moisture conditions is not known.

The presence of earthworms should enhance N_2O production from nitrification and nitrifier-denitrification because earthworm activity stimulates N mineralization and nitrification (Costello and Lamberti, 2009; Lubbers et al., 2011). Nitrification was the source of 12%–85% of the N_2O production in soil containing *Aporrectodea turgida* alone or in a mixed population with *Lumbricus terrestris*, and there was about 30 times more N_2O production from earthworm-worked soil than the control without earthworms (Speratti and Whalen, 2008). Considering that denitrification is a major source of soil N_2O emissions (Kool et al., 2011), how earthworms affect the activity and composition of microbial denitrifier communities needs to be considered. For instance, denitrifying activity is affected by access to labile carbon, so earthworm activities that increase soil labile carbon could change the N_2O/N_2 ratio (Miller et al., 2008; Nebert et al., 2011). Soils with low mineral N (especially NO_3^-) and high moisture often favor N_2O consumption, since NO_3^- is preferred as an electron acceptor over N_2O (Chapuis-Lardy et al., 2007; Rosenkranz et al., 2006; Ruser et al., 2006), so earthworm activities that result in nitrification and therefore high NO_3^- concentration are expected to produce N_2O and increase N_2O emissions from soil. If earthworm intestinal tract or biostructures are favorable micro-habitats for denitrifying bacteria that lack nitrous oxide reductase (N_2OR, synthesized by the *nosZ* gene), the terminal reaction product would be N_2O (Chapuis-Lardy et al., 2010; Depkat-Jakob et al., 2013; Nebert et al., 2011; Zumft and Körner, 2007). Still, there have been relatively few studies to investigate denitrifiers in earthworm-worked soil, and none that have studied earthworm–denitrifier interactions under fluctuating soil moisture conditions.

The objective of this study was to measure the earthworm-induced N_2O emissions under constant soil moisture, both aerobic and anaerobic conditions, and in soils with repeated wetting and drying cycles. A secondary objective was to determine how earthworms influenced the activity of soil denitrifiers, and whether this was related to the N_2O emissions. This laboratory mesocosm experiment was conducted with a mixed population of endogeic (*A. turgida*) and anecic (*L. terrestris*) earthworms, since these species typically co-habit soils in our region.

2. Materials and methods

2.1. Soil and earthworm collection

Individuals of *A. turgida* and *L. terrestris* were extracted with dilute (0.5%) formaldehyde solution from a red clover (*Trifolium pretense* L.) field at the Macdonald Campus Research Farm, Ste-Anne-de-Bellevue, Quebec, Canada (45°28′ N, 73°45′ W). Earthworms were washed several times with tap water to remove formaldehyde on the body surface and then transferred into 37 L culture boxes for at least one month. Earthworms were fed with grass-based plant compost from the Macdonald Campus Research Farm. Soil for earthworm culture and the incubation study was Chateauguay clay loam soil (fine, mixed, nonacid, frigid, Hapludalf), with 36.8 g organic C kg^{-1} and a pH of 6.5.

2.2. Experimental design

This experiment used a completely randomized factorial design with 2 earthworm treatments (with and without earthworms, referred as EW and nEW, respectively) and 3 soil moisture conditions (constant 97% WFPS, constant 33% WFPS, and wetting–drying cycles (WD) from 97% WFPS to 33% WFPS) (Table 1). The experiment was conducted in mesocosms, 1.57 L polyvinyl chloride plastic tubes with 10 cm diameter and a height of 20 cm. Soil (sieved < 6 mm mesh) was packed to 15 cm height at a bulk density of 1.20 ± 0.003 g cm^{-3}, leaving 5 cm of headspace. Although the redistribution of water may occur in a 15 cm tall soil core (Guo et al., 2013), the cores needed to be sufficiently large to accommodate earthworm movement, including possible vertical displacement in response to the WD treatment. Although the natural burrowing habits of *L. terrestris* would be better simulated in cores tall enough to hold 1 m of soil (Shipitalo and Bayon, 2004), a taller soil core was not selected because soil moisture at the surface and at soil depths lower than 20 cm would be significantly differently (Paul et al., 2012), which would affect the estimation of earthworm effects on N_2O emissions under different soil moisture conditions. Each soil moisture treatment was repeated in 15 mesocosms, which included undisturbed EW ($n = 5$) and nEW ($n = 5$) treatments for gas sampling as well as a disturbed EW treatment ($n = 5$), where earthworms were removed periodically to assess their survival and biomass, giving 45 mesocosms in total.

After soil was added, the moisture content was adjusted to 33% WFPS in 30 mesocosms (for the 33% WFPS and WD treatments) and 97% WFPS in 15 mesocosms that were then pre-incubated for 4 d at constant temperature (20 °C) in the dark to achieve a stable N_2O flux rate. Then, the earthworm treatment was added to mesocosms in the undisturbed and disturbed EW treatments. Each earthworm treatment included 3 adult *A. turgida*, 1 juvenile *L. terrestris* and 1 adult *L. terrestris*, giving 382 individuals m^{-2} of endogeic and 255 individuals m^{-2} of anecic earthworms. This earthworm density is greater than field populations in this region, which range from 46 to 422 individuals m^{-2} (Eriksen-Hamel et al., 2009; Whalen, 2004; Whalen et al., 2012). Two days before adding the earthworm treatment, we removed all earthworms from culture boxes, washed

Table 1
Soil inorganic N (mean ± standard errors) in a mesocosm experiment, as affected by earthworms (with earthworms, EW; without earthworms, nEW) and soil moisture (constant 33% water-filled pore space (WFPS), constant 97% WFPS and wetting-drying cycles (WD) that went from 97% to 33% WFPS). Values within a column followed by different letters are significantly different ($P < 0.05$).

Treatment	NH_4^+–N (mg kg^{-1} soil)	NO_3^-–N (mg kg^{-1} soil)
EW-33% WFPS	3.25 ± 1.03 b	152 ± 14.6 a
nEW-33% WFPS	1.98 ± 0.55 b	96.1 ± 67.4 a, b
EW-97% WFPS	62.6 ± 5.18 a	33.5 ± 8.23 b, c
nEW-97% WFPS	56.1 ± 5.53 a	26.2 ± 27.4 c
EW-WD	2.80 ± 1.00 b	15.7 ± 5.39 c
nEW-WD	0.362 ± 0.116 b	27.3 ± 13.8 c
AVOVA (*P* value)		
Earthworm	0.011*	0.182
Soil moisture	<0.001***	<0.001***
Earthworm × soil moisture	0.213	0.098

*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

them with ddH_2O and left them on moist Kimwipe tissue without food for 48 h, and recorded the initial biomass (gut cleared) of the group of individuals placed in each mesocosm. After earthworms had burrowed into the mesocosm, earthworm food was added on top of all 45 mesocosms (both EW and nEW treatments) as a mixture of 2 g grass-based plant compost (433 g kg^{-1} C and 39 g kg^{-1} N) and 1 g Magic Worm Food (a *sphagnum* peat moss base material, 388 g kg^{-1} C and 12 g kg^{-1} N, Magic Products Inc. Amherst Junction, Wisconsin, United States), provided the total of 159 g C and 11 g N m^{-2}. Finally, all mesocosms were covered with a 1.5 mm mesh wire screen, secured with an elastic band to prevent earthworm escape and permit gas exchange. All mesocosms were left in the dark at 20 °C for an additional 4 d pre-incubation after adding the earthworm treatment.

Soil water content was maintained by weighing each mesocosm daily and adding water as necessary, during the pre-incubation phase and the rest of the experiment. Following the 8 d pre-incubation, mesocosms in the WD treatment were wetted by adding water to reach 97% WPFS, which counted as day 1 of the experiment. A dehumidifier was set up inside the incubator to speed water evaporation, such that mesocosms in WD treatment were permitted to dry to 33% WFPS before they were wetted to 97% WFPS. A total of three WD cycles occurred during the experiment, which lasted for 69 d.

2.3. The N_2O measurement

The N_2O measurement was taken from all the undisturbed mesocosms on the first and second day after rewetting the WD treatment, and then once every 2–3 d until the end of each cycle. For gas sampling, each mesocosm was sealed using a polyethylene lid equipped with rubber septa. After 2 h, 9 mL of headspace gas was removed from each mesocosm and injected into a 5.9 mL vacuumed exetainer (Labco, High Wycombe, UK) with an extra mil teflon-silicone septa (National Scientific, Rockwood, TN, USA). Background N_2O concentration was determined by taking an air sample from the incubator room at the beginning of each gas sampling period; since there was gas exchange between screen-covered mesocosms and the incubator room, the N_2O concentration was representative of the initial N_2O concentration in mesocosm headspace at the beginning of the 2 h measurement period. The N_2O concentration was analyzed by a gas chromatograph (Model 6890, Hewlett Packard, Avondale, PA, USA) equipped with a HP-PLOT/Q column (32.5 m × 535 μm × 40.0 μm, Agilent Technologies Inc, Santa Clara, CA) and detected with a micro-electron capture detector at 300 °C. Carrier gases were helium at 4.0 mL/min and ultrahigh purity nitrogen at 15.0 mL/min. The N_2O–N production from a mesocosm was calculated according to Drury et al. (2007). The cumulative N_2O–N emissions from a mesocosm was calculated based on the average N_2O production during the 2 h sampling period, interpolated between sampling events by assuming a linear change in N_2O emissions between each successive sampling event.

2.4. Earthworm survival and biomass

The disturbed EW treatment ($n = 5$) was used to determine the earthworm survival and biomass at days 22 (the middle of the 1st WD cycle), 34, 51, and 69 (the end of the 1st, 2nd, and 3rd WD cycles, respectively). Soil was removed, earthworms were collected and counted, their biomass (g fresh weight) was determined after gut clearance for 24 h on wet filter paper, then earthworms were returned to the same mesocosm after repacking the soil. At the end of the experiment (day 69), the undisturbed mesocosms were also destructively sampled, and the final earthworm survival and biomass were the values only from disturbed mesocosms ($n = 5$).

2.5. Soil analyses

At the end of the 69 d incubation, soil from each undisturbed mesocosm was mixed thoroughly and subsamples were taken for chemical and biological analyses including inorganic N, DEA and denitrifier gene copies. Inorganic N was extracted in 2 M KCl and the NH_4^+–N and NO_3^-–N concentrations were determined colorimetrically with the indophenol blue method (Sims et al., 1995) on a BIO-TEK EL312 Microplate Reader (BIO-TEK Instruments Inc, Winooski, VT, USA). The DEA was measured with an acetylene block assay as described by Drury et al. (2007). Briefly, 25 g of soil was put into 250 mL flask and 25 mL of solution containing 300 mg glucose-C kg^{-1} soil and 50 mg NO_3^-–N kg^{-1} soil was added. The flask was closed by a rubber septum, flushed with argon gas for 30 min, and 10% of the headspace was replaced by acetylene. Flasks were put into a rotary shaker at 225 revolutions min^{-1}. After 1, 2, 3, and 5 h, 9 mL headspace gas was removed and stored into a 5.9 mL vacuumed exetainer (Labco, High Wycombe, UK) with an extra mil teflon–silicone septa (National Scientific, Rockwood, TN, USA). The N_2O concentration was analyzed by gas chromatography as described above. The N_2O–N production from each flask at each sampling time was calculated according to Drury et al. (2007), and the DEA was determined from the slope of the best fit line calculated when plotting N_2O–N production against time.

2.6. DNA extraction and quantitative PCR (qPCR) analyses

For qPCR, subsample of soil was stored at −80 °C for DNA extraction with a PowerSoil® DNA Isolation Kit (MO BIO Laboratories, Inc., CA, USA). The qPCR reactions were performed in triplicate on Stratagene Mx3005P QPCR Systems (Agilent Technologies, Santa Clara, CA, United States). Each reaction consisted of 5 μL of Brilliant III Ultra-Fast SYBR Green QPCR Master Mix (Agilent Technologies, Santa Clara, CA, United States), 0.4 μL of 10 μM of each forward and reverse primers (final concentration of 400 pM), 2.2 μL nuclease-free H_2O, and 2 μL of template DNA. The primers were 1055f–1392r for bacterial 16S rRNA gene (Harms et al., 2003) at an annealing temperature of 59 °C, nirS1F-nirS3R for *nirS* gene (Braker et al., 1998) at an annealing temperature of 59 °C, and nosZ1527f–norZ1773r for *nosZ* gene (Scala and Kerkhof, 1998) at an annealing temperature of 57 °C. The PCR procedure was as follows, 5 min at 95 °C; 40 cycles of 30 s at 95 °C, 40 s at the annealing temperature for the primers, and 72 °C for 1 min.

A dissociation curve was obtained at the end of each PCR reaction, with the protocol of 1 min at 95 °C, 30 s at 55 °C and 30 s at

95 °C. The single peak of dissociation curve indicated the specificity of PCR products. Standard curves for 16S rRNA were generated by amplifying a fragment of 16S rRNA from *Escherichia coli* genomic DNA. Similarly, the standard curves for *nirS* and *nosZ* were developed by amplifying a plasmid DNA containing a fragment of the *nirS* gene and *nosZ* gene (Siciliano et al., 2000). Each assay contained a 10-fold serial standard dilution, soil DNA, and no template controls. The quantification of *nosZ* and *nirS* had a detection limit of 10^2 copies per assay, and the quantification of 16S rRNA had a detection limit of 10^3 copies per assay. The presence of PCR inhibitors in the soil samples was tested by a serial dilution of soil DNA extract. No inhibition was detected in any case. The PCR efficiency and copy number were determined by MxPro software (Agilent Technologies, Santa Clara, CA, USA).

2.7. Statistical analyses

The effects of earthworm and soil moisture treatments, and the earthworm × moisture interaction on cumulative N_2O emissions, soil inorganic N, DEA and denitrifier gene copies were analyzed using a two-way ANOVA with SAS 9.3 (SAS Institute Inc., Cary, NC, USA). Least square mean values of significant ($P < 0.05$) main effects and interactive effects were compared with a Tukey–Kramer test. The cumulative N_2O emissions were log-transformed prior to ANOVA analysis to satisfy the assumption of normality with Shapiro–Wilk test and homoscedasticity with Levene's test. Pearson's correlation coefficients were used to describe the relationship among cumulative N_2O emissions, inorganic N, and denitrifier gene copies.

3. Results

3.1. Earthworm survival and biomass

From the visual observation, earthworms were present on the surface soil after rewetting events, but they disappeared within one or two days. Fresh casts and middens appeared on the soil surface after rewetting events. Earthworm burrows were observed in the 33% WFPS and WD treatments, especially large subsurface burrows by *L. terrestris*, but not in tubes kept at 97% WFPS.

Earthworm survival in the 33% WFPS and WD treatments was 100% and 93% for *A. turgida* as well as 80% and 70% for *L. terrestris*. The lowest survival was in the 97% WFPS treatment, which had 3 mesocosms with 0 or 1 remaining earthworm from day 0 to day 69 of the experiment (Fig. 1). The earthworm survival and biomass from disturbed mesocosms were listed in Table S1, which indicated the possible bias from disturbed mesocosms and undisturbed mesocosms based on the survival rate of the two species.

3.2. The N_2O emissions

Cumulative N_2O emissions were affected by soil moisture ($P < 0.001$), with 1025–2055 times more N_2O released from the WD treatment than the 33% WFPS and 97% WFPS treatments (Fig. 2). Although the 33% WFPS and 97% WFPS treatments had cumulative N_2O emissions in the same range, adding earthworms increased by 50% the amount of soil N_2O produced in the 33% WFPS treatment but decreased by 34% the amount of soil N_2O produced under constant 97% WFPS (Fig. 2).

The N_2O emission rate increased more than 30000 times in nEW treatment and more than 6000 times in EW treatment after the rewetting event in the second WD cycle, while N_2O emission rate increased 133 times and 16 times in nEW and EW treatments after the rewetting event in the third wetting-drying cycle (Fig. 3). The cumulative N_2O emissions after three WD cycles were 82% lower in

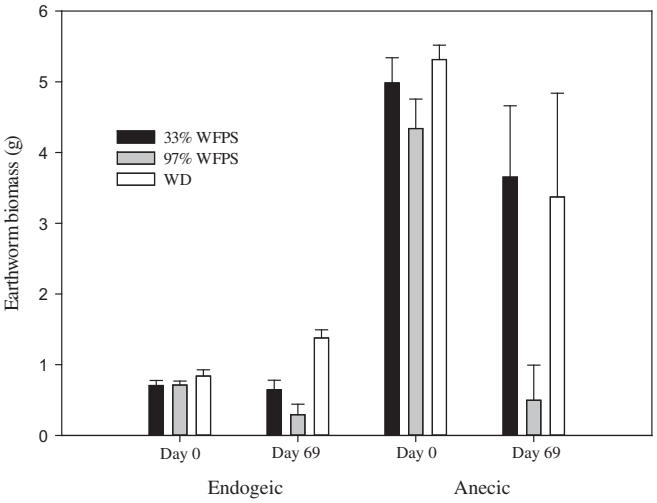

Fig. 1. Changes of earthworm total biomass (mean ± standard error) from the beginning (Day 0) and the end (Day 69) of mesocosm experiment with three soil moisture treatments –33% water-filled pore space (WFPS), 97% WFPS and wetting–drying cycles (WD) incubation. At the end of the experiment (Day 69), endogeic earthworms had an average survival of 100%, 20% and 93% in the treatments of 33% WFPS, 97% WFPS and WD, respectively, while anecic earthworms had an average survival of 80%, 20%, and 70% in the treatments of 33% WFPS, 97% WFPS and WD, respectively. Both earthworm total biomass and survival data were obtained from undisturbed mesocosms.

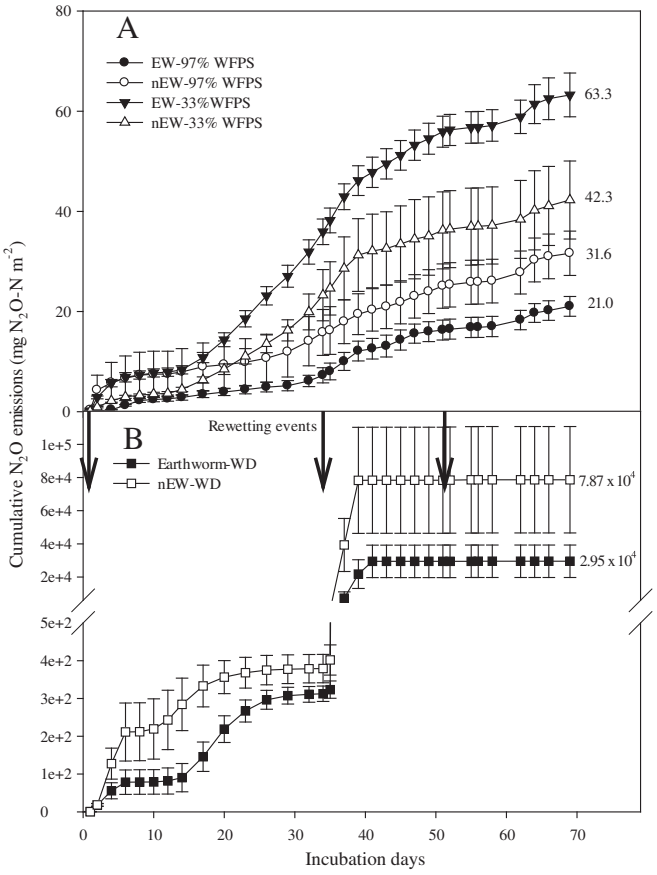

Fig. 2. Cumulative N_2O emissions (mean ± standard error) during 69 d mesocosm experiment from the soils with and without earthworm (EW and nEW, respectively) at (A) constant soil moisture (33% water-filled pore space (WFPS) and 97% WFPS) and (B) wetting-drying cycles (WD). Arrows indicate the rewetting events.

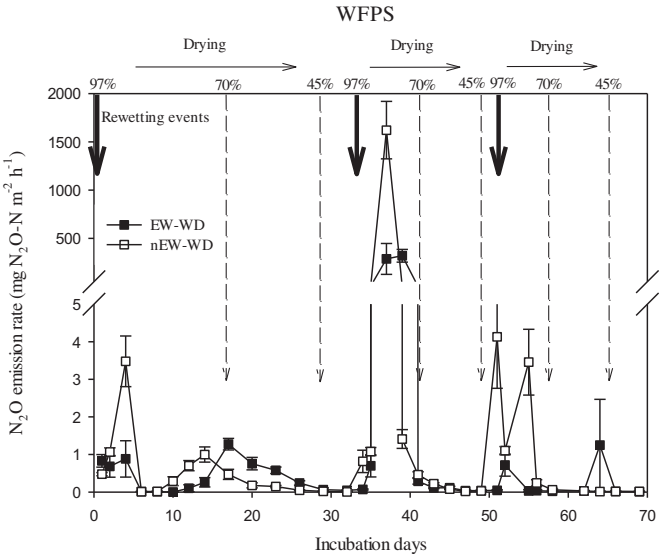

Fig. 3. The N_2O emission rate (mean ± standard error) during 69 d mesocosm experiment from the soils with and without earthworm (EW and nEW, respectively) at wetting–drying cycles (WD). Solid arrows indicate the rewetting events, while dash arrows indicate the change of soil moisture during WD (WFPS = water-filled pore space).

the EW treatment compared to the nEW treatment ($P < 0.05$, Fig. 3). However, the N_2O emissions were greater in the EW than nEW treatment during the drying phase of the WD cycle, when soil moisture was less than 70% WFPS in the first WD cycle, less than 50% WFPS in the second WD cycle, and less than the 45% WFPS in the third WD cycle (Fig. 3).

3.3. The DEA and quantification of 16S rRNA, nirS, and nosZ genes

Earthworms increased the DEA significantly ($P < 0.05$), by 7 times at 33% WFPS and 2-fold in the 97% WFPS treatment and by 5 times in the WD treatment (Fig. 4). There were also more DEA in mesocosms with 97% WFPS and WD treatments than the 33% WFPS

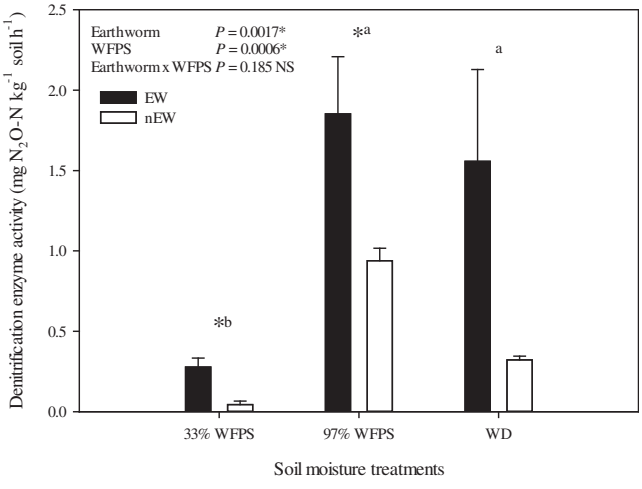

Fig. 4. Earthworm effects on denitrification enzyme activity (DEA) (mean ± standard error) at 33% water-filled pore space (WFPS), 97% WFPS and wetting-drying cycles (WD) after 69 d mesocosm experiment from the soils with and without earthworm (EW and nEW, respectively). Values followed by different letters indicates difference in DEA between soil moisture levels ($P < 0.05$). An asterisk (*) is used when earthworm treatment within a moisture level is significant at $P < 0.05$. NS = not significant.

treatment (Fig. 4). There was a significant ($P < 0.05$) earthworm × soil moisture effect on 16S rRNA, *nirS* and *nosZ* genes, such that earthworms and the WD treatment gave the greatest 16S rRNA gene and *nosZ* gene copies (Fig. 5). There were more *nirS* gene copies in mesocosms without earthworms that were kept at 33% WFPS than in the other treatments (Fig. 5).

3.4. Relationship between cumulative N_2O emissions, DEA, bacterial gene copies and inorganic nitrogen

Cumulative N_2O emissions were negatively correlated ($P < 0.01$) with NH_4^+-N and inorganic N concentrations, but positively correlated with bacterial 16S rRNA gene and *nosZ* gene copies (Table 2). The DEA was positively correlated with NH_4^+–N and negatively correlated with NO_3^-–N concentration (Table 2). A positive correlation between *nosZ* and bacterial 16S rRNA genes was also noted (Table 2).

4. Discussion

4.1. Earthworm effects on N_2O emissions in dry soil

Earthworms stimulated N_2O emissions from soil held at constant 33% WFPS, with 1.5 times more cumulative N_2O emissions in earthworm-worked soil than in the absence of earthworms. It seems likely that N_2O production in dry soil was a byproduct of the nitrification process, which means it was released during hydroxylamine oxidation to nitrite by ammonia oxidizing microorganisms, namely bacteria and archaea (Kool et al., 2011; Leininger et al., 2006). This assumption is supported by the tendency for higher NO_3^-–N concentration and bacterial 16S rRNA gene copies in mesocosms with earthworms than without earthworms at 33% WFPS; in addition, the DEA was lower in soils kept at 33% WFPS than the other soil moisture levels.

Nitrification was already proposed as a source of N_2O in soil microcosms containing *A. turgida* alone or *A. turgida* plus *L. terrestris*, where the soil moisture was maintained at 40% WFPS (Speratti and Whalen, 2008). Our results are also consistent with the 57% increase in N_2O in field soils with *L. terrestris* at 47% WFPS (Borken et al., 2000). There is a considerable body of literature describing how earthworms increase N mineralization and nitrification in well-aerated soils (Costello and Lamberti, 2009; Lubbers et al., 2011; Rizhiya et al., 2007), and it appears that these processes lead to N_2O emissions as well. Future studies should focus on earthworm–nitrifier interactions and their effects on N_2O production, especially under dry soil conditions. The qPCR-based studies could help to estimate the earthworm influences on microbial communities (Saunders et al., 2012), and the earthworm effects on N_2O sources can be detected by isotope tracing studies (Kool et al., 2011).

4.2. Earthworm effects on N_2O emissions in wet soil

Earthworms reduced N_2O emissions from soil held at constant 97% WFPS, with 1.5 times lower cumulative N_2O emissions in earthworm-worked soil than in the absence of earthworms. This result differs from Rizhiya et al. (2007), who reported that *A. longa* and *Lumbricus rubellus* increased N_2O emissions from soil kept at 100% WFPS for 90 d. There are several possible explanations. First, it could be that poor survival of earthworms in the 97% WFPS treatment reduced their interaction with soil microorganisms responsible for denitrification. However, this argumentation cannot explain the lower N_2O emissions in the presence of earthworms. Besides, there was no difference in the number of *nirS*, *nosZ* and 16S rRNA gene copies between EW and nEW treatments at 97% WFPS,

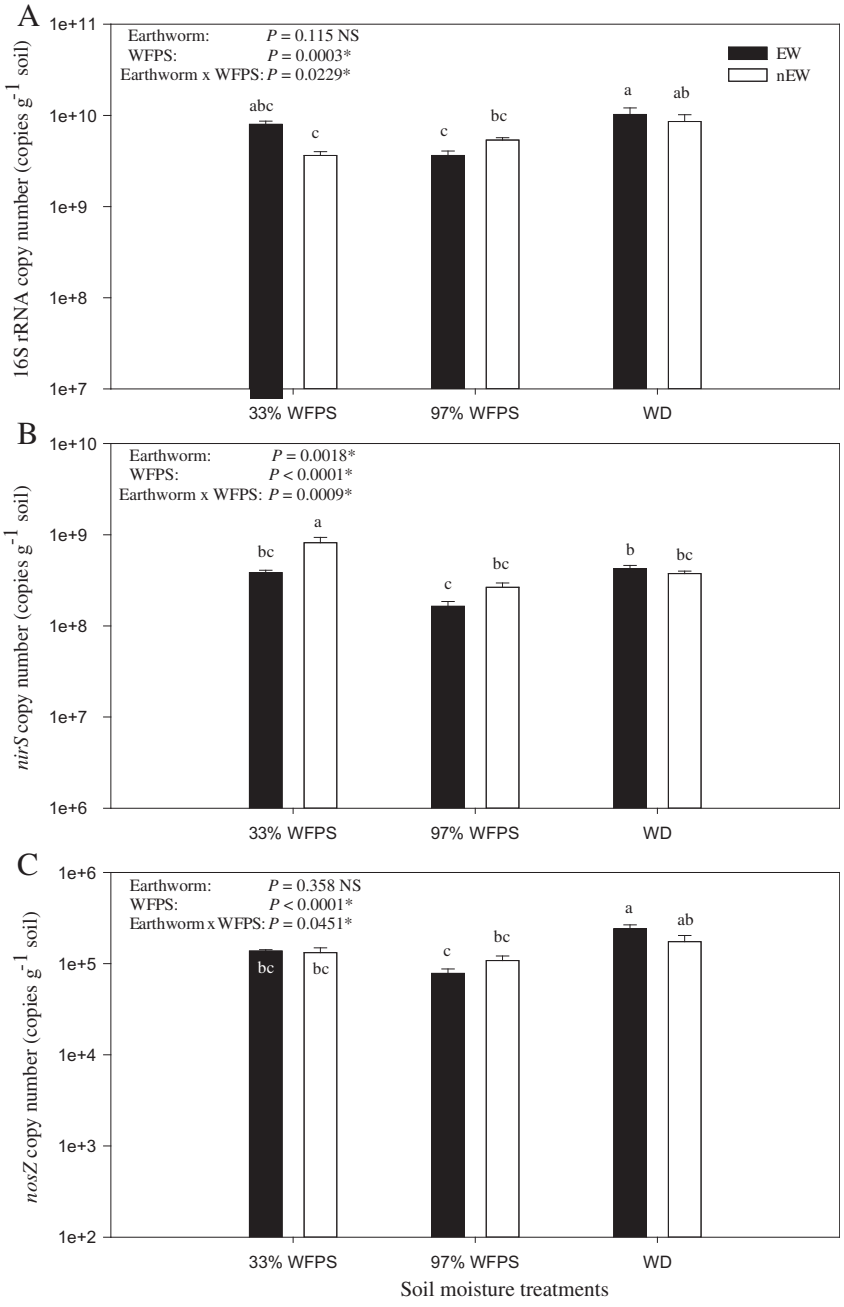

Fig. 5. Earthworm effects on (A) 16S rRNA, (B) *nirS* and (C) *nosZ* gene copy numbers (mean ± standard error) at 33% water-filled pore space (WFPS), 97% WFPS and wetting–drying cycles (WD) after 69 d mesocosm experiment from the soils with and without earthworm (EW and nEW, respectively). Slopes, efficiencies and R^2: 16S rRNA copy numbers: slope = −3.220 to −3.626, efficiencies = 88.7%–104.4%, R^2 = 0.959 to 0.996; *nirS* copy numbers: slope = −3.524 to −3.644, efficiencies = 88.1%–92.2%, R^2 = 0.982 to 0.983; *nosZ* copy numbers: slope = −3.434 to −3.463, efficiencies = 94.4%–95.5%, R^2 = 0.966 to 0.998. Values followed by different letters are significantly different ($P < 0.05$). NS = not significant.

Table 2
Pearson correlation coefficients (r) between cumulative N_2O emissions, denitrification enzyme activity (DEA), bacterial gene copies (16S rRNA, *nirS*, and *nosZ*), and inorganic N in a 69 d mesocosm experiment with earthworm and soil moisture treatments.

Parameter	NH_4^+–N	NO_3^-–N	Inorganic N	DEA	16S rRNA	*nirS*	*nosZ*
Cumulative N_2O emissions	−0.397*	−0.352	−0.556**	−0.021	0.419*	0.003	0.511**
NH_4^+–N		−0.366	0.120	0.445*	−0.419*	−0.605***	−0.540**
NO_3^-–N			0.884***	−0.429*	−0.156	0.175	−0.211
Inorganic N				−0.193	−0.307	−0.161	−0.436*
DEA					0.007	−0.415*	0.129
16S rRNA						−0.150	0.787***
nirS							0.200

*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

which also seems to eliminate that possibility. Second, the presence of earthworms could favor more N_2O consumption than without earthworms. Since there was ample NO_3^-–N for denitrification and two-fold more DEA in the 97% WFPS treatment with earthworms, this suggests that N_2O was completely reduced to N_2 by denitrifiers when earthworms were present. Indirect evidence that reducing conditions existed in the 97% WFPS treatment comes from the high NH_4^+–N concentration in soil after 69 d, suggesting that dissimilatory nitrate reduction to ammonium also occurred in those mesocosms, while NH_4^+-N would also come from the mineralized dead earthworm tissues (Christensen, 1988; Whalen et al., 1999). Third, the anecic earthworm *L. terrestris* can promote reduction N_2O to N_2 due to incorporation of residues into the subsurface of soil, while the slow movement of the N_2O diffusivity within soil profile makes it conversion to N_2 more likely before gas release from the soil surface (Paul et al., 2012). Nevertheless, the soil depth in the cores was quite shallow (15 cm), which indicates that the results would underestimate N_2O production under field conditions.

Acetylene blocking is often used to assess the DEA in the earthworm intestinal tract and in earthworm biostructures (Bradley et al., 2011; Chapuis-Lardy et al., 2010; Horn et al., 2006; Nebert et al., 2011); the results are reported as the amount of N_2O produced because N_2 production cannot be detected accurately unless stable isotopes are used. However, when anaerobic conditions are sustained, earthworm–microbial interactions will consume N_2O and emit N_2 as the end product (Rosenkranz et al., 2006; Ruser et al., 2006). There are a few possibilities that could explain this finding, such as: (i) earthworms release more labile carbon, which is an energy source for denitrifiers, (ii) earthworms alter soil microenvironments to create more favorable habitat or facilitate substrate transfer to denitrifiers, and (iii) earthworms alter soil microenvironments to slow gas diffusion, therefore N_2O is reduced to N_2 before it exits the soil matrix. Further research is necessary to determine which of these mechanisms is the most plausible across a range of soil types.

4.3. Earthworm effects on N_2O emissions in WD

Earthworm effects on N_2O emissions in WD could be classified in two phases: the rewetting phase and drying phase. In the rewetting phase, earthworms reduced the intensity of the N_2O pulse after rewetting, with 21-fold lower N_2O emissions, on average, in earthworm-worked soil than in the absence of earthworm. There are several possible explanations. First, earthworm burrowing activities after rewetting events could increase the aeration and partly inhibit denitrification (Beare et al., 2009; Kim et al., 2012; Kool et al., 2011). However, the significantly higher DEA in WD soil with earthworms eliminated this possibility. Second, earthworm activities could alter the bacterial community composition and favor denitrifiers that consume N_2O (Chapuis-Lardy et al., 2007; Nebert et al., 2011).

In the drying phase, earthworms are expected to cause a switch in N_2O production, gradually increasing N_2O emissions as the soil gets drier, with the switch point occurring from 70% to 45% WFPS, based on data from Fig. 3. However, the source of N_2O remains unclear. On one hand, based on the description in wet soils, earthworm would stimulate N_2O from denitrification since more earthworm biostructues after rewetting stimulate soil N mineralization and denitrification (Chapuis-Lardy et al., 2007; Rizhiya et al., 2007). One the other hand, earthworms also would stimulate the N_2O from nitrification process, as proposed to explain greater N_2O emissions with earthworms in the 33% WFPS treatment. The net effect of earthworms on N_2O emissions during rewetting-drying cycles thus depends on the "switch point" and the duration of earthworm interactions with denitrifiers and ammonia oxidizers. Other experiments using soil moisture levels in the "switch" range found an increase in N_2O production due to earthworms at 61% WFPS (Giannopoulos et al., 2010; Lubbers et al., 2011), 66% WFPS by Rizhiya et al. (2007), but no earthworm effect at 64% WFPS by Chapuis-Lardy et al. (2010). Our explanation of earthworm influences on soil N_2O emissions under rewetting-drying conditions provides a framework for interpreting experimental results around the "switch" range, which determines whether earthworms increase, decrease or have no effect on N_2O production. We encourage other researchers to evaluate earthworm–microbial interactions across the entire spectrum of soil moisture conditions that may be observed in the field, including drying and rewetting.

We acknowledge that earthworm populations in our repacked soil cores exceeded naturally-occurring populations in this area, which limits direct extrapolation of our findings to the field. We also acknowledge the small mesocosm size would inhibit earthworm activities, especially since anecic earthworms could make deep vertical burrows extending past 1 m in the soil profile under field conditions (Capowiez et al., 2006; Shipitalo and Bayon, 2004). Regarding the feeding behaviors of anecic species, they would incorporate residues into the deeper soils, which make the reduction of N_2O to N_2 possible. Thus, the negative effects of earthworms on N_2O emissions are likely to be underestimated in lab studies compared to the field situation. Regarding the denitrifier genes, the *nirS*-containing bacteria represent a subset of the entire bacterial denitrifier that reduce nitrite to nitric oxide. The other nitrite reductase gene, *nirK*, was not detected in this study (data not shown). These results are consistence with other studies (Dong et al., 2009; Nebert et al., 2011), which show that *nirS*-containing bacteria are more widespread in bacterial communities. Moreover, the *nosZ* primers cannot target all of the *nosZ*-containing bacteria, which would underestimated the abundance of *nosZ*-denitrifiers and partly affect the results. Nevertheless, our research provides evidence that the influence of earthworms on N_2O production would depend on the soil moisture conditions. Our results suggest that fields with larger earthworm populations would produce more N_2O than fields without earthworm under dry soil condition, but would produce less N_2O than fields without earthworms when soil undergoes rewetting-drying or is saturated.

Acknowledgments

We would like to acknowledge Dr. Charles W. Greer for providing the plasmids containing denitrifying gene fragments. We would also like to thank Mr. Hicham Benslim for his technical assistance with gas chromatograph analysis. Funding for this work was provided by Natural Sciences and Engineering Research Council of Canada (NSERC). C. Chen was supported by postgraduate awards from the China Scholarship Council Joint Scholarship Program and Marian & Ralph Sketch Fellowship.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.soilbio.2013.09.020.

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