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# Effect of the endogeic earthworm *Pontoscolex corethrurus* on the microbial structure and activity related to $CO_2$ and $N_2O$ fluxes from a tropical soil (Madagascar)

L. Chapuis-Lardy[a,*], A. Brauman[b], L. Bernard[b], A.L. Pablo[b], J. Toucet[b], M.J. Mano[b], L. Weber[c], D. Brunet[b], T. Razafimbelo[d], J.L. Chotte[b], E. Blanchart[b]

[a] *IRD, UMR 210 Eco&Sols c/o LRI, Université d'Antananarivo, BP 434, 101 Antananarivo, Madagascar*
[b] *IRD, UMR 210 Eco&Sols, 2 Place Viala, 34060 Montpellier Cedex 01, France*
[c] *Institute of Plant Sciences, Swiss Federal Institute of Zürich (ETH), Eschikon 33, CH-8315 Lindau, Switzerland*
[d] *LRI-SRA, Laboratoire des Radio-isotopes, Université d'Antananarivo, Route d'Andraisoro, BP 3383, 101 Antananarivo, Madagascar*

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ABSTRACT

The objective of this laboratory study was to determine the influence of a tropical endogeic earthworm, *Pontoscolex corethrurus*, on $CO_2$ and $N_2O$ fluxes from a tropical Ferralsol and microorganisms potentially involved in these gases emissions. $CO_2$ and $N_2O$ fluxes were measured during 35 days from soil mesocosms with and without earthworms. At the end of the incubation, 7% of soil was egested as cast in the earthworm treatment. Then, casts which may be aged from few hours to 35 days old were isolated from non-ingested soil. Different descriptive parameters (activity, density, and structure) of the microbial communities were investigated in the control, the non-ingested soils, and the casts. Quantitative PCR of denitrification genes encoding the nitrite (*nirK*) and nitrous oxide (*nosZ*) reductases was used to study denitrifier density in the earthworm casts. The presence of *P. corethrurus* induced a significant increase in $CO_2$ emissions but did not affect $N_2O$ fluxes when measured at mesocosm level. Despite the absence of significant differences in C and N contents between soils and casts, the near infra-red spectra analysis clearly underlined a specific organic signature for the casts. Fungal and bacterial biomass significantly decreased (~2-fold) in casts compared to parent soil, but the fungal-to-bacterial ratio was not modified by the earthworm casting activity. Data suggested that bacterial communities, especially denitrifiers, were modified in casts. The relative abundance of *nirK* and *nosZ* genes increased in the casts while the genetic structures of total bacteria and denitrifying communities were slightly modified in the casts. This study highlighted the importance of earthworm casts as a specific soil habitat where a subset of soil functional bacterial communities (such as denitrifiers) found favourable condition for their growth. However the effect of *P. corethrurus* was less evident when up-scaling from casts to mesocosm level.



## 1. Introduction

Soils represent one of the major global sources of carbon dioxide ($CO_2$) and nitrous oxide ($N_2O$), important greenhouse gases linked to climate change (Intergovernmental Panel on Climate Change, 2007). Production of $CO_2$ is the natural final step of the mineralization of organic matter, which returns a part of the C originally fixed by photosynthesis to the atmosphere. $N_2O$ is produced during numerous nitrogen transformations in soils (Robertson and Tiedje, 1987), but on most occasions denitrification and nitrification are the main processes. These processes are regulated by several soil physical, chemical and biological factors and their interactions. Earthworm activity is recognized as an important factor in regulating carbon (C) and nitrogen (N) cycles in the soil (e.g. Lavelle et al., 1998; Marhan et al., 2007), and $CO_2$ and $N_2O$ fluxes from the soil to the atmosphere (Bertora et al., 2007; Speratti and Whalen, 2008). In Malagasy soils under no-till systems, earthworm abundance can reach 119 individuals per square meter on average (Coq et al., 2007). Cast production of the endogeic earthworm *Pontoscolex corethrurus* in these soils can reach high levels up to an equivalent of 250 Mg of soil ingested per year and hectare (Chapuis-Lardy et al., 2009) while all ages of casts can be present in the field at one time. Endogeic, geophagous earthworms are generally reported to increase C mineralization in the short term (Martin, 1991; Burtelow et al., 1998), and to favour C storage through the stabilization of SOM in stable micro-aggregates in the long term (e.g. Lavelle and Martin, 1992; Guggenberger et al., 1996; Bossuyt et al., 2005). In a different body of literature, earthworm activity has been reported to be responsi-

* Corresponding author. Tel.: +261 20 22 330 98; fax: +261 20 22 369 82.
*E-mail address:* lydie.lardy@ird.fr (L. Chapuis-Lardy).

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ble for increased $N_2O$ emissions (Borken et al., 2000; Rizhiya et al., 2007). These effects result from strong interactions between earthworms and microorganisms. Microorganisms are the main agents of biochemical decomposition, whereas endogeic earthworms are involved in the stimulation of microbial populations through a mutualistic relationship, e.g., mucus addition and increase of the contact of microorganisms with organic matter during gut transit (Lavelle et al., 1995). Earthworms also modify the microbial populations through digestion and dispersion in casts (e.g. Edwards and Fletcher, 1988; Brown et al., 2000; Edwards, 2004). While earthworms do not seem to harbour specific procaryotic communities (Egert et al., 2004), several studies highlighted that bacterial communities of the ingested material were strongly modified by the passage through earthworm gut (Scheu et al., 2002; Sen and Chandra, 2009).

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 ingested denitrifiers, leading to $N_2O$ emissions from the worms themselves (Karsten and Drake, 1997; Horn et al., 2003; Drake and Horn, 2006) and the fresh casts they produced (Svensson et al., 1986; Matthies et al., 1999; Bertora et al., 2007). While denitrification is not specific to any one phylogenetic group, literature suggested that the composition and density of soil denitrifier communities may be factors affecting denitrification at a significant extent (e.g. Cavigelli and Robertson, 2001; Holtan-Hartwig et al., 2000; Wallenstein et al., 2006). So far, the impact of earthworm activities on the functional bacterial communities involved in $CO_2$ and $N_2O$ emission remains unclear.

The objective of this laboratory study was to determine the influence of the endogeic, earthworm *P. corethrurus* on $CO_2$ and $N_2O$ fluxes from a tropical soil, and to precise which descriptive parameters of the functional microbial communities (activity, density or genetic structure) are impacted by earthworm activities. The aim was not to evaluate the direct effect of the gut passage through examination of gut content or freshly produced casts and its dynamic in ageing process, but to examine earthworm effects from an overall point of view by analysing an average sample of casts of various ages collected at one time ("snapshot") in resembling natural conditions. In a mesocosm study, with or without earthworms, we regularly measured $CO_2$ and $N_2O$ fluxes during a 35-day period and determine afterwards from soil without earthworms, from non-ingested soil and from casts: (i) substrate induced respiration and potential denitrification, (ii) soil characteristics (C and N contents, NIRS spectra), (iii) microbial structure (EL-FAME), (iv) genetic structure of 16S and *nirK* bacterial communities (DGGE), and (v) relative density of *nirK* and *nosZ* functional genes (quantitative PCR).

## 2. Materials and methods

### 2.1. Sample collection and experimental setup

Samples of earthworms and the surrounding soil (0–10 cm layer) necessary for the mesocosm study were collected under *Aristida* sp. fallows at Andranomanelatra in the Malagasy Highlands, near the city of Antsirabe (19°47′S, 47°06′E, 1600 m above sea level). The area is under an altitude tropical climate with a dry/cold season from May to October and a humid/warm one from November to April. The mean annual rainfall is 1300 mm and the mean annual temperature 16 °C. The soil was developed on volcano-lacustrine alluvia (Raunet, 1981), and classified as a clayey Andic Dystrustept in the Soil Taxonomy (Soil Survey Staff, 2003) or a Ferralsol in the FAO classification (FAO, 1998). Soil subsamples were collected from the upper layer (0–10 cm), air-dried and subsequently sieved through a 2 mm sieve. It contained 620 g $kg^{-1}$ soil 1:1 clays, 46.9 g organic C $kg^{-1}$ soil and 3.7 g total N $kg^{-1}$ soil, with a pH ($H_2O$) of 4.9 (Razafimbelo et al., 2008). The available ($NaHCO_3$-extractable) P content was 13.9 mg $kg^{-1}$ soil, and iron and aluminium oxides contents were 47 and 17 g $kg^{-1}$ soil, respectively.

*P. corethrurus* (Glossoscolecidae), an endogeic, geophagous earthworm species commonly found in tropical agricultural soils was used in the laboratory experiment. The hand-collected individuals were kept in the fallow soil at 25 °C until the experiment started. A total of 14 mesocosms (2.7 L metallic boxes) were filled with 1.8 kg of dry soil packed to simulate bulk density (~1 g $cm^{-3}$) observed at field in the 0–10 cm soil layer. The soil was humidified with 650 ml of demineralized water to reach a 35% water content and kept for a 7-day preincubation at 25 °C. On Day 0, soil water content was adjusted to 40% and earthworm treatments were randomly assigned to seven boxes. Two adults (on average 1.2 g of living material) of *P. corethrurus* were added in each of the boxes dedicated to the worm treatments. Such a density corresponds to about 119 individuals $m^{-2}$ as observed in direct seeding mulch-based cropping systems in the area (Coq et al., 2007). The remaining seven boxes were kept without worms to serve as control. Fluxes of $CO_2$ and $N_2O$ from the mesocosms were measured at 7 dates during the experiment period on Days 0, 3, 7, 10, 17, 25 and 35. For gas collection, boxes were closed with a gas-tight septum for a total sampling period of 90 min. Hourly fluxes were calculated assuming a linear increase in gas concentration in the box headspace with time. This linear increase was occasionally checked for each treatment by measuring the concentration every 30 min during the 90 min period. Gas samples were taken from the chamber headspace using gas-tight syringes and analyzed for $CO_2$ on a Varian 3900 gas micro-chromatographer and for $N_2O$ on a Varian 3800 gas chromatographer equipped with an electron capture detector. For each individual mesocosm, soil moisture was maintained at 40% water content by weight adjustment every 2–4 days, and always after a gas measurement session.

Soil samples were collected after 35 days of incubation. In the treatment with earthworms, casts produced during the entire incubation in the soil and at the soil surface were manually collected in the fraction >2000 μm according to their globular shape. The average cast sample built from each box encompassed materials egested few hours to 35 days before collection. Further analyses were performed on three types of samples: control soil (Ctrl) from mesocosms without earthworms, and casts and non-ingested (NI) soil from boxes with earthworms.

### 2.2. Chemical and spectral characterization of soils and casts after incubation

Fifty grams of fresh soil (10 g for casts) were mixed in 1 M KCl (soil:solution ratio 1:3.4) for the determination of nitrate content by the colorimetric method of Anderson and Ingram (1989). Carbon and nitrogen contents of soil and cast samples were determined by a CHN combustion analyser (Carlo Erba NA 2000). The near infra-red spectroscopy (NIRS) characterizes the molecular composition of organic matter in soil samples by analysing reflected spectra of material exposed to radiative energy in the infra-red region (e.g. Joffre et al., 2001; Brunet et al., 2007). Samples were processed according to Brunet et al. (2007). Briefly, 5 g of each sample (2-mm sieved) were packed into a quartz-glass cell and scanned with a NIRSystems analyzer 5000 spectrophotometer (NIRSystems, Silverspring, USA). Fifteen reflectance measurements of monochromatic light were made from 1100 to 2500 nm to produce an average spectrum with data points at 2 nm intervals over this range. Reflectance ($R$) is converted to absorbance ($A$) using the following

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equation: $A = \log(1/R)$. Spectral data were processed with the ISI Software System (Shenk and Westerhaus, 1991).

### 2.3. Microbial communities' analysis after incubation

#### 2.3.1. Microbial activity potentials

The potential respiration (substrate induced respiration, SIR) and potential denitrification (denitrifying enzyme activity, DEA) were measured on the seven replicates of each treatment. The term 'potential' (Tiedje, 1982) implies that the microbial activity was measured under optimal conditions for specific enzymes, i.e., with the enzyme concentration as the sole limiting factor. Beside direct $N_2O$ flux measurements, potential denitrification provides additional information due to its relationship with denitrification genes (*nirK* and *nosZ*) densities (Baudoin et al., 2009). Substrate induced respiration (SIR), a general indicator of microbial metabolism, was assayed using the method from Anderson and Domsch (1978) modified as follows. For each sample, 10 g of soil (or casts) were placed into a 150 ml plasma-flask. A solution containing 1 mg of C (glucose) per g of soil was added to ensure 80% of the soil water holding capacity. The flasks were then incubated at 25 °C and the headspace volume was sampled after 2 and 4 h and analyzed for $CO_2$ using a gas micro-chromatograph (Varian 3900-GC).

Denitrifying enzyme activity (DEA) was measured using the method described by Lensi et al. (1991). Briefly, 20 g of soil (1 g for casts) were put in a 150 ml (7 ml for casts) airtight vial and humidified with a nutritive solution containing $KNO_3$ (0.2 mg N $g^{-1}$ soil), glucose (1 mg C $g^{-1}$ soil) and glutamic acid (1 mg C $g^{-1}$ soil) to reach water saturation. The atmosphere of each vessel was evacuated and replaced by a 90:10 He:$C_2H_2$ mixture to provide anaerobic conditions and to inhibit $N_2O$ reductase activity. The headspace air of the vials was sampled after 24 and 28 h of incubation at 25 °C and $N_2O$ was quantified by gas chromatography. Samples were assayed in triplicate.

#### 2.3.2. Structure and biomass of the microbial communities

Microbial community structure and biomass were assessed by fatty acid methyl ester (FAME) analysis on the seven replicates per treatment. Fatty acids were extracted from soil samples by using the ester-linked FAMEs method (modified from Schutter and Dick, 2000). Three grams of soil or casts were mixed with 15 ml of 0.2 M KOH in methanol, and the preparation was incubated for 1 h at 37 °C, during which ester-linked fatty acids were released from soil microorganisms and methylated. 2.65 ml of 1.0 M acetic acid were added to neutralize the pH. FAMEs were extracted into a hexane organic phase by addition of 10 ml hexane, and the sample was centrifuged at 800 × *g* for 20 min to separate the aqueous and hexane phases. The hexane layer was transferred to a clean tube, and the hexane was evaporated under a stream of $N_2$, after which FAMEs were resuspended in 170 μl hexane, methyl tert butyl ether (1:1) with an 30 μl internal standard (methyl stearate 0.01 M, 18:0). Individual FAMEs were separated and quantified by gas chromatography by using a model Agilent 6890N GC (Agilent Inc., Palo Alto, CA). The GC capillary column was a medium polar cyanopropyl (DB 23) with 60 m long, an internal diameter of 0.25 mm and film thickness of 0.15 μm. Flame ionization detection (FID) was achieved at a temperature of 280 °C using a carrier gas of helium at a flow rate of 30 ml $min^{-1}$. Identification of the FAME was performed automatically using the Agilent Chemstation software (Agilent, Inc.) in combination with the Agilent Retention Time Locking (RTL) library for FAME. The FAME database was extended with a mixture of 24 bacterial FAMEs (Bacterial Acid Methyl Esters Mix 47080-U; Supelco, Inc.) and 10-Me 16:0 (Matreya, Pleasant Gap, PA). The concentrations of FAMEs (nmol $g^{-1}$ soil) calculated from peak areas and reported as typical of fungi, Gram-negative bacteria, Gram-positive bacteria, and actinobacteria were used as signatures for these microbial groups (Zelles, 1999 and references therein).

#### 2.3.3. Genetic structure of the bacterial communities

*2.3.3.1. Whole bacterial community genetic structure (16S rRNA gene).* The genetic structure of bacteria community was determined after rRNA gene amplification and DGGE fingerprinting performed on five randomly chosen replicates (out of seven). Nucleic acids were extracted from the soil and casts samples according to the procedure of Martin-Laurent et al. (2001). PCR amplification was performed (mastercycler Epgradient, Eppendorf, France) according to Assigbetse et al. (2005) using primers 518R and GC338F previously designed to target the V3 region of 16S rRNA gene (Muyzer et al., 1993). Amplicons were resolved by DGGE using 8% (w/v) acrylamide gels (acrylamide–bisacrylamide 40%, 37.5:1 from Sigma–Aldrich, St. Quentin Fallavier, France) and a gradient of 45–70% denaturant (Muyzer et al., 1993) in 1 × TAE buffer (40 mM Tris (pH 8.0), 20 mM acetic acid, 1 mM EDTA) with the Ingeny phorU system (Ingeny International, Goes, The Netherlands) at 60 °C and 100 V for 17 h. The gel images were captured using Bio-capt software (Ets Vilbert Lourmat France). The 16S rRNA gene-DGGE band patterns were analyzed using TotalLab TLV120 software (Nonlinear dynamics, Newcastle, UK) to obtain matrices banding profiles with the intensity of each bands.

*2.3.3.2. Denitrifier bacterial community structure.* The genetic structure of the denitrifier bacteria community was determined by DGGE fingerprinting of the nitrite reducing communities (*nirK* genes). For technical reasons, the analysis was performed on three replicates only. DNA was extracted from 0.5 g of soil (or casts) samples with the FastDNA® SPIN Kit for soil according to the manufacturer's instructions (MP Biomedicals, France) with a humic acid purification step with guanidine thiocyanate 5.5 M. Partial sequences of *nirK* were amplified with the primer pairs F1aCu/R3Cu (Throbäck et al., 2004). Reverse primers R3Cu carried a 33-bp GC-clamp to further resolve amplicons by DGGE. Amplifications were carried out in a total volume of 25 μL with puReTaq® Ready-To-Go® PCR beads (Amersham-Biosciences, Orsay, France). Final mixture for *nirK* amplification contained 2.5 U of DNA polymerase, 1.5 mM $MgCl_2$, 200 mM of each dNTP, 0.5 μM of each primer, 600 ng BSA $\mu L^{-1}$ and 5 ng of purified soil DNA. Amplifications were performed with mastercycler Epgradient with the following conditions: initial denaturation at 94 °C for 2 min, afterwards 10 touchdown cycles were performed as follow: 30 s at 94 °C for denaturation, 30 s at 62 °C for annealing (−0.5 °C every cycle down to 55 °C) and 30 s at 72 °C for extension. Finally, 30 additional cycles were performed at a constant annealing temperature of 57 °C, with a final extension step of 15 min at 72 °C. Size specificity of the amplicons was checked by 1.5% agarose electrophoresis after staining with ethidium bromide. Amplicon was resolved by DGGE using the same protocol described above.

#### 2.3.4. Density of functional bacterial communities (quantitative PCR assay)

The quantitative PCR assay was carried out (on an aliquot of the sample replicates used for DGGE analysis) by using SYBR green as the detection system in a reaction mixture of 25 μl containing: 0.5 μM (each) primer, 12.5 μl of SYBR green PCR master mix, including HotStar Taq DNA polymerase, QuantiTect SYBR green PCR buffer, deoxynucleoside triphosphate mix with dUTP, SYBR green I, ROX, and 5 mM $MgCl_2$ (Absolute QPCR SYBR Green Rox ABgene, UK), and 2 μl of DNA-diluted template corresponding to about 10 ng of total DNA. T4gp32 (500 ng/reaction; Q-BIOgene, France) was used to enhance PCR efficiency. Thermal cycling conditions for the 16S rRNA, *nirK*, and *nosZ* genes were as described in the others studies (e.g. Henry et al., 2004). Thermal cycling, fluorescent data collec-

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**Table 1**
Repeated measures analysis of variance of the effect of earthworm treatment (with or without *P. corethrurus*) and sampling date on $CO_2$ and $N_2O$ fluxes from mesocosms ($n = 7$) during the 35-day study.

| Effect | $CO_2$ flux ($\mu g\ C\text{-}CO_2\ kg^{-1}\ h^{-1}$) | | | $N_2O$ flux ($\mu g\ N\text{-}N_2O\ kg^{-1}\ h^{-1}$) | | |
|---|---|---|---|---|---|---|
| | df | *F*-value | *P* | df | *F*-value | *P* |
| Date | 6, 152 | 140.15 | <0.0001 | 6, 152 | 35.32 | <0.0001 |
| Treatment | 1, 152 | 5.07 | 0.026 | 1, 152 | 0.36 | 0.549 |
| Date × treatment | 6, 152 | 0.64 | 0.697 | 6, 152 | 1.14 | 0.343 |

tion, and data analysis were carried out with an ABI Prism 7900 (Applied Biosystems) sequence detection system according to the manufacturer's instructions. Standard curves were obtained with serial plasmid dilutions of a known amount of plasmid DNA containing a fragment of the 16S rRNA, *nirK*, or *nosZ* gene (Henry et al., 2004, 2006). The presence of PCR inhibitors in DNA extracted from soil was estimated by mixing a known amount of standard DNA with soil DNA extract prior to q PCR. No inhibition was detected. Since the number of 16S rRNA operon per cells is variable, the 16S rRNA gene copy data were not converted into cells numbers and our results were expressed as gene copy numbers per gram of soil after verifying that the DNA extraction yield was similar for each soil condition (Ctrl, NI and casts). To obtain an estimate of the relative abundance of the different taxa within the total bacterial community in the samples, data were also expressed as the ratio of taxon-specific 16S rRNA gene copy.

### 2.4. Data analysis

Data were tested for normality and log-transformed before statistical analysis when necessary. The effect of earthworm treatment on $CO_2$ and $N_2O$ fluxes was analyzed by repeated measures analysis of variance (RM-ANOVA) using Statistica software. Means were compared using the least significant difference (LSD) test at $P < 0.05$.

A principal component analysis (PCA) of spectral data obtained by NIRS was computed to make an ordination of the samples. Dendrogram of similarity was built with TotalLab TLV120 software from the DGGE data sets using the *Dice* similarity coefficient and the unweighted pairwise grouping method with mathematical averages (UPGMA).

## 3. Results

### 3.1. $CO_2$ and $N_2O$ fluxes

The highest $CO_2$ and $N_2O$ fluxes were measured at the beginning of the experiment in both treatments with and without earthworms (182–236 $\mu g\ C\text{-}CO_2\ kg^{-1}\ h^{-1}$ and 6.4–7.2 $\mu g\ N\text{-}N_2O\ kg^{-1}\ h^{-1}$, respectively on Days 0 and 3, with no statistical difference between these two dates). Then, the fluxes decreased to reach a minimum between Day 7 and the end of the 35-day incubation period (Fig. 1A and B). The RM-ANOVA for $CO_2$ fluxes showed that the earthworm treatment and the gas sampling date significantly ($P < 0.05$) affected the $CO_2$ fluxes, but the treatment × sampling date interaction was not significant (Table 1). The $N_2O$ fluxes were only affected by sampling date, but neither by earthworm treatment nor by treatment × date interaction (Table 1).

### 3.2. Post-incubation chemical and spectral analyses of soil and casts

Earthworms were in good health and active after the incubation period. During the 35-day period, they gained weight (+14% per individual on average), produced cocoons and casts (60.6 g per worm on average) in all boxes (data not shown). Thus, the proportion of soil ingested by earthworm during the experiment was ~7% (w/w).

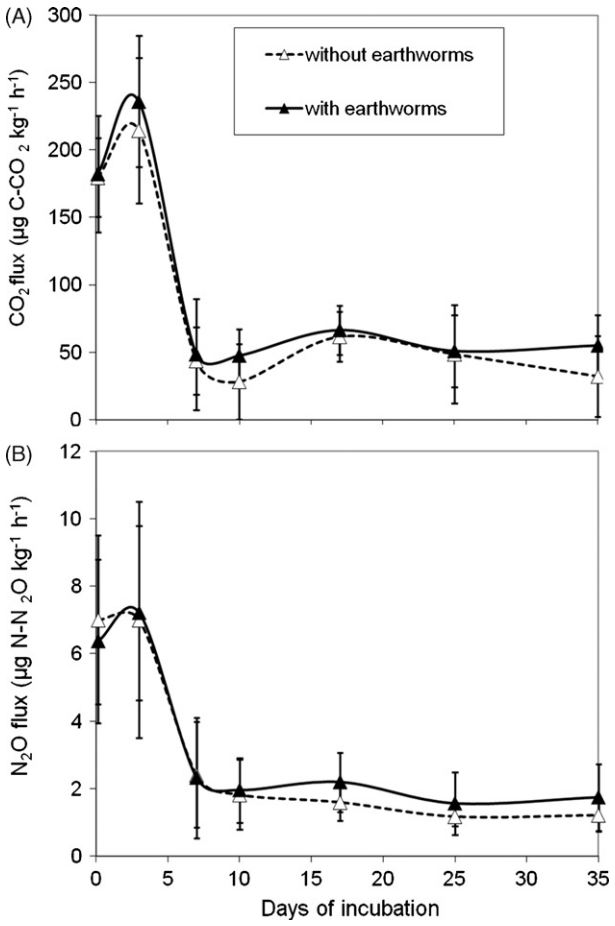


**Fig. 1.** Dynamics of $CO_2$ (A) and $N_2O$ (B) fluxes from mesocosms without earthworms (control) and with added earthworms (*P. corethrurus*). Bars on data points are the standard deviation of the mean ($n = 7$).

Principal component analysis was applied on the spectral data obtained by NIRS. The first and second axes of the NIRS matrix PCA absorbed 98.6% and 1.35% of the total inertia, respectively (Fig. 2). Owing to the sharp decrease of eigenvalues, no other axes were retained for interpretation. Earthworm casts were clearly separated from control and non-ingested soil samples along the first axis.

The casting activity of *P. corethrurus* had no significant effect on soil total carbon or nitrogen contents whereas the $N\text{-}NO_3^-$ content was significantly higher in the casts than in the control (Crtl) or the non-ingested (NI) soils (Table 2).

**Table 2**
Carbon content, total and nitrate N contents, substrate induced respiration (SIR, $mg\ C\text{-}CO_2\ kg^{-1}\ h^{-1}$), and denitrifying enzyme activity (DEA, $\mu g\ N\text{-}N_2O\ kg^{-1}\ h^{-1}$) in the control soil (Ctrl), and the non-ingested soil (NI) and casts derived from the earthworm treatments with *P. corethrurus* after 35-day study (mean and standard deviation, $n = 7$).

| | Ctrl | NI | Casts |
|---|---|---|---|
| C ($mg\ kg^{-1}$) | 47.1 (1.1) a | 46.5 (0.6) a | 48.0 (0.1) a |
| N ($mg\ kg^{-1}$) | 3.8 (0.1) a | 3.7 (0.0) a | 3.8 (0.1)a |
| $N\text{-}NO_3^-$ ($mg\ kg^{-1}$) | 147 (7) a | 162 (8) a | 312 (110) b |
| SIR ($mg\ C\ kg^{-1}\ h^{-1}$) | 2.2 (0.4) a | 2.2 (0.3) a | 1.7 (0.1) b |
| DEA ($\mu g\ N\ kg^{-1}\ h^{-1}$) | 79.2 (7.6) a | 68.9 (8.9) a | 88.4 (36.9) a |

Different suffix letters within a row indicate significant differences between soils and casts at $P < 0.05$.

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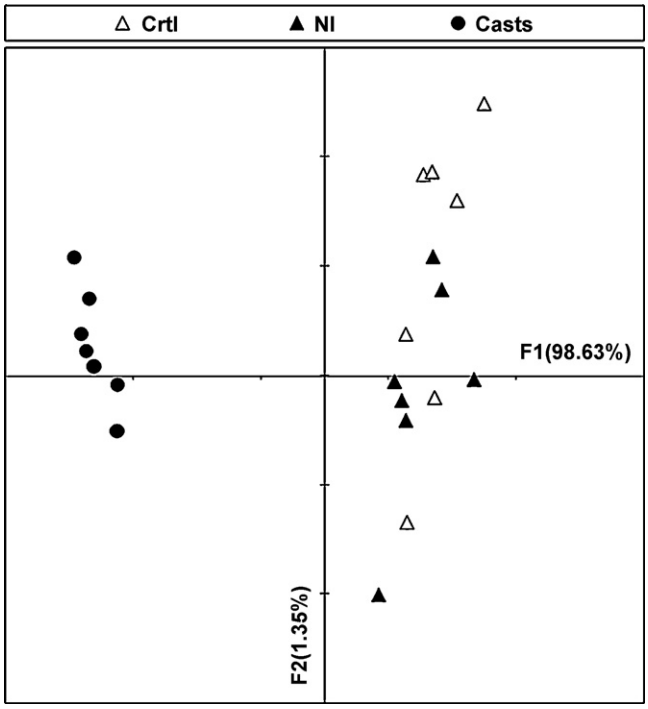


**Fig. 2.** Principal component analysis (PCA) of data from all sample sites based on NIRS spectra; control soil (Ctrl) as derived from mesocosms without earthworms; non-ingested (NI) soil and casts from mesocosms with earthworms (*P. corethrurus*) ($n = 7$).

### 3.3. Microbial communities' analyses in soil and casts

Substrate induced respiration was significantly lower in casts than in soils (1.7 and 2.2 mg C $kg^{-1}$ $h^{-1}$, respectively) while soil potential denitrification was unchanged by the earthworm activity (Table 2).

The FAME concentrations in the casts significantly differed from those of soils (Ctrl and NI) while the relative proportion of biomarkers (used as an index of the structure of the community) did not differ between soils and derived casts (Fig. 3). The amount of FAMEs ranged between 0.5 and 17.3 nmol $g^{-1}$ soil depending on the microbial community. The concentrations of all biomarkers, for fungi, Gram-positive and Gram-negative bacteria communities, were significantly lower in the casts while no significant difference was observed between the control (Ctrl) and the non-ingested (NI) soil

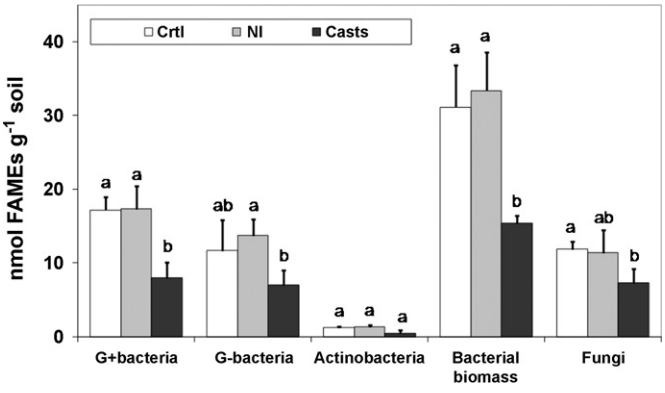


**Fig. 3.** Amounts of fatty acid methyl ester (FAME) biomarkers (nmol FAMEs $g^{-1}$ soil) characteristics for individual groups of microorganisms in the control (Ctrl), non-ingested (NI) soils and the casts of *P. corethrurus*. Different letters indicate significant differences between treatments (Ctrl, NI, and casts) for each group ($P < 0.05$). Means ($n = 7$) and standard deviations.

**Table 3**
Nucleic acid yield, copy numbers of the 16S rRNA gene and mean relative densities of *nirK* and *nosZ* gene fragments in the control (Ctrl), non-ingested (NI) soils and the casts of *P. corethrurus* ($n = 5$).

| | Ctrl | NI | Casts |
|---|---|---|---|
| Nucleic acid yield ($\mu g\ g^{-1}$ soil) | 12.69 a | 16.36 a | 14.11 a |
| 16S rRNA gene density (no. of gene copies $g^{-1}$ soil) | $3.9 \times 10^8$ a | $4.7 \times 10^8$ a | $2.2 \times 10^8$ b |
| Denitrification genes[a] (% of 16S rRNA gene density) | | | |
| *nirK* | 0.18 a | 0.16 a | 0.26 b |
| *nosZ* | 0.04 a | 0.03a | 0.06 b |
| *nosZ*:*nirK* | 0.21 ab | 0.16 b | 0.24 a |

Different suffix letters within a row indicate significant differences between soils and casts at $P < 0.05$.

[a] Based on the gene copy number per ng of nucleic acid material.

(Fig. 3). The relative proportions of biomarkers did not significantly differ between the soil and the casts: G+ bacteria (36–40%), G− bacteria (27–31%), actinobacteria (2–3%) and fungi (26–32%). As both bacteria and fungi biomass was decreased in casts, the fungal-to-bacterial biomass index ratio was not significantly different between the soils and the casts, respectively 0.34 and 0.49.

The total bacterial community, measured as the 16S rRNA gene copy number, ranged from $2.2 \times 10^8$ to $4.7 \times 10^8$ copies per gram of soil, with significantly lower values in the casts than in the soils (Table 3). As the ingestion of soil by earthworms may affect the relative abundances of denitrifiers to total bacteria, we therefore calculated ratios of denitrification genes to the 16S rRNA gene from the total bacteria and obtained proportions from 0.16 to 0.26% and 0.03 to 0.06% for *nirK* and *nosZ*, respectively (Table 3). The relative abundances of *nirK* and *nosZ* showed a significant increase from the soil to the casts.

The data from DGGE analysis were analyzed by direct similarity comparison using the unweighted pairwise grouping method with mathematical averages to formulate dendrogram (Fig. 4A and B). Discrete reproducible differences in the community composition due to soil ingestion and egestion by *P. corethrurus* were evident as the casts clustered separately from the soil they were derived from.

## 4. Discussion

### 4.1. Effect of earthworms on gas emissions from the mesocosms

The mean $CO_2$ flux was increased in the presence of earthworms as highlighted by a significant treatment effect in RM-ANOVA. This can be explained by (i) the respiration of earthworms, and/or (ii) an increase of microbial activity in presence of earthworms. However, the effect of earthworms on soil respiration was not consistent during the study period (non-significant effect of date × treatment), suggesting that respiration was more strongly influenced by the fluctuations in environmental (microclimate, substrate availability) conditions than the earthworm activities. Moreover, earthworms may differentially affect $CO_2$ emissions depending on the functional groups. Speratti and Whalen (2008) reported a greater mean $CO_2$ flux from microcosms with a combination of *Lumbricus terrestris* and *Aporrectodea caliginosa* than from the single species treatments, suggesting a stimulation of microbial respiration as a result from interactions between anecic and endogeic functional groups. Postma-Blaauw et al. (2006) reported similar results.

*P. corethrurus* had no effect on the mean $N_2O$ flux from microcosms in this study, which is in agreement with results reported by Speratti and Whalen (2008) for another endogeic species (*A. caliginosa*), but in contrast to other reports from studies that focused other functional groups of earthworms or mixed endogeic with

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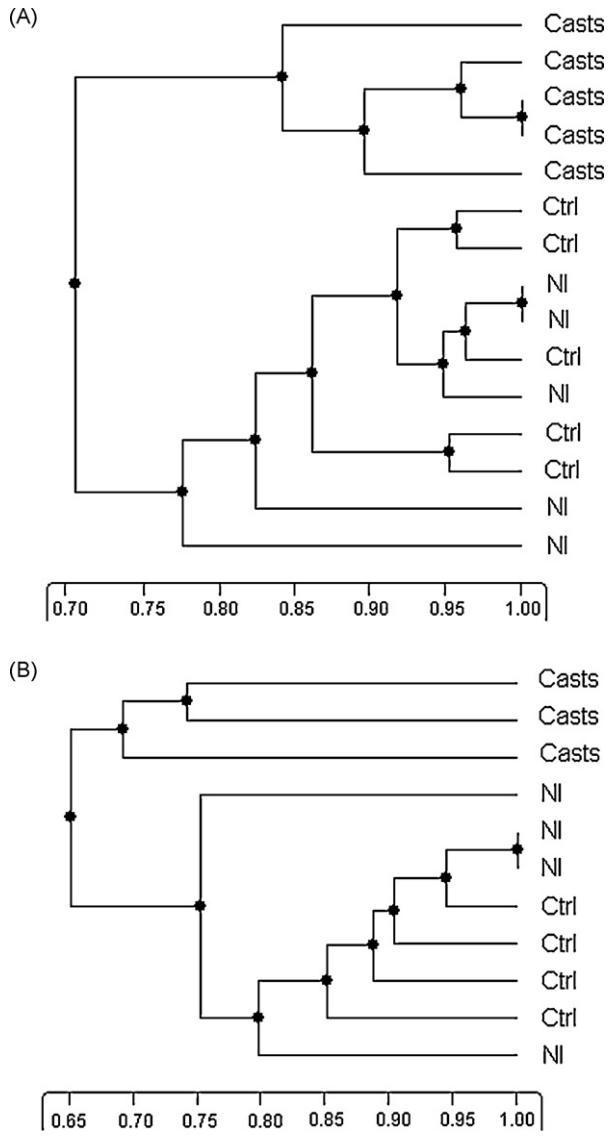


**Fig. 4.** Dendrograms of similarity derived from DGGE analyses of 16S rRNA gene fragments (A) and *nirK* genes (B) amplified from the control (Ctrl) and non-ingested (NI) soil samples and from the casts of *P. corethrurus* (*Dice* coefficient and UPGMA used to formulate dendrogram).

individuals from others groups (Karsten and Drake, 1997; Matthies et al., 1999; Borken et al., 2000; Bertora et al., 2007; Rizhiya et al., 2007). As a whole, the contrasted results from the present study and literature suggested that $CO_2$ and $N_2O$ fluxes from microcosms may depend on soil type, environmental conditions and earthworm functional groups. Moreover, while direct $N_2O$ emissions by *P. corethrurus* individuals was insignificant ($\sim$pg N-$N_2O$ $h^{-1}$ $g^{-1}$ worm biomass; pers. com.), the proportion of soil ingested by earthworms (7%) during the 35-day period, and egested as casts, was probably not large enough to significantly impact the direct fluxes measurement at the scale of the mesocosm.

### 4.2. Effect of earthworms on soil physicochemical properties

NIRS spectra may be considered as fingerprints of the OM composition (Joffre et al., 2001), particle-size distribution (Sørensen and Dalsgaard, 2005; Madari et al., 2006) and biological properties (Cécillon et al., 2008). The use of PCA analysis clearly underlined that the modifications of soil OM caused by the earthworms result in specific organic fingerprints in their casts. However, neither quantitative differences in C and N contents nor modification of particle-size distribution (results not shown) were observed between the casts and the soil they derived from. The difference emphasized by the PCA may merely be linked to the variability in OM biochemical composition and distribution (Zhang et al., 2003). Barois et al. (1993) showed for *P. corethrurus* that ingested soil was completely dispersed during the gut transit and that the soil particles were fully reorganized, resulting in newly formed aggregates and specific microstructure in the casts. Consequently, soil protected organic components become exposed to microbial decomposition, while accessible organic components might be protected during cast formation (Barois et al., 1993; Bossuyt et al., 2005). Moreover, the addition of intestinal mucus during biostructure production is further reflected in NIRS signatures (Hedde et al., 2005). Specific microbial activities in the gut of some engineer species and in their fresh biostructures probably affect the OM molecular composition via the production of specific mucigel and cellular compounds (Martin and Marinissen, 1993; Lavelle et al., 1998). Cast material had 2 times more mineral N than un-ingested soil. Higher concentrations of extractable nutrients in the casts than in the surrounding soil material are usually attributed to the preferential feeding by earthworms on plant residues (with higher nutrient contents) in the soil and a flush of mineralization of soil organic matter (and release of mineral N and P) in the gut and fresh casts (Lee, 1985).

### 4.3. Effect of earthworms on soil microbial density, activity and community structure

The FAME concentrations were lower in the casts than in the bulk soil whatever the microbial community. Fungal hyphae are said to be disrupted during the gut transit and part of the diet of earthworms may consist of fungal hyphae (Wolter and Scheu, 1999; Bonkowski et al., 2000). Bonkowski et al. (2000) demonstrated that the preferences of five earthworm species for a range of soil fungi followed a general pattern, irrespective of ecological groups. The microbial biomass indicators obtained either by FAMEs or SIR methods decreased in the casts, showing that the overall growth conditions were less favourable for microorganisms in the casts (which in our study aged from 1 to 35 days old) than in the soils. Scheu (1987) reported no substantial alteration of microbial biomass after the passage of soil through the gut of the endogeic *A. caliginosa*, but it declined in the ageing faeces. Similar patterns of the microbial biomass in ageing casts occurred for epigeic (Daniel and Anderson, 1992) and anecic earthworm species (Tiunov and Scheu, 2000). Along with the changes in the cast microenvironment when ageing, the decline in microbial biomass might also have been caused by enhanced grazing of protozoa, nematodes or other microbivores, which have been shown to be abundant in the earthworm casts (e.g. Brown, 1995; Winding et al., 1997). Other mechanisms, presumably antagonistic interactions between members of the faecal microbial community might explain the observed decline in microbial biomass. The DGGE analyses revealed that the structures of total bacteria and denitrifying communities were modified in casts. The differences detected with the clustering statistics approach between the casts and the parent soil were rather small, but consistent among the replicate samples, and therefore significant. Several studies have also shown that bacterial and fungal species are differentially affected by earthworm gut passage (e.g. Pedersen and Hendriksen, 1993; Moody et al., 1996). Several reasons for the differences between the total bacteria and denitrifying community structure of the soil and casts are possible: (i) selective feeding (Tiunov and Scheu, 2000) (ii) anoxic conditions in the gut favouring microorganisms able to grow under anaerobic condition (Karsten and Drake, 1995; Horn et al., 2003); (iii) proliferation of microorganisms effectively exploiting the particular

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physicochemical conditions of gut (Furlong et al., 2002; Horn et al., 2003) and persisting in the casts; (iv) differential lyses of microbes by digestive enzymes secreted by the earthworm (Edwards and Fletcher, 1988); and (v) inhibition of bacteria by inhibitory substances secreted by other bacteria (Brown, 1995; Furlong et al., 2002). However, while the gut microbiota of the epigeic *Lumbricus rubellus* was shown to be substantially affected by the food source ingested (Knapp et al., 2009), the existence of abundant indigenous gut microbiota appeared unlikely (e.g. Ihssen et al., 2003; Horn et al., 2006). Ihssen et al. (2003) suggested that the gut denitrifiers were soil derived, and Horn et al. (2006) concluded from comparative sequence analysis that denitrifying populations found in the gut content of four lumbricid species were regular soil microorganisms rather than endemic to the gut. Some studies have underlined the potential role of the composition of the denitrifying community among the factors leading to $N_2O$ emission (Cavigelli and Robertson, 2001; Henry et al., 2004). Moreover, an unknown percentage of denitrifying bacteria lacks the genes encoding $N_2O$ reductase (Wood et al., 2001) and will emit $N_2O$ as an end-product in denitrifying conditions, irrespective of soil physicochemical characteristics. Thus, it is important to know the relative abundance of bacteria able to reduce $N_2O$ in order to better understand the key drivers of $N_2O$ emissions from soil. The *nosZ*-to-*nirK* ratio was higher in the casts than in the non-ingested soil, suggesting that the denitrification process might be more complete in the casts and $N_2$ emitted rather than $N_2O$ as the end-product. The percentages of *nirK* and *nosZ* genes to 16S rRNA gene were increased in the casts. This is in line with the activation of denitrification genes observed by Horn et al. (2006) after passage of soil through earthworm gut. Several authors observed a higher denitrification potential of soil in the earthworm gut (Karsten and Drake, 1997; Matthies et al., 1999; Horn et al., 2003; Ihssen et al., 2003). In our study, DEA was unchanged despite changes in microbial structure and density and an increase of mineral $NO_3^-$ content in the casts, suggesting that the denitrification potential was not impacted by the size of the denitrifying community or that the effect was counterbalanced by the ageing process of casts after egestion.

## 5. Conclusion

Even if *P. corethrurus* did not affect $N_2O$ fluxes when measured at the given spatial and temporal scales its casting activity in the studied soil stimulated a subset of ingested soil microorganisms, including denitrifying bacteria. Produced casts represent a very specific habitat for soil microorganisms. However, the denitrification potential in the casts remained unchanged, indicating that such a parameter measured alone may not reflect the complex interactions of earthworms with the microbial community. Soil ingestion and subsequent casting positively influenced the relative abundances of denitrification genes. Understanding of the links between gene expression and process even on a microscale is still challenging. While our study is the first documenting the actual density of denitrification genes in tropical earthworm casts, further studies on denitrifying bacteria should compare freshly egested to old casts to distinguish changes occurring during the gut passage from those occurring during cast ageing.

Denitrifiers showed a shift in their community structure and relative density in the casts but when scaling-up to the mesocosm level, the gaseous production was not affected by earthworms. This suggests: (i) that primary drivers were others (edaphic parameters; specific physical properties of cast), (ii) or that soil ingestion by earthworms may affect the density and structure of the inactive or moribund part of the community and not active populations, and (iii) or that the duration of experiment was not longer enough to produce significant amounts of casts that will in turn impact the $N_2O$ flux measurement at mesocosm scale.

The results presented here cannot be enlarged to other earthworm/soil pairs while scaling-up inevitably creates problems given the amount of ingested soil in short-time studies. Modelling earthworms casting activity and consequences of cast properties and cast ageing will help to address up-scaling when considering nutrient cycling (Blanchart et al., 2009). It is particularly important to address spatial and temporal scaling issues in further research which includes a wide gamut of species and ecological strategies comprising communities, thus properly estimating the functional roles of earthworms in terms of gas emissions.

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