Biology and Fertility of Soils

Emission of greenhouse gases and soil changes in casts of a giant Brazilian earthworm

--Manuscript Draft--

Manuscript Number:	BFSO-D-20-00772R2
Full Title:	Emission of greenhouse gases and soil changes in casts of a giant Brazilian earthworm
Article Type:	Original Paper
Keywords:	Rhinodrilus alatus; macronutrients; microbial biomass; Methane; Nitrous Oxide
Corresponding Author:	George G. Brown, PhD Empresa Brasileira de Pesquisa Agropecuaria Florestas BRAZIL
Corresponding Author Secondary Information:	
Corresponding Author's Institution:	Empresa Brasileira de Pesquisa Agropecuaria Florestas
Corresponding Author's Secondary Institution:	
First Author:	Alessandra Santos
First Author Secondary Information:	
Order of Authors:	Alessandra Santos
	Marcus Vinicius Cremonoesi, PhD
	Josiléia Acordi Zanatta, PhD
	Luis Neves Cunha, PhD
	Harold L. Drake, PhD
	George G. Brown, PhD
Order of Authors Secondary Information:	

Funding Information:

Conselho Nacional de Desenvolvimento Científico e Tecnológico (310690/2017-0)	Mr. George G. Brown
Conselho Nacional de Desenvolvimento Científico e Tecnológico (401824/2013-6)	Mr. George G. Brown
Conselho Nacional de Desenvolvimento Científico e Tecnológico (307486/2013-3)	Mr. George G. Brown
Fundação Araucária	Mrs. Alessandra Santos
Fundação para a Ciência e a Tecnologia (CEECIND/01986/2017)	Mr. Luis Neves Cunha
Coordenação de Aperfeiçoamento de Pessoal de Nível Superior	Mr. Marcus Vinicius Cremonoesi

Abstract:

Greenhouse gas emissions (CO_2, N_2O, CH_4), chemical, physical and microbiological properties (pH, macro and micronutrients, texture, moisture, exchangeable NH_4^+, NO_3^-, total C and N, organic-C, microbial biomass C and metabolic coefficient) were monitored in casts of a large, endogeic native Brazilian species *Rhinodrilus alatus* and from non-ingested control soil incubated for up to 32 days. Earthworm casts represented a significantly different chemical and microbiological environment, with higher soil moisture, pH, H+Al, NH_3, Cu, Fe and Mn contents, lower microbial biomass-C and higher metabolic quotient (qCO_2), but with few differences in CO_2, N_2O and CH_4 emissions compared with non-ingested control soil. Nonetheless, fermenting, methanogenic, and nitrate reducing

	microbes encountered ideal conditions for sustained anaerobic activity in the clayey, dense and moist castings of *R. alatus*, maintaining emission of N_2O and CH_4 and confirming previous results observed using gut contents. The high exchangeable NH_4 and H_2O contents influenced the oxy-reduction processes, affected GHG emissions and N transformations, and modified soil microbial biomass and activity. In addition, selective ingestion concentrates C and N contents in the casts and transformation processes affect the availability of important plant nutrients, topics that deserve further attention, considering the widespread collection of this species for use as fish-bait in Brazil.
Suggested Reviewers:	Lydie Chapuis-Lardy lydie.lardy@ird.fr
	Marcus Horn horn@ifmb.uni-hannover.de
	Ingrid Lubbers ingrid.lubbers@wur.nl
	Joann Whalen joann.whalen@mcgill.ca

Replies to Editor of BFSO-D-20-00772

Emission of greenhouse gases and soil changes in casts of a giant Brazilian earthworm

Dear Paolo:

We thank you once again for helping us improve this manuscript and for your careful revision of the revised text! We have taken into consideration all of your suggestions, and have attached the revised version of the manuscript including all revisions marked (using track changes in word), as well as the final manuscript (with changes accepted).

We hope that the manuscript is now acceptable for publication and look forward to hearing back from you soon.

We send you our very best wishes of much health to you and all your loved ones in this delicate time worldwide.

Yours sincerely,
George (on behalf of all co-authors)

Emission of greenhouse gases and soil changes in casts of a giant Brazilian earthworm

Alessandra Santos[1], Marcus Vinicius Cremonesi[1], Josiléia Acordi Zanatta[2], Luis Cunha[3,4], Harold L. Drake[5], George Gardner Brown[1,2*]

[1] Soil Science Departament, Federal University of Paraná, Curitiba, Paraná, Brasil;

[2] Embrapa Forestry, Colombo, Paraná, Brasil

[3]University of Coimbra, Centre for Functional Ecology, Department of Life Sciences, Calçada Martim de Freitas, 3000-456 Coimbra, Portugal;

[4]School of Applied Sciences, University of South Wales, Pontypridd, Wales, CF37 4BD UK;

[5]Department of Environmental Microbiology, University of Bayreuth, Bayreuth, Germany

Alessandra: ORCID: 0000-0002-5800-1749

Marcus: ORCID: 0000-0001-6898-6513

George: ORCID: 0000-0001-9550-6909

Josileia: ORCID 0000-0002-3214-4864

Luis ORCID: 0000-0002-5870-2537

*Author for Correspondence:

Dr. George Gardner Brown

george.brown@embrapa.br

Acknowledgments

Support for this study was provided by the National Council for Scientific and Technological Development (CNPq grants 310690/2017-0, 401824/2013-6 and 307486/2013-3), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), the Araucária Foundation, Embrapa Forestry and the Federal University of Paraná. Luis Cunha was supported by Fundação para a Ciência e Tecnologia (CEECIND/01986/2017). The authors also thank Anne Dotaf, Marie Bartz, Lilianne Maia, Siu Tsai, Glaciela Kaschuk, Marcela Guiotoku, Peter Depkat-Jacob, Timo Oppermann, Antonio Carlos Motta, Jeferson Dieckow and the Paropeba National Forest staff for help in various phases of this study.

Abstract

Greenhouse gas emissions (CO_2, N_2O, CH_4), chemical, physical and microbiological properties (pH, macro and micronutrients, texture, moisture, exchangeable NH_4^+, NO_3^-, total C and N, organic-C, microbial biomass C and metabolic coefficient) were monitored in casts of a large, endogeic native Brazilian species *Rhinodrilus alatus* and from non-ingested control soil incubated for up to 32 days. Earthworm casts represented a significantly different chemical and microbiological environment, with higher soil moisture, pH, H+Al, NH_3, Cu, Fe and Mn contents, lower microbial biomass-C and higher metabolic quotient (qCO_2), but with few differences in CO_2, N_2O and CH_4 emissions compared with non-ingested control soil. Nonetheless, fermenting, methanogenic, and nitrate reducing microbes encountered ideal conditions for sustained anaerobic activity in the clayey, dense and moist castings of *R. alatus*, maintaining emission of N_2O and CH_4 and confirming previous results observed using gut contents. The high exchangeable NH_4 and H_2O contents influenced the oxy-reduction processes, affected GHG emissions and N transformations, and modified soil microbial biomass and activity. In addition, selective ingestion concentrates C and N contents in the casts and transformation processes affect the availability of important plant nutrients, topics that deserve further attention, considering the widespread collection of this species for use as fish-bait in Brazil.

Keywords: *Rhinodrilus alatus;* macronutrients; microbial biomass; methane; nitrous oxide

Introduction

Earthworms represent the highest proportion of soil invertebrate biomass in many ecosystems (Lavelle 1984) and act as ecosystem engineers (Lavelle et al. 1997), contributing to various soil ecosystem services important for the survival of human beings on the planet (Lavelle et al. 2006). Their bodies, feeding, burrowing and casting activities constitute the drilosphere (Bouché 1977), a hotspot of microbial activity, nutrient mineralization and greenhouse gas (GHG) emission (Brown et al. 2000; Lubbers et al. 2013). However, earthworm activities in soils vary depending on the species and their ecological category, and endogeic (geophagous) species are responsible for greater soil movement and casting than epigeic and anecic species, that feed more on litter or litter-soil mixtures, respectively (Lavelle 1981; 1988). In fact, tropical endogeic species can ingest large amounts of mineral soil and organic material annually, reaching cast production values of more than 300 t ha^{-1} in Mexican pastures and up to 1250 t ha^{-1} in Lamto (Ivory Coast) savannas, most of which are subsurface casts (Lavelle 1988).

Earthworm casts often contain higher levels of available nutrients for plants (inorganic P, Ca, Mg, K, Na) as well as higher organic C and N content than non-ingested soil (Hulugalle and Ezumah 1991; van Groenigen et al. 2019), which can be due to selective feeding on C and N-rich soil fractions, finer soil particles (e.g., more clay) or decomposing plant material (Barois et al. 1999; van Groenigen et al. 2019). Moreover, casts are also zones of intense microbial activity (Scheu 1987; Deviglier and Verstrasse 1997), that stimulate N and P mineralization, as well as CO_2 and N_2O emissions (Eriksen-Hemel and Whalen 2007; Lubbers et al. 2011).

The overall effects of earthworms on soil C and N mineralization and GHG emission is dependent on gut processes, and the rate of emission of these gases also depends on the species involved and feeding source (Depkat-Jacob et al. 2013). Furthermore, these processes also depend on the earthworm casting habit and morphology, matric potential, gut retention times, and rate of gut mucus production. Castings with higher water contents may form anoxic zones that are conducive to denitrification, while soil compacting species (Blanchart et al., 1997) form casts that take longer to dry out, and also have lower O_2 contents, particularly in finer textured soils (Blanchart et al. 1999; Drake and Horn 2007). Fermenting, methanogenic and nitrate reducing microbes are activated in the earthworm's digestive tract by earthworm feeding and mucus secretion (Zeibich et al. 2019b), and may remain highly active in moist earthworm castings containing numerous anoxic microzones, compared to bulk soils (Drake and Horn 2007). Under low redox potential conditions GHG emissions may continue for long periods in earthworm casts (Elliott et al. 1991).

Over 30 years ago, earthworm activities were pointed out as important sources of GHG emissions, particularly N_2O and CO_2 (Svensson et al. 1986; Elliott et al. 1990), but CH_4 emission was only recently detected in three Brazilian earthworms (Depkat-Jacob et al. 2012), contrasting with a lack of it in several European species (Sustr and Simek 2009). However, methane emission from earthworm casts has not yet been reported. Although neutral effects have been observed (Koubová et al. 2012), the impact of earthworms on overall substrate/soil methane emission appears to be biased towards methanotrophy rather than methanogenesis (Moon et al. 2010; Kernecker et al. 2014; Mitra and Kaneko 2014), mainly due to the soil mixing/aeration effects of earthworms that reduce methane emissions, particularly in water-saturated conditions.

More than 50 species of large earthworms (>30 cm length) are known from Brazil (James and Brown 2006), and their casting activities result in major soil bioturbation, and impacts on nutrient cycling and plant production (Kuczak et al. 2006; Fiuza et al. 2011). *Rhinodrilus alatus* Righi, 1971 (Fig. 1A) is of the most well-known Brazilian giant earthworms, as it has been intensively collected and commercialized for over 80 years (Drumond et al. 2015). Adults of this species measure around 60 cm length (but may reach 1.2 m) and inhabit an area of around 20 thousand km^2, in the Cerrado region of central Minas Gerais (Drumond et al. 2012). Although its life-history has been relatively well studied (Drumond et al. 2013; 2015), little is known of the impacts of this species on soil properties and processes. This species was shown to emit N_2, N_2O and small amounts of CH_4 *in vivo* and from its gut contents (Depkat-Jacob et al. 2012; 2013), but GHG emissions have not yet been studied in its large castings (Figure 1B). Hence, the present study was undertaken to evaluate: (1) GHG emissions from *R. alatus* castings; (2) the influence of *R. alatus* casts on some soil chemical and physical properties, and (3) the influence of cast aging on GHG emission potential and mineral N contents.

Material and Methods

Earthworms and soil for laboratory incubations

The soil and earthworms were collected in August 2016 under native Cerrado vegetation in the Paraopeba National Forest (19°15'17.2"S, 44°24'04.9"W; Fig 1). We collected 120 kg of a yellow Latosol (Ferralsol; IUSS 2015), which was sieved (2 mm) and kept in the laboratory at 22 ºC until use. Earthworms were identified using the species description (Righi 1971) and had total body lengths between 56.5 and 62.5 cm (Figure 1A). All individuals were kept in separate containers in their soil of origin, in the dark at 22ºC until further use.

Earthworms were placed individually into plastic containers (boxes of 24 x 17 x 10 cm height, with a lid) containing 500 g sieved soil, and control boxes without earthworms prepared in the same manner. Both treatments (boxes with and without earthworms) were replicated five times. All casts produced in a period of less than 24 h were collected and used for chemical and physical analyses. Control soil samples incubated over the same time period (<24 h) were also collected. Furthermore, fresh casts, i.e., produced in less than a 2 h period, from each container were incubated for 10 distinct time-periods (0, 4, 8, 12, 24, 48, 96 hours and 8, 16 and 32 days) for GHG emission, microbial biomass-C (MBC), metabolic quotient (qCO_2), inorganic N and moisture measurements. Each incubation time-period was run using separate cast samples, as not enough casts could be obtained within a single 2 h period to use for all time-periods. After fresh cast production, the earthworms were removed from each box, and the boxes with the surface casts returned to the incubator. As controls for the casts, boxes with non-ingested soil were incubated in parallel. The experiment was carried out in an incubator in the dark at a temperature of 22 ± 1 °C.

Greenhouse gas emissions

Cast and control samples collected at each incubation time-interval were immediately used to evaluate GHG emission. Air samples were collected using a modified method based on Kusel and Drake (1995), in which approximately 5 g of castings or soil were incubated in 38 ml hermetically sealed glass vials. The glass vials were kept closed for 24 h at which point a 10 ml gas sample was taken and stored in exetainers until analysis. An initial time point (0 h) gas sample was taken in the same way to determine the initial 0 h emission rates. The cumulative production of GHG after a 24 h period was then determined by injecting 2.5 ml gas samples into a Trace 1310 gas chromatograph at Embrapa Forestry in Colombo-PR. For each experimental time period, the values of CO_2, CH_4 and N_2O concentrations were taken by subtracting the cumulative emission rates at 24 h by the 0 h value, and GHG emissions calculated as nmol g dry soil^{-1} d^{-1} basis.

Soil Analysis

Control soil and castings (<24 h old) taken from each box (five replicates of each) were dried for 48 h at 40 °C, and soil analyses (pH, P, K, Ca, Mg, Al, H+Al, Na, Mn, Fe, Zn, Cu) performed following methods described in Hue and Evans (1986): pH in $CaCl_2$ 0.01M (10 g with 1:2.5 soil/solution ratio); nonexchangeable potential acidity (H+Al) with 10 g soil and pH 7 0.5 mol L^{-1} Ca acetate; exchangeable Ca^{2+}, Mg^{2+} and Al^{3+} with 10 g soil and 1 mol L^{-1} KCl; and available P, Mn^{2+}, Fe^{3+}, Zn^{2+} and Cu^{2+} and exchangeable K^+ and Na^+ measured using 10 g soil and 0.05 mol L^{-1} H_2SO_4 and 0.025 mol L^{-1} HCl (Mehlich-1). Similarly, total C, N and H analyses were performed on finely-ground (<212 µm) dried cast and control soil samples (0.025 g) by combustion in a Vario EL III CHN analyser. Soil particle size analysis was performed using 20 g soil and the pipette method, after removal of organic matter with 30 % (v/v) H_2O_2, with the first sampling of silt+clay at 10 cm by using a pipette and the second at 5 cm after 3 h and 28 min to collect clay (Gee and Bauder 1986). Field capacity of the sieved control soil was measured following the method described in the Tropical Soil Biology and Fertility (TSBF) Programme manual (Anderson and Ingram 1993). Gravimetric soil moisture, MBC, qCO_2 and inorganic N were measured on fresh (<2 h) casts/control soil and the 10 incubation time-periods. Exchangeable NH_4^+ and NO_3^- were analysed by ultraviolet absorption spectrophotometry using 10 g soil (Mulvaney 1996), while MBC was analyzed using 50 g soil, following the fumigation incubation method described in Jenkinson and Powlson (1976), and qCO_2 was calculated by dividing C-CO_2 evolution by MBC (Anderson and Domsch 1993).

Statistical analyses and data availability

The GHG emission values and those of selected soil properties (exchangeable NH_4^+, NO_3^-, MBC, qCO_2) from predetermined time-intervals were analyzed by regression, while all other parameters were analyzed by T-test (with a $p < 0.05$) using Statistica v.7.0 (StatSoft 2006) and Sigma Plot 12. A principal component analysis (PCA) was used to evaluate relationships between GHG emissions (24 h-old casts and control soil), chemical, physical and biological (24 h) properties of castings and control soil, including a Monte Carlo permutation test to evaluate significance ($p < 0.05$), using the ADE-4 package for R (Dray and Dufour 2007). Linear correlations (Pearson's coefficient) were also explored between all the variables obtained for 24 h-old casts and control soil using the same data as for the PCA. All data generated or analysed during this study are included in this published article and its supplementary information files (Online Supplementary Tables 1 to 8).

Results

Soil and fresh cast chemical and physical properties

The soil used for the experiment was an extremely acidic (pH <4) fine-textured (clayey) Latosol (Ferralsol), with over 50% clay and around 45% silt (Table 1). When excreted, the earthworm casts were a humid paste, deposited in a globular form (Fig. 1B). Soil moisture contents in casts were significantly higher than in the non-ingested soil throughout most of the ageing process, except at 4 and 8 days (Fig. 2). Field moisture capacity of the soil was 25% H_2O, and casts generally had >25% gravimetric water content. In fact, fresh (0 h) casts had twice the field moisture water contents (51%) and 63% higher moisture than the control soils, but over time they became progressively drier so that after 32 days, they had about half their original moisture content (26%, i.e., approximately field capacity). Control soil moisture contents ranged from a maximum of 27% (close to field capacity) at 8 days to a minimum of 17% at 32 days but were generally below 20% H_2O throughout the experimental period.

Casts <24 h old also had a minimal (2%) but significant pH increase, and a moderate increase in H+Al (12%), organic C (14%) and Cu (15%) (Table 1). Total N, total C and total H contents were also significantly affected by gut transit, being 20%, 9% and 10% higher in casts compared with control soil, respectively. Important increases were also observed in both available Fe (37%) and Mn (43%) in casts. The other elements analyzed, such as Al, K, P, Ca and Mg, Zn, C/N and texture did not show any differences (Table 1).

The PCA with GHG emissions, soil chemical, physical and biological properties explained 67% of the data variability in the first two axes (Fig. 3), being 45% by axis 1 (PC1) and 22% by axis 2 (PC2). The PC1 separated control soil and cast samples mainly due to chemical results, particularly pH, total C, N and H, available Cu, Mn, Fe, NH_4, NO_3 and organic matter contents but also soil moisture (all of these except NO_3 significantly higher in the casts) and MBC (significantly higher in control soil). The second axis (PC2) was mainly related to Ca, Mg, Al, CEC and soil base contents and saturation (V%) which were higher in one of the five cast samples (Online Supplemantary Table 1).

Using all data (control and casts), the only significant correlations (R^2= 0.7, p< 0.05) between GHG emissions (24 h data) and soil properties were between N_2O and soil Fe contents and between CH_4 and total soil N (correlation matrix available in Online Supplementary Table 6). On the other hand, when only control soil data were used, significant negative correlations were observed between base saturation (V%) and CH_4 and N_2O emissions (R^2 = -0.88 and -0.96, respectively), and between Al saturation (m%) and CO_2 emissions (R^2 = -0.93; Online Supplementary Table 7). Conversly, when only cast data were used, significant correlations were observed between soil moisture and CH_4 emissions (R^2 = 0.98), as well as between MBC and qCO_2 and N_2O emissions (R^2 = 0.9 and -0.92, respectively; Online Supplementary Table 8).

Mineral-N and microbial analyses at different time intervals

MBC in non-ingested soil ranged from 494 to 595 mg C kg^{-1} soil and was significantly higher than in the casts (247 to 387 mg C kg^{-1}) at all time intervals (Fig. 4A). Metabolic quotient values ranged from 4.6 to 18 mg C-CO_2 g^{-1} MBC h^{-1} for control soil, and between 11.4 and 37 mg C-CO_2 g^{-1} MBC h^{-1} for casts, and were significantly higher in the fresh casts at the beginning of the incubation period (0 h), 12 h old casts and in all casts older than 48 h (Fig. 4B). There was a significant decrease (quadratic regression coefficient R^2 = 0.83, p<0.01) in qCO_2 values in the control soil over time, with lowest values at 32 days. In the casts, qCO_2 values varied over time, with three peaks, at 12, 48 and 96 h, and lowest value at 32 days.

Mineral-N concentrations were highest in earthworm casts, and consisted mainly of exchangeable NH_4, with significantly higher values than the control soil for all cast ages analyzed (Fig. 5A). Casts had a minimum of 121 mg kg^{-1} exchangeable NH_4^+, while in the control maximum values reached only 25 mg kg^{-1}. Nitrate was present in very low values in the castings (Fig. 5B), generally <1 mg kg^{-1} NO_3^-, and were significantly lower than in the control soil at all incubation times, except at 4 and 8 h (2.5 mg kg^{-1}). Lowest NO_3^- content in the control was observed at 0 h (1.6 mg kg^{-1}) and there was a significant trend (quadratic regression coefficient R^2 = 0.84, p <0.01) for increasing concentration over time, with highest NO_3^- at 32 days (3.4 mg kg^{-1}).

GHG production over time

Methane emission rates were generally negative, indicating methanotrophy in both the control soil and earthworm casts. However, methane sink tended to be higher in the control (ranging from -0.4895 to -0.0339 nmol g^{-1}) than in the casts, except at 4 h, although it was significantly higher only at 8 days (Fig. 6A). Methane emission from casts was detected on two occasions (0 and 12 h), this being the first record of CH_4 emission reported from earthworm castings that we are aware of.

After the first 12 h of incubation, control soil and cast respiration rates (CO_2 emission) were similar, with a significant trend of decreased emissions over time and lowest values reported at 32 days. The reduction in CO_2 emissions for the control and the casts was 700 and 1,600%, respectively (Fig. 6B). Significant differences between cast and control were detected only at 8 h, when emissions were higher in the control soil.

Nitrous oxide emissions tended to be low and close to 0 in the control soil, while in casts they tended to be higher, although significant differences were detected only at 8 h (Fig. 6C). In fresh casts (0 h), emissions were 0.23 nmol N_2O g^{-1}, reaching 1.61 nmol N_2O g^{-1} in 8 h.

Discussion

Most of the casts of *R. alatus* are deposited within the soil, and only a small fraction is excreted on the soil surface (Drumond et al. 2015), a feature commonly observed in tropical endogeic earthworm species (Lavelle 1988). Within the soil matrix, earthworm casts will be subjected to different conditions than on the soil surface, as evaluated in the present study and most other studies on earthworm castings (with a few exceptions; e.g., Bouché and Al-Addan 1997; Jégou et al. 1998; Mariani et al. 2007; Bottinelli et al. 2010). Microbes can compete for resources in casts (as evidenced by qCO_2 in the present study), and this may be further exarcebated by the presence of other soil organisms (of all sizes), as well as plant roots within the soil matrix around the casts deposited in field conditions, factors which may greatly change the overall processes measured over time in laboratory experiments (Jiménez et al. 2004) such as the present one. Nonetheless, we provide evidence here that the giant earthworm *R. alatus* can have important impacts on soil chemical and microbiological properties that deserve further attention, in order to properly value its potential contribution to soil ecosystem processes and services, as well as GHG emissions *in-situ*.

Cast physical and chemical properties

It is well known that the drilosphere (including casts) represents a different physical and chemical environment than non-ingested soil, and that this reflects itself on the microbiological and biochemical processes that occur therein (Brown et al. 2000). These are the result of transformation processes that occur in the earthworm gut (gut-associated processes) and concentration/enrichment processes occurring as a result of earthworm feeding habits, that often include food selection (Devliegher and Verstraete 1997; van Groenigen et al. 2019). In the present case, the giant earthworm species *R. alatus* was shown to do both: there was a significant increase in soil organic-C, total C and N in casts compared with the non-ingested control soil, indicating selective feeding of earthworms on richer soil particles (as observed for other earthworms in the temperate and tropical regions; Barois et al. 1999; van Groenigen et al. 2019), and increases in several chemical properties in casts due to nutrient transformation processes. The C concentration factor means that the species behaves as a polyhumic endogeic (sensu Lavelle 1981) in laboratory cultures, feeding on C-richer portions of the soil.

Soil pH increased after transit through the intestinal tract of *R. alatus*, a phenomenon which has been observed for various other earthworm species, both large and small in size, and of various ecological categories (Fiuza et al. 2011; Clause et al. 2014; Hmar and Ramaneijam 2014; van Groenigen et al. 2019). Earthworms can change soil pH by the excretion of NH_3 from the nephridia into the gut, or by calcium carbonate ($CaCO_3$) secretions, added to the ingested soil in the oesophagous of species that have calciferous glands (Piearce 1972). *R. alatus* has three pairs of calciferous glands that secrete $CaCO_3$ into the oesophagous (Righi 1971), but no differences were found in cast Ca contents compared with the uningested control soil. Hence, the increase in pH may be more attributed to the increase in ammonia excreted by the earthworm, which is rapidly transformed to NH_4^+ in casts (Parle 1963b).

Although *R. alatus* is an exonephridial (holonephric) species, that excretes NH_3 through the body wall via the nephridiopores and not into the intestine (Righi 1971), ammonium content of casts was still very high. Furthermore, NH_4^+ contents remained high up to 32 days, with noticeable increase in nitrate values only on two occasions at the beginning of the incubation process (4 and 8 h). As casts age, NH_4^+ is usually transformed to NO_3^- by nitrifying microbes (Lavelle et al. 1992; Parkin and Berry 1994; Decaëns et al. 1999; Kawaguchi et al. 2011). However, this did not occur with the dense, clayey and moist casts of *R. alatus*. The lack of NO_3^- production may be due to the low O_2 contents of the casts, denitrification and/or the incorporation/use of mineral N by the microbiome (Lavelle and Martin 1992).

High mineral-N contents have been observed in castings of both ecto and endo-nephridial species, with values ranging from 144 to over 1,000 mg kg^{-1}, depending on the soil type and the species (Barois et al. 1999; Hernández-Castellanos et al. 2010). Hence, other mechanisms must be involved in order to explain the high values in ectonephridial species casts, possibly including NH_3 diffusion from the nephridia to the gut (earthworms excrete urea and ammonia; Bahl 1947), NH_4^+ production in the gut by mineralization of organic compounds (van Groenigen et al. 2019), or even N_2 fixation (Barois et al. 1987). These processes have been little studied, although mineral-N contents have been evaluated in many earthworm species (van Groenigen et al. 2019).

The pH increase in casts can have significant effects on soil processes as well as the availability of nutrients that are pH-sensitive, as showed in the present study. The availability of several micronutrients (Mn, Fe, Cu) was higher in casts than control soil, confirming reports for other earthworm species (Bityutskii and Kaidun 2008; Bartz et al. 2010; Bityutskii et al. 2012). The higher moisture level in worm casts may favor the solubilization of these elements as they are exposed to greater hydrolysis and complexation by organic acids in the gut. Also, oxy-reduction reactions can be important. For example, *R. alatus* increased Fe availability by 37%, possibly by reduction reaction of Fe^{3+} to Fe^{2+} in the intestine, promoted by the anoxic and high H_2O environment of the gut. Nevertheless, Fe speciation changes in earthworm guts and castings are little known and represent an important topic for further research.

Although most studies show that earthworms increase P values in casts (Chapuis-Lardy et al. 1998; Jiménez et al. 2003; Bayon and Binet 2006; Kuczak et al. 2006; Vos et al. 2019), there was no increase in extractable P in *R. alatus* casts. Relative increases in earthworm cast P contents can be due to various processes such as increased P mineralization in the gut and casts, preferential feeding on clay particles or organic-matter rich substrates (e.g., plant litter), changes in soil pH that affect P adsorption to minerals, and competitive adsorption between orthophosphate and elevated concentration of DOC (van Groenigen et al. 2019; Vos et al. 2019). However, we measured only one form of extractable-P (Mehlich-1, the standard in Brazil for acid soils), and various forms of P in soil are affected by earthworm species (Vos et al. 2019), so further studies on different P-forms in *R. alatus* casts are needed.

Microbial biomass C and CO_2 emission

Inside fresh casts, microorganisms initially find an anoxic and moist niche which is rich in water-soluble organic matter, derived in part from the remainder of intestinal mucus secretions and ingested microbial cells, DNA, RNA and proteins (Drake and Horn 2007; Zeibich et al. 2018; 2019a; 2019c) that were not digested or assimilated by the worms. Ingested soil microbes are activated in the gut of many earthworm species, including *R. alatus* (Depkat-Jacob et al. 2012; 2013) where the gut-transit time of approximately 24 hours is sufficient for microbial activation. Therefore, we hypothesized that microbial activation also occured in their casts. However, casts of *R. alatus* had generally very little difference in CO_2 evolution over time compared with control soil, and fresh and ageing casts displayed lower soil MBC, indicating that despite the higher organic-C content of fresh casts, there was no concomitant increase in MBC. Lower MBC has been detected in casts of several endogeic species (Scheu et al. 2002; Chapuis-Lardy et al. 2010), although in some conditions endogeic earthworms may increase soil MBC, particularly in freshly deposited casts (Sheu 1987), or in topsoils with litter added on the soil surface (Chang et al. 2016). As castings age, microbial activity reduces (Scheu 1987) and may be inhibited, particularly in drier, more compact casts, where C may be protected and thus sequestered in microaggregates within the casts (Martin 1991; Six et al. 2004).

Some earthworms are known to feed on soil microorganisms such as protozoa, nematodes, fungi and algae (Brown and Doube 2004), but nothing is known of the feeding habits of *R. alatus*, besides that it ingests large amounts of soil and that it concentrates soil organic-C and N (Table 1). Different forms of organic carbon (e.g., long-chain fatty acids, probably derived from the membranous lipids of digested bacteria) can be used by earthworms (Drake and Horn 2007). *R. alatus* has a very large and muscular gizzard, and a very long intestine (Righi 1971), and microorganisms with high cell volumes are preferentially destroyed during passage through the earthworm gut and gizzard (Drake and Horn 2007). However, the present experiment did not study ingested microbial species and the impacts of *R. alatus* on the microbial community in casts. The results obtained showed that *R. alatus* has a negative effect on MBC, which requires further investigation in order to ascertain any functional impacts on the soil ecosystem.

The higher qCO_2 in earthworm casts, found at several times along the ageing process, may suggest a higher stress or "younger" environment for microbes in casts than in control soil (Anderson and Domsch 1993). Although MBC was reduced by almost half in casts compared with the control, the remaining microorganisms were still very active (considering the emission rates of CO_2, that were generally similar to non-ingested soil), although less efficient in their use of C for microbial growth. The ratio of bacteria to fungi in the casts and the control soil (not evaluated in the present experiment) may be a factor worth further investigation, since fungi incorporate more C than bacteria (Nannipieri et al. 2003), and varying responses have been observed in bacterial populations and activity in endogeic earthworm casts (Medina-Sauza et al. 2019).

Emission of CH_4 and N_2O

Contrary to what had been observed in previous studies, methane emission was detected in *R. alatus* casts on two occasions, this being the first known case of CH_4 emission from earthworm casts. Although this may have been expected from previous studies (Drake et al. 2006; Depkat-Jacob et al. 2012) it was never confirmed. Furthermore, conditions for methane emission generally occur only in selected niches such as invertebrate guts (Sustr and Simek 2009) and wetland environments (Mehring et al. 2017). We have shown here that this niche can be extended to the castings of *R. alatus* and could be expected from castings of other earthworm species, particularly compacting species and those inhabiting wetlands, where casts will be mostly water saturated and with limited O_2. Interestingly, CH_4 emission was highly and positively correled to cast moisture contents. However, in most cases methane oxidation occurred both in casts and non-ingested soil over time and when methane was emitted, rates were very low when compared to studies on earthworm gut content emissions. Depkat-Jakob et al. (2012) measured methane emissions by *Eudrilus eugeniae* (an epigeic species) gut contents with values up to 41 nmol CH_4 g^{-1} in 5 h incubations, while those of *R. alatus* were only around 1 nmol CH_4 g^{-1} in its natural soil (from where the worms were collected). In the present study, methane emissions in *R. alatus* casts did not exceed 0.06 nmol CH_4 g^{-1} day^{-1}. Overall, CH_4 was more consumed rather than produced in both casts and control soil, probably due to the greater activity of the methanotrophic microbes in relation to the methanogenic bacteria. However, further work on the organisms active in earthworm casts under both oxic and anoxic conditions are needed, in order to better understand these phenomena.

Nitrifying and denitrifying microbes activated in the anaerobic gut of *R. alatus* appeared to remain active in their casts as well, considering the production of N_2O measured on several occasions in *R. alatus* casts, confirming results obtained for many other earthworm species (Braga et al. 2016; Elliott et al. 1990; Lubbers et al. 2011; 2013). The gut contents and individuals of *R. alatus* are known to emit both N_2O and N_2 (Depkat-Jacob et al. 2013), but N_2 emission from their casts has still not been evaluated. The emission of N_2O in the first stages of cast incubation (0 to 24 h) was expected, as the high moisture and mineral-N content of the casts would continue to stimulate the activity of the nitrate reducers, stimulated by gut passage (Depkat-Jacob et al. 2013) and egested in the casts. Most denitrifiers have the ability to produce and consume N_2O and the net release of N_2O during denitrification is regulated by factors like moisture content, temperature, pH and also the concentrations of N-ions, all found in high concentrations in the casts of *R. alatus*. Interestingly, the rate of N_2O emission was highly correlated with MBC in *R. alatus* casts. The production of N_2O by nitrate-assimilating bacteria is favored in systems that contain high levels of organic-C, however some nitrifiers are able to use nitrate or nitrite as electron receptors, and can produce N_2O or N_2 under conditions of limited oxygen (Fischer et al. 1997). Nitrate-dissimilating bacteria are abundant in earthworm intestines, where 15 of the 25 distinct and isolated taxa were nitrate dissimilating and only five were denitrifying bacteria (Drake and Horn 2007). However, the N_2O production rates of nitrate reducers are 30 times lower than that of denitrifiers, indicating that denitrification is still the main N_2O generator in earthworm guts and probably also in *R. alatus* casts. Further efforts are needed to characterize the methanogenic and methonotropic bacteria, as well as the denitrifyers in *R. alatus* guts and casts, and to elucidate the potential importance of these GHG emissions and cast production for both C and N cycling in their native environments.

Conclusions

Earthworm (*R. alatus*) casts are microbiologically and chemically different than non-ingested soil, with high moisture contents, high exchangeable NH_4^+, higher organic C, total C and N, pH and some micronutrients. These conditions allow for the emission of low amounts of N_2O and CH_4 in younger-aged casts. As casts age, all GHG emissions tend to decrease, and methanotrophy is prevalent. Lower microbial biomass and a higher qCO_2 in casts than in control soil indicates that earthworms are digesting soil microorganisms in the intestine and that the casts represent zones of high microbial activity but also stress, hindering bacterial growth, since CO_2 emission remained similar to control soil.

The large, dense and moisture-rich castings of *R. alatus* appear to function as an extension of the gut zone, especially in this fine-textured soil where low O_2 contents create a special soil niche with complex biochemical and microbial processes. The high NH_4 and H_2O contents influence oxy-reduction processes, GHG emissions and N transformations, modifying soil microbial biomass and activity. Furthermore, selective soil ingestion by earthworms concentrates organic-C content in their casts and transformation processes affect the availability of important plant nutrients, factors which deserve further attention considering the widespread use and collection of this species *in-situ* for use as fish-bait in Brazil.

References

Anderson JM, Ingram JSI (1993) Tropical Soil Biological and Fertility – A Handbook of Methods. 2nd ed. CABI, Wallingford, UK.

Anderson JPE, Domsch KH (1993) The metabolic quotient of CO_2 (qCO_2) as a specific activity paramenter to assess the effects of environmental condition, such as pH, on the microbial of forest soil. Soil Biol Biochem 25:393-395

Bahl KN (1947) Excretion in the Oligochaeta. Biol Rev Cambridge Philos Soc 22:109-47

Barois I, Lavelle P, Brossard M, Tondoh J, Martinez MA, Rossi JP, Senapati BK, Angeles A, Fragoso C, Jimenez JJ, Decaens T, Lattaud C, Kanyonyo J, Blanchart E, Chapuis L, Brown G, Moreno A (1999) Ecology of earthworm species with large environmental tolerance and/ or extended distributions. In: Lavelle P, Hendrix P, Brussard L (Eds) Earthworm management in tropical agroecosystems. CAB International, Wallingford, UK, pp 57–86

Barois I, Verdier B, Kaiser P, Mariotti A, Rangel P, Lavelle P (1987) Influence of the tropical earthworm *Pontoscolex corethrurus* (Glossoscolecidae) on the fixation and mineralization of nitrogen. In: Omodeo P, Bonvicini AM (Eds) On earthworms. Mucchi, Bologna, pp 151–158

Bartz MLC, Costa ACS, Souza IG, Brown GG (2010) Micronutrientes e óxidos de ferro em coprólitos de minhocas produzidos em um Latossolo Vermelho distroférrico (Oxisol) sob diferentes sistemas de manejo. Acta Zool Mex 26:281-294

Bayon RC, Binet F (2006) Earthworms change the distribution and availability of phosphorous in inorganic substrates. Soil Biol Biochem 38:235-246

Bityutskii NP, Kaidun PI (2008) The influence of earthworms on the mobility of microelements in soil and their availability for plants. Euras Soil Sci 41:1306–1313

Bityutskii NP, Kaidun PI, Yakkonem KL (2012) The earthworm (*Aporrectodea caliginosa*) primes the release of mobile and available micronutrients in soil. Pedobiologia 55:93-99

Blanchart E, Lavelle P, Braudeau E, Le Bissonnais Y, Valentin C (1997) Regulation of soil structure by geophagous earthworm activities in humid Savannas of Côte d'Ivoire. Soil Biol Biochem 29:431-439

Blanchart E, Albrecht A, Alegre J, Duboisset A, Gilot C, Pashanasi B, Lavelle P, Brussaard L (1999) Effects of earthworms on soil structure and physical properties. In: Lavelle P, Brussaard L, Hendrix P (Eds) Earthworm management in tropical agroecosystems. CAB International, Wallingford, UK, pp 149-172

Bottinelli N, Hallaire V, Menasseri-Aubry S, Le Guillou C, Cluzeau D (2010) Abundance and stability of belowground earthworm casts influenced by tillage intensity and depth. Soil Till Res 106:263–267

Bouché MB (1977) Strategies lombriciennes. In: Lohm U, Persson T (Eds) Soil organisms as components of ecosystems. Ecological Bulletins vol 25, Stockholm, Sweden, pp 122–132

Bouché MB, Al-Addan F (1997) Earthworms, water infiltration and soil stability: some new assessments. Soil Biol Biochem 29:441-452

Braga LP, Yoshiura CA, Borges CD, Horn MA, Brown GG, Drake HL, Tsai SM (2016) Disentangling the influence of earthworms in sugarcane rhizosphere. Sci Rep 6:38923

Brown GG, Barois I, Lavelle P (2000) Regulation of soil organic matter dynamics and microbial activity in the drilosphere and the role of interactions with other edaphic functional domains. Eur J Soil Biol 36:1–23

Brown GG, Doube BM (2004) Functional interactions between earthworms, microorganisms, organic matter, and plants. In: Edwards CA (Ed) Earthworm ecology. CRC Press, Boca Raton, FL, pp 213–39

Chang CH, Szlavecz K, Buyer JS (2016) Species-specific effects of earthworms on microbial communities and the fate of litter-derived carbon. Soil Biol Biochem 100:129–139

Chapuis-Lardy L, Brauman A, Bernard AL, Pablo J, Toucet MJ, Mano L, Weber L, Brunet D, Razafimbelo T, Chotte JL Blanchart E (2010) 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). Appl Soil Ecol 45:201–208

Chapuis-Lardy L, Brossard M, Lavelle P, Schouller E (1998) Phosphorus transformations in a Ferralsol through ingestion by *Pontoscolex corethrurus*, a geophagous earthworm. Eur J Soil Biol 34:61–67

Cheng J, Wong HM (2010) Effects of earthworms on Zn fractionation in soil. Biol Fertil Soils 36:72-78

Clause J, Barot S, Richard B, Decaëns T, Forey E (2014) The interactions between soil type and earthworm species determine the properties of earthworm casts. Appl Soil Ecol 83:149-158

Decaëns T, Rangel AF, Asakawa N, Thomas RJ (1999) Carbon and nitrogen dynamics in ageing earthworm casts in grasslands of the eastern plains of Colombia. Biol Fertil Soils 30:20–28

Depkat-Jakob PS, Brown GG, Tsai SM, Horn M, Drake HL (2013) Emission of nitrous oxide and dinitrogen by diverse earthworm families from Brazil and resolution of associated denitrifying and nitrate-dissimilating taxa. FEMS Microb Ecol 83:375-391 doi:doi.org/10.1111/j.1574-6941.2012.01476.x

Depkat-Jakob PS, Hunger S, Schulz K, Brown GG, Tsai SM, Drake HL (2012) Emission of methane by *Eudrilus eugeniae* and other earthworms from Brazil. Appl Environ Microb 78: 3014-3019 doi:http://doi.org/10.1128/AEM.07949-11

Devliegher W, Verstraete W (1997) Microorganisms and soil physicochemical conditions in the drilosphere of *Lumbricus terrestris*. Soil Biol Biochem 29:1721–1729

Drake H, Schramm A, Horn M (2006) Earthworm gut microbial biomes: their importance to soil microorganisms, denitrification, and the terrestrial production of the greenhouse gas N_2O. In: König H, Varma A (Eds) Intestinal microorganisms of termites and other invertebrates. Soil Biology, vol 6, Springer, Berlin, pp 65-87 doi:doi.org/10.1007/3-540-28185-1_3

Drake HL, Horn MA (2007) As the worm turns: the earthworm gut as a transient habitat for soil microbial biomes. Annu Rev Microb 61:169-189 doi:doi.org/10.1146/annurev.micro.61.080706.093139

Dray S, Dufour AB (2007) The ade4 Package: implementing the duality diagram for ecologists. J Stat Soft 22:1–20.

Drumond MA, Brown GG, Marini-Filho O (2012) Avaliação do risco de extinção do minhocuçu *Rhinodrilus alatus* Righi, 1971. Biodiversidade Brasileira 2:125-130

Drumond MA, Guimarães AQ, El Bizri HR, Giovanetti LC, Sepúlveda DG, Martins RP (2013) Life history, distribution and abundance of the giant earthworm *Rhinodrilus alatus* RIGHI 1971: conservation and management implications. Braz J Biol 73:699-708

Drumond MA, Guimarães AQ, Silva RHP (2015) The hole of local knowledge and traditional extraction practices in the management of giant earthworms in Brazil. PLoS One 10:e0123913

Elliott PW, Knight D Anderson JM (1990) Denitrification in earthworm casts and soil from pasture under different fertilizer and drainage regimes. Soil Biol Biochem 22:601-605

Elliott PW, Knight D Anderson JM (1991) Variables controlling denitrification from earthworm casts and soil in permanent pasture. Biol Fertil Soils 11:24-29

Eriksen-Hamel NS, Whalen JK (2007) Impacts of earthworms on soil nutrients and plant growth in soybean and maize agroecosystms. Agric Ecosyst Environ 120:442-448

Fischer K, Hahn D, Honerlage W, Zeyer J (1997) Effect of passage through the gut of the earthworm *Lumbricus terrestris* L. on *Bacillus megaterium* studied by whole cell hybridization. Soil Biol Biochem 29:1149-1152

Fiuza SS, Kusdra JF, Furtado DT (2011) Caracterização química e atividade microbiana de coprólitos de *Chibui bari* (Oligochaeta) e do solo adjacente. Rev Bras Ciên Solo 35:723-728

Heinzmann FX, Miyazawa M, Pavan MA (1984) Determinação de nitrato em extratos de solos ácidos por espectrofotometria de absorção ultravioleta. Rev Bras Ciên Solo 8:159-163

Hernández-Castellanos B, Barois I, Brown GG, García-Pérez JÁ (2010) Modificaciones químicas inducidas por dos especies de lombrices geófagas em suleos de Veracruz, México. Acta Zool Mex (n.s.) 26:295-308

Hmar L, Ramanujam S (2014) Earthworm cast production and physic-chemical properties in two agroforestry systems of Mizoram (India). Trop Ecol 55:77-86

Hue NV, Evans CE (1986) Procedures used for soil and plant analysis by the Auburn University soil testing laboratory no. 106. Department of Agronomy and Soils, Auburn, AL.

Hulugalle NR, Ezumah HC (1991) Effects of cassava-based cropping systems on physic-chemical properties of soil and earthworm casts in a tropical Alfisol. Agric Ecosyst Environ 35:55-63

IUSS (2015) World Reference Base for Soil Resources 2014, update 2015. International soil classification system for naming soils and creating legends for soil maps. World Soil Resources Reports No. 106. FAO, Rome

James SW, Brown GG (2006) Earthworm ecology and diversity in Brazil. In: Moreira FMS, Siqueira JO, Brussaard L (Eds) Soil biodiversity in Amazonian and other Brazilian ecosystems, CABI, Wallingford, UK, pp 56-116

Jégou D, Cluzeau D, Balesdent J, Tréhen P (1998) Effects of four ecological categories of earthworms on carbon transfer in soil. Appl Soil Ecol 9 249-255

Jenkinson DS, Powlson DS (1976) The effects of biocidal treatments on metabolism in soil V. A method for measuring soil biomass. Soil Biol Biochem 8:209-213

Jiménez JJ, Cepeda A, Decaëns T, Oberson A, Friesen DK (2003) Phosphorus fractions and dynamics in surface earthworm casts under native and improved grasslands in a Colombian savanna Oxisol. Soil Biol Biochem 35:715–727

Kawaguchi T, Kyoshima T, Kaneko N (2011) Mineral nitrogen dynamics in the casts of epigeic earthworms (*Metaphire hilgendorfi*: Megascolecidae). Soil Sci Plant Nutr 57:387-39

Kernecker M, Whalen JK, Bradley RL (2014) Endogeic earthworms lower net methane production in saturated riparian soils. Biol Fertil Soils 51:271-275

Koubova A, Goberna M, Simek M, Chronakova A, Pizl V, Insam H, Elhottova D (2012) Effects of the earthworm *Eisenia andrei* on methanogens in a cattle-impacted soil: a microcosm study. Eur J Soil Biol 48:32-40

Kuczak CN, Fernandes ECM, Lehmann J, Rondon MA, Luizão FJ (2006) Inorganic and organic phosphorus pools in earthworm casts (Glossoscolecidae) and a Brazilian rainforest Oxisol. Soil Biol Biochem 38:553–560

Kusel K. Drake HL (1995) Effects of environmental parameters on the formation and turnover of acetate by forest soils. Appl Environ Microbiol 61:3667-3675

Lavelle P (1981) Stratégies de reproduction chez les vers de terre. Acta Oecol Oec Gen 2:117–133

Lavelle P (1984) The soil system in the humid tropics. Biol Intern 9:4-19

Lavelle P (1988) Earthworm activities and the soil system, Biol Fertil Soils 6:237–251

Lavelle P (1997) Faunal activities and soil process: strategies that determine ecosystem function. Adv Ecol Res 37:93-132

Lavelle P, Bignell D, Lepage M, Wolters V, Roger P, Ineson P, Heal OW, Ghillion S (1997) Soil function in a changing world: the role of invertebrate ecosystem engineers. Eur J Soil Biol 33:159–193

Lavelle P, Decaëns T, Aubert M, Barot S, Blouin M, Bureau F, Margerie P, Mora P, Rossi JP (2006) Soil invertebrates and ecosystem services. Eur J Soil Biol 42:S3-S15

Lavelle P, Martin A (1992) Small-scale and large-scale effects of endogeic earthworms on soil organic matter dynamics in soils of the humid tropics. Soil Biol Biochem 24:1491-1498

Lavelle P, Melendez G, Pashanasi B, Schaefer R (1992) Nitrogen mineralization and reorganization in casts of the geophagous tropical earthworm *Pontoscolex corethrurus* (Glossoscolecidae). Biol Fertil Soils 14:49–53

Lubbers IM, Brussaard L, Otten W, Groenigen JW (2011) Earthworm-induced N mineralization in fertilized grassland increases both N_2O emission and crop-N uptake. Eur J Soil Sci 62:152-161

Lubbers IM, van Groenigen KJ, Fonte SJ, Six J, Brussaard L, van Groenigen JW (2013) Greenhouse-gas emissions from soils increased by earthworms. Nat Climate Change 3:187–194

Mariani L, Jiménez JJ, Asakawa N, Thomas RJ, Decaëns T (2007) What happens to earthworm casts in the soil? A field study of carbon and nitrogen dynamics in Neotropical savannahs. Soil Biol Biochem 39:757–767

Martin A (1991) Short-term and long-term effect of the endogeic earthworm *Millsonia anomala* (Omodeo) (Megascolecidae, Oligochaeta) of a tropical savanna, on soil organic matter. Biol Fertil Soils 11:234-238

Medina-Sauza RM, Álvarez-Jiménez M, Delhal A, Reverchon F, Blouin M, Guerrero-Analco JA, Cerdán CR, Guevara R, Villain L and Barois I (2019) Earthworms building up soil microbiota, a review. Front Environ Sci 7:81 doi: 10.3389/fenvs.2019.00081

Mehring AS, Cook PLM, Evrard V (2017) Pollution-tolerant invertebrates enhance greenhouse gas flux in urban wetlands. Ecol Appl 27:1852-1861

Mitra P, Kaneko N (2017) Impact of aquatic earthworms on methane emission reduction from the paddy field soil in Japan. J Agric Sci 9:36-46

Moon KE, Lee SY, Lee SH, Ryu HW, Cho KS (2010) Earthworm cast as a promising filter bed material and its methanotrophic contribution to methane removal. J Hazard Mater 176:131–138

Mulvaney RL (1996) Nitrogen – Inorganics forms. In: Bartels JM, Bigham JM, Sparks DL, Page AL, Helmke PA, Loeppert RH, Soltampam PN, Tabatai MA, Johnson CT, Summer ME (Eds) Methods of soil analysis, Part 3 - Chemical methods, 5 edn. Soil Science Society of American Book Series, Madison, WI, pp 1123-1184

Nannipieri P, Ascher J, Ceccherini MT, Landi L, Pietramellara G, Renella G (2003) Microbial diversity and soil functions. Eur J Soil Sci 54:655-670

Nascimento CWA, Leite PVV, Nascimento RSMP, Mello EEC (2005) Influência da calagem no fracionamento e na disponibilidade de manganês em solos de Pernambuco. Agropec Tec 26:22-28

Parkin TB, Berry EC (1994) Nitrogen transformations associated with earthworm casts. Soil Biol Biochem 26:1233–1238

Parle JN (1963a) Micro-organisms in the intestine of earthworms. J Gen Microb 31:1-11

Parle JN (1963b) A microbiological study of earthworm casts. J Gen Microb 31:13-22

Piearce TG (1972) The calcium relations of selected Lumbricidae. J Animal Ecol 41:167-188

Righi G (1971) Sobre a Familia Glossoscolecidae (Oligochaeta) no Brasil. Arq Zool 20:1–96

Scheu S (1987) Microbial activity and nutrient dynamics in earthworm casts (Lumbricidae). Biol Fertil Soils 5:230–234

Scheu S, Schlitt N, Tiunov AV, Newington JE, Jones TH (2002) Effects of the presence and community composition of earthworms on microbial community functioning. Oecologia 133:254–260

Six J, Bossuyt H, Degryze S, Denef K (2004) A history of research on the link between (micro)aggregates, soil biota, and soil organic matter dynamics. Soil Till Res 79:7–31

Šustr V, Šimek M (2009) Methane release from millipedes and other soil invertebrates in Central Europe. Soil Biol Biochem 41:1684 –1688

Svensson BH, Boström U, Klemedtson L (1986) Potential for higher rates of denitrification in earthworm casts than in the surrounding soil. Biol Fertil Soils 2:147–149

Van Groenigen JW, Van Groenigen KJ, Koopmans GF, Stokkermans L, Vos HJJ, Lubbers IM (2019) How fertile are earthworm casts? A meta-analysis. Geoderma 338:525-535

Vos HMJ, Koopmans GF, Beezemer L, de Goede RGM, Hiemstra T, van Groenigen JW (2019) Large variations in readily-available phosphorus in casts of eight earthworm species are linked to cast properties. Soil Biol Biochem 138:107583

Zeibich L, Schmidt O, Drake HL (2018) Protein- and RNA-enhanced fermentation by gut microbiota of the earthworm *Lumbricus terrestris*. Appl Environ Microbiol 84:657-18

Zeibich L, Schmidt O, Drake HL (2019a) Dietary polysaccharides: fermentation potentials of a primitive gut ecosystem. Environ Microbiol 21:1436–1451

Zeibich L, Schmidt O, Drake HL (2019b) Fermenters in the earthworm gut: do transients matter? FEMS Microbiol Ecol 95:221

Zeibich L, Staege M, Schmidt O, Drake HL (2019c) Aminoacids and ribose: drivers of protein and RNA fermentation by ingested bacteria of a primitive gut ecosystem. Appl Environ Microbiol 85:01297-19

Figure legends

Fig 1. An adult individual of *Rhinodrilus alatus* Righi, 1971 in its experimental plastic box (A) and its large castings (B), deposited in the yellow Latosol from the Cerrado in Paraopeba, Minas Gerais, Brazil.

Fig 2. Moisture variation (%) in casts of *R. alatus* and control soil over a 32-day incubation (ageing) period. *Significant differences between casts and control soil, with t-Test at $p < 0.05$.

Fig 3. Principal Component Analysis (PCA) of *R. alatus* casts and control soil, including chemical (pH, P, K, Ca, Mg, Al, H+Al, Na, Mn, Fe, Zn, Cu; red arrows), physical (gravimetric moisture, % sand, silt and clay; blue arrows) and biological (MBC, qCO_2; black arrows) variables, as well as GHG emissions (CH_4, N_2O and CO_2; black arrows) for 24 h old castings. Correlation circle representing the correlation between individual variables and the first two PCA axes (A) and position of individual replicates (control or cast samples) on the plane defined by the first two PCA axes (B). Significance of Monte Carlo permutation test $p < 0.004$.

Fig 4. Soil microbial biomass C (MB-C) and metabolic quotient (qCO_2) values at various time intervals over a 32-day incubation (ageing) period for *R. alatus* castings and control soil. *Significant differences between casts and control soil, with t-Test at $p < 0.05$.

Fig 5. Concentration of $N\text{-}NO_3^-$ (nitrate) and exchangeable NH_4^+ (ammonium) in *R. alatus* casts and control soil at various time intervals over a 32-day incubation (ageing) period. *Significant differences between casts and control soil, with t-Test at $p < 0.05$.

Fig 6. Emission of greenhouse gases CH_4 (methane), CO_2 (carbon dioxide) and N_2O (nitrous oxide) at various time intervals over a 32-day incubation (ageing) period. Values shown represent the cumulative emission of one full day (24 h period). *Significant differences between casts and control soil, with t-Test at $p < 0.05$.

Supplementary Online Material

Supplementary Tables 1 to 8 containing full data on each variable measured in both control soil and *Rhinodrilus alatus* castings, in all replicates and incubation periods (when applicable).

Supplementary Table 1. Soil chemical and physical data of *Rhinodrilus alatus* casts and control soil (<24 h). Sum = Sum of bases; CEC = effective cation exchange capacity; C_{org} = organic carbon; m = Al saturation; V = base saturation; Rep = individual replicate number

Supplementary Table 2. Inorganic N (ammonium, nitrate), total C, N and H (obtained by combustion), gravimetric moisture content, microbial biomass C (MBC) and metabolic quotient (qCO_2) of earthworm (*R. alatus*) casts and control soils after incubation periods ranging from 0 h to 32 d.

Supplementary Table 3. Emission of methane (CH_4) from earthworm (*R. alatus*) casts and control soils at each of the incubation periods ranging from 0 h to 32 d. Values shown represent the emissions rates obtained from 0 h and 24 h incubation periods, and the cumulative emission rates (24 h - 0 h) of one full-day period.

Supplementary Table 4. Emission of carbon dioxide (CO_2) from earthworm (*R. alatus*) casts and control soils at each of the incubation periods ranging from 0 h to 32 d. Values shown represent the emissions rates obtained from 0 h and 24 h incubation periods, and the cumulative emission rates (24 h - 0 h) of one full-day period. (-) values below detection limit

Supplementary Table 5. Emission of nitrous oxide (N_2O) from earthworm (*R. alatus*) casts and control soils at each of the incubation periods ranging from 0 h to 32 d. Values shown represent the emissions rates obtained from 0 h and 24 h incubation periods, and the cumulative emission rates (24 h - 0 h) of one full-day period. (-) values below detection limit

Supplementary Table 6. Pearson's linear correlation coefficient matrix for all soil properties (chemical, physical, biological) and GHG emissions (CH_4, N_2O, CO_2) using results for 24 h-old *R. alatus* casts and control soil. CEC = effective cation exchange capacity; Corg = organic carbon; m% = Al saturation; V% = base saturation; N, C, H = total N, C and H by combustion; Mois. = gravimetric moisture content, MBC = microbial biomass C; qCO_2 = metabolic quotient.

Supplementary Table 7. Pearson's linear correlation coefficient matrix for all soil properties (chemical, physical, biological) and GHG emissions (CH_4, N_2O, CO_2) using results for 24 h-old *R. alatus* casts only. H+AL = exchangeable acidity; CEC = effective cation exchange capacity; Corg = organic carbon; m% = Al saturation; V% = base saturation; N, C, H = total N, C and H by combustion; Mois. = gravimetric moisture content, MBC = microbial biomass C; qCO_2 = metabolic quotient.

Supplementary Table 8. Pearson's linear correlation coefficient matrix for all soil properties (chemical, physical, biological) and GHG emissions (CH_4, N_2O, CO_2) using results for control soil only. H+AL = exchangeable acidity; CEC = effective cation exchange capacity; Corg = organic carbon; m% = Al saturation; V% = base saturation; N, C, H = total N, C and H by combustion; Mois. = gravimetric moisture content, MBC = microbial biomass C; qCO_2 = metabolic quotient.


A
B
4.5 cm

Figure 2

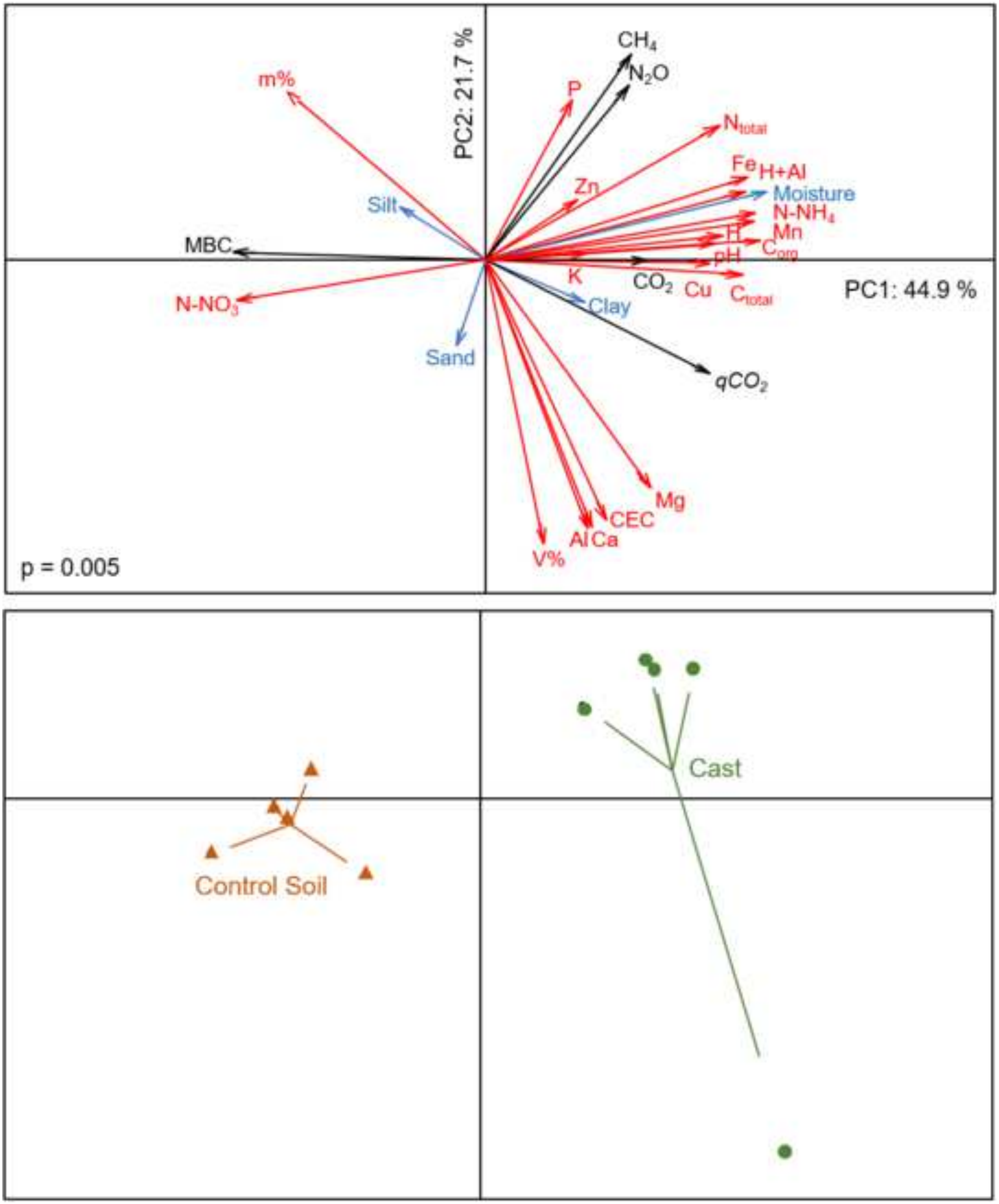
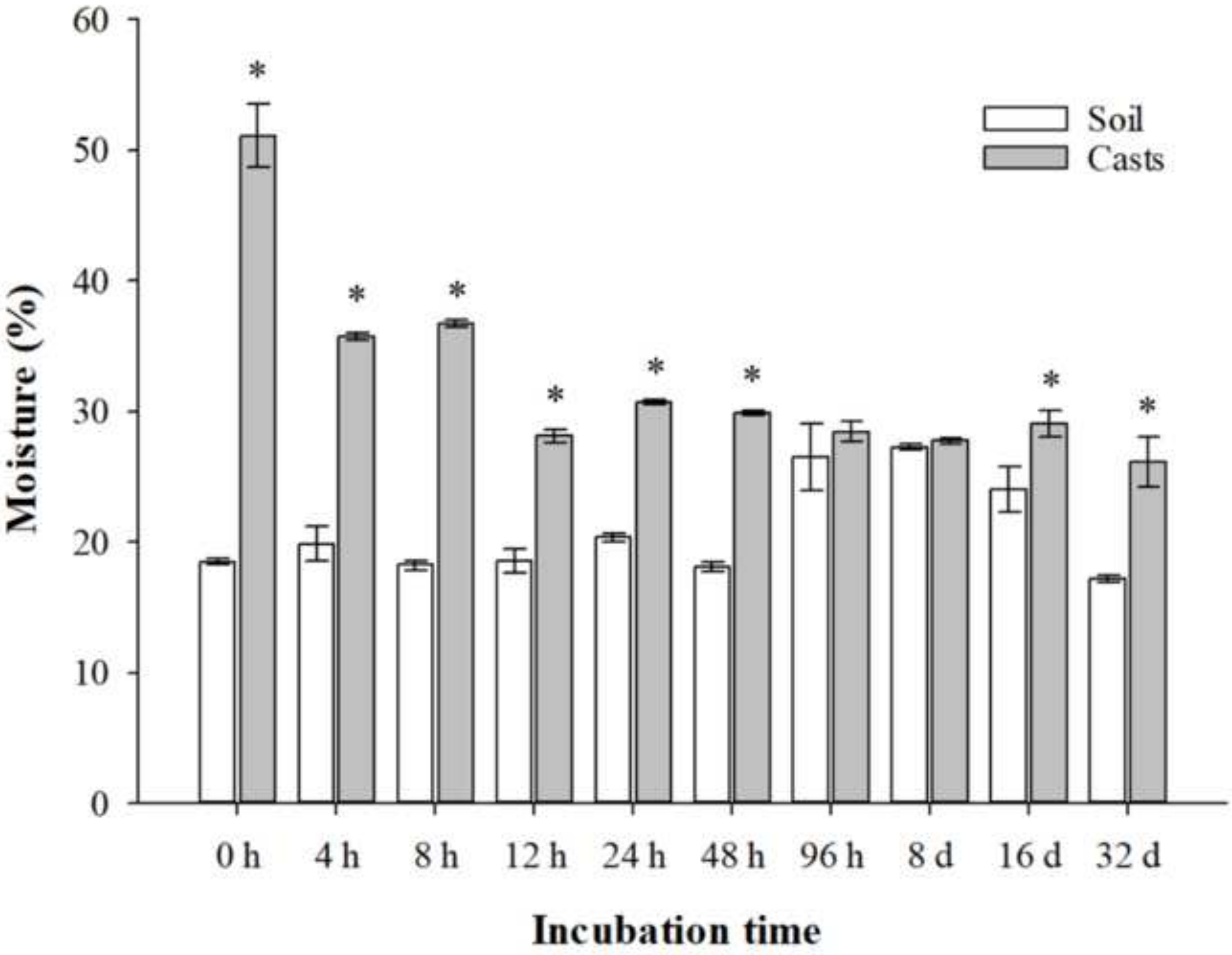

PC2: 21.7 %
CH$_4$
N$_2$O
m%
P
N$_{total}$
Fe
H+Al
Zn
Moisture
Silt
N-NH$_4$
H
Mn
MBC
C$_{org}$
pH
K
CO$_2$
Cu
C$_{total}$
PC1: 44.9 %
N-NO$_3$
Clay
Sand
qCO$_2$
Mg
CEC
Al
Ca
V%
p = 0.005
Cast
Control Soil

Figure 3

Click here to access/download;Figure;Fig3.tiff..tif



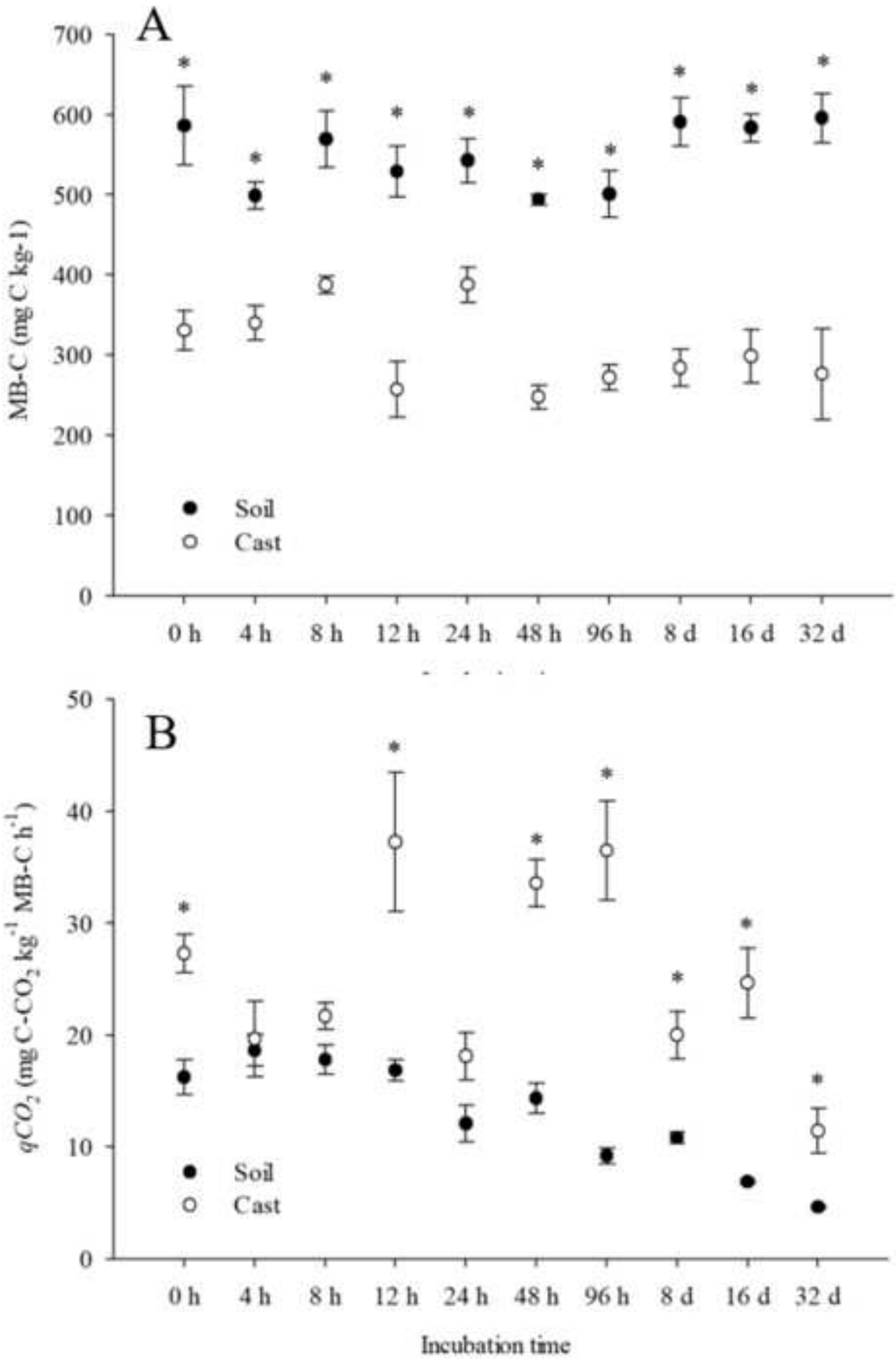

A
MB-C (mg C kg-1)
700
600
500
400
300
200
100
0
Soil
Cast
0 h
4 h
8 h
12 h
24 h
48 h
96 h
8 d
16 d
32 d
B
qCO_2 (mg C-CO$_2$ kg^{-1} MB-C h^{-1})
50
40
30
20
10
0
Soil
Cast
0 h
4 h
8 h
12 h
24 h
48 h
96 h
8 d
16 d
32 d
Incubation time

Figure 5

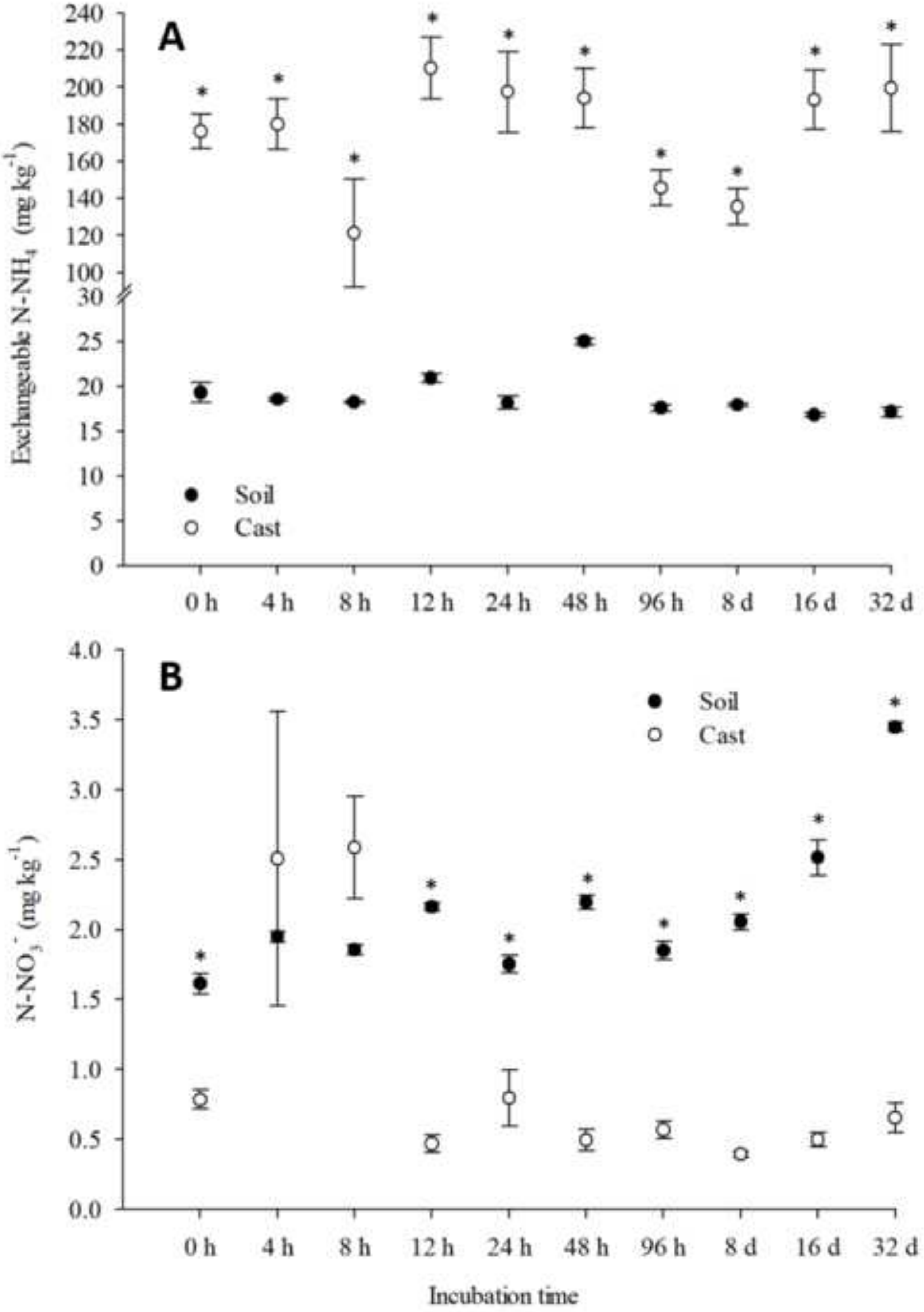
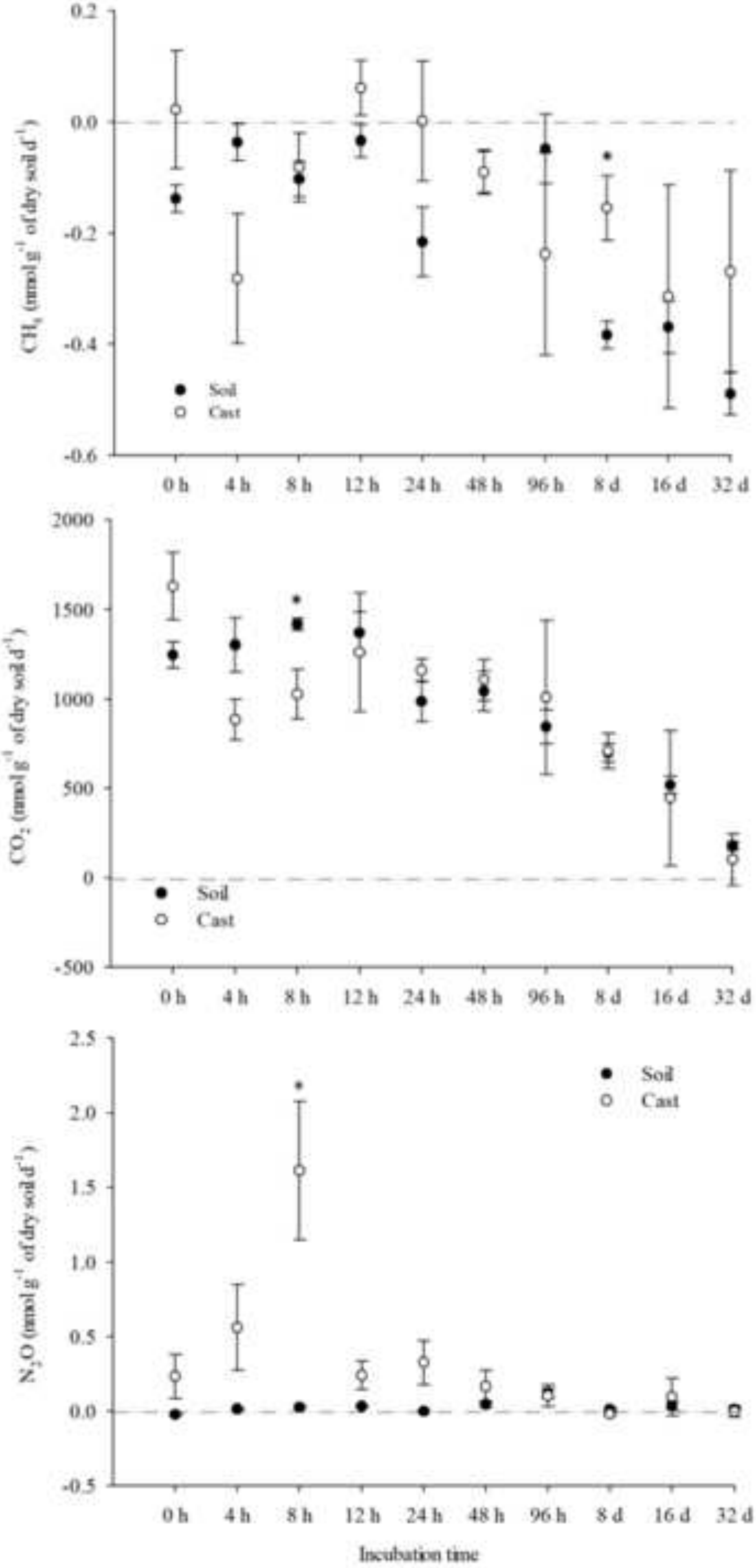

A
Exchangeable N-NH$_4$ (mg kg^{-1})
240
220
200
180
160
140
120
100
30
25
20
15
10
5
0
● Soil
○ Cast
*
0 h
4 h
8 h
12 h
24 h
48 h
96 h
8 d
16 d
32 d
B
N-NO$_3^-$ (mg kg^{-1})
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
● Soil
○ Cast
*
0 h
4 h
8 h
12 h
24 h
48 h
96 h
8 d
16 d
32 d
Incubation time

Figure 6



0.2
0.0
-0.2
-0.4
-0.6
CH4 (nmol g-1 of dry soil d-1)
Soil
Cast
*
0 h
4 h
8 h
12 h
24 h
48 h
96 h
8 d
16 d
32 d
2000
1500
1000
500
0
-500
CO2 (nmol g-1 of dry soil d-1)
Soil
Cast
*
0 h
4 h
8 h
12 h
24 h
48 h
96 h
8 d
16 d
32 d
2.5
2.0
1.5
1.0
0.5
0.0
-0.5
N2O (nmol g-1 of dry soil d-1)
Soil
Cast
*
0 h
4 h
8 h
12 h
24 h
48 h
96 h
8 d
16 d
32 d
Incubation time

Table 1. Results (means ± standard errors) of soil chemical and physical properties of control soil and casts of *Rhinodrilus alatus*. CEC = effective cation exchange capacity, C_{org} = organic carbon, V = base saturation, m = Al saturation. Statistically significant differences ($p<0.05$) are shown in bold for the significantly higher values.

Parameter	Control soil		*R. alatus* casts	
pH ($CaCl_2$)	3.95	± 0.01	**4.03**	**± 0.00**
		cmol$_c$ dm^{-3}		
H+Al	9.28	± 0.17	**10.50**	**± 0.00**
Al	1.92	± 0.05	2.05	± 0.24
Ca	0.76	± 0.02	0.86	± 0.12
Mg	0.40	± 0.00	0.52	± 0.10
K	0.07	± 0.00	0.07	± 0.00
Na	0.00	± 0.00	0.00	± 0.00
Sum of bases	1.23	± 0.02	1.42	± 0.22
P	2.66	± 0.09	2.86	± 0.15
CEC	3.15	± 0.07	3.47	± 0.45
		mg dm^{-3}		
Mn	2.94	± 0.31	**5.20**	**± 0.25**
Zn	0.78	± 0.07	0.86	± 0.02
Fe	93.96	± 3.07	**149.60**	**± 5.66**
Cu	1.36	± 0.02	**1.60**	**± 0.05**
C_{org}	17.42	± 0.58	**20.42**	**± 1.00**
		%		
Sand	3.47	± 0.14	3.47	± 0.08
Silt	45.56	± 0.90	45.18	± 0.24
Clay	50.96	± 0.81	51.44	± 0.28
V	11.7	± 0.3	11.8	± 1.4
m	60.9	± 0.2	59.5	± 0.6
		g kg^{-1}		
TC	21.28	± 0.24	**23.26**	**± 0.45**
TN	1.64	± 0.02	**1.98**	**± 0.06**
H	9.23	± 0.19	**10.18**	**± 0.08**
C/N	10.62	± 0.26	10.34	± 0.50

Online Supplementary Tables 1 to 8