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Changes in nutrient pools, microbial biomass and microbial activity in soils after transit through the gut of three endogeic earthworm species of the genus *Postandrilus* Qui and Bouché, 1998

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Abstract
Purpose Endogeic earthworms play a significant role in biogeochemical cycles due to the large amount of soil they ingest, and because after transit through their guts, casts usually show differences in nutrient contents and microbial populations with bulk soil. Here, we studied how three endogeic earthworm species, *Postandrilus majorcanus*, *Postandrilus sapkarevi* and *Postandrilus palmensis*, inhabiting soils in Majorca island (Balearic Islands, W Mediterranean), modify nutrient pools and microbial communities of soil.
Materials and methods To do this, we analysed C, N and P pools, microbial biomass (phospholipid fatty acids, PLFA) and microbial activity (fluorescein diacetate hydrolysis, FDA) in paired samples of bulk soil and fresh casts.
Results and discussion The mineral and organic N contents were generally enhanced in casts produced by all three earthworm species. However, inorganic P and organic C contents were only higher in *P. sapkarevi* (32 %, only P) and *P. majorcanus* casts (100 % for both soil nutrient pools) than in bulk soil. Bacterial and fungal biomass were only higher than in bulk soil in *P. majorcanus* casts (65 and 100 %, respectively), but without effects on microbial activity, that was lower in *P. palmensis* casts (26 %). Earthworm gut transit strongly influenced the soil microbial community structure, resulting in differences between casts and soils.
Conclusions The increased nutrient mineralization (6-, 1.3- and 1.4-fold for N, C and P, respectively) in casts produced by these earthworm species is of particular importance because of the amount of casts released and the seasonal variations in earthworm activity, which may favour plant growth.

Keywords Casting · Endogeic earthworms · FDA · Microbial biomass · PLFA · *Postandrilus*

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1 Introduction

Earthworms represent the largest animal biomass component of soil in most temperate terrestrial ecosystems (Edwards 2004). By ingesting soil, earthworms significantly enhance the decomposition of soil organic matter and modify microbial biomass and activity (Lavelle and Spain 2001; Edwards 2004). More importantly, most of these effects occur directly or indirectly through earthworm casting and cast ageing processes (Aira et al. 2003, 2005, 2010). Earthworm casts are nutrient-enriched faecal structures in which microbial populations can flourish or decline. These effects on microorganisms depend on both the microorganism studied and earthworm species and diet (Egert et al. 2004; Horn et al. 2003; Knapp et al. 2009; Singleton et al. 2003). In temperate zones, cast production ranges between 36 and 108 Mg Ha^{-1} $year^{-1}$ (Lavelle and Spain 2001) and is therefore a key process in biogeochemical cycles. This may also be relevant to plant growth due to high seasonal variations in earthworm casting, which is mainly restricted to late winter–early spring in the Mediterranean region, when high levels of soil moisture trigger earthworm activity.

Endogeic earthworm species live in nonpermanent horizontal burrows in the upper soil layer, ingest large amounts of soil (Edwards 2004) and selectively forage organic-matter-rich soil (Marhan and Scheu 2006). The genus *Postandrilus*, described by Qiu and Bouche (1998), is restricted to the

Balearic Islands (W Mediterranean Sea). This earthworm genus, which inhabit agricultural and forest soils, comprises five species of sizes ranging between 120 mm (*Postandrilus palmensis*) and 500 mm (*Postandrilus majorcanus*); the other three species are *Postandrilus medoakus*, *Postandrilus lavellei* and *Postandrilus sapkarevi*. The main objective of this study was to monitor the short-time changes in soil chemical and microbiological properties due to earthworm activity, i.e. after passage through the gut of three species of the genus *Postandrilus* (*P. palmensis*, *P. majorcanus* and *P. sapkarevi*), by studying the differences between fresh casts and the surrounding bulk soil. We monitored the changes in available pools of C, N and P; overall microbial biomass; bacterial and fungal biomass and microbial activity.

2 Material and methods

2.1 Site description and experimental design

Casts and soils were sampled in Majorca (Balearic Islands, W Mediterranean Sea) in early March. The climate of the island is Mediterranean, with mean rainfall of 26 mm and mean temperature around 13 °C in this month for the last 30 years. January and February has slightly lower temperatures (~12 °C) and higher precipitations (~38 mm). Majorcan soils are Haplic Calcisols (Soil Atlas of Europe 2005). We selected three locations on the island with well-established monospecific populations of the earthworm species *P. palmensis*, *P. sapkarevi* and *P. majorcanus* (Pérez-Losada et al. 2011). The sampling sites for casts produced by *P. palmensis* and *P. sapkarevi* had similar dominant vegetation, which was composed by carob trees (*Ceratonia siliqua*) and holm oaks (*Quercus ilex*), whereas the sampling site for *P. majorcanus* casts was dominated by holm oaks. In all these sites, the earthworms produce massive amounts of casts at the soil surface. To ensure that casts were produced by only one of each earthworm species, we sampled earthworms by using the formalin extraction method (area 0.5 m^2, $n=5$; Raw 1959) in areas were cast density was high. In all three sites, we found only one of each of the three *Postandrilus* species. We sampled the surrounding (bulk) soil at a distance of no more than 20 cm from the earthworm casts. We chose fresh casts, i.e. those that were not there the day before. Casts and soils were sampled the last day of a sampling trip and conserved at ambient temperature (15 °C) because it was shown that temperature did not influence microbial community structure whenever storage time is no longer than 14 days (Lauber et al. 2010). Casts and the bulk soil samples ($n=5$ for each earthworm species) were sieved (2 mm) prior to analysis.

2.2 Analytical methods

The organic matter content was determined as weight loss after heating at 550 °C for 4 h. The pH was recorded from a suspension of the samples in distilled water 1:20 (fresh weight/volume) sample to extractant with a CRISON pH BASIC 20 (Aira et al. 2003). Inorganic N forms ($N\text{-}NH_4^+$ and $N\text{-}NO_3^-$) were determined in 0.5 M K_2SO_4 extracts (1:5 weight/volume) using a modified indophenol blue technique (Sims et al. 1995) with a Bio-Rad Microplate Reader 550. Total Extractable N was determined in 0.5 M K_2SO_4 extracts, after oxidation with $K_2S_2O_8$, as described by Cabrera and Beare (1993), and the dissolved organic nitrogen (DON) content was calculated as (total Extractable N)−($N\text{-}NH_4^+$+$N\text{-}NO_3^-$). Dissolved organic C (DOC) was determined colorimetrically after moist digestion ($K_2Cr_2O_7$ and H_2SO_4) of aliquots of 0.5 M K_2SO_4 extracts of the samples. Phosphate was extracted from soil samples (2 g dw) with acetic acid (2.5 %), filtered and read at 700 nm after the addition of ammonium molibdate (0.1 M) and tin chloride (Allen et al. 1986).

Microbial communities were analysed by phospholipid fatty acid (PLFA) analysis (Gómez-Brandón et al. 2010). Total lipids were extracted from 2 g (dry weight) of soil samples with 60 ml of chloroform–methanol, 2:1 (*v*/*v*). The mixture was then filtered and evaporated under a stream of N_2 gas. The total lipid extract was then dissolved in chloroform (3×1 ml). Lipids were separated on silicic acid columns (Strata SI-1 Silica) (55 μm, 70 Å; 500 mg/6 ml) into neutral, glycol- and phospholipids, with chloroform, acetone and methanol, respectively. The fraction containing phospholipids was evaporated under a N_2 stream and redissolved in 500 μl of methyl-tert-butyl ether. One hundred microliters of this solution were placed in a 1.5-ml vial with 50 μl of the derivatizing agent (trimethylsulfonium hydroxide, TMSH), and the mixture was then vortexed for 30 s and allowed to react for 30 min; 10 μl of nonadecanoic acid methyl ester was then added as an internal standard. The chromatographic conditions are described elsewhere (Gómez-Brandón et al. 2010). The fatty acids were identified and quantified by comparing the retention times and mass spectra with those obtained for known standard mixtures or pure PLFAs.

The PLFAs used as biomarkers are described by Frostegård and Bååth (1996) and Bååth (2003). Total live microbial biomass was determined as the sum of all extracted PLFAs expressed as microgram per gram dry weight. Relative abundances of bacteria were determined by the abundance of specific biomarkers commonly used for this group. The sum of PLFAs considered to be predominantly of bacterial origin were further classified as Gram-positive bacterial (G+) PLFAs (i14:0, i15:0, a15:0, i16:0, i17:0 and a17:0) and Gram-negative bacterial (G−) PLFAs (16:1ω7c, cy17:0, 17:1ω8, 18:1ω7c and cy19:0) (Frostegård and Bååth 1996). Fungi were determined using c18:1ω9c and c18:2ω6c PLFAs (F) (Bååth 2003; Frostegård and Bååth 1996).

Microbial activity was determined with the fluorescein diacetate (FDA) hydrolysis, as proposed by Adam and Duncan (2001). Briefly, 2 g of soil was incubated 20 min at 30 °C with 15 ml of 60 mM potassium phosphate and 0.5 ml of a stock solution (0.2 ml 1000 μg of FDA ml^{-1}). To stop the reaction, 15 ml of chloroform–methanol (2:1 *v*/*v*) was added, and the mixture was gently mixed and then centrifuged (425 *g*, 3 min). The supernatant was filtered (Whatman No2) and read at 490 nm.

2.3 Statistical analysis

Means of casts and surrounding soil of three earthworm species were separated using paired *t* test, with the *t* test function of R environment, with previous assessment of normality (Shapiro-Wilk test, Shapiro.test function) and homogeneity of variance (Bartlett's test, bartlett.test function) (R Development Core Team 2007). Data from the PLFA analysis were also subjected to discriminant analysis with the discrimin function of the ade4 library (Dray and Dufour 2007).

3 Results

Organic matter content was higher in *P. majorcanus* cast than in the bulk soil, whereas it was lower in *P. palmensis* casts than in the bulk soil (Table 1); *P. palmensis* and *P. sapkarevi* casts were significantly more acidic than in the surrounding soil (Table 1). Cast produced by all three *Postandrilus* species were enriched in $N\text{-}NH_4^+$, with an eighteen-fold increase in the *P. sapkarevi* casts, a nine-fold increase in the *P. majorcanus* casts and a four-fold increase in the *P. palmensis* casts, relative to the respective bulk soil (Table 1). Nitrate contents were significantly higher (two times higher) in *P. palmensis* and *P. sapkarevi* casts than in the surrounding soil, in direct contrast to the difference between the *P. majorcanus* cast and the surrounding soil (Table 1). The dissolved organic nitrogen contents were enhanced five-fold (in *P. palmensis* and *P. sapkarevi* casts) and two-fold (in *P. majorcanus* casts), relative to the bulk soil (Table 1). Casts produced by *P. majorcanus* contained twice as much dissolved organic carbon than the surrounding soil, with no differences in casts produced by the other two earthworm species (Table 1). The phosphate content was slightly enhanced in *P. sapkarevi* casts, and was increased by two-fold in *P. majorcanus* casts, relative to the bulk soil (Table 1).

Casts produced by *P. majorcanus* contained 1.6 times more microbial biomass than the surrounding soil, and there were no differences between the bulk soil and the casts produced by the other two earthworm species (Table 1). More specifically, the fungal biomass doubled and microbial biomass increased by 1.6 times (Table 1). Discriminant analysis of the 22 identified PLFAs revealed large differences among the microbial communities of the samples, which explained 46 % of the data variance (Fig. 1). Samples were significantly discriminated into six groups according to their precedence (Monte Carlo test based on 999 simulations, *P* value=0.006). The largest differences between bulk soil and casts corresponded to those produced by *P. majorcanus* and *P. palmensis*. They were due to increases in bacterial PLFAs positively related to root 1 (*P. majorcanus*) and negatively related (*P. palmensis*) to root 2 (see table in Fig. 1). Microbial activity was 1.3 times lower in *P. palmensis* casts than in the bulk soil but was unchanged in *P. sapkarevi* and *P. majorcanus* casts (Table 1).

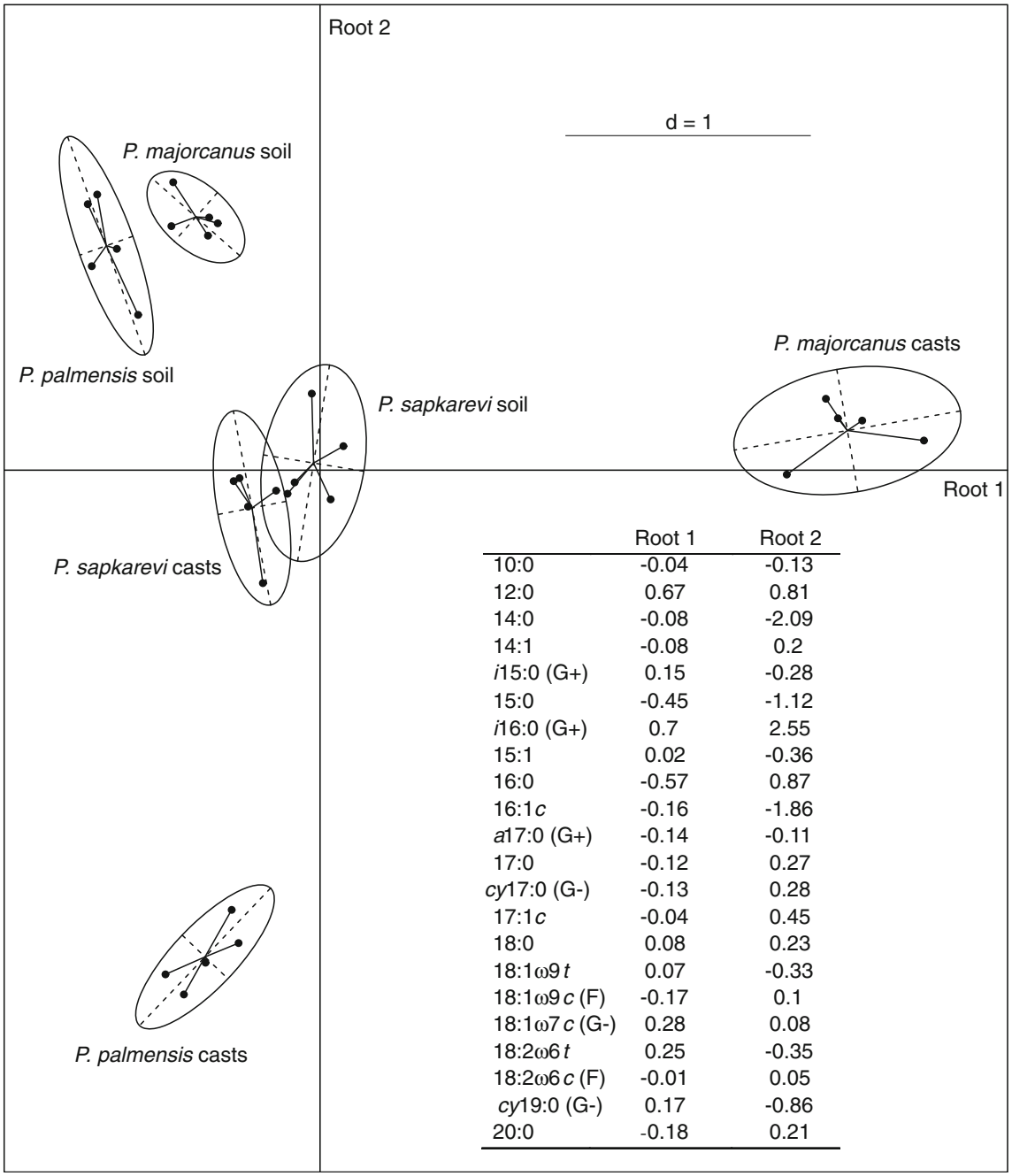
Table 1 Chemical and biochemical characteristics of the bulk soil and casts produced by three earthworm species of the genus *Postandrilus*

	Postandrilus palmensis		*Postandrilus sapkarevi*		*Postandrilus majorcanus*	
	Soil	Cast	Soil	Cast	Soil	Cast
Organic matter (%)	23.9±0.3	20.9±1.2*	24.4±0.3	25.9±0.8	11.1±0.3	19.7±2.6*
pH	6.2±0.3	4.1±0.1***	5.7±0.2	4.4±0.1*	5.6±0.1	5.5±0.3
$N\text{-}NH_4^+$ (μg g^{-1} dw)	2.6±0.5	12.2±0.6***	0.7±0.3	12.9±0.3***	0.7±0.1	6.3±0.5**
$N\text{-}NO_3^-$ (μg g^{-1} dw)	7.5±0.6	16.9±1.5**	6.6±1.1	12.7±1.4*	7.3±1.1	3.8±0.9*
DON (μg g^{-1} dw)	6.9±1.6	35.4±5.3**	7.8±3.3	38.9±10.2*	9.4±2.1	21.2±4.3*
DOC (mg g^{-1} dw)	0.67±0.12	0.53±0.05	0.75±0.07	0.67±0.11	0.5±0.1	1.2±0.2*
$P\text{-}PO_4^+$ (μg g^{-1} dw)	21.1±0.9	23.4±1.3	22.3±2.9	29.4±2.4*	11.4±1.7	25.9±2.7*
FDA (μg g^{-1} dw)	95.8±3.7	75.6±5.4*	61.5±6.5	70.2±7.2	79.4±10.6	135.9±10.5
Total PLFAs (μg g^{-1} dw)	19.1±1.2	28.4±3.7	24.9±1.7	20.9±2.6	18.1±0.6	29.2±0.9***
Bacterial PLFAs (μg g^{-1} dw)	16.6±0.9	24.1±3.1	22.3±1.5	17.9±2.5	15.9±0.5	26.3±0.8***
Fungal PLFAs (μg g^{-1} dw)	0.7±0.1	0.5±0.1	0.6±0.1	0.5±0.1	0.4±0.1	0.8±0.1***

Mean values and standard error (n=5)

DON dissolved organic nitrogen, *DOC* dissolved organic carbon, *FDA* fluorescein diacetate hydrolysis

*P<0.05; **P<0.01; ***P<0.001 paired *t* test



	Root 1	Root 2
10:0	-0.04	-0.13
12:0	0.67	0.81
14:0	-0.08	-2.09
14:1	-0.08	0.2
*i*15:0 (G+)	0.15	-0.28
15:0	-0.45	-1.12
*i*16:0 (G+)	0.7	2.55
15:1	0.02	-0.36
16:0	-0.57	0.87
16:1*c*	-0.16	-1.86
*a*17:0 (G+)	-0.14	-0.11
17:0	-0.12	0.27
*cy*17:0 (G-)	-0.13	0.28
17:1*c*	-0.04	0.45
18:0	0.08	0.23
18:1ω9*t*	0.07	-0.33
18:1ω9*c* (F)	-0.17	0.1
18:1ω7*c* (G-)	0.28	0.08
18:2ω6*t*	0.25	-0.35
18:2ω6*c* (F)	-0.01	0.05
*cy*19:0 (G-)	0.17	-0.86
20:0	-0.18	0.21

Fig. 1 Discriminant function analysis of 22 PLFAs of bacterial and fungal origin, from samples of casts of *Postandrilus majorcanus* (cPm), *P. sapkarevi* (cPs), *P. palmensis* (cPp) and from the corresponding surrounding soils (sPm, sPs and sPp), which are labelled inside their 67 % inertia ellipses. The *five points inside each inertia ellipse* are the five replicates per treatment (*d* is the scale of the root 1 and root 2 axes). The values of canonical correlations of each PLFA used in the analysis with both roots are shown. Gram-positive bacterial (*G*+), Gram-negative bacterial (*G*−) and fungi PLFAs (F) are indicate in the inset table. *Root 1* represents 23 % of the variance and *Root 2* represents 23 % of the variance

4 Discussion

We found that the soil chemical and microbiological properties were modified in very different ways by the three earthworm species. Thus, nutrient pools were enhanced after passage of the soil through the guts of all the three earthworm species. However, the microbial populations were only modified by *P. majorcanus* (i.e. enriched bacterial and fungal biomass in casts). Moreover, earthworm gut transit strongly shaped soil microbial community structure, resulting in differences between casts and the surrounding soil and between casts produced by all three earthworm species.

The concentrations of NH_4^+-N and NO_3^--N (except for *P. majorcanus* casts) were higher in casts produced by all three

earthworm species than those in the surrounding soil, as usually found in other endogeic species such as *Aporrectodea caliginosa*, *Aporrectodea molleri* and *Pontoscolex corethurus* (Aira et al. 2003; Chapuis-Lardy et al. 2010; Parkin and Berry 1994) inhabiting very different types of soil. The dissolved organic nitrogen (DON) content was also enhanced in the earthworm casts, although the differences were greater for *P. palmensis* and *P. sapkarevi* casts than those for *P. majorcanus* casts. These data contrast with the decreased DON observed in casts of *A. caliginosa* and *A. molleri* (Aira et al. 2003), which were attributed to digestion of the N by earthworms (Jensen 1997). However, the present data suggest that mucus excreted by earthworms may account for high levels of DON (Brown et al. 2000) at least in dry soils such as the Majorcan soils, where mucus secretion should ameliorate low moisture of soils. The increased P contents in *P. sapkarevi* and *P. majorcanus* casts correspond with those reported for casts of endogeic earthworms *Pontoscolex corethurus*, *Octolasion cyaneum* and *Octolasion tyrtaeum* (Bisht et al. 2006; Buck et al. 1999; Chapuis-Lardy et al. 2009). However, no changes in P pools were found in casts produced by other endogeic species such as *P. corethurus*, *A. caliginosa* and *A. molleri* (Aira et al. 2003; Chapuis-Lardy et al. 1998; Le Bayon and Binet 2006). Thus, despite the general trend of producing nutrient-enriched casts, it seems that modifications of soil nutrient pools will be the outcome of interactions among earthworm digestive capabilities and the composition of soil and gut microbiota.

Microbial biomass and activity are usually enhanced in casts (Tiunov and Scheu 2000a; Zhang and Hendrix 1995). However, we found that the total microbial biomass and the bacterial and fungal biomass were only significantly enhanced in *P. majorcanus* casts. This finding is similar to those obtained for casts of *A. caliginosa* (Aira et al. 2003, 2005, 2010) feeding in a different soil. Earthworms digest fungi (reviewed in Brown and Doube 2004) and modify fungal diversity in the soil (Tiunov and Scheu 2000b), although higher fungal biomass has been observed in casts than in the surrounding soil (Tiunov and Scheu 2000a)—as we also found in *P. majorcanus* casts. The lack of changes in the microbiological parameters in casts produced by the other two species may be due to the formation of stable aggregates of organic matter in the casts, which is common in endogeic earthworm species (Lavelle and Spain 2001). This implies the retention and gradual release of nutrients to the microorganisms (Edwards 2004), which are thus able to remain active for longer than in the undigested soil, thus creating hotspots of microbial activity. However, we did not find any such increase in microbial activity, which even decreased significantly in *P. palmensis* casts. This may be explained by the differences in nutrient content of casts and bulk soil. The enhancement of microbial biomass after passage through the gut of *P. majorcanus* may be attributed to increased N, C and P contents in the casts produced by this species. According to this, the decreased microbial activity in casts of *P. palmensis* may be due to the lack of differences in C and P pools, which could have limited microbial growth (Tate 2000). We found clear differences in microbial community structure between casts and soil samples. Interestingly, the huge dissimilarity between microbial communities of *P. palmensis* and *P. majorcanus* casts, which came from relatively similar soil microbial communities, resulted from diverging patterns of PLFA variation. Curiously, microbial communities of *P. sapkarevi* casts resembled more to those of bulk soil, even though changes in nutrient pools between casts and soil were comparable to the other two earthworm species. This suggests that microbial resistance to digestion and earthworm digestive capabilities, as well as the composition of gut microorganisms, could be more responsible than changes on soil nutrient content occurred during gut transit in the modifications on soil microbial community structure. Earthworms largely impact the microbial community structure, and there is a growing evidence that this effect depends on earthworm species and type of soil (Aira et al. 2010; Domínguez et al. 2010; Egert et al. 2004; Knapp et al. 2009; Thakuria et al. 2009; Tiunov and Scheu 2000a). However, here we cannot separate these two factors because the three earthworms live in different areas that probably harbour different microbial communities (Fig. 1).

5 Conclusions

In summary, our data show that soil nutrient mineralization is enhanced and soil microbial community structure is altered after passage of the soil through the guts of earthworms of the genus *Postandrilus*. This is of key importance because of the massive amount of casts released by these earthworm species and the seasonal variation in their feeding activities, which are restricted during most of the year by temperature and soil moisture content. Soil nutrients are mobilised at the same time as germination of plant seeds, thus favouring plant growth.

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