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Detritivorous earthworms modify microbial community structure and accelerate plant residue decomposition

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ABSTRACT

The impact of detritivorous earthworms (*Eisenia andrei*) on microbial community structure and function in grape marc, a lignocellulosic enriched plant residue, was investigated in a mesocosm experiment. Analysis of carbon and nitrogen pools was also carried out in order to evaluate how changes in microbial communities affect plant residue decomposition. The grape marc was completely processed after fifteen days as a result of the high density of earthworms present and the rapid gut transit time. *Eisenia andrei* had a large impact on the structure of the microbial community, as revealed by phospholipid fatty acid analysis. Earthworm activity reduced the abundance of both bacterial (except for Gram-negative bacteria) and fungal PLFA biomarkers relative to the control values. Decreases in microbial activity and in protease and cellulase activities were also attributable to the presence of earthworms. Moreover, earthworms strongly modified the substrate utilization patterns of microbial communities, as revealed by BIOLOG analysis. The presence of earthworms led to an increase in the utilization of some amino acids and polymers, which reached a higher substrate diversity value than that in the control mesocosm. The differences in microbial communities were accompanied by a reduction in the total C content and the labile C pool, relative to the control, although there were no significant differences in either cellulose or hemicellulose contents. However, total N content increased in both mesocosms – with and without earthworms – and the concentration of NH_4^+ was also enhanced by earthworm activity. The results indicate that detritivorous earthworms play a key role in decomposing fresh plant residues in the short term via their intensive interactions with microbial communities.

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1. Introduction

In most terrestrial ecosystems the above-ground net primary production, around 80–90%, enters the soil food web as dead plant material (Bardgett, 2005). Bacteria and fungi, which form up to 90% of the soil microbial biomass, are the primary litter decomposers (Berg and Laskowski, 2005), but their activity is strongly affected by the soil fauna that live alongside them (Cragg and Bardgett, 2001; Wardle, 2006).

Earthworms represent the major soil animal biomass in most terrestrial temperate ecosystems (Edwards and Bohlen, 1996). They are known to play a key role in plant material decomposition thus increasing the rate of disappearance of organic matter (Suárez et al., 2006; Milcu et al., 2008; Ernst et al., 2009) and modifying microbial biomass and activity (Seeber et al., 2006; Butenschoen et al., 2007). Earthworms are involved in the indirect stimulation of microbial populations through comminution of organic matter, which results in a greater surface area available for microbial colonization and further decomposition (Seeber et al., 2008). They also modify microbial biomass and activity through digestion, stimulation and dispersion in casts (Domínguez, 2004). Moreover earthworm casts contain nutrient and microbial populations different from those contained in the material prior to ingestion (Furlong et al., 2002; Haynes et al., 2003; Singleton et al., 2003; Knapp et al., 2009), which could enable a better exploitation of resources either because of the appearance of microbial species in the fresh substrate or the pool of readily assimilable compounds in the casts (Brown and Doube, 2004).

Studies investigating the effects of earthworms on microorganisms are required, particularly with epigeic earthworm species, for a better understanding of how the interactions between detritivore earthworms and microorganisms affect plant residue decomposition. Moreover, most of the current knowledge regarding interactions between earthworms and microorganisms relates to soil-dwelling endogeic and anecic species. Epigeic earthworms are litter dwellers and litter transformers; they live in organic

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horizons, in or near the surface litter, where they feed primarily on coarse particulate organic matter, ingest large amounts of undecomposed litter and excrete holorganic faecal pellets (Lavelle and Spain, 2001). They interact closely with other biological components of the soil system, and in turn can affect the structure and function of microfauna and microflora (McLean and Parkinson, 1998, 2000a,b; Lores et al., 2006; Aira et al., 2008; Monroy et al., 2008). In the present study we investigated the short-term effects of the epigeic earthworm species *Eisenia andrei* on the microbial community structure and function of a plant residue, in comparison with those in a control treatment. We investigated the impact of this earthworm species on the abundance of different microbial groups, its influence on total microbial activity and on the activity of enzymes involved in C and N cycles after fifteen days, and also how the action of this earthworm species affected the community-level physiological profiles. Moreover, we evaluated how such changes in microbial community structure and function affected plant residue decomposition by analysing carbon and nitrogen pools. We monitored the changes in labile carbon pool (dissolved organic carbon) and other more slowly decomposable compounds (cellulose, hemicellulose and lignin). We also determined the changes in the concentration of total N and inorganic N (NH_4^+ and NO_3^-).

There is recent evidence in the literature suggesting that the digestion of the organic material by epigeic earthworms has negative effects on microbial biomass (revised in Domínguez et al., 2009). The activity of these detritivorous earthworms can reduce microbial biomass directly by selective feeding on bacteria and fungi, or indirectly by accelerating the depletion of resources for the microbes (Domínguez, 2004). We hypothesized that this might affect the abundance of the different microbial groups, and result in a reduced total microbial activity, and in lower enzyme activities in comparison with the control (no earthworms) due to the minor substrate availability as a result of the acceleration of the depletion of resources for the microbes. We also hypothesized that these changes in microbial biomass and activity will result in concurrent changes in the potential ability of the microbial community to utilize certain carbon sources.

2. Materials and methods

2.1. Substrate and experimental design

We chose grape marc as the substrate. Grape marc is a lignocellulosic enriched residue that consists of the stalks, skin, pulp and seeds remaining after the grape crushing and pressing stages in wine production (Flavel et al., 2005). This substrate was chosen to study the changes produced by the earthworm activity in a wide range of recalcitrance of carbon compounds to decomposition. The grape marc was obtained from a vineyard in Pontevedra (Galicia, NW Spain), homogenized, stored at 5 °C until use, and was turned (for aeration) and moistened with water during the two days prior to the experiment.

The impact of detritivorous earthworms on plant residue decomposition was determined in a mesocosm experiment with the epigeic earthworm species *E. andrei*, which is widely distributed and easy to manage under laboratory conditions. Specimens of *E. andrei* were collected from a stock maintained in the laboratory for one month, during which grape marc was provided as a food source. Plastic containers (2 L) were filled to three quarters of the capacity with moistened (80% moisture content) and mature vermicompost in order to ensure survival of the earthworms. Five hundred juvenile and adult specimens of the epigeic earthworm species *E. andrei* (220 ± 14 g fresh weight per container) were placed on the surface of the vermicompost. One kilogram (fresh weight) of grape marc was placed on a mesh (5 mm pore size) on the surface of the vermicompost and was rewetted by spraying with 20 mL of tap water. The use of plastic mesh avoids mixing the grape marc and the vermicompost bedding and also facilitates the removal of grape marc after being processed by the earthworms. The containers were covered with perforated lids, and placed in an incubation chamber at 20 °C and 90% relative humidity. We also included a control treatment that consisted of the grape marc incubated without earthworms. Each treatment was replicated five times. The time taken by earthworms to degrade a residue depends on the species and density of earthworms, and the rates at which they ingest and process the residue (Domínguez, 2004). The high density of earthworms used and the relatively rapid gut transit time of the epigeic earthworm species *E. andrei*, around 2.5–7 h (Hartenstein et al., 1981), resulted in the plant residue being completely processed by the earthworms in fifteen days. After this time the remaining material that is left after having been processed by the earthworms was collected from the surface of the vermicompost, and the biomass of earthworms was determined (233 ± 12 g fw per container). A sample from the control containers (ca. 200 g fresh weight) was also collected for analysis at the end of the incubation period.

Samples were sieved (<5 mm) in order to remove the stalks and seeds, and several parameters were determined, as detailed below.

2.2. Microbial community structure

Microbial community structure was assessed by phospholipid fatty acid (PLFA) analysis. Briefly, the total lipidic extract was obtained from 200 mg of each freeze-dried sample with 60 mL of chloroform–methanol (2:1, v/v), following the method described by Folch et al. (1957) and modified for highly organic samples by Gómez-Brandón et al. (2008). The lipid extract was then fractionated into neutral lipids, glycolipids and phospholipids with chloroform (5 mL), acetone (10 mL) and methanol (5 mL), on silicic acid columns (Strata SI-1 Silica (55 μm, 70 Å), 500 mg/6 mL). The fraction containing phospholipids was subjected to alkaline methanolysis (White and Ringelberg, 1998) to obtain the fatty acid methyl esters (FAMEs), and analysed by gas chromatography–mass spectrometry (GC–MS). The detailed GC-MS experimental conditions have been described by the authors elsewhere (Gómez-Brandón et al., 2008). In order to identify the FAMEs, the retention times and the mass spectra were compared with those obtained from the standards. FAMEs were quantified by an internal standard calibration procedure (see Gómez-Brandón et al., 2008). The calibration levels of the FAMEs varied in the range 0.4–250 $\mu g\ mL^{-1}$. The coefficients of determination (R^2) were higher than 0.99 for all calibration curves. FAMEs were described by the standard ω-nomenclature A:BωC (IUPAC-IUB, 1977).

The sum of all identified PLFAs (total PLFAs) was used to estimate the viable microbial biomass (Zelles, 1999). Certain PLFAs were used as biomarkers to determine the presence and abundance of specific microbial groups (Frostegård and Bååth, 1996; Zelles, 1997; Joergensen and Wichern, 2008). The sum of PLFAs characteristic of Gram-positive (i15:0, a15:0, i16:0 and a17:0), and Gram-negative bacteria (16:1ω7c, 17:1ω7c, cy17:0 and cy19:0), and actinobacteria (10Me18:0) was chosen to represent bacterial PLFAs, and the sum of PLFAs 18:1ω9c and 18:2ω6c to indicate fungal PLFAs. The fungal to bacterial (F/B) PLFA ratio was also calculated.

2.3. Microbial activity

Total microbial activity was assessed as basal respiration, by measuring the rate of evolution of CO_2 (Anderson, 1982), as modified by Aira et al. (2007a) for solid organic samples. Protease activity was measured by determining the amino acids released, after incubating the samples (1 g fresh weight) with sodium caseinate (2%) for 2 h at 50 °C, with Folin-Ciocalteu reagent, in a Microplate Reader at 700 nm (Ladd and Butler, 1972). Cellulase

activity was estimated by determining the reducing sugars released after incubating the samples (5 g fresh weight) with carboxymethyl cellulose sodium salt (0.7%) for 24 h at 50 °C, in a Microplate Reader at 690 nm (Schinner and von Mersi, 1990).

2.4. Community-level physiological profiles

The potential ability of the microbial community to utilize select carbon sources was assessed by determining the community-level physiological profiles (CLPPs) with the Biolog® Ecoplate microplate identification system (BIOLOG Inc., Hayward, CA, USA), which consists of thirty different sole carbon sources plus a non-C control contained in 96-well microtiter plates (Hitzl et al., 1997; Insam, 1997). Briefly, the plates were incubated at 20 °C, and the optical density was read in a Microplate Reader at 595 nm after incubation for 24, 48, 96 and 120 h. The 48 h absorbance data were used for the analysis because this was the time necessary for the microbial growth and colour development, and at this time a positive response for microorganisms was observed in 75% or more of the wells (Ibekwe and Kennedy, 1998). The substrate diversity calculated by the Shannon's diversity (H') index was estimated after an incubation time of 48 h.

2.5. Chemical analyses

Electrical conductivity (EC) and pH were measured in aqueous extracts (1:10, w/v). Total C and N contents were analyzed in a Carlo Erba 1500 C/N analyzer with dried samples. Dissolved organic carbon (DOC) was determined colorimetrically in microplates after moist digestion ($K_2Cr_2O_7$ and H_2SO_4) of aliquots of 0.5 M K_2SO_4 extracts. Inorganic nitrogen (NH_4^+ and NO_3^-) was determined in 2N KCl extracts by acid–base titration with 0.01N HCl, in a Büchi distillation unit. Cellulose, hemicellulose and lignin contents were determined by use of the FibreBag System® (Gerhardt, Königswinter, Germany) according to the method of Goering and Van Soest (1970).

2.6. Statistical analysis

One-way analysis of variance (ANOVA) and comparison of means based on Tukey's test were used to determine significant differences between the samples. We included the initial grape marc in the statistical analysis to evaluate the changes in its microbiological and chemical properties after incubation for fifteen days with and without earthworms. A discriminant function analysis was also used to analyze the PLFA and BIOLOG data to assess overall differences in the microbial community structure and CLPP profiles of grape marc derived from the earthworm activity. The standard statistic Wilks' lambda was used to denote the statistical significance of the discriminatory power of this analysis, with values ranging from 1.0 to 0.0 (the closer the values to 0.0 the higher the discriminatory power). This analysis also enabled us to determine which PLFAs and C sources are primarily responsible for these overall differences by calculating their discriminant scores with the canonical discriminant functions. The normality and the variance homogeneity of the data were tested prior to ANOVA and discriminant analyses. All statistical tests were evaluated at the 95% confidence level. Statistical analysis of the data was carried out with the SPSS 14.0 software program.

3. Results

3.1. Microbial community structure

The discriminant analysis of the PLFA data clearly distinguished between the initial grape marc, the grape marc processed by earthworms and the control without earthworms (Fig. 1a, Wilk's $\lambda = 0.0001$, $P = 0.002$). The discriminant functions associated with the first and second eigenvalues, DF1 and DF2, accounted for 82% and 18% of the variance for a total explained variance of 100% (Fig. 1a). Twelve PLFAs were selected by the analysis as the most important in terms of the overall changes in the microbial community structure. Discriminant scores of each PLFA with DF1 and DF2 are plotted in Fig. 1b. The first discriminant function largely separated the initial grape marc from the earthworm treatment, which shifted negatively along this function. Thus earthworm activity led to a decrease in the abundance of the PLFA 15:1ω5c and the G− bacterial and fungal biomarkers 17:1ω7c and 18:1ω9c, which were strongly associated with the positive side of DF1, and to an increase in the abundance of PLFAs 17:0 and 14:1ω5c, negatively associated with this function, as shown in Table 1. Other PLFAs such as 12:0, 14:0, 18:0 and the fungal biomarker 18:2ω6c also contributed to the separation along this axis; their concentration was lower with earthworm presence than that in the initial residue (Table 1). The second discriminant function separated the initial residue and earthworm treatment from the control, mainly due to a higher abundance of PLFAs 15:0 and 16:0 in the control (Table 1). Although not detected by the discriminant analysis, a reduction was also observed in the abundance of the G+ bacterial biomarkers as a result of the earthworm activity, as shown in Table 1.

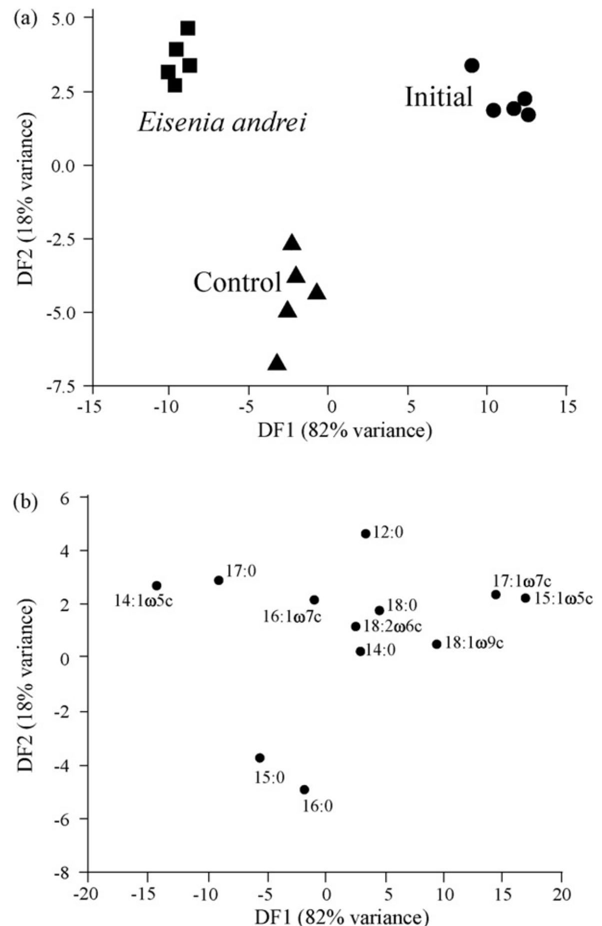

Fig. 1. Changes in the microbial community structure of the grape marc after fifteen days. (a) Discriminant function analysis performed on the twenty-one PLFAs identified in the initial grape marc, and the substrates obtained after incubation for fifteen days without earthworms (control), and in the presence of the epigeic earthworm species *Eisenia andrei*; (b) discriminant scores of the twelve PLFAs selected by the analysis as the most important in terms of the overall differences in the microbial community structure.

Table 1
PLFA yields (μg mL^{-1}) in the initial grape marc and in the substrates obtained after incubation for fifteen days without earthworms (control), and in the presence of the epigeic earthworm species *Eisenia andrei*.

	Initial	Processed substrates	
		Control	*Eisenia andrei*
PLFA biomarkers			
G+ bacteria			
i15:0	9.41 ± 0.36a	9.23 ± 0.87a	5.89 ± 0.82b
a15:0	2.58 ± 0.19ab	3.51 ± 0.33a	1.95 ± 0.33b
i16:0	4.96 ± 0.26a	4.93 ± 0.35a	2.47 ± 0.28b
a17:0	2.96 ± 0.08a	2.78 ± 0.15a	1.67 ± 0.22b
G− bacteria			
16:1ω7c	2.32 ± 0.19	2.07 ± 0.15	2.96 ± 0.36
17:1ω7c	1.95 ± 0.01a	1.82 ± 0.05a	1.04 ± 0.20b
cy17:0	21.40 ± 2.09	21.98 ± 2.23	20.87 ± 2.94
cy19:0	18.57 ± 1.02	18.66 ± 1.25	18.55 ± 1.02
Actinobacteria			
10Me18:0	32.18 ± 3.63a	28.35 ± 1.30b	25.18 ± 2.65b
Fungi			
18:1ω9c	2.81 ± 0.33a	2.21 ± 0.21ab	1.83 ± 0.36b
18:2ω6c	8.45 ± 0.71a	6.32 ± 0.84a	3.37 ± 0.29b
Other microbial PLFAs			
12:0	0.26 ± 0.08a	0.24 ± 0.07a	0.12 ± 0.07b
14:0	0.40 ± 0.11	0.43 ± 0.03	0.34 ± 0.06
15:0	0.34 ± 0.08b	0.55 ± 0.03a	0.32 ± 0.04b
16:0	5.30 ± 0.28ab	6.55 ± 0.28a	5.06 ± 0.47b
17:0	0.37 ± 0.21b	0.44 ± 0.04ab	0.64 ± 0.09a
18:0	1.59 ± 0.20a	1.26 ± 0.10ab	1.04 ± 0.10b
14:1ω5c	0.72 ± 0.09b	0.67 ± 0.16b	0.97 ± 0.04a
15:1ω5c	0.77 ± 0.10a	0.73 ± 0.05a	0.40 ± 0.12b
18:3ω6c	0.16 ± 0.07	0.17 ± 0.01	0.14 ± 0.05
18:3ω3c	0.46 ± 0.02a	0.42 ± 0.03ab	0.34 ± 0.01b

Values are means ± standard error. (a–c) Means within the same row denote significant differences between samples (Tukey HSD test; α = 0.05).

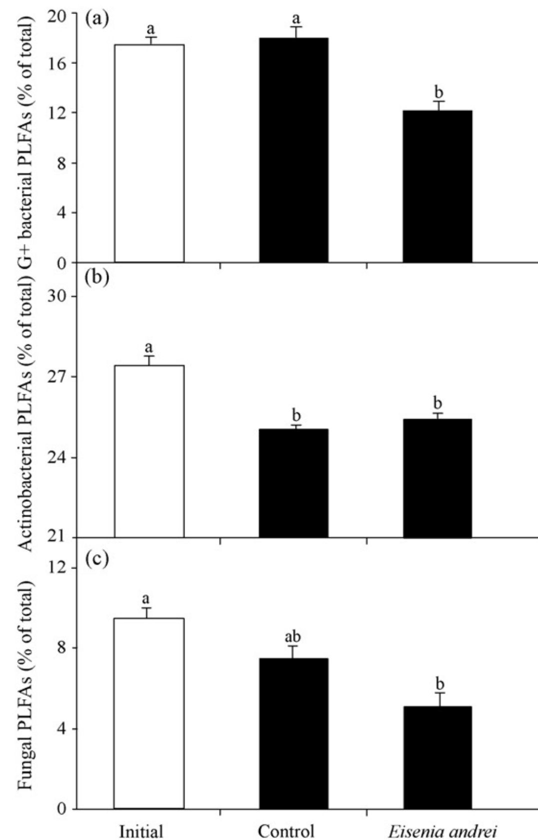

Fig. 2. Relative abundance (% of total) of specific PLFAs used as biomarkers of different microbial groups from the initial grape marc, and the substrates obtained after incubation for fifteen days without earthworms (control), and in the presence of the epigeic earthworm species *Eisenia andrei*: (a) Gram-positive bacterial PLFAs; (b) actinobacterial PLFAs; (c) fungal PLFAs. Values are means ± standard error. Different letters indicate significant differences between samples (Tukey HSD test; α = 0.05).

Earthworm activity reduced the viable microbial biomass measured as total PLFAs relative to the control without earthworms (ANOVA $F_{2,12}$ = 26.99, P = 0.0001; 96.90 ± 1.04 μg mL^{-1} and 113.60 ± 3.44 μg mL^{-1} for treatments with and without earthworms). The same pattern was observed with G+ bacterial PLFAs (Fig. 2a; ANOVA $F_{2,12}$ = 17.18, P = 0.0001). However, there were no significant differences between samples in relation to G− bacterial PLFAs (ANOVA $F_{2,12}$ = 0.17, P = 0.84). A decrease in the abundance of actinobacterial PLFAs was observed in treatments with and without earthworms (Fig. 2b; ANOVA $F_{2,12}$ = 5.81, P = 0.02). Earthworm activity also reduced the abundance of fungal PLFAs (Fig. 2c; ANOVA $F_{2,12}$ = 8.49, P = 0.005), and consequently the fungal to bacterial PLFA ratio (ANOVA $F_{2,12}$ = 4.30, P = 0.04).

3.2. Microbial activity

The total microbial activity measured as basal respiration was about 1.7 times lower in the earthworm treatment than that in the control without earthworms (Fig. 3a; ANOVA $F_{2,12}$ = 110.7, P = 0.0001). Earthworm activity also greatly reduced the activities of the protease (Fig. 3b; ANOVA $F_{2,12}$ = 5.76, P = 0.02) and cellulase enzymes (Fig. 3c; ANOVA $F_{2,12}$ = 10.16, P = 0.003) in comparison with the control.

3.3. CLLP profiles

The activity of earthworms led to higher values of the H' index relative to the control (ANOVA $F_{2,12}$ = 19.5, P = 0.02; 3.22 ± 0.02 and 3.11 ± 0.03 for treatments with and without earthworms respectively). The discriminant analysis of the data from Biolog® Ecoplate clearly distinguished the samples (Fig. 4a; Wilk's λ = 0.0001, P = 0.0001). The first and second eigenvalues, DF1 and DF2, accounted for 96% and 4% of the variance for a total explained variance of 100% (Fig. 4a). Twelve C sources were selected by this analysis as the most important in terms of the overall changes in the substrate utilization patterns of the microbial populations of grape marc. Discriminant scores of each C source with DF1 and DF2 are plotted in Fig. 4b. The first discriminant function mainly differentiated the initial grape marc from the earthworm treatment, which shifted negatively along this function. Thus earthworm activity led to a reduction in the potential utilization of carbohydrates β-methyl-D-glucoside and D-mannitol, and in the utilization of carboxylic acids D-galacturonic and γ-hydroxybutyric, which strongly influenced the positive side of this function, as shown in Table 2. The activity of earthworms was also related to an increase in the potential use of carbon sources N-acetyl-D-glucosamine and D-galactonic acid, negatively associated with this function, as shown in Table 2. Other sources such as D-cellobiose, α-D-lactose, D-xylose also contributed to the separation along this axis; their utilization was lower with earthworm presence than that in the initial residue (Table 2). Although the second discriminant function explained a very low percentage of the total variance, it contributed to differentiating the initial residue and earthworm treatment from the control—mainly because of a higher potential utilization of the carbohydrate i-erythritol and the pyruvic acid methyl ester in the control substrate. Although not selected by the discriminant analysis, an increase was also observed in the utilization of some amino acids and polymers as a result of the earthworm activity, as shown in Table 2.

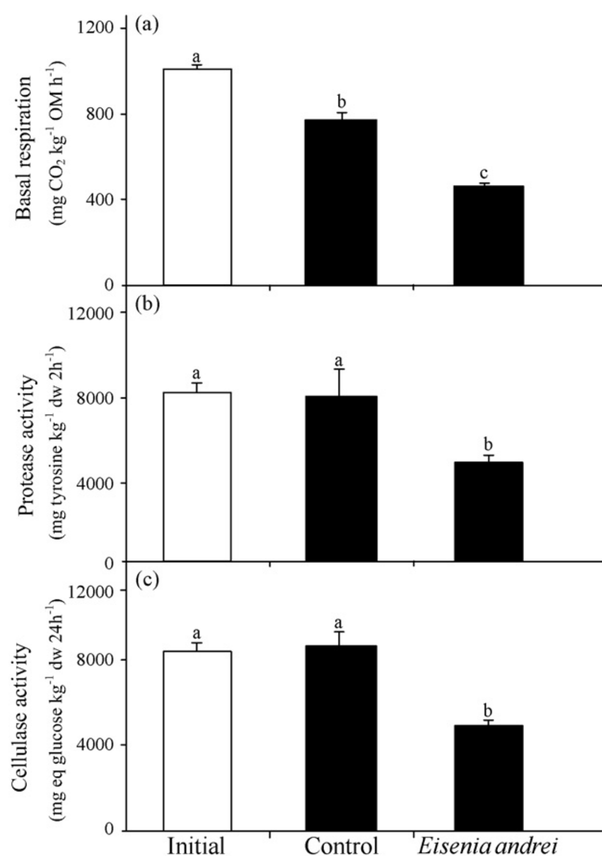

Fig. 3. Microbial activity of the initial grape marc, and the substrates obtained after incubation for fifteen days without earthworms (control), and in the presence of the epigeic earthworm species *Eisenia andrei*. (a) Total microbial activity measured as basal respiration; (b) protease activity; (c) cellulase activity. Values are means ± standard error. Different letters indicate significant differences between samples (Tukey HSD test; $\alpha = 0.05$).

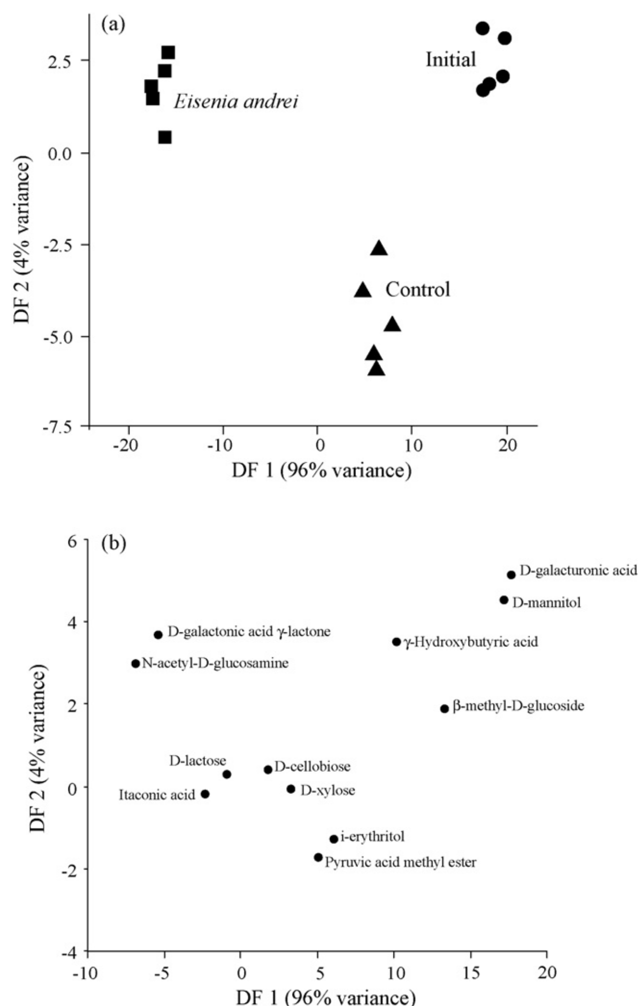

Fig. 4. Changes in the potential utilization of carbon sources in the grape marc after fifteen days. (a) Discriminant function analysis performed on the thirty carbon sources identified in the initial grape marc, and the substrates obtained after incubation for fifteen days without earthworms (control), and in the presence of the epigeic earthworm species *Eisenia andrei*; (b) discriminant scores of the twelve carbon sources selected by the analysis as the most important in terms of the overall differences in community-level physiological profiles.

3.4. Chemical properties

The activity of earthworms led to an increase in the pH of the grape marc, which reached a value of 8.12, whereas it did not change after incubation for fifteen days without earthworms, and its value was 7.77 (Table 3). There were no significant differences in the EC between samples (Table 3). Earthworm activity led to a decrease, relative to the control, in the labile C pool (DOC) and the total C concentration of the initial grape marc (Table 3). A reduction was also observed in the concentration of cellulose and hemicellulose, relative to the initial residue, as a result of the earthworm activity (Table 3). However, there were no significant differences between samples as regards lignin content (Table 3). An increase in the total N concentration was observed in treatments with and without earthworms (Table 3). Earthworm activity increased the concentration of NH_4^+ relative to the control without earthworms, but no changes were detected in NO_3^- concentration (Table 3).

4. Discussion

In this study we evaluated the short-term effects of epigeic earthworms on microbial community structure and function and on plant residue decomposition. Despite the short duration of the study, important changes were found to have occurred as a result of earthworm activity. Phospholipid fatty acid analysis of microbial communities revealed that the epigeic earthworm species *E. andrei* had a great impact on the structure of the grape marc microbial community. This is consistent with previous findings based on PLFA profiles, which indicate that earthworm activity strongly modifies the structure of the microbial community in soil samples incubated with different plant materials (Clapperton et al., 2001; Enami et al., 2001; Butenschoen et al., 2007; Elfstrand et al., 2008). Although these earlier studies concern soil-dwelling endogeic and anecic species, epigeic earthworms are known to interact closely with microorganisms (Aira et al., 2008), and thus, in turn, affect the structure of microbial communities (Lores et al., 2006).

As expected, the activity of epigeic earthworms resulted in a lower abundance of bacteria (except for Gram-negative bacteria) and fungi. Previous studies of the effects of earthworms on microorganisms have also shown decreases in the abundance of characteristic bacterial PLFAs (Butenschoen et al., 2007; Pawlett et al., 2009) and fungi (Saetre, 1998; Butenschoen et al., 2007). However, other studies have shown an increase (Enami et al., 2001) or scarce changes (Marhan et al., 2007) in these PLFA biomarkers. The contrasting results for microbial populations in the presence of earthworms may be related to the duration of the experiments, and to the earthworm species involved and/or the kind of food source, as shown by Knapp et al. (2008, 2009). The latter authors observed that the gut and cast microbiota of the epigeic earthworm species

Table 2
Values ($A_{595\ nm}$) of utilization of sole carbon sources in the initial grape marc and in the substrates obtained after incubation for fifteen days without earthworms (control), and in the presence of the epigeic earthworm species *Eisenia andrei*.

Carbon source	Initial	Processed substrates	
		Control	*Eisenia andrei*
Carbohydrates			
D-Cellobiose	2.35 ± 0.10a	2.25 ± 0.02a	1.84 ± 0.09b
α-D-Lactose	1.41 ± 0.95a	0.88 ± 0.05b	1.11 ± 0.15ab
β-Methyl-D-glucoside	2.10 ± 0.14a	1.99 ± 0.07a	1.65 ± 0.05b
D-Xylose	0.62 ± 0.08ab	0.76 ± 0.09a	0.20 ± 0.04b
i-Erythritol	0.47 ± 0.08ab	0.59 ± 0.04a	0.34 ± 0.04b
D-Mannitol	1.95 ± 0.23a	1.81 ± 0.12a	1.49 ± 0.12b
N-Acetyl-D-glucosamine	1.71 ± 0.09b	1.92 ± 0.11a	2.37 ± 0.08ab
Carboxylic acids			
Pyruvic acid methyl ester	1.34 ± 0.09ab	1.45 ± 0.07a	0.92 ± 0.04b
D-Galactonic acid γ-lactone	1.74 ± 0.05b	1.91 ± 0.08ab	2.04 ± 0.05a
D-Galacturonic acid	2.47 ± 0.26a	1.56 ± 0.06b	1.45 ± 0.09b
γ-Hydroxybutyric acid	0.72 ± 0.10a	0.45 ± 0.05b	0.42 ± 0.07b
Itaconic acid	0.68 ± 0.17ab	0.77 ± 0.03a	0.50 ± 0.01b
α-Ketobutyric acid	0.28 ± 0.02	0.24 ± 0.04	0.24 ± 0.06
D-Malic acid	1.09 ± 0.11	1.23 ± 0.11	1.40 ± 0.20
Amino acids			
L-Arginine	0.47 ± 0.03	0.45 ± 0.07	0.50 ± 0.03
L-Asparagine	1.09 ± 0.16b	1.81 ± 0.16a	1.36 ± 0.06ab
L-Phenylalanine	0.45 ± 0.02a	0.24 ± 0.04b	0.26 ± 0.04b
L-Serine	1.20 ± 0.09b	1.35 ± 0.03ab	1.64 ± 0.07a
L-Threonine	0.41 ± 0.03ab	0.25 ± 0.09b	0.62 ± 0.13a
Glycyl-L-glutamic acid	0.31 ± 0.03ab	0.21 ± 0.04b	0.42 ± 0.02a
Amines/amides			
Phenylethylamine	0.87 ± 0.06a	0.31 ± 0.03b	0.29 ± 0.07b
Putrescine	0.64 ± 0.07b	1.03 ± 0.06a	0.83 ± 0.07ab
Polymers			
Tween 40	0.87 ± 0.05	0.99 ± 0.03	1.12 ± 0.08
Tween 80	1.05 ± 0.04	1.07 ± 0.07	0.99 ± 0.11
α-Cyclodextrin	0.50 ± 0.05b	0.15 ± 0.04c	1.02 ± 0.13a
Glycogen	0.88 ± 0.13b	0.98 ± 0.03b	2.46 ± 0.15a
Miscellaneous compounds			
Glucose-1-phosphate	1.44 ± 0.12a	1.62 ± 0.30a	0.98 ± 0.13b
D,L-α-Glycerol phosphate	0.62 ± 0.05	0.50 ± 0.03	0.58 ± 0.04
2-Hydroxybenzoic acid	0.05 ± 0.02	0.03 ± 0.01	0.04 ± 0.01
4-Hydroxybenzoic acid	0.87 ± 0.07	1.07 ± 0.01	0.80 ± 0.02

Values are means ± standard error. (a–c) Means within the same row denote significant differences between samples (Tukey HSD test; $\alpha = 0.05$).

Lumbricus rubellus were strongly influenced by the food source ingested. Aira et al. (2008) also found that the effects of epigeic earthworms on microbial community structure depended on earthworm density. These authors detected a significant increase in the fungal biomass of pig slurry, measured as ergosterol content, at intermediate and high densities of earthworms (50 and 100 earthworms per mesocosm, respectively), which suggests that there may be a threshold density of earthworms at which fungal growth is triggered. Despite the high density of earthworms used in the present study (500 earthworms per mesocosm), the fungal biomass was lower than in the control mesocosm. Such differences may be due to the type of substrate (pig slurry or grape marc) and/or to the method used to measure fungal biomass (ergosterol or fungal PLFAs). Discrepancies between these measurements have been reported by Högberg (2006).

The presence of epigeic earthworms also led to a decrease in the total microbial activity of grape marc, to a greater extent than in the control mesocosm. Similar decreases in microbial activity measured as basal respiration were reported in short-term experiments with epigeic earthworm species (Aira et al., 2002, 2006; Aira and Domínguez, 2009). Contrary to the present findings, other authors have reported an increase (Parthasarathi and Ranganathan, 1999) or no changes (Aira and Domínguez, 2009) in microbial activity in the presence of epigeic earthworms. As mentioned previously with respect to microbial biomass, the differences in microbial activity may also be attributable to the duration of the experiments, and to the earthworm species involved and/or the type of food source. Indeed, Aira et al. (2006) observed a reduction in microbial activity in casts of *Eudrilus eugeniae* fed with pig manure, whereas Aira and Domínguez (2009) did not detect any changes in this parameter in the presence of *Eisenia fetida*. However, the latter authors observed a reduction in microbial activity when *E. fetida* fed on cow manure rather than pig slurry. Furthermore, Aira and Domínguez (2009) observed differences depending on whether microbial activity was measured as basal respiration or dehydrogenase activity. The latter technique only accounted for a limited percentage of respiration since oxygen is a better electron acceptor than triphenyltetrazolium chloride, which is usually used as a substrate for dehydrogenase activity (Nannipieri et al., 1990).

As we found for total microbial activity, there was also a decrease in the activities of protease and cellulase enzymes in relation to earthworm presence, whereas no such reduction was observed in the control mesocosm. Aira et al. (2007b) observed high correlations between the microbial biomass and protease and cellulase activities, which indicate that microorganisms play an important role in shaping the patterns of these two enzymes during the course of residue degradation. Thus, the reduction in both enzyme activities relative to the control may be due to the lower microbial biomass as a result of earthworm activity, which probably affected enzyme production. Aira et al. (2006) also reported a reduction in the activity of protease enzyme in a short-term experiment with epigeic earthworms, but did not find any differences in cellulase activity. Extracellular enzymes may remain active for long periods when protected in humic complexes (Benítez et al., 2005). However, the present study was not of long

Table 3
Chemical properties of the initial grape marc, and the substrates obtained after incubation for fifteen days without earthworms (control), and in the presence of the epigeic earthworm species *Eisenia andrei*.

	Initial	Processed substrates		ANOVA	
		Control	*Eisenia andrei*	*F*-ratio	*P*-value
pH	7.77 ± 0.01b	7.77 ± 0.03b	8.12 ± 0.09a	14.08	**0.001**
EC ($mS\,cm^{-2}$)	0.28 ± 0.01	0.26 ± 0.01	0.27 ± 0.01	0.34	0.72
Total C ($g\,kg^{-1}$)	503 ± 2a	486 ± 3a	459 ± 6b	13.62	**0.001**
DOC ($mg\,kg^{-1}$)	5078 ± 284a	5170 ± 467a	4050 ± 167b	3.91	**0.049**
Cellulose ($g\,kg^{-1}$)	175 ± 4a	169 ± 4ab	148 ± 5b	8.84	**0.004**
Hemicellulose ($g\,kg^{-1}$)	69 ± 5a	51 ± 8ab	40 ± 6b	7.45	**0.008**
Lignin ($g\,kg^{-1}$)	517 ± 3	531 ± 14	543 ± 8	3.99	0.07
Total N ($g\,kg^{-1}$)	35 ± 3b	47 ± 1a	47 ± 2a	6.08	**0.02**
NH_4^+ ($mg\,kg^{-1}$)	170 ± 13ab	127 ± 12b	193 ± 21a	6.79	**0.01**
NO_3^- ($mg\,kg^{-1}$)	78 ± 6	80 ± 6	80 ± 4	1.33	0.30

Values are means ± standard error. *P*-values in bold denote a significant effect at 95% confidence level; in that case, different letters were used to indicate significant differences between samples (Tukey HSD test).

enough duration to achieve the high degree of organic matter stabilization necessary for formation of humus. Earthworms may also affect the activity of these enzymes by modifying the availability of C and N pools. Thus, the total C content and the labile carbon pool were significantly lower than in the control mesocosm. The concentrations of cellulose and hemicellulose were also lower in the mesocosm with earthworms, although the values were not statistically different from the control values. The depletion of C pools was primarily due to microbial biomass uptake and, to a lesser extent, to assimilation by earthworms, because there were no significant differences in earthworm biomass after fifteen days. However, there was an increase in the total N content of the grape marc in the mesocosms incubated for fifteen days with and without earthworms. This may be due to the concentration effect caused by the weight loss associated with mineralization of the organic matter. The concentration of NH_4^+ also increased, relative to the control, as a result of earthworm activity, probably because NH_4^+ is one of the excretion products of earthworms (Lee, 1985). Therefore, as the increase in the N pool was attributable to earthworm presence, the reduction in protease activity may be related to the decrease in microbial biomass.

Utilization of the different C pools is associated with different groups of microorganisms, resulting in microbial succession that is linked to the changes in residue chemistry during decomposition (McMahon et al., 2005). The further depletion of C pools in relation to the presence of earthworms therefore reduced the availability of some carbon sources for microorganisms, and in turn affected the substrate utilization patterns of microbial populations. The activity of earthworms led to lower utilization of most carbohydrates and carboxylic acids, except for N-acetyl-D-glucosamine and D-galactonic acid γ-lactone, which may stem from the production of mucus, which is rich in mucopolysaccharides (Brown et al., 2000). Earthworm activity therefore favoured the appearance of other microbial populations with the capacity to utilize polymers and amino acids, and a higher substrate diversity value was reached than in the control mesocosm. This is consistent with the findings of previous studies, i.e. that epigeic earthworms have a great impact on the substrate utilization patterns of microbial populations, as assessed by Biolog® Ecoplate (Scheu et al., 2002; Aira et al., 2006, 2007a; Sheehan et al., 2008; Sen and Chandra, 2009). Although this technique has been widely used in environmental research to evaluate changes in microbial communities, it does not reflect the functional abilities of the entire microbial community (Smalla et al., 1998). Recently, Ros et al. (2008) used molecular tools to analyse the microbial populations growing on particular carbon substrates after inoculating Biolog™ plates with extracts from two soils differing in CLPP patterns and microbial community structure. These authors observed a low microbial diversity in all suspensions of carbon sources that they analysed, from both soils, thus confirming that the method reflects only a very limited subset of microbial genera. This approach is therefore only recommended as a screening method, and its use for analysis of microbial communities should be accompanied by other complementary techniques.

As we expected, the activity of the epigeic earthworm species *E. andrei* greatly modified microbial community structure and function, and in turn the rate of decomposition of the organic matter. These findings indicate that detritivorous earthworms play a key role in the decomposition of plant residues in the short term, via their intense interactions with decomposer communities.

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