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Abstract

Earthworms in interaction with soil microorganisms play a key role in litter decomposition. Moreover, as soil engineers, earthworms modify microbial communities and their enzymatic activities. Most studies focusing on earthworms and soil enzymatic compare distinct ecological categories of earthworms whereas their contributions and interactions within a given ecological category remain largely unknown. In this context, the aims of the present study were to determine and compare the contribution of (1) three strict-anecic earthworm species, (2) three epi-anecic earthworm species and (3) the pairwise interactions between these different species on *Lolium perenne* leaf litter decomposition and soil microbial activity. After 30 days of incubation, the surface litter mass loss and five soil enzymatic activities (FDAse, β-D-glucosidase, cellobiohydrolase, leucine amino-peptidase and acid phosphatase) were measured in both earthworm burrows and middens. In mono-specific assemblages, leaf litter mass loss and enzymatic activities were significantly higher in the presence of epi-anecic compared to strict-anecic species, whatever the species identity. These differences were higher for the β-D-glucosidase, leucine amino-peptidase and FDAse (+78%, +57% and +34%, respectively). Earthworm species interactions at both intra- and inter-ecological sub-categories did not enhance leaf litter mass loss and enzymatic activities. Interestingly, FDAse activity was higher in earthworm burrows whereas acid phosphatase activity was higher in earthworm middens. These results indicate that the two anecic ecological sub-categories have different impacts on soil functioning and each of them regroups earthworm species with similar behaviour. This functional distinction highlights the key role of epi-anecic earthworms in fresh surface litter burial and decomposition, featuring their importance on nutrient cycling in soil and for microbial activities stimulation through resource availability.

1. Introduction

Organic matter decomposition is a key ecosystem function due to its importance for carbon (C) and nutrient cycling (Bardgett et al., 2005; Wall, 2012), soil structure (Oades, 1984; Soane, 1990), and water storage (Tisdall and Oades, 1982; Hudson, 1994). As roughly 90 % of the global terrestrial plant production enters the dead organic matter pool (Cebrian, 1999), the decomposition of plant material is one of the most crucial processes in terrestrial ecosystems (Tiessen et al., 1994). At the fine scale, soil organic matter (SOM) decomposition is performed by saprotrophic microorganisms that catalyze several biochemical reactions involved in the mineralization process (Coûteaux et al., 1995). Both soil fungi and bacteria secrete enzymes (Dick and Tabatabai, 1992; Dick et al., 2000; Tabatabai, 2003) acting on specific chemical bounds to hydrolyze complex molecules and polymers into low molecular weight compounds (Burns and Dick, 2002). Unlike many bacteria which tend to focus on one substrate at a time, fungi can secrete enzymes targeting several compounds simultaneously and are known to degrade more complex molecules than bacteria (Adl, 2003; Burns et al., 2013). Soil enzymatic activity measurements are used as soil quality indicators (Karaca et al., 2010; Schloter et al., 2018) since both enzyme production and enzymatic activity efficiencies are known to be highly sensitive to soil texture, pH, organic matter content, climate and microbial biomass changes (Bandick and Dick, 1999; Acosta-Martínez and Tabatabai, 2000; Tabatabai, 2003). Despite it is well established that microbial communities and the processes they drive depend strongly on their interactions with soil fauna (Lussenhop, 1992; Brown, 1995; Tiunov and Dobrovolskaya, 2002; Jouquet et al., 2005), our knowledge about the extent to which the soil microbial enzymes react to these interactions is still limited.

Earthworms are soil ecosystem engineers able to modify soil physical, chemical and biological properties (Lavelle, 1988; Jones et al., 1994; Blouin et al., 2013) with consecutive effects on soil microorganisms including changes in microbial biomass, respiration, or enzymatic activities (Ross and Cairns, 1982; Brown, 1995; Binet et al., 1998; Tiunov and Scheu, 1999; Brown et al., 2000; Tiunov et al., 2001; Aira et al., 2009). According to their physiological, morphological and behavioral characteristics, earthworms are classified into three main ecological categories: epigeic, anecic and endogeic (Bouché, 1972, 1977). Among those, anecic earthworms play a key role in SOM degradation (Edwards, 2004; Curry and Schmidt, 2007) since they consume a mixture of organic matter with mineral soil and bury fresh leaf litter into their vertical burrows. Moreover, during their activity, anecic earthworms often create middens at the entrance of their burrows (Bouché, 1977; Brown et al., 2000) which are a surface structure made up of a mix of soil, casts, mucus and buried organic matter fragments (Nielsen and Hole, 1964). Within anecic earthworms, several studies have highlighted two ecological sub-categories according to earthworm morphological traits (Briones and Álvarez-Otero, 2018), burrowing (Jégou et al., 2000, 2001; Bastardie et al., 2005) and feeding behaviors (Ferrière, 1980; Eisenhauer et al., 2008; Andriuzzi et al., 2016; Larsen et al., 2016; Hoeffner et al., 2018). Thus, epi-anecic earthworms preferentially consume fresh leaf litter at the soil surface which is then buried into the soil profile whereas strict-anecic earthworms consume a mix of leaf litter and humified organic matter within the soil profile (Cortez et al., 1989; Jégou et al., 1998; Stromberger et al., 2012; Larsen et al., 2016; Hoeffner et al., 2018).

The average fresh individual biomass of each earthworm species within these two ecological sub-categories can vary greatly, from 0.5 g for *Lumbricus rubellus* to 15.0 g for *Lumbricus terrestris* within epi-anecic earthworms and from 0.3 g for *Aporrectodea caliginosa*

meridionalis to 3.2 g for *Aporrectodea giardi* within strict-anecic earthworms (Bouché, 1972). This huge variability of biomasses between earthworm species of a same ecological sub-category suggests different metabolic needs and capacities to bury leaf litter in their burrows (Hoeffner et al., 2018). Thus, depending on both their belonging to the epi- or strict-anecic sub-categories and their individual biomass, anecic earthworm species could influence soil microorganisms. For example, Jégou et al. (2001) reported a higher dehydrogenase activity in *L. terrestris* (epi-anecic earthworm) than in *A. giardi* (strict-anecic earthworm) burrow walls but no difference between these two species was observed on acid and alkaline phosphatase activities. Moreover, Hoeffner et al. (2018) reported that fungal communities were differentially impacted by four epi-anecic earthworm species. However, to our knowledge, no previous study compared the specific impact of these two ecological sub-categories on soil enzymatic activities involved in the main biochemical cycles such as those of C or N.

Several authors reported that anecic earthworm communities can be composed from one to six different species (Gerard, 1967; Poier and Richter, 1992; Boag et al., 1997; Margerie et al., 2001; Decaëns et al., 2008). Interactions between earthworm species within and between ecological categories can thus potentially modify their contributions to litter burial and their impacts on soil microbial communities and activities. For example, Xia et al. (2011) observed a synergistic effect on surface leaf litter mass loss between *L. rubellus* (epi-anecic earthworm) and *Octalasion lacteum* (endogeic earthworm). Within epi-anecic earthworms, Postma-Blaauw et al., (2006) reported that the interaction between *L. rubellus* and *L. terrestris* reduced mineral nitrogen concentration derived from SOM and increased bacterial biomass probably due to an increase of litter incorporation. However, to our knowledge, no previous studies attempted to demonstrate if both strict- and epi-anecic earthworms could interact together in driving soil enzymatic activities.

The present study aims to determine the effects of strict- and epi-anecic earthworm species in addition to their interactions on leaf litter mass loss and soil microbial enzymatic activities. We used a block design experiment with three strict-anecic earthworm species (*Aporrectodea caliginosa meridionalis*, *Aporrectodea nocturna* and *Aporrectodea giardi*) and three epi-anecic earthworm species (*Lumbricus rubellus*, *Lumbricus centralis* and *Lumbricus terrestris*) in all possible pairwise combinations of two individuals (6 mono- and 15 bi-specific pairs). After 30 days of incubation, surface leaf litter mass loss was determined and five enzymatic activities were measured in earthworm middens and burrows. Since epi-anecic earthworms bury higher amount of leaf litter than strict-anecic earthworms, we first hypothesized greater leaf litter mass loss and enzymatic activities in the presence of epi- than strict-anecic earthworms. Secondly, we hypothesized a synergistic effect on leaf litter mass loss and enzymatic activities with bi-specific pairs, an effect that should be increased when combining one strict- and one epi-anecic species.

2. Materials and methods

2.1. Material collection

Soil (5-20 cm depth) was collected in a temporary grassland near Trans-La-Forêt, France (48°50’ N, -1°58’ W) in the Long Term Ecological Research (LTER) site ‘‘Zone Atelier Armorique’’. The climate of the region is oceanic with a mean annual temperature of 11.7 °C, a mean annual rainfall of 815.0 mm and a mean annual relative humidity of 80.9 % (mean values over the period 2010-2016, data from Météo France). The collected soil was hand-sieved at 4 mm and homogenized. The soil was identified as a loam Cambisol (IUSS Working Group, 2015) with 48% sand, 38% silt and 14% clay, characterized by 1.7% organic carbon, 0.2% total nitrogen, a

C:N ratio of 9.5 and a pH of 6.4 (data from INRA SAS, Arras, France). The soil was pre-incubated for one week at 12 °C under a 12 h:12 h light: dark regime with a water content adjusted to 29% w/w by addition of deionized water prior to the experiment.

Fresh leaf litter of *Lolium perenne* L., a typical grassland species, was collected from a temporary grassland close to the soil sampling location. The leaf litter was air-dried and stored at room temperature until the beginning of the experiment. The leaf litter characteristics of *L. perenne* were determined according to the protocols described in Hoeffner et al. (2018). The leaf litter was characterized by 43.1% organic carbon, 3.1% total nitrogen, 3.4% phenolics, a C:N ratio of 13.9, a water holding capacity of 392.6% and a specific leaf area of 330.2 cm^2 g^{-1}.

Adult earthworms were collected in temporary grasslands around the soil sampling location. Three strict-anecic earthworm species, *A. caliginosa meridionalis* (Bouché, 1972; hereafter called AM), *A. nocturna* (Ude, 1885; hereafter called AN), *A. giardi* (Savigny, 1826; hereafter called AG); and three epi-anecic earthworm species, *L. rubellus rubellus* (Hoffmeister, 1843; hereafter called LR), *L. centralis* (Bouché, 1972; hereafter called LC), *L. terrestris*, (Linné, 1758; hereafter called LT) were studied. The six earthworm species were hand collected two weeks before the beginning of the experiment, grouped in mono-specific boxes containing the hand-sieved soil, and fed with air-dried leaves of *L. perenne* studied here.

2.2. Experimental setup

From the three strict-anecic and the three epi-anecic earthworm species selected, 21 pairwise combinations of two individuals were performed in five replicates following 5 earthworm assemblages (Supplementary Fig. S1): i) mono-specific assemblages of 2 individuals of strict-anecic earthworms (3 treatments), ii) mono-specific assemblages of 2 individuals of epi-anecic

earthworms (3 treatments), iii) bi-specific assemblages of 2 individuals within strict-anecic earthworms (3 treatments), iv) bi-specific assemblages of 2 individuals within epi-anecic earthworms (3 treatments), and v) bi-specific assemblages of 2 individuals including one strict- and one epi-anecic earthworm (9 treatments). Control without earthworm accounting for the leaf litter mass loss due to microbial decomposition and leaching was performed in 5 replicates.

Each mesocosm (PVC cylinder, 30 cm high, 10 cm diameter sealed at the base) was filled with 4.9 kg of fresh soil. The soil was placed in the mesocosm and compacted to a bulk density of 1.3 $g.cm^{-3}$ in two steps. A plastic grille (mesh size 1.2 cm) was placed on top of the soil and 3.5 g of air-dried leaves of *L. perenne* cut into sections of approximately 7 cm length were deposited just before the start of the experiment on the grille and re-humidified with deionized water. In parallel, 5 replicates of 3.5 g of leaves were dried at 72 °C during 48 h to determine the initial leaf litter dry weight. Earthworms were gut voided (36 h starving on a moist sponge in a plastic box) and weighed before being placed in the corresponding mesocosms. Individual earthworms presented an initial fresh biomass gradient varying from 0.62 g for LR to 4.72 g for LT (Supplementary Fig. S2). Mesocosms were closed with a mesh of 1 mm to avoid earthworms' escape during the experiment. The mesocosms were incubated in a climatic chamber at 12 °C, with a relative humidity of 85% and a 12 h: 12 h light: dark regime. Soil moisture was maintained by spraying deionized water at the soil surface twice per week. Given the large number of mesocosms, different sets of replicates were launched one day apart leading to 5 blocks of 22 mesocosms.

After 30 days, the mesocosms were destructively sampled. The leaf litter remaining on the grille at the soil surface was collected and the earthworms. In the mesocosms containing earthworms, two soil microsites were sampled and analyzed separately: the surface middens between +3 and -2 cm and a 2 mm soil layer from the inside (i.e. close to the burrow lumen) of the

entire burrow network (regardless of the earthworm species) between -3 and -15 cm deep using a thin spatula. Strict-anecic earthworms do not form middens *sensu stricto* (Nielsen and Hole, 1964), however we sampled the entrance of the burrow constituted of casts and mucus. In the control mesocosms, the bulk soil was collected. These fresh soil samples were stored at 4 °C prior to enzymatic activity analyses performed within the following 14 days.

The remaining leaf litter was first dried at 72 °C for 48 h to determine leaf litter dry mass and then burned at 550 °C for 6 h to determine the leaf litter ash content. Based on the ash-free dry mass (AFDM) of the plant litter, leaf litter mass loss in each mesocosm was determined as the difference between initial and final AFDM.

During the experiment, 4 earthworms died (2% of the whole community): two LR, one LC and one AM. Whatever the treatment, the surviving earthworms were adults (with a turgid clitellum) and no juveniles were detected. All earthworms made burrows of a similar morphology, open at the soil surface, although the burrow diameters of LT, LC, AG were larger than those of LR, AC, AN (personal observations).

2.3. Enzymatic activity analyses

Five enzymatic activities commonly used as indicators for soil quality and involved in C, N and P cycles were studied (Bandick and Dick, 1999; Dodor and Tabatabai, 2003; Baldrian, 2009): fluorescein diacetate hydrolase (EC 3.1.1.x, FDAse for broad-spectrum indicator of soil activity), β-D-glucosidase (EC 3.2.1.21, C cycle), cellobiohydrolase (EC 3.2.1.91, C cycle), leucine-aminopeptidase (EC 3.4.11.1, N cycle) and acid phosphatase (EC 3.1.3.2, P cycle).

β-D-glucosidase, cellobiohydrolase, leucine-aminopeptidase and acid phosphatase assays were adapted from Marx et al. (2001) and FDAse from Green et al. (2006) using microplate assays.

For each enzyme, the corresponding substrate and standard were obtained in crystalline form from Sigma-Aldrich (MI, USA). Fluorescence-based soil assays for FDAse, β-D-glucosidase, cellobiohydrolase, leucine aminopeptidase and acid phosphatase were based on protocols using the respective following substrates: Fluorescein diacetate, 4-methylumbelliferyl β-d-glucopyranoside, 4-methylumbelliferyl β-d-cellobioside, l-leucine 7-amido-4-methylcoumarin hydrochloride, and 4-methylumbelliferyl phosphate. Stock solutions of the substrates and calibration solutions of 4-methylumbelliferone (MUB), amino-4-methylcoumarin (AMC) and fluorescein were prepared in appropriate diluents (Marx et al., 2001; Green et al., 2006) and subsequently used. Substrate-saturating concentrations were assayed from 5 random soil samples, and substrate concentrations were adapted for the measurements.

Briefly, 0.5 g of fresh soil were suspended and mixed in 50 mL of appropriate buffer for 5 min using an orbital shaker and then sonicated for 120 s. Aliquots of 50 µL were dispensed into black 96-well microplates in 3 replicates (Greiner bio-one, Kremsmünster, Austria). The specific substrate solutions were added to reach a final volume of 200 µL, and the microplates were stored at 30 °C in the dark. Fluorescence was read from each well every 60 min during a 300 min period (spectrophotometer Safas Monaco Xenius) at excitation and emission wavelengths of 490 and 514 nm for FDAse, 360 and 450 nm for MUB-substrates and 380 and 440 nm for AMC-substrates, respectively. Enzymatic activities were expressed as FDA, MUB or AMC released g^{-1} soil h^{-1}.

2.4. Statistical analyses

The 4 soil mesocosms that contained dead earthworms were removed from the data processing. Statistical analyses were performed using the R software 3.2.3 (R. Core Team, 2017). Significance was evaluated at $P < 0.05$. Data met the conditions of normality and homoscedasticity.

The differences between the initial biomass of the six earthworm species were assessed by one-way ANOVA, followed by Tukey HSD tests for post hoc pairwise comparisons ("agricolae" package).

Litter mass loss was first analyzed by one-way ANOVA, followed by Tukey HSD tests for post hoc pairwise comparisons, to assess the differences between litter mass loss in the presence of mono-specific assemblages of strict- and epi-anecic earthworms and the control treatment (i.e. without earthworm). Second, a three-way ANOVA was applied, followed by Tukey HSD tests for post hoc pairwise comparisons, to test the effects of earthworm assemblages, earthworm pairs, and blocks on litter mass loss.

Enzymatic activities were first analyzed by one-way ANOVAs, followed by Tukey HSD tests for post hoc pairwise comparisons to assess the differences between mono-specific assemblages of strict- and epi-anecic earthworms and the control treatment (independently of the middens and the burrows). Second, 4-way ANOVAs, followed by Tukey HSD tests for post hoc pairwise comparisons were used to test the effects of earthworm assemblages, earthworm pairs, microsites, and block on each enzymatic activity.

3. Results

3.1. Litter mass loss

In mesocosms with mono-specific assemblages, litter mass loss was 57% higher in the presence of epi-anecic earthworms compared to the control, whereas no significant effect was observed in the presence of strict-anecic earthworms (1.65 ± 0.04, 2.59 ± 0.09 and 1.81 ± 0.03 g for control, epi- and strict-anecic earthworms, respectively, $F = 45.10$, $P < 0.001$). Within the

mono-specific assemblages of epi-anecic earthworms, litter mass loss was 23% and 17% higher with LT compared to LR and LC, respectively, whereas no difference was observed between strict-anecic earthworm species ($F = 9.94$, $P < 0.001$, Supplementary Table S1, Fig. 1a).

In the mesocosms with bi-specific assemblages of either strict- or epi-anecic earthworms, the mean litter mass losses were similar to those observed with the corresponding mono-specific assemblages ($F = 97.55$, $P < 0.001$, Supplementary Table S1, Fig. 1b). The mean litter mass losses in the presence of bi-specific assemblages including one strict- and one epi-anecic earthworm were between those observed in the presence of the corresponding mono-specific assemblages ($F = 97.55$, $P < 0.001$, Supplementary Table S1, Fig. 1b). Additionally, litter mass loss in the presence of mono- and bi-specific assemblages of epi-anecic earthworms correlated to their initial biomass (monospecific assemblage: $R^2 = 0.81$, $P < 0.001$; bispecific assemblage: $R^2 = 0.73$, $P < 0.001$; Supplementary Fig. S3).

3.2. Enzymatic activities

Compared to the bulk soil of the control, the five enzymatic activities were enhanced in soil microsites from mesocosms with mono-specific assemblages of epi-anecic earthworms ($F = 8.49$ to 93.46, $P < 0.001$, Supplementary Fig. S4). In soil microsites from mesocosms with mono-specific assemblages of strict-anecic earthworms, only the β-D-glucosidase was enhanced by 50% compared to bulk soil ($F = 93.46$, $P < 0.001$, Supplementary Fig. S4b).

Except for the acid phosphatase, enzymatic activities were significantly higher in soil microsites from mesocosms with mono- and bi-specific assemblages of epi-anecic earthworms than in those of strict-anecic earthworms ($F = 19.02$ to 44.01, $P < 0.001$, Supplementary Table S2, Fig. 2f-j). These differences were higher for the β-D-glucosidase (+75%; Fig. 2g), leucine-

aminopeptidase (+58%; Fig. 2i) and FDAse (+32%; Fig. 2f) than for the cellobiohydrolase activity (+14%; Fig. 2h).

Overall, the enzymatic activity levels in soil microsites from mesocosms with bi-specific assemblages of either strict- or epi-anecic earthworms were similar to those observed in the presence of their respective mono-specific assemblages (Supplementary Table S2, Fig. 2f-j). In the presence of mono-specific assemblages within each sub-category, the enzymatic activity levels were similar whatever the earthworm species considered (Fig. 2a-e). However, within these mono-specific assemblages, either strong differences or slight similarities were observed between strict- and epi-anecic pairs (Fig. 2a-e). For example, the β-D-glucosidase and leucine-aminopeptidase activities in the presence of LC were +77% and +59% higher than those observed in the presence of mono-specific assemblages of strict-anecic earthworms (Fig. 2b and d), respectively. Conversely, the FDAse and cellobiohydrolase activities in the presence of LC were similar to those observed in the presence of mono-specific assemblages of strict-anecic earthworms (Fig. 2a and c).

In the presence of bi-specific assemblages composed of one strict- and one epi-anecic earthworm, the enzymatic activity levels were generally intermediate between those observed in the respective mono-specific assemblages (Supplementary Table S2, Fig. 2f-j). Thus, the activities of FDAse, β-D-glucosidase, cellobiohydrolase and leucine-aminopeptidase in the presence of both strict- and epi-anecic earthworms were significantly higher than in the presence of strict-anecic and lower than in the presence of epi-anecic earthworms (Supplementary Table S2, Fig. 2f-j). In addition, some differences were observed between the earthworm pairs composed of one epi- and one strict-anecic earthworm. For example, the activities of the β-D-glucosidase, cellobiohydrolase

and leucine-aminopeptidase in the presence of the AG/LT pair were significantly higher than those of the AG/LR pair (F = 2.03 to 3.09, P < 0.02, Supplementary Table S2).

Concerning the two soil microsites, the FDAse activity was significantly 16% higher in middens than in burrows (F = 41.53, P < 0.001, Supplementary Table S2, Fig. 3a), conversely to the acid phosphatase activity that was significantly 3% lower in middens than in burrows (F = 5.19, P = 0.024, Supplementary Table S2, Fig. 3e). The β-D-glucosidase and leucine-aminopeptidase activities were similar in the two soil microsites (F = 0.38 and 0.95, P > 0.331, Supplementary Table S2, Fig. 3b and d). With the exception of the cellobiohydrolase activity, most enzymatic activities did not differ between the microsites of mono- and bi-specific assemblages of either strict- or epi-anecic earthworms, (Fig. 3c). This enzymatic activity (cellobiohydrolase) was indeed higher in the middens obtained in the presence of the AG/LT pair than in both middens and burrows in the presence of the AG/LR and AG/LC pairs (significant EW pairs × Site interaction, F = 1.79, P = 0.039, Supplementary Table S2, Supplementary Fig. S5).

4. Discussion

4.1. Mono-specific assemblages

In this study, we observed that only epi-anecic earthworms contributed to surface litter mass loss of *L. perenne* and, as previously observed by Hoeffner et al. (2018), this contribution was strongly correlated to the initial earthworm biomass. The rate of surface litter mass loss in the presence of *L. terrestris* was 4.9 mg g^{-1} day^{-1} which is similar to previous studies that reported rates ranging from 2.4 (Binet and Trehen, 1992) to 10.4 mg g^{-1} day^{-1} (Curry and Bolger 1984). Moreover, in the present study, rate of litter mass loss with *L. centralis* was 5.3 mg g^{-1} day^{-1} confirming the

first observation of Hoeffner et al. (2018). In the presence of *L. rubellus*, the rate of litter mass loss was 17.0 mg g^{-1} day^{-1}, which was slightly lower than the 23.1 mg g^{-1} day^{-1} observed by Xia et al. (2011) with *Liriodendron tulipifera* litter. Conversely, no significant contribution of the strict-anecic earthworms to the surface litter mass loss was observed after 30 days of experiment. Previous studies on the feeding behaviour of strict-anecic earthworms reported inconsistent results. Some of them highlighted rates of fresh litter mass loss varying from 7.1 mg g^{-1} day^{-1} with *A. giardi* fed *Triticum aestivum* (Cortez et al., 1989) to 16.3 mg g^{-1} day^{-1} with *A. caliginosa meridionalis* fed *Castanea sativa* (Cortez and Bouché, 2001). However, it is not clear if these studies took into account the litter mass loss resulting from microbial activity while their contribution can be substantial and should not be overlooked (Coûteaux et al., 1995). In the meantime and in line with our findings, Eisenhauer et al. (2008) observed that *Aporrectodea longa* (strict-anecic earthworm) did not contribute to surface litter mass loss and other studies, using isotopic markers, noticed that *A. longa* and *A. giardi* preferred to feed on humified SOM rather than on fresh surface litter (Cortez et al., 1989; Jégou et al., 1998, 2000; Andriuzzi et al., 2016; Larsen et al., 2016).

Such different behaviour between strict- and epi-anecic earthworms could induce differences in their interactions with soil microorganisms through a specific effect on microbial resources. Contrary to strict-anecic earthworms, epi-anecic earthworms, by contributing to surface litter mass loss, concentrate fresh leaf litter in their burrows (Jégou et al., 1998, 2000; Andriuzzi et al., 2016) and their gut contents were reported to be richer in litter than those of strict-anecic earthworms (Bouché and Kretzschmar, 1974; Piearce, 1978; Ferrière, 1980). Consequently, several studies reported enhanced contents of total C, N and P in the burrows (Parkin and Berry, 1999; Tiunov and Scheu, 2000; Hoang et al., 2017) and the middens of epi-anecic earthworms compared to the surrounding soil (Subler and Kirsch, 1998; Wilcox et al., 2002; Aira et al., 2009). The feeding

activity of epi-anecic earthworms and the subsequent transfer of litter into the mineral soil is of key importance to enhance soil microbial activity (Binet et al., 1998; Subler and Kirsch, 1998; Zimmer et al., 2005; Hoang et al., 2016). Thus, in the present study, the five enzymatic activities measured were enhanced in the presence of epi-anecic earthworms. Several studies have also observed higher enzymatic activities in *L. terrestris* burrows or middens compared to the surrounding soil, including β-D-glucosidase (Don et al., 2008; Lipiec et al., 2016; Hoang et al., 2016; Athmann et al., 2017), leucine aminopeptidase (Athmann et al., 2017), acid and alkaline phosphatase (Jégou et al., 2001; Schrader and Seibel, 2001; Lipiec et al., 2016; Hoang et al., 2016) and cellobiohydrolase (Don et al., 2008; Hoang et al., 2016). However, for the first time, we report here that both *L. rubellus* and *L. centralis* also enhanced enzymatic activities in their burrows and middens. Interestingly, within the three epi-anecic earthworms studied, although the contribution of *L. terrestris* to litter mass loss was the biggest, corresponding to the heaviest species, it did not induce a higher increase of soil enzymatic activities compared to the smaller *L. rubellus* or *L. centralis*. This finding suggests that the stimulation of enzymatic activities by epi-anecic earthworms does not depend on earthworm individual biomass. In the present study, β-D-glucosidase (C cycle) and leucine-aminopeptidase (N cycle) were the most stimulated activities, which is in line with the presence of easily available compounds (cellulose or hemicellulose) in the *L. perenne* leaves and their low C:N ratio, respectively (Hoeffner et al., 2018).

Even if they did not contribute to surface litter mass loss, the strict-anecic earthworms stimulated the β-D-glucosidase activity. Jégou et al. (2001) also reported a specific enhancing effect of the presence of strict-anecic earthworms on the dehydrogenase activity in the burrows of *A. giardi* while the acid and alkaline phosphatase activities were not affected. Strict-anecic earthworms, by feeding mainly on SOM, might enrich their burrows and middens with their mucus,

urine and cast deposits that should be concentrated in organic compounds as observed for *L. terrestris* (Needham, 1957; Laverack, 1963) and increase soil moisture. Thus, they might stimulate other enzymatic activities involved in the hydrolysis of more recalcitrant compounds, such as lignin or cutin which represent a significant part of plant litter input into the soil but were not measured in the present study (Gleixner et al., 2001). Moreover, the burrow network of strict-anecic earthworms is more expanded and broadened than that of epi-anecic earthworms which build only one or two main burrows (Jégou et al., 2000, 2001; Bastardie et al., 2005). This could dilute the organic matter inputs in strict-anecic earthworms burrows compared to those of epi-anecic earthworms, reducing the observed effects on soil microbial enzymatic activities.

4.2. Bi-specific assemblages

In contrast to our second hypothesis of synergistic effect between species, the litter mass loss in the presence of earthworms from different species within and between each anecic earthworm ecological sub-categories was simply additive. Similar results were observed for the enzymatic activities that were additive in pairwise interaction within each sub-categories while few exceptions were observed in some strict- and epi-anecic assemblages. For example, when *A. giardi* was paired with *L. terrestris*, the level of several enzymatic activities was higher than when it was paired with *L. rubellus*, suggesting that some specific earthworm combinations promote enzymatic activities. Such specific effects might be explained by the enhanced decomposition of relative recalcitrant organic compounds when easily decomposable organic matter is present due to mechanisms of facilitation or complementary resource used by diverse detritivores (Wardle et al., 1997; Hättenschwiler and Gasser, 2005).

According to the differences in the feeding behaviour observed between the two earthworm sub-categories, one could have expected an increase in surface litter mass loss induced by some commensal interactions. The epi-anecic earthworms, by actively burying fresh litter, should indeed provide SOM for the strict-anecic earthworms without being impaired. For example, Xia et al. (2011) previously observed a synergistic effect on surface litter mass loss in the presence of *L. rubellus* (epi-anecic earthworm) and *Octolasion lacteum* (endogeic earthworm), i.e. between two different earthworm ecological categories, with endogeic earthworms feeding on SOM. Based on the literature, synergistic effects on litter mass loss might be expected when organisms show high functional dissimilarity between their feeding behavior (Heemsbergen et al., 2004; Zimmer et al., 2005; De Oliveira et al., 2010; Coulis et al., 2015). It seems that, in the present study, strict- and epi-anecic earthworms, even if they relied on different nutrient sources (fresh surface litter *vs.* SOM), should not be functionally dissimilar enough to synergistically interact. Thus, pairwise species interactions between the two sub-categories led mainly to additive effects on both surface litter mass loss and enzymatic activities. Moreover, as previously reported by Hoeffner et al. (2018), our study confirms that litter mass loss may in certain ecological contexts be predicted by epi-anecic earthworm biomass in the field.

4.3. Anecic earthworm microsites and enzymatic activities

Very few studies have analysed the variability of enzymatic activities within the drilosphere (Hoang et al., 2016; Athmann et al., 2017). Hoang et al. (2016) observed that within the network of burrows of *L. terrestris*, activities of the acid phosphatase, chitinase and β-D-glucosidase were more stimulated in hotspots and more frequently stimulated than in the surrounding soil. However, to our knowledge, no studies compared these activities in the different soil microsites created by

earthworms (i.e. middens and burrows) while they present differences in their physical, biological and chemical properties due to the way they are constructed (Nielsen and Hole, 1964). In this study, enzymatic activities were differently affected according to these two microsites created by earthworms. FDAse, which is an indicator of global soil activity, was significantly higher in anecic earthworm middens than in their burrows. Middens are a mixture of buried litter, casts, and mucus that together make them a hotspot of biological activity thus stimulated soil microorganisms (Subler and Kirsch, 1998; Schrader and Seibel, 2001).

The β-D-glucosidase and leucine-aminopeptidase activities that are involved in C and N cycles, respectively, showed a trend to higher values in the earthworm middens compared to the burrows, while the opposite was observed for the acid phosphatase activity. These results might reflect contrasting nutrient requirements for soil microorganisms in anecic earthworm burrows and middens. Enzymatic activities are indeed commonly considered as indicators of microbial nutrient demand and do not necessarily indicate a greater amount of available elements (Olander and Vitousek, 2000; Moorhead and Sinsabaugh, 2006; Sinsabaugh et al., 2008). Thus, by being composed of higher amount of cast compared to the burrow, middens should be characterized by lower C and N availability that enhance the enzymatic activities involved in C and N cycling. Moreover, earthworm casts are known to be enriched in P (Tiwari et al., 1989; Basker et al., 1993) derived from plant litter, that is released during the earthworm gut transit (Mansell et al., 1981) and should thus end up in middens, inhibiting the activity of enzymes involved in this element cycling. Scheu (1987) also observed that P was not limiting in casts from *A. caliginosa* (endogeic earthworm) while it was in burrow or in soil. This higher need of P for soil microorganisms in earthworm burrows should have enhanced the acid phosphatase activity in this soil microsite.

Conclusion

Many studies have observed that anecic earthworms contribute to litter burying and thus improve litter decomposition through microbial enhancement, but these studies did neither take into account earthworm species diversity within this anecic earthworm category nor their interactions. Here, we report that epi-anecic earthworms (*Lumbricus* sp.) contributed to surface litter mass loss and stimulated five soil enzymatic activities measured in both burrows and middens conversely to strict-anecic earthworms (*Aporrectodea* sp.). These results reflected differences in the behaviour of these two earthworm sub-categories, epi-anecic earthworms consuming essentially fresh plant litter and strict-anecic earthworms feeding mainly on humified SOM. Moreover, we report for the first time that pairwise species interactions within and between strict- and epi-anecic earthworms did not enhance surface litter mass loss and enzymatic activities. Independently of earthworm species, enzymatic activities were differently affected in earthworm middens and burrows, probably reflecting some different nutrient requirements. This study highlights that within anecic earthworms, strict- and epi-anecic earthworms have different impact on litter decomposition and confirms the functional differentiation of these ecological sub-categories.

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Figure legends

Fig. 1. Litter mass loss of *Lolium perenne* after 30 days of experiment according to the 6 anecic earthworm species in mono-specific assemblages (a) and to the combinations of earthworm species within and between the two ecological sub-categories (b). Values are means ± SD; initial litter mass = 3.5 g. Different letters denote significant differences among earthworm pairs with a > b > c (Tukey HSD test results). AM = *A. caliginosa meridionalis*; AN = *A. nocturna*; AG = *A. giardi*; LR = *L. rubellus rubellus*; LF = *L. festivus*; LC = *L. centralis*; LT = *L. terrestris*. n = 5 for each anecic earthworm species in monospecific assemblages (panel a). SAn1 = Mono-specific assemblages of strict-anecic, n=14; SAn2 = Bi-specific assemblages of strict-anecic, n=15; SAn/EpA = Bi-specific assemblages with one strict- and one epi-anecic, n=44; EpA2 = Bi-specific assemblages of epi-anecic, n=13; and EpA1 = Mono-specific assemblages of epi-anecic, n=15.

Fig. 2. Enzymatic activities of FDAse (a, f), β-D-glucosidase (b, g), cellobiohydrolase (c, h), leucine-aminopeptidase (d, i) and acid phosphatase activities (e, j) after 30 days of experiment according to the 6 anecic earthworm species in mono-specific assemblages (a, b, c, d, e) and to the combinations of earthworm species within and between the two ecological sub-categories (f, g, h, i, j). Enzymatic activities were expressed in nmol of substrate consumed h^{-1} g^{-1} dry soil. Values are mean ± SD across burrows and middens. Different letters denote significant differences among earthworm assemblages with a > b > c (Tukey HSD test results). AM = *A. caliginosa meridionalis*; AN = *A. nocturna*; AG = *A. giardi*; LR = *L. rubellus rubellus*; LF = *L. festivus*; LC = *L. centralis*; LT = *L. terrestris*. n = 8 to 10 for each anecic earthworm species in monospecific assemblage (panels a, b, c, d and e). SAn1 = Mono-specific assemblages of strict-anecic, n=28; SAn2 = Bi-specific assemblages of strict-anecic, n=30; SAn/EpA = Bi-specific assemblages with one strict-

and one epi-anecic, n=88; EpA2 = Bi-specific assemblages of epi-anecic, n=26; and EpA1 = Mono-specific assemblages of epi-anecic, n=30.

Fig. 3. Activities of (a) FDAse, (b) β-D-glucosidase, (c) cellobiohydrolase, (d) leucine-aminopeptidase and (e) acid phosphatase activities after 30 days of experiment in anecic microsites (middens and burrows). Enzymatic activities were expressed in nmol of substrate consumed h^{-1} g^{-1} dry soil. Values are mean ± SD; n = 101. Different letters denote significant differences among anecic microsites with a > b (Tukey HSD test results).

Fig. 1

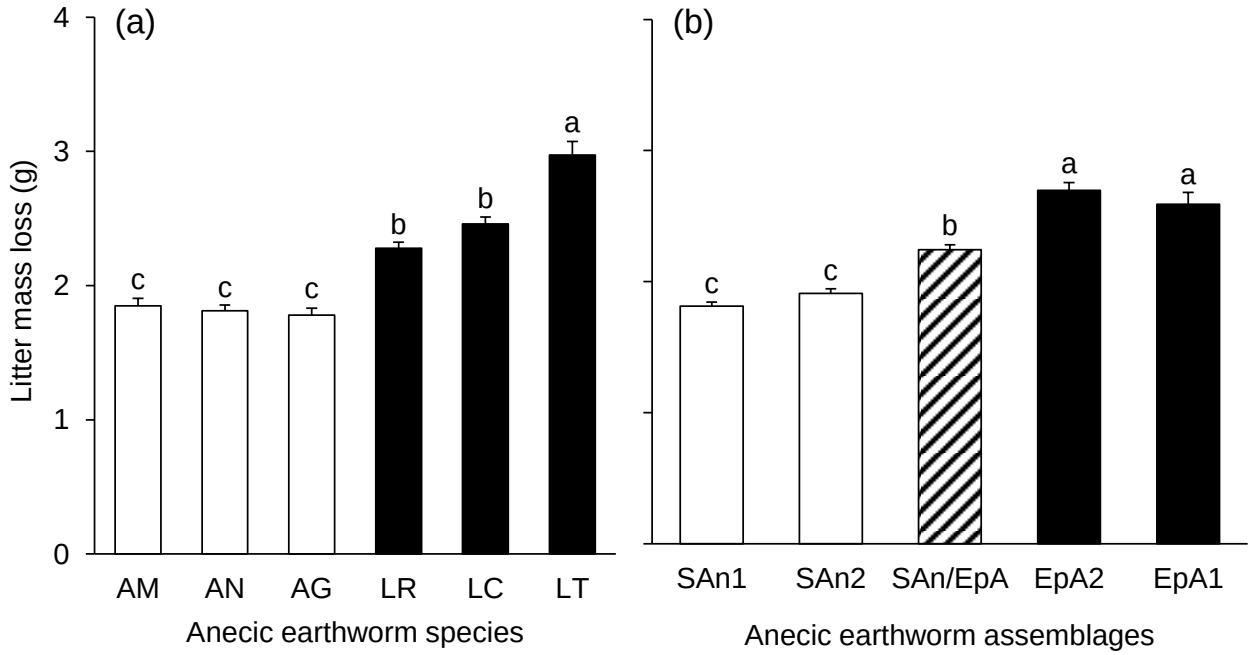

(a)
(b)
Litter mass loss (g)
0
1
2
3
4
c
c
c
b
b
a
AM
AN
AG
LR
LC
LT
Anecic earthworm species
c
c
b
a
a
SAn1
SAn2
SAn/EpA
EpA2
EpA1
Anecic earthworm assemblages

Fig. 2.

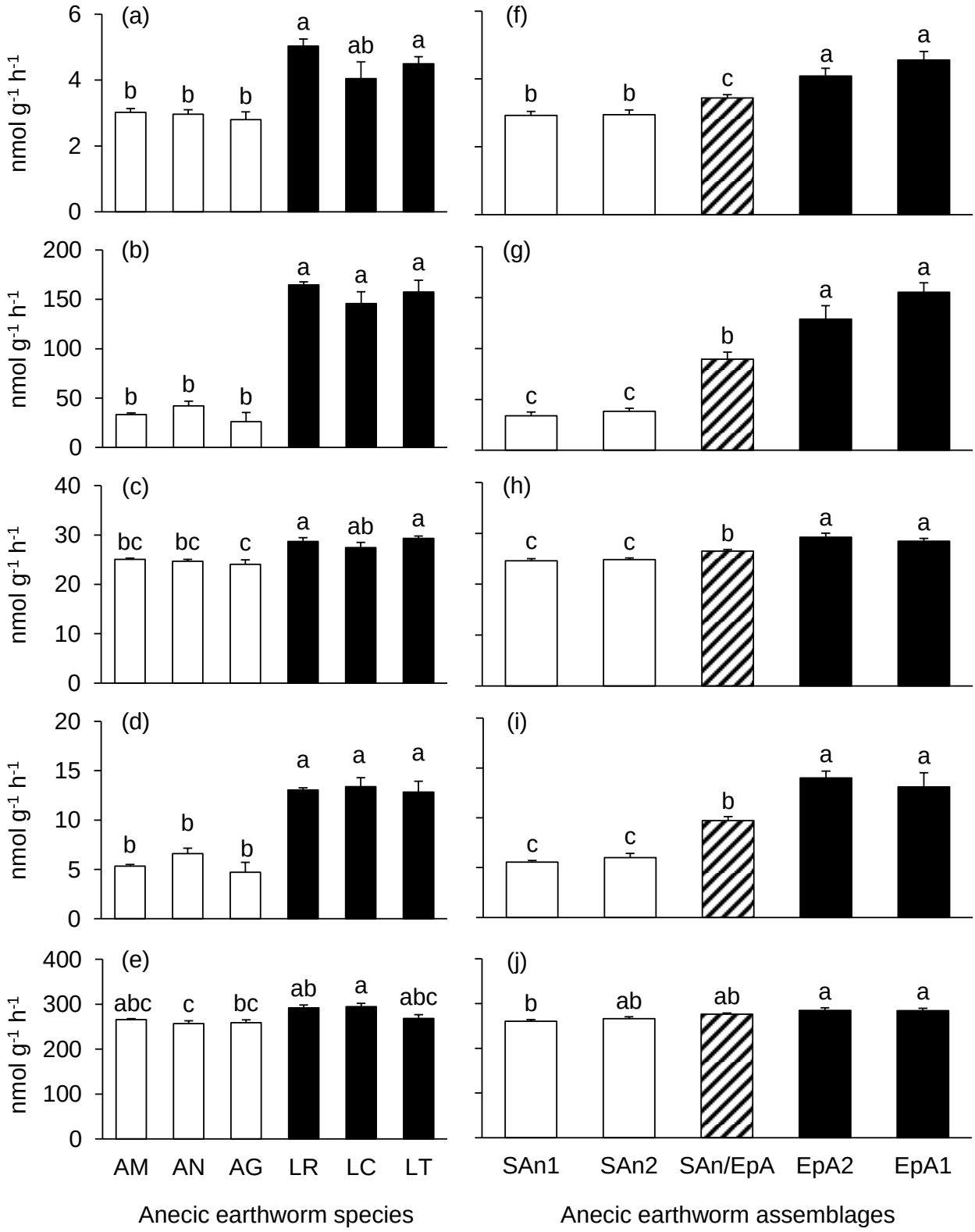
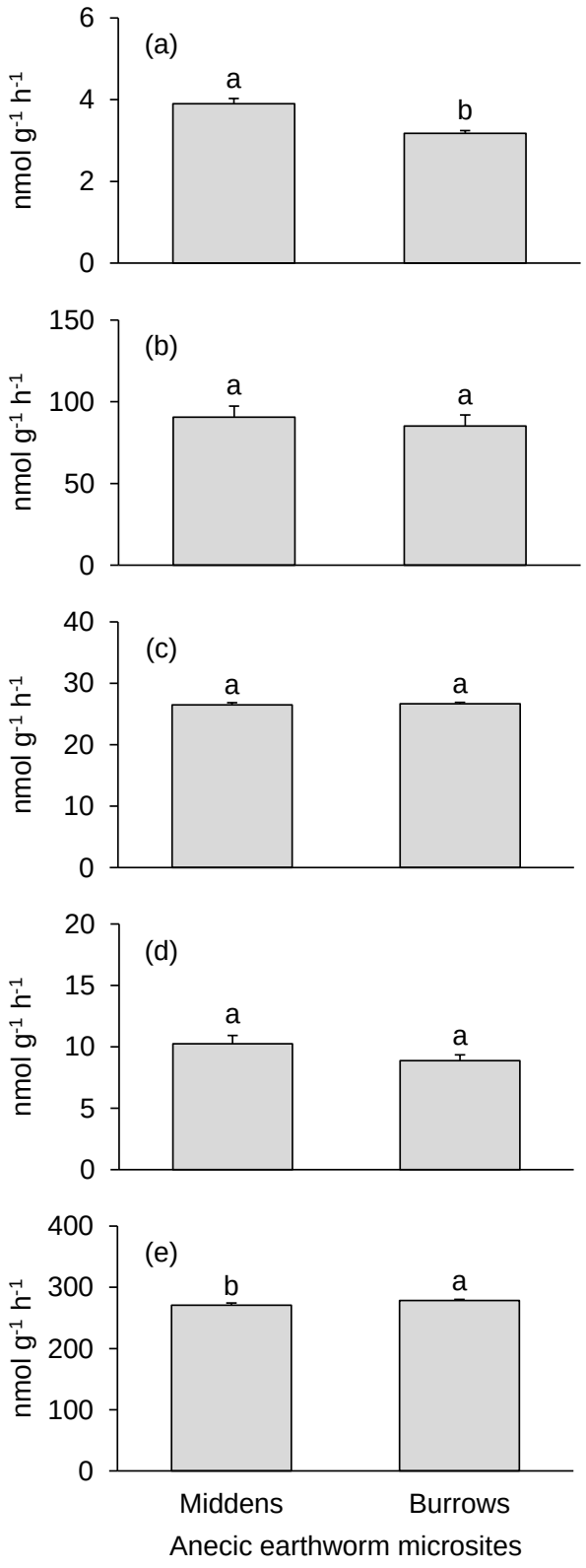

(a)
(b)
(c)
(d)
(e)
(f)
(g)
(h)
(i)
(j)
nmol g-1 h-1
AM
AN
AG
LR
LC
LT
SAn1
SAn2
SAn/EpA
EpA2
EpA1
Anecic earthworm species
Anecic earthworm assemblages

Fig. 3.


(a)
(b)
(c)
(d)
(e)
nmol g-1 h-1
a
b
Middens
Burrows
Anecic earthworm microsites