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Incorporation of ^{13}C labelled shoot residues in *Lumbricus terrestris* casts: A combination of transmission electron microscopy and nanoscale secondary ion mass spectrometry

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ABSTRACT

Earthworms transform organo-mineral associations in soil, especially by incorporating fresh residues inside casts where the microbial abundance and activity are enhanced. The heterogeneous distribution of organic carbon in these structures influences decomposition levels at the microscale. The incorporation of ^{13}C labelled plant residues by *Lumbricus terrestris* inside cast was investigated, through the innovative combination of two fine scale imaging techniques: transmission electron microscopy and nanoscale secondary ion mass spectrometry (NanoSIMS). The association of these methods sheds new lights on organo-mineral structures. Different types of organic matter (plant residues, microbial remains) were identified in the casts and the freshly incorporated residues could be differentiated from the indigenous organic matter thanks to $\delta^{13}\text{C}$ NanoSIMS mapping. ^{13}C labelled bacteria and fungi abundance and diversity highlight their preeminent role in litter decomposition within casts. Labelled plant residues observed at various stages of decomposition and microorganisms presented highly variable $\delta^{13}\text{C}$ values, emphasizing the complexity of organic matter dynamics and the importance of microscale analyses to describe this variability. Thus, the combination of NanoSIMS and TEM shows great potential to relate organic matter stages of decomposition with their ^{13}C signature.

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1. Introduction

Organic matter is a key resource for soil fauna and microorganisms. Earthworms account for the main invertebrate biomass in soils (Edwards, 2004) and are recognized as essential soil engineers (Lavelle et al., 1998). These saprophagous invertebrates ingest both organic (plant residues and microorganisms) and mineral (soil particles) materials, in different proportions depending on their ecological category. Anecic earthworms, which are the dominant ecological category in European ecosystems, feed on surface litter

which is dragged into their burrows (Lee, 1985). During ingestion, soil and plant residues are mixed, intimately associated with mucus and excreted along burrows or at the soil surface in the form of casts (Guggenberger et al., 1996; Six et al., 2004). This diet influences organic matter evolution within soil, i.e. incorporation, degradation and sequestration (Lee, 1985). Indeed, when plant residues are deposited on the soil surface, they can either be mineralized, releasing CO_2 to the atmosphere, or transferred into the soil as various organic compounds. Earthworms favour the transfer of carbon into soil aggregates (Fonte et al., 2012; Arai et al., 2013), casts and burrows (Jégou et al., 2000; Stromberger et al., 2012). In general, casts, burrow walls and their surroundings present larger carbon concentrations compared to bulk soil or aggregates formed by physical or microbial processes (Jégou et al., 2000; Fonte et al., 2012). However, the impact of earthworms on soil and

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Table 1

Composition of the soil used in the experiment.

Characteristics	Units	Values
Clay (<2 µm)	g kg ⁻¹	189
Loam (2–50 µm)	g kg ⁻¹	248
Sand (50–2000 µm)	g kg ⁻¹	563
Total carbonates (CaCO ₃)	g kg ⁻¹	19.0
CEC Metson	cmol kg ⁻¹	9.90
Organic carbon	g kg ⁻¹	0.12
Nitrogen	g kg ⁻¹	0.01

casts carbon stock is variable, depending on the studied time scales (Lubbers et al., 2013). In the presence of earthworms, a short term mineralization, decreasing the stock of carbon, is usually followed by a long term protection of carbon in soils (Brown et al., 2000). The initial mineralization step is mainly induced by an increase in the microbial activity due to the presence of readily available carbon from mucus or plant residues (Brown, 1995). After few months or years, the drying and ageing of casts tighten the bonds between organic matter, mucus and mineral particles (Brown et al., 2000). This phenomenon leads to the formation of organo-mineral aggregates with higher stability (Six et al., 2004; Zangerlé et al., 2011) where recalcitrant organic matter is integrated and protected from decomposition (Shipitalo and Protz, 1989). The effect of earthworms on carbon cycling has been widely studied using biochemical methods (Hong et al., 2011; Zangerlé et al., 2011). In addition, artificial ¹³C labelling litter has been used to follow the fate of carbon in the soil in the presence of earthworms (Fonte et al., 2007; Fahey et al., 2013). The large scale of analyses used in these studies does not give the possibility to visualize the interaction between soil–plant–microorganisms. However, the heterogeneous distribution of organic carbon in soil structures induces contrasted microbial activity areas. This distribution inside casts using *in situ* fine scale imaging has been little investigated, despite the high potential of this approach.

Nanoscale secondary ion mass spectrometry (NanoSIMS) provides elemental and isotopic maps of organic and/or mineral materials at high spatial resolution (submicron). NanoSIMS brings the capacity to spatially track an isotopic label, hence identifying specific locations of components (Clode et al., 2009; Vogel et al., 2014). It has mainly been used in cosmochemistry, material science, biology and geology (McMahon et al., 2006; Herrmann et al., 2007a; Hoppe et al., 2013). It has recently been applied to soil science, first focussing on soil microorganisms (Herrmann et al., 2007b) and then to characterize organo-mineral associations in soil (Hatton et al., 2012; Heister et al., 2012; Keiluweit et al., 2012; Mueller et al., 2012; Remusat et al., 2012; Mueller et al., 2013; Vogel et al., 2014; Rumpel et al., 2015). Most of the efforts have focused on developing methodologies for calibration (Hatton et al., 2012), sample preparation (Mueller et al., 2012) or quantification of compounds in an artificial soil (Heister et al., 2012). Vogel et al. (2014) went a step further by reporting encouraging results on the proportion of OM associated to mineral particles in soil in the presence of labelled litter. A recent study compared the incorporation, after three years, of ¹³C and ¹⁵N labelled roots at two soil depths and demonstrated, thanks to the NanoSIMS, contrasting processes of stabilization depending on soil depths (Rumpel et al., 2015). Studies using NanoSIMS to understand the role of earthworms in soil are scarce. Gicquel et al. (2013) have double labelled earthworms with N and S to follow their fate in the earthworm intestinal epithelium and in the burrows of a peat soil using NanoSIMS. Thus, NanoSIMS shows great potential to investigate both physical and chemical roles of earthworms at the nanoscale.

Despite the high spatial resolution of the NanoSIMS, the identification of soil organic matter as plant residues or

microorganisms, such as bacteria or fungi, remains challenging. Combinations of NanoSIMS with other microscopic techniques are required (Moore et al., 2012; Remusat et al., 2012; Poch and Virto, 2014). The coupling of NanoSIMS with scanning electron microscopy (SEM) (Heister et al., 2012) or scanning transmission X-ray microscopy (STXM) and near edge X-ray absorption fine structure spectroscopy (NEXAFS) (Remusat et al., 2012) is helpful to identify the nature of the organic material sampled by NanoSIMS. The combination with the nm-scale resolution of the transmission electron microscopy (TEM) can be very powerful in identifying microstructures (Moore et al., 2012). It has also proven its efficiency to investigate organo-mineral associations in soil micro-aggregates (Watteau et al., 2006, 2012) and earthworm burrows (Pey et al., 2013) and casts (Pey et al., 2014). TEM has been used with NanoSIMS to study the structural and chemical properties in plant physiology (Clode et al., 2009; Misson et al., 2009; Smart et al., 2010; Moore et al., 2011).

This work aimed at investigating the incorporation and decomposition of plant residues in soil at the microscale using imaging techniques. To meet this objective, we analyzed two contrasted samples: (1) artificially ¹³C labelled litter prior to its incorporation inside casts and (2) plant residues incorporated inside the structurally complex earthworm casts and the microorganisms implied in their decomposition. We used, for the first time in this field of study, the innovative combination of two fine-scale imaging techniques, namely TEM and NanoSIMS, to characterize the nature of organic matter and to locate and determine the origin of incorporated organic matter thanks to ¹³C labelling.

2. Materials and methods

2.1. The experimental setup

The labelling experiment was performed using a mesocosm filled with approximately 75 L of a loamy-sand soil collected in a pasture (Oise, France). The soil characteristics were obtained from the Laboratoire d'Analyses des Sols (LSA) in Arras (France) (Table 1). The mesocosm was placed in a greenhouse where the soil humidity and temperature were maintained at 23% and 13 °C, respectively. Six anecic earthworms of the *Lumbricus terrestris* species were deposited onto the mesocosm.

Plants of Italian Ryegrass (*Lolium multiflorum*) were artificially labelled in ¹³C (2.9 atom %) at the Alternative Energies and Atomic Energy Commission (CEA) in Cadarache (France). Plants were grown under a controlled and constant ¹³CO₂ enriched atmosphere. Plant shoots were dried and subsequently mixed and homogenized during 40 s with a laboratory blender (Waring Commercial) in order to obtain few millimetre sticks. 250 g of such residues were deposited on the soil surface. After six months of experiment, residues were no longer visible at the soil surface. Some of the recognizable casts were randomly collected at the soil surface, on the same day, creating a composite sample. The initial litter deposited at the soil surface at the beginning of the experiment and the casts were analyzed for carbon and nitrogen using a Vario

Table 2

Initial litter and cast composition. n: Replicate number of analyses. Numbers in brackets indicate the standard error.

Measurements	Organic carbon	Nitrogen	C:N
Units	g kg ⁻¹	g kg ⁻¹	–
Litter (n = 13)	4.1 (0.7)	0.28 (0.2)	14.8 (1.2)
Casts (n = 3)	0.3 (0.2)	0.03 (0.0)	9.8 (0.1)

Fig. 1. TEM micrographs of initial shoots of *Lolium multiflorum*. cp, Chloroplast area; cw, cell wall; ep, epidermis; sg, starch grain.

pyrocube – Micromass Isoprime® installed at the UMR IEES (UPMC, Paris) (Table 2).

2.2. Ultrastructural analyses by TEM

Initial litter and fresh casts (2 g) were chemically fixed with osmium tetroxide and cast structure was physically preserved with agar (Watteau et al., 2006). About ten cubes of few mm³ were cut in the core of the composite cast sample, dehydrated in graded acetone series, and embedded in epoxy resin (Epon 812) until complete polymerization. Five repetitions were thus obtained for the litter and cast samples. Ultrathin sections of 80–100 nm were obtained using a diamond knife on a Leica Ultracut S ultramicrotome. The sections were stained with uranyl acetate and lead citrate and analyzed with a JEOL EMXII transmission electron microscope operating at 80 kV. Each section was investigated in order to obtain a general view of the considered section and the occurrence of representative structures.

2.3. Nano-scale analyses by NanoSIMS

For NanoSIMS analyses, ultrathin sections of 200 nm from the same block of dehydrated osmium-fixed samples as for TEM were used in order to obtain twin sections, making possible the identification of the structures with nanoSIMS. To ensure charge

dissipation, samples were coated with 10 nm of gold. Images were acquired using the NanoSIMS 50 (Cameca, France) located at Museum National d'Histoire Naturelle in Paris, France. Sample surface was sputtered by a 1.5 pA Cs⁺ beam to obtain a 20 × 20 μm² images divided into 256 × 256 pixels, at 1 ms/pixel raster speed, with an approximate spatial resolution of 100 nm. Secondary images of ¹²C[−], ¹²C¹⁴N[−], ¹³C¹⁴N[−], ¹⁶O[−] and ²⁸Si[−] were simultaneously collected; between 50 and 70 frames were accumulated. It must be noted that the beam never got through the 200 nm thick samples, implying a very low sputtering rate (less than 0.04 nm/s). Prior to each acquisition, the sample surface was presputtered over 30 × 30 μm² using a 100 pA Cs⁺ beam for 10 min to remove gold coating and surface contamination and to reach sputtering steady-state. The images were processed using the L'IMAGE® software (L. Nittler, Carnegie Institution, USA). The ¹³C¹⁴N[−]/¹²C¹⁴N[−] ratio was used to generate ¹³C isotopic maps, relative to the PDB standard. For the sake of clarity, the values will be turned δ¹³C in the following. It must be noted that ¹²C¹⁴N[−] emission from the epoxy resin is negligible compared to the organic matter signal. A kerogen standard was used to check for instrumental stability over the course of the analytical session. As the instrumental fractionation was lower than 5‰ during the session, it was neglected for the δ¹³C correction. The resin was masked on the δ¹³C maps using a threshold defined thanks to the ¹²C¹⁴N pixel distribution. In brief, we determined that resin count rate was about 10,000 counts/s. Thus, we could mask the resin-rich areas and kept, for the isotopic images, only areas with the largest count rate. Regions of interest (ROI) were manually drawn to isolate microorganisms or plant residues and determine their isotopic composition. A total of seven NanoSIMS images were acquired on samples from the labelled experiment. The initial labelled litter was analyzed and images were used for the interpretation of cast images.

3. Results

3.1. Labelled litter characterization

Labelled litter TEM images revealed plant tissues such as thin cell walls, chlorophyll parenchyma and epidermis cells (Fig. 1). Within the cells of the parenchyma, chloroplast areas containing starch grains were recognized. Similar features were identified on NanoSIMS images (Fig. 2). The mean δ¹³C value for plant tissue was 1004‰. Cell walls and chloroplasts presented different mean δ¹³C

Fig. 2. NanoSIMS images of the ¹²C¹⁴N[−] and δ¹³C in initial shoots of *Lolium multiflorum*. For the isotopic image, a mask was applied to remove the resin contribution, using a defined threshold for the ¹²C¹⁴N[−] signal. Black regions hence represent either a hole on the section or resin. cp, Chloroplast area; cw, cell wall; ep, epidermis cell; sg, starch grain.

Fig. 3. TEM micrographs of casts of *Lumbricus terrestris*. (a) Differently-sized organic and mineral particles, isolated or associated; (b) packed plant cells; (c) degraded cell walls; (d) Organo-mineral aggregate; (e) Organo-mineral and bacterial aggregates and (f) Fungi. aom, amorphous organic matter; b, bacteria; ba, bacterial aggregate; br, bacterial residue; cw, cell wall; f, fungi; fr, fungal residue; h, hole; m, mineral; s, bacterial spore.

values, with 804 (standard error: 120) and 1041 (standard error: 57) ‰, respectively.

3.2. Earthworm casts characterization

The investigated cast contained organic and mineral particles with different sizes, either isolated or associated into aggregates (Fig. 3a). Aggregates showed variable proportion of organic matter – plant residues and microorganisms – and minerals. Organic constituents presented a large diversity of nature. Plant tissues could still be identified and they exhibited various degradation stages as a function of their fragmentation and degradation level (Fig. 3b and c). Vegetal tissues were still recognizable even though cells were packed (Fig. 3b) or cell walls degraded (Fig. 3c). Many long and twisting ‘laces’ (Fig. 3a) resembling to thin cell walls of initial litter (Fig. 1), were therefore recognized as cell walls residues. Cell walls residues were either isolated (Fig. 3a) or included in aggregates with quartz or clays (Fig. 3a and d). Minerals were also

present as isolated particles (Fig. 3a and d). Microorganisms were also frequently observed. As plant tissues were not completely degraded, bacteria colonized these residues (Fig. 3c). Bacteria could also be observed either isolated or forming colonies (Fig. 3a) and/or associated within aggregates (Fig. 3a and d). Amorphous organic matter, likely including exopolymers produced by bacteria was associated with minerals to form typical bacterial aggregates (Fig. 3e and f). Most of these bacteria still appeared intact, but some residues were present as bacterial cell walls (Fig. 3a and f) and spores, recognized for their dark core and coat (Willey et al., 2008) (Fig. 3c and e). Some fungi, intact or as residues were identified, based on their size (>bacteria) and/or their thick cell wall as in extant organisms (Fig. 3f).

The different organic components in casts were identified on NanoSIMS images with a high degree of confidence thanks to their typical size and shape as observed on TEM images. In addition the high $^{12}\text{C}^{14}\text{N}$ confirmed the biological origin of the observed structures. The ‘laces’ identified as plant cell walls on TEM images

Fig. 4. NanoSIMS maps of ^{16}O -, ^{28}Si -, $^{12}\text{C}^{14}\text{N}$ - and $\delta^{13}\text{C}$ illustrating the organic matter diversity in casts of *Lumbricus terrestris*. For the isotopic image, a mask was applied to remove the epoxy resin contribution, using a defined threshold for the $^{12}\text{C}^{14}\text{N}$ - element. b, Bacteria; cw, cell wall; f, fungi.

(Fig. 3a) were recognizable in NanoSIMS images (Fig. 4), along with structures identified as bacteria, isolated or forming colonies in aggregates and few fungi (Figs. 4 and 5). The organic nature of the identified structures was confirmed by the high carbon and nitrogen content evidenced by the $^{12}\text{C}^{14}\text{N}$ - signal (Figs. 4 and 5). The ^{28}Si and $^{12}\text{C}^{14}\text{N}$ images clearly indicated the presence of organo-mineral associations as observed on TEM images. The $\delta^{13}\text{C}$ maps revealed the presence of labelled and unlabelled organic matter, which were heterogeneously distributed in earthworm casts (Figs. 4 and 5). Both plant residues and microorganisms were labelled. Labelled cell walls from plant residues were easily recognizable (Fig. 4). Structures identified as labelled microorganisms were mainly represented by individual bacteria in aggregates (Fig. 4) or gathered as colonies (Fig. 5). Labelled fungi were also observed (Fig. 4). For example, on Fig. 4, the $\delta^{13}\text{C}$ for plant residues, bacteria and fungi reached 279, 304 and 191‰, respectively.

For the seven images obtained on the investigated casts, the mean $\delta^{13}\text{C}$ was 54‰, varying from –5 to 148‰ (Table 3). A total of 38 and 66 ROI were drawn from the images, for plant residues and bacteria, respectively. Thus, we obtained the isotopic values for each selected ROI. No significant differences in $\delta^{13}\text{C}$ were observed between labelled material in plant residues, bacteria and fungi. Plant residues and bacteria presented a mean $\delta^{13}\text{C}$ of 238 and 334‰, respectively. 70% of the labelled bacteria presented a $\delta^{13}\text{C}$ between 150 and 350‰, whereas plant residues were more uniformly distributed between 0 and 550‰ (Fig. 6).

4. Discussion

4.1. Incorporation of plant residues in casts

TEM and NanoSIMS images reveal a complex spatial organization of organic and mineral constituents in the earthworm casts. Casts are known to be rich in organic matter from many studies using biochemical (Bossuyt et al., 2004, 2005; Pulleman et al., 2005; Oyedele et al., 2006; Fahey et al., 2013) and TEM (Pey et al., 2014) approaches. The association of TEM and NanoSIMS analyses enables to distinguish, on a same sample, the different constituents inside the casts and to determine if they originated from the ^{13}C labelled litter deposited on the soil surface.

As expected, the observations of the image allow the visualization of the incorporation of plant residues by earthworms inside casts. Moreover we identified plant residues in various stages of degradation (Fig. 3), originating either from residues previously incorporated in the soil ($\delta^{13}\text{C} < 0$) or from the added labelled litter (Figs. 4 and 5). The time of residence, the localization and the way the litter was incorporated in the soil structure could influence decomposition of the plant residues. Thus, the different stages of degradation of plant residues could result from degradation having occurred before the beginning of the experiment or from differences in residue localization on, or in, the soil structure. The various degradation levels could also result from different litter incorporation techniques by earthworms. Indeed, residues can be

Fig. 5. NanoSIMS maps of ^{16}O -, ^{28}Si -, $^{12}\text{C}^{14}\text{N}$ - and $\delta^{13}\text{C}$ in casts of *Lumbricus terrestris*, highlighting the presence of individual bacteria (b) and bacterial aggregate (ba). For the isotopic image, a mask was applied to remove the epoxy resin contribution, using a defined threshold for the $^{12}\text{C}^{14}\text{N}$ element.

incorporated directly or indirectly by earthworms. During the direct incorporation by ingestion, residues are highly fragmented by the grazing activity of earthworms and grinded during the transit, especially passing the gizzard where they are submitted to strong muscular contractions in the presence of mineral particles (Shipitalo and Protz, 1989). The indirect incorporation consists in a pre-oral mechanism, so-called ‘ploughing-in’ of residues, where litter is embedded by fresh casts which initiate microbial decomposition (Cortez and Bouché, 1998). The presence of hardly, partially or totally decomposed plant cells on TEM and NanoSIMS images could suggest that both mechanisms took place in the studied casts.

4.2. Abundant microorganisms in casts

Cast images obtained in this study show an important microbial population as observed in previous studies using TEM (Barois et al., 1993; Pey et al., 2014) or microbial analysis (Postma-Blaauw et al., 2006; Frouz et al., 2011). TEM and NanoSIMS images of the earthworm casts reveal the presence of bacteria both in active and spore forms, as observed by Pey et al. (2014). The presence of few spores may indicate that some active bacteria encountered unsuitable environmental condition during the transit in the mobile anoxic microarea formed by the earthworm gut inducing sporulation (Brown, 1995; Drake and Horn, 2007). As the gut transit time is long for anecic earthworms, especially for *L. terrestris* (from 8 to 20 h)

(Hartenstein and Amico, 1983), some microorganisms get more time for adaptation and multiplication (Brown, 1995). Surviving cells and spores can then rapidly multiply and recover their activity in casts (Brown, 1995). Indeed, the increased surface area of plant residues incorporated in casts and the high concentration of readily available carbon contained in mucus secreted by earthworms, favours proliferation of bacteria and their further decomposition of residues (Parle, 1963; Brown, 1995; Amador and Göres, 2007; Frouz et al., 2011). Bacteria occur in the investigated cast either isolated or in cluster inside aggregates (Fig. 5). The presence of bacterial cluster was reported to be frequent in casts compared to bulk soil and interpreted as a consequence of higher organic matter content (Kristufek et al., 1994; Frouz et al., 2011). We also observed fungi in the casts (Figs. 3 and 4). Tiwari and Mishra (1993) have shown that fungi are more abundant and present a higher diversity in casts compared to bulk soil. More specifically, anecic earthworms generally favour the development of rapidly growing fungi that can easily assimilate materials in casts (Moody et al., 1995). As in the case of bacteria, this development can be explained by the favourable growth conditions in casts (Brown, 1995).

4.3. Residues decomposition and the role of microorganisms in casts

The $\delta^{13}\text{C}$ values of the plant residues observed in earthworm casts are lower than those of the initial litter (238 vs 1004‰). Two

Table 3
 $\delta^{13}\text{C}$ values for the total NanoSIMS image, labelled plant residues, bacteria and fungi. The total $\delta^{13}\text{C}$ values of NanoSIMS images were obtained using a same threshold defined thanks to the $^{12}\text{C}^{14}\text{N}^-$ distribution. For each category; n: number of manually drawn ROI used to obtain the mean $\delta^{13}\text{C}$ values, the minimum and maximum of the $\delta^{13}\text{C}$ values are also indicated. The images 5 and 6 correspond to Figs. 4 and 5, respectively.

Images id	Total	Plant residues	Bacteria	Fungi
Units	‰	‰ (min; max)	‰ (min; max)	‰
1	72	250 (n = 4) (22; 401)	186 (n = 13) (14; 497)	NA
2	104	253 (n = 12) (20; 509)	395 (n = 4) (122; 597)	NA
3	–5	129 (n = 1) –	408 (n = 7) (109; 943)	NA
4	47	176 (n = 6) (45; 326)	483 (n = 2) (258; 707)	NA
5	10	279 (n = 3) (201; 358)	304 (n = 17) (109; 723)	191 (n = 1)
6	3	158 (n = 4) (31; 270)	236 (n = 12) (144; 489)	NA
7	148	421 (n = 8) (281; 633)	326 (n = 11) (115; 665)	NA

explanations can be put forward to account for this difference. First, heterogeneity of labelling cannot be excluded as Nguyen Tu et al. (2013) have demonstrated that leaves from labelled Ryegrass grown under constant $^{13}\text{CO}_2$ -enriched atmosphere present important isotope variability, among and within leaves. In this respect, the fragments of residues incorporated by earthworms in the investigated casts would originate from poorly labelled litter fragments, as observed on the nanoSIMS image of initial litter (Fig. 2). However, the isotope variability between initial litter and plant residues in casts is much larger than observed by Nguyen Tu et al. (2013) among and within leaves (76% vs 20%). Thus, this hypothesis would at most count for a minor contribution to this difference. Secondly, selective degradation of plant residues may have resulted in ^{13}C depletion as non-structural compounds from litter such as sugars, which are rapidly lost as dissolved carbon, are more strongly labelled than structural compounds such as lignin (Benner et al., 1987; Fahey et al., 2011). Thus, degradation would result in a decrease of the $\delta^{13}\text{C}$ of remaining plant residues (Benner et al., 1987). This hypothesis is supported by the presence of lignin-rich plant cell walls as the main recognizable labelled plant residues on NanoSIMS images of the casts (Fig. 4), the cell constituents having been rapidly degraded. This selective degradation may take place during assimilation by microorganism or by earthworms during ingestion. An indicator of the microbial decomposition is the labelling of microorganisms in casts (Figs. 4 and 5). Indeed, microorganisms are not originally abundant in the initial litter and their mean $\delta^{13}\text{C}$ value is of 334‰ in the casts, highlighting their role in residue decomposition. Furthermore, it has been demonstrated that the high microbial biomass, diversity and activity in casts lead to the recycling of plant residue constituents by microorganisms and their mineralization (Postma-Blaauw et al., 2006). Labile plant

constituents are the main source of microbial products (Cotrufo et al., 2013) because they are efficiently assimilated by microbes and litter cell contents present higher lability than cell walls (Fahey et al., 2011). Thus, microorganisms have probably used the labelled dissolved carbon contained in plant residues. The presence of bacterial cluster in the casts (Fig. 5) confirms that it stands for a ‘hotspot’ for microbial activity. Indeed, the presence of labile substrate creates favourable conditions for microbial development and activity, as recently reviewed by Kuzyakov and Blagodatskaya (2015). The selective degradation of plant residues may also occur upon ingestion by earthworms. Indeed, the latter preferentially assimilate labile carbon and generally little digest and assimilate cell wall constituents (Brown and Double, 2004; Curry and Schmidt, 2007).

In addition to the mean decrease of $\delta^{13}\text{C}$ between initial litter and plant residues in casts, a large variability in values was observed for plant residues present in casts (from 20 to 633‰). Again, this may be explained by the labelled heterogeneity or, more likely, the presence of residues of various decomposition stages in cast. We also observed a high variability of $\delta^{13}\text{C}$ value for bacteria (from 14 to 943‰). Clode et al. (2009) interpreted this variability as the presence of different species that incorporated ^{13}C at different rates or by different cellular activity at the sampling time. As TEM images showed various living states of bacteria, the ‘cellular activity’ hypothesis is likely.

5. Conclusion

Earthworms are recognized as soil engineers that create organo-mineral structures influencing organic carbon incorporation and protection in the soil. The heterogeneous distribution of organic carbon in these structures induces areas with contrasting decomposition levels at the microscale. The combination of TEM and NanoSIMS imaging highlighted the complex microscale organization in earthworm casts. By comparing morphologies on the NanoSIMS and TEM images, the different organic components (plant residues and microorganisms) could be identified within the casts and their associations with mineral particles could be described. This allowed us to assess the influence of earthworm activity on the organic carbon incorporation in casts. Images also revealed that casts are populated with abundant and diverse microorganisms, which actively participate in litter decomposition, as demonstrated by their high labelling. Thanks to ^{13}C labelling and NanoSIMS imaging, we identified the labelled litter incorporated in casts by earthworms. We also observed a significant decrease of $\delta^{13}\text{C}$ from the intact litter to plant residues incorporated in the casts, suggesting decomposition between this two contrasted samples. The various stages of plant residues decomposition and the high variability of $\delta^{13}\text{C}$ values for plant residues and microorganisms highlighted the complexity of organic matter dynamics

Fig. 6. $\delta^{13}\text{C}$ histograms for bacteria and plant residues, with a 50‰ interval. The number of manually drawn ROI was 38 and 66, for plant residues and bacteria, respectively.

and the importance of microscale analyses to identify and explain this variability. The combination of NanoSIMS and TEM shows great potential to relate organic matter states of decomposition with their ^{13}C signature. Further work is in process to develop this potential.

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