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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 $\delta^{13}C$ 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 $\delta^{13}C$ 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.

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 ^{13}C 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 ^{13}C and ^{15}N 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 ^{13}C 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 ^{13}C labelling.

2. Materials and methods

2.1. The experimental setup

The labelling experiment was performed using a mesocosm filled with approximatively 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 ^{13}C (2.9 atom %) at the Alternative Energies and Atomic Energy Commission (CEA) in Cadarache (France). Plants were grown under a controlled and constant $^{13}CO_2$ 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)