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Use of X-ray microcomputed tomography for characterizing earthworm-derived belowground soil aggregates

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Abstract

Soil structure is closely linked to biological activities. However, identifying, describing and quantifying soil aggregates remain challenging. X-ray micro computed tomography (X-ray μCT) provides a detailed view of the physical structure at a spatial resolution of a few microns. It could be a useful tool to discriminate soil aggregates, their origin and their formation processes for a better comprehension of soil structure properties and genesis. Our study aims to (a) determine different X-ray μCT-based aggregate parameters for differentiating earthworm casts belowground (earthworm aggregates) from aggregates that are not formed by earthworms (non-earthworm aggregates), and (b) to evaluate if these parameters can also serve as specific "tomographic signatures" for the studied earthworm species. For this purpose, we set up a microcosm experiment under controlled conditions during 8 weeks, including three species of earthworms tested separately: the epigeic *Lumbricus rubellus*, the anecic *Lumbricus terrestris* and the endogeic *Allolobophora chlorotica*. Our results show that X-ray μCT analysis helps distinguish earthworm aggregates from non-earthworm ones using (a) the relative volume of the components within aggregates and (b) the volumetric mass of aggregates and their global volume. In particular, the volume ratio of mineral grains within the aggregates is significantly different according to earthworm species. So, X-ray μCT is a powerful and promising tool for studying the composition of earthworm casts and their formation. However, future research is needed to take into account the shapes and spatial distribution of the aggregates' components, in particular the different states of organic matter decomposition.

Highlights

- Can earthworm belowground casts be differentiated from other soil aggregates using X-ray μCT?
- The use of X-ray μCT for characterizing soil aggregates at a spatial resolution of a few microns.
- A combination of X-ray μCT variables discriminates earthworm casts from non-earthworm aggregates.

- X-ray μCT, used alone, is relevant for defining species-specific signatures of earthworm casts.

KEYWORDS

Allolobophora chlorotica, belowground casts, earthworms, *Lumbricus rubellus*, *Lumbricus terrestris*, mesocosm, soil aggregates, X-ray microcomputed tomography

1 | INTRODUCTION

Soil structure is defined as the spatial arrangement of mineral and organic particles and their associated pores into groupings called aggregates (Oades, 1993). These latter define porosity, which regulates, among other things, water retention and movement, aeration and temperature. Hence, structure plays a key role in several soil functions and ecosystem services, such as water cycling regulation, plant germination and root growth, as well as resistance to erosion. As a general rule, soil structure results from an interplay between physical (drying/wetting cycles and/or freezing/thawing alternations), chemical (inorganic binding between particles) and biological processes (bioturbation by soil biota, production of gluing components) (Blouin et al., 2013; Piron et al., 2017; Pulleman, Six, Uyl, Marinissen, & Jongmans, 2005; Pulleman, Six, Van Breemen, & Jongmans, 2005; Totsche et al., 2018).

Focusing on soil fauna, earthworms are the main agents of soil structure formation and stabilization in temperate ecosystems (Lavelle et al., 1997). Their effects result mostly from their moving through soil, feeding on preferred organic items, ingesting soil particles, mixing them with organic matter in their gut and finally producing biogenic structures (e.g., burrows and casts) (Lavelle et al., 1997; Le Bayon et al., 2017; Six, Bossuyt, Degryze, & Denef, 2004). Casts are produced on the soil surface and/or belowground in burrows and they differ according to their size (less than 1 mm to a few centimetres), shape (spherical, ovoid, etc.) and stability (from slightly stable to compact) depending on earthworm size and feeding habits (Lee, 1985). Consequently, earthworms affect soil aggregation depending on their behaviour and their interactions. Earthworms have been divided into three ecological categories according to Bouché (1977): (a) epigeics, which mainly live in holorganic soil layers and thus have a small effect on soil structure; (b) endogeics, which feed on belowground organic matter and actively move into the soil layers to satisfy their dietary requirements, thereby becoming major actors of aggregation (Lavelle et al., 2016); and (c) anecics, which incorporate pieces of organic matter deep into the soil, mix them with mineral particles and excrete large amounts of casts both inside and onto the soil surface. Processes inside earthworm guts enhance the formation and stabilization of macroaggregates (>250 μm) (Guggenberger, Thomas, & Zech, 1996; Parmelee et al., 1990; Scullion & Malik, 2000) and also of microaggregates (53–250 μm) inside macroaggregates (Barois, Villemin, Lavelle, & Toutain, 1993; Shipitalo & Protz, 1989). According to Angst et al. (2019), earthworm mucus boosts the microbial activities, which, in turn, accelerate the decomposition of plant-derived organic matter and build-up of microbial necromass. This increase of necromass contributes to the stabilization of organic matter in soil.

Due to the enrichment in mucus during gut transit, freshly emitted casts are less stable than the bulk soil (Schrader & Zhang, 1997). However, they tend to stabilize with time through a combination of physical (thixotropic hardening), chemical (secretion of calcium humate as a binding agent) and biological mechanisms (polysaccharides from gut microflora), as reviewed by Six et al. (2004) and Six and Paustian (2014). Such strengthening enhances the overall stability of the soil structure and hence its resistance to soil erosion.

Although earthworms are major agents of soil structure, it is not clear so far how aggregates are formed. Studies of processes that produce soil structure encounter several methodological challenges for identifying, describing and quantifying both the soil structure and components of the aggregates. Several methods have been developed for identifying the origins of soil aggregates and discriminating the role of biological agents. For example, Hedde, Lavelle, Joffre, Jiménez, and Decaëns (2005) proposed near infrared spectroscopy (NIRS) spectra as fingerprints for identifying macro-invertebrates (earthworms, termites and ants) responsible for soil aggregation. Using the same method, Zangerlé, Hissler, McKey, and Lavelle (2016) established the contribution of different earthworm species to macro-aggregation in three temperate sites (forest, grassland and crop) and demonstrated that each ecological category or species produces casts with a specific NIRS spectral signature. However, the discrimination among casts with this approach is better for young casts than for aged ones and a reference database of the NIR spectral signature is required for each field site. Consequently, reliable signatures are not yet clearly established in field conditions

(Bottinelli, Capowiez, Hallaire, Ranger, & Jouquet, 2013) or in experimental conditions (Schomburg et al., 2018; unpublished data).

Physical fractionation of aggregates is often performed to quantify the percentage of water-stable aggregates (WSA), a robust indicator for estimating soil structural stability in wet conditions (Kemper & Rosenau, 1986; Six et al., 2004). However, Six and Paustian (2014) argued that WSA should be viewed as a complementary tool to other techniques such as thin sectioning and tomography. Since the 1970s, thin sectioning has allowed investigation of the spatial arrangement and the nature of components in aggregates and contributes to understanding the effect of soil biota (i.e., mainly plants, enchytraeids and earthworms) on structure formation (Guenat, Bureau, Weber, & Toutain, 1999; Pulleman, Six, Uyl, et al., 2005; Pulleman, Six, Van Breemen, & Jongmans, 2005). Shipitalo and Protz (1989) quantified the contribution of particulate organic matter in both macro- and microaggregates to the identification of the effects of earthworms on structure formation in differently managed fields. The analysis of images generated on thin sections has been used to quantify, in a semi-automatic way, several properties (number, shape and surface) of aggregates (Jangorzo, Schwartz, & Watteau, 2014). By combining transmission electron microscopy (TEM) and nanoscale secondary ion mass spectrometry (NanoSIMS) imaging, Vidal, Remusat, Watteau, Derenne, and Quenea (2016) assessed the influence of earthworm activities on incorporation of ^{13}C-labelled organic matter in casts. However, even though thin sectioning allows chemical information to be obtained with spectromicroscopic techniques, it is a destructive technique and only allows a two-dimensional visualization, a limitation that can be partly overcome by making a series of successive thin sections (Le Couteulx, Wolf, Hallaire, & Pérès, 2015).

During the last three decades, 3D X-ray computed tomography (CT) has gained increasing importance in analysing the biophysical interactions and structural development in soil systems (see the reviews of Helliwell et al., 2013 and Baveye et al., 2018). These non-destructive analyses are especially used to study earthworm burrows and are based on both undisturbed soil cores (Capowiez, Pierret, Daniel, Monestiez, & Kretzschmar, 1998; Daniel, Kretzschmar, Capowiez, Kohli, & Zeyer, 2008; Koestel & Schlüter, 2019; Liernur et al., 2017; Schomburg et al., 2018; Zhang, Xu, Li, Hou, & Ren, 2017) and repacked soil samples in microcosms (Amossé, Turberg, Kohler-Milleret, Gobat, & Le Bayon, 2015; Capowiez, Bottinelli, Sammartino, Michel, & Jouquet, 2015; Capowiez, Sammartino, & Michel, 2011; Schomburg et al., 2019). For example, Capowiez et al. (2015) demonstrated that some burrow system characteristics clearly differentiate anecic from endogeic earthworms. Concerning the identification of the different soil components, Helliwell et al. (2013) underlined that organic matter, such as plant roots or decomposing residues, are far more difficult to detect than pores. Moreover, Hapca, Baveye, Wilson, Lark, and Otten (2015) developed a method to generate 3D maps of soil chemical properties at the microscale by combining 2D scanning electron microscopy with energy dispersive X-ray (SEM–EDX) data with 3D X-ray computed tomography images. The spatial correlation between the X-ray greyscale intensities and the chemical maps allowed prediction of the 3D chemical composition. However, the prediction was relevant for elements sparsely distributed in a soil sample such as iron, but decreased for homogenously distributed elements, such as carbon, silicon or oxygen.

However, few studies are dedicated to soil aggregates. Garbout, Munkholm, and Hansen (2013) proposed morphometric parameters (volume, surface area, thickness and sphericity) for describing aggregate structure but it appeared that quantifying features needs a higher resolution (e.g., down to 30 μm in size). To solve this problem, the recent use of X-ray microcomputed tomography (X-ray μCT) promises a spatial resolution of a few microns. It thus allows better investigation of not only the porosity and the pore size distribution of soil aggregates (Malobane, Nciizah, Mudau, & Wakindiki, 2019; Nakano et al., 2015; Yu, Fu, & Lu, 2017; Zhao, Xu, Liu, Zhang, & Tuo, 2016) but also the spatial distribution of organic matter in aggregates (Arai et al., 2019; Kravchenko et al., 2014; Kravchenko, Negassa, Guber, & Schmidt, 2014; Peth et al., 2014). By staining soil aggregates with osmium in a vapour phase, Peth et al. (2014) mapped organic matter distribution in undisturbed 5-mm-sized soil aggregates, and recently, Arai et al. (2019) improved this method by combining staining with a liquid-phase osmium with resin embedding.

X-ray μCT is therefore a strong and promising tool for studying soil structure, especially the visualization and quantification of the different components at the aggregate scale. It may thus provide additional information on the origin and formation processes of soil aggregates, whether they were formed by soil fauna or not. For this reason, we investigated the suitability of this very high-resolution technique used alone (i.e., not combined with other methods) for the characterization of earthworm belowground aggregates.

Our study aims to (a) determine whether some X-ray μCT parameters may help differentiate earthworm casts belowground (earthworm aggregates) from aggregates that are not formed by earthworms (non-earthworm aggregates), and (b) evaluate if these parameters may also serve as specific "tomographic signatures" for the studied earthworm species.

For this purpose, we set up a microcosm experiment under controlled conditions for 8 weeks, including three species of earthworms tested separately: the epigeic *Lumbricus rubellus*, the anecic *Lumbricus terrestris* and the endogeic *Allolobophora chlorotica*.

2 | MATERIAL AND METHODS

2.1 | Experimental design

An in-vitro experimental design was set up according to Schomburg et al. (2018). Briefly, mesocosms of 35 cm in height and 14.5 cm in diameter were filled up with 18.5 L of silty sediment sieved at 1 mm and mixed with 0.01% (w/w) of catgrass (*Cyperus alternifolius zumula*; pieces 3–5 mm in length) for feeding earthworms during the experiment (Table 1). The sediment was collected from a recent alluvial deposit from a Calcaric Fluvisol (IUSS Working Group WRB, 2015) in the Sarine River floodplain near Grandvillard (7°04′E, 46°32′N). Earthworm sampling was performed using hot mustard (Lawrence & Bowers, 2002) and three species were selected relative to their ecological behaviour: the epigeic *Lumbricus rubellus* (R), the endogeic *Allolobophora chlorotica* (C) and the anecic *Lumbricus terrestris* (T), all of which are encountered in floodplains (Fournier, Samaritani, Shrestha, Mitchell, & Le Bayon, 2012; Salomé, Guenat, Bullinger-Weber, Gobat, & Le Bayon, 2011). All mesocosms were prepared similarly and randomly allocated to four treatments with earthworms (*L. rubellus*, *A. chlorotica* and *L. terrestris*) and without any earthworms (K). For each treatment, five replicates were set up and a group of three adult earthworms of similar biomass was added to the corresponding mesocosms. The 20 mesocosms were incubated in a climate chamber with day/night cycles of 16/8 hours and 18°C/14°C, respectively. Humidity in the pots was controlled once a week to keep the soil moisture content at field capacity. After 8 weeks of incubation, aggregates formed by earthworms (*L. rubellus, A. chlorotica or L. terrestris*) inside the soil columns were visually selected and carefully sampled using a laboratory spatula before being air-dried. Reference aggregates (control) were obtained by sampling soil from the control pots (K) without earthworms. Some of the aggregates were used for stability measurements (WSA%), and others for the first micro-CT-tomography analyses (see 2.2 for details).

TABLE 1 Initial soil physicochemical parameters measured in the sieved soil and in the catgrass before starting the experiment

Physicochemical parameters of sediment and catgrass	
Sediment	
Silt (%)	67.34 ± 0.96
Sand (%)	20.02 ± 1.13
Clay (%)	12.63 ± 0.24
pH_{water}	7.7 ± 0.03
TOC content (%)	3.92 ± 0.07
Total carbonate content (%)	39.77 ± 4.51
Catgrass	
TOC content (%)	34.42 ± 0.41
N content (%)	0.32 ± 0.01

2.2 | Physicochemical analyses of the initial material

Table 1 summarizes chemical analyses performed on initial sediment and catgrass. Grain size distribution was obtained using a LS 13320 Laser Diffraction Particle Size Analyzer equipped with an APS Auto Prep Station (Beckman Coulter, Brea, CA). A combined pH meter/conductometer (914 pH Meter/conductometer, Metrohm, Herisau, Switzerland) allowed measurement of pH with a soil/water ratio of 1:2.5. Total carbonate content was obtained using a Bernard Calcimeter described by Vatan (1967) and Rock-Eval pyrolysis was used to measure total organic carbon content (TOC) in both sediment and catgrass. Total nitrogen in catgrass was measured using a CHN analyser (Flash 2000 Organic Elemental Analyzer, Thermo Scientific, Waltham, MA). The percentage of water-stable aggregates (WSA) was determined by plunging the earthworm and non-earthworm macroaggregates (between 1 and 2 mm size) into demineralized water for 5 min (Kemper & Rosenau, 1986) using an automatic sieve-immersion apparatus described in Murer et al. (1993), and calculated with correction for the coarse sand content.

2.3 | X-ray µCT analyses of aggregates

A total of 47 individual aggregates were analysed by X-ray micro-CT according to the experimental design in Table 2.

Each individual soil aggregate was air-dried, weighed and placed at the end of a small carbon rod before being scanned with an X-ray micro-CT scanner (Ultratom, RX-Solutions, Chavanod, France). The scanning protocol and parameters were identical for all aggregates. A Hamamatsu high-power X-ray tube was used, in reflexion mode, with a 0.2-mm-thick copper filter and a tungsten target. The acquisition was performed in cone beam mode with a current of 210 µA and a voltage of 55 kV. During volume data acquisition, the soil aggregate was rotated by 360° and 1,632 projections were taken by steps of 0.22° to ensure a very precise volume reconstruction. The X-ray beam attenuation was registered by an XL Varian 2,530 plane detector with 2,176 × 1,792 pixels. The projections were then processed (X-act RX-Solutions, Filtered Back-projection) to reconstruct a corrected volume composed of some hundreds of slices in 16-bit Tiff format (typically between 800 and 1,500 slices). Voxel dimensions were typically of 0.011 mm × 0.011 mm × 0.011 mm.

Images were then processed with AVIZO v. 9.3.0 (FEI, 2018). First, they were segmented by intensity thresholding to differentiate three components of the aggregates that were clearly visually identified on the images: (a) the high attenuation elements that we interpreted as high-density mineral grains (G), (b) the low attenuation elements that we interpreted as the low-density organic matter and the voids and (c) the intermediate attenuation elements that we interpreted as the undifferentiated matrix of the aggregates. Thanks to the significant attenuation contrasts between these three components, the segmentation thresholds could be kept identical for all aggregates. However, to get a segmentation of mineral grains and organic matter + voids that best fit to the visual observation of these components and that did not include undesired voxels (e.g., noise), these thresholds were kept rather conservative and, in a second step, the volume of each of these two components was increased by one voxel in each direction. An example of this segmentation is shown in Figure 1 for a soil aggregate (K4-3).

Secondly, the volume of the segmented components was calculated for each aggregate and gave the contribution in volume percent of grains (GVol), of organic

TABLE 2 Experimental design for the analysis of aggregates by X-ray micro-CT

Studied factor	Code	Mesocosms	Subsamples	Observations
Allolobophora chlorotica	C	3	3	9
Lumbricus terrestris	T	4	3	12
Lumbricus rubellus	R	4	3	12
Without earthworm: control	K	5	3 (except one with only 2)	14
Total		16	12	47

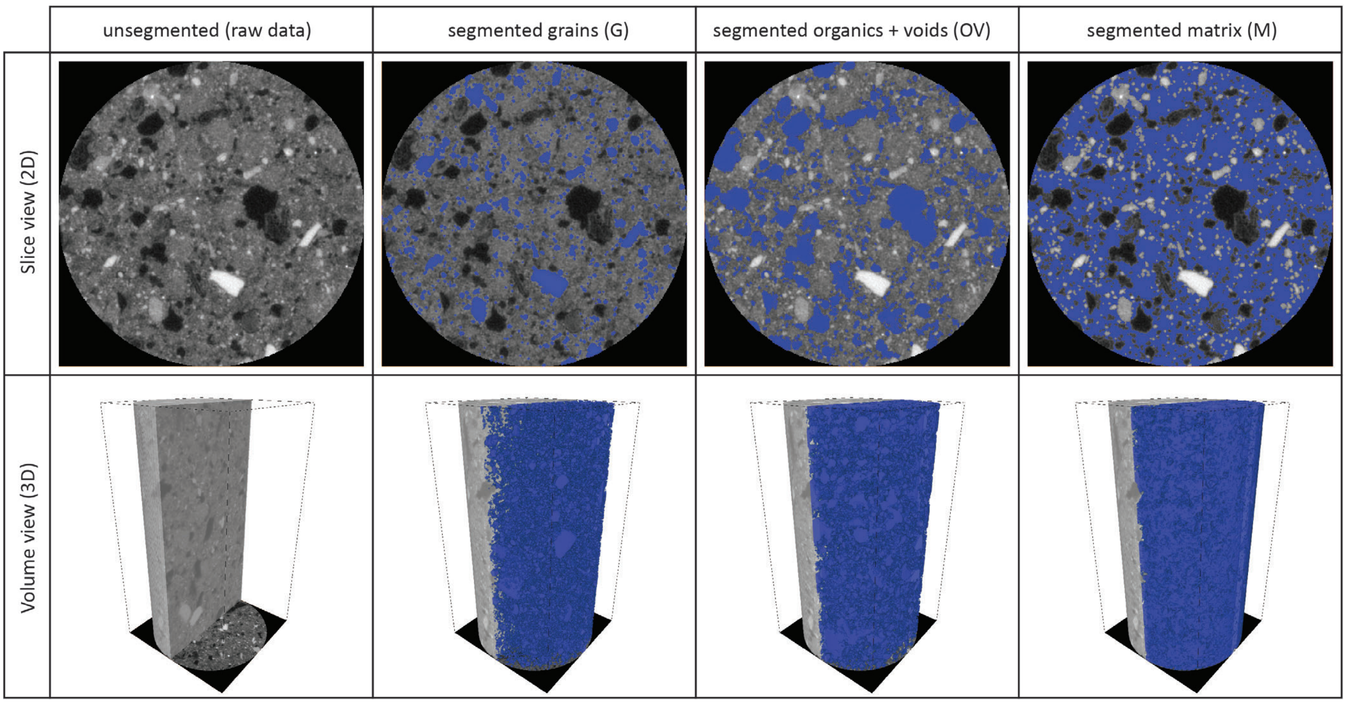

FIGURE 1 Example of the segmentation into three components: grains (G), organic matter and voids (OV) and undifferentiated matrix (M) for the soil aggregate K4-3 ("K" type) in the region of interest #1 (ROI#1). Top: in 2D (selected slice 495 of the ROI#1), the segmented surfaces (in blue) represent 13.9% for G, 26.9% for OV and 59.2% for M. Bottom: in 3D (whole ROI#1 volume), the segmented volumes represent 15.6% for G, 31.9% for OV and 52.5% for M [Color figure can be viewed at wileyonlinelibrary.com]

matter and voids (OVVol) and of matrix (MVol). Moreover, the two following volume ratios were calculated: matrix to grain ratio (MGRat) and organic matter and voids to (matrix + grain) ratio (OVMGRat). It should be noted that this segmentation protocol was not directly applied to the whole aggregate but to three sub-regions of interest (ROI) within each aggregate (except for a very small aggregate, R4, where only two ROIs were used for the calculation). This ROI method was chosen to select as far as possible intact aggregate volumes and, in particular, to avoid including open cracks, due to the drying of the aggregates, in the calculation of the OV volume. The results obtained for each of these three ROIs were then added together into a single volume (VolROI) for subsequent analyses. Finally, the irregular volume of each aggregate (VolAgg) was calculated from the micro-CT-based 3D reconstruction (Figure 2) and, with the weight of the aggregate (WghtAgg), led to a precise calculation of the aggregate density in g/cm^3 (MVolAgg).

2.4 | Statistical analyses

The experimental design is a one-factor design with subsampling. The studied factor is "Earthworm (EW)" with four treatments: C (*A. chlorotica*), R (*L. rubellus*), T (*L. terrestris*) and K (control, without earthworms). The experimental unit is a monospecific mesocosm. A total of 20 mesocosms (five replicates for each treatment) were allocated at random to the four factor levels. The numbers of replicates and subsamples were decided according to the time and material available for the experiment.

Despite suitable experimental conditions, the amount of material was not sufficient to ensure replicates for CT analyses. Thus, analyses of aggregates are based on 16 available mesocosms: C with three replicates, K with five replicates, R with four replicates and T also with four replicates. In every mesocosm, three random subsamples were collected and considered separately for μCT analyses. For testing the effect of factor EW, the mesocosm replicates are true replicates and subsamples within mesocosms for μCT analysis are pseudo-replicates. Several aggregate variables were considered (Table 2). Only five were chosen for the final analysis (GVol, MVol, MGRat, VolAgg and MVolAgg).

The statistical analysis was performed with the software R (R Core Team, 2018). The issue of pseudo-replication was solved differently in univariate and multivariate approaches. In univariate analyses, the lme model used is testing factor earthworm (EW) at the treatment of the subsamples average per mesocosm, giving the correct number of degrees of freedom.

An ANOVA was applied for testing the effect of EW on the individual aggregate variables.

The tested null hypotheses are:

- The effect of factor EW on every individual variable is equal to zero,
- The effect of factor EW on the five individual variables taken as a multivariate system is equal to zero.

In both cases, two-tailed tests were used. We used Tukey multiple comparisons tests for comparing the four EW treatments, only in the case of a significant EW effect. One contrast was of particular interest and tested: the comparison between the control K and the average of the three earthworm treatments (*L. rubellus*, *A. chlorotica* and *L. terrestris*). In the multivariate case, the analysis was based directly on the average values of the mesocosms for the treatment. For redundancy analysis (RDA), data were standardized, allowing comparisons between variables with different units. No data were excluded. The repeatability of the whole measurement protocol (micro-CT measure and image segmentation) was estimated by repeating 10 times the same micro-CT measure on the same aggregate, then processing the 10-image series with the same segmentation protocol (see section 2.3). The means, standard deviations and coefficients of variation of the calculated components for these repetitions are, respectively, 10.88% ± 0.48 (CV = 4.4%) for GVol, 12.47% ± 0.48 (CV = 3.8%) for OVVol and 76.66% ± 0.54 (CV = 0.7%) for MVol.

3 | RESULTS

3.1 | Percentage of water-stable aggregates (WSA)

The effect of earthworms on the percentage of WSA was highly significant ($p < 0.001$). The average percentage of WSA was significantly lower in the presence of earthworms compared to the control without earthworms, regardless of the species of earthworms. In addition, there was no significant difference between the three treatments with earthworms (Table 3).

3.2 | Tomography of aggregates

The X-ray μCT analysis of the studied aggregates shows that all of them are very heterogeneous and made of a complex and organized mixture of components, which varies in terms of nature and dimensions. The difference in nature is indicated by significant attenuation contrasts between components that allows differentiating (segmenting) the aggregate material into three categories: mineral grains, (organic material + voids) and undifferentiated aggregate matrix. Due to their relatively strong attenuation, the mineral grains (G) are the easiest components to segment. At the other end, the voids and the organic matter are both low attenuating and, in some cases, very difficult to differentiate on their intensity values alone. For this reason, we had to consider these two components as a single one (OV) to apply the same categorization method to all the studied aggregates. The OV will thus represent the porosity of the aggregate material and the presence of both organic matter contained in the initial material and organic matter

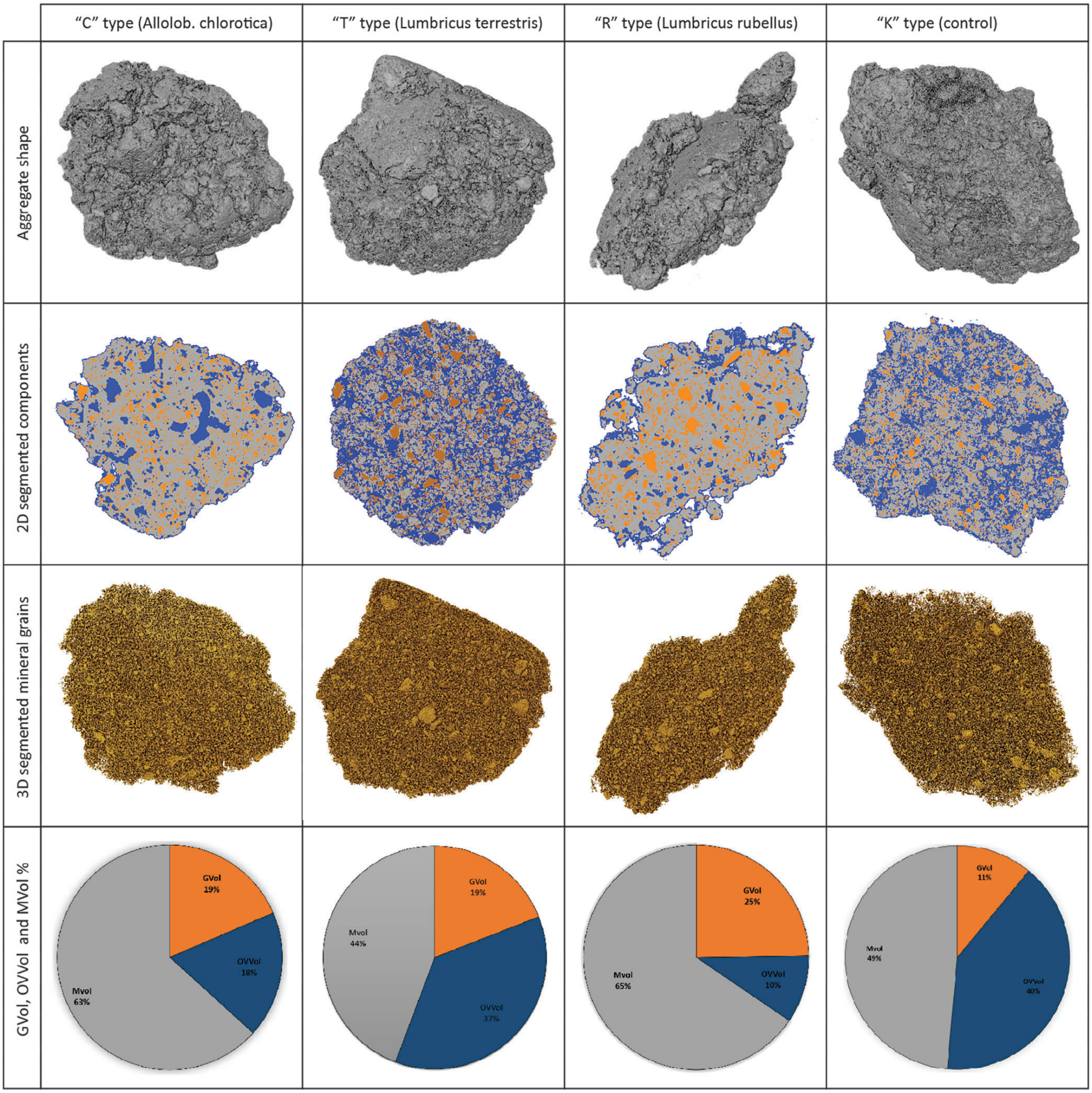

FIGURE 2 Micro-computed tomography(CT) imaging results on four aggregates: "C" type (*Allolobophora chlorotica*, CH5-2), "T" type (*Lumbricus terrestris*, LT1-2), "R" type (*Lumbricus rubellus*, LR5-3) and "K" type (control sample, K2-2). For each aggregate are shown: (a) the aggregate full shape (volume rendering), (b) a selected slice with the segmentation of grains (G, in orange), organic matter and voids (OV, in blue) and matrix (M, in grey), (c) the 3D spatial distribution of the segmented grains (GVol) for the whole aggregate and (d) the percentage in volume of the three segmented components (GVol, OVVol, MVol) for the whole aggregate [Color figure can be viewed at wileyonlinelibrary.com]

ingested by earthworms. The matrix (M) is composed of any material whose attenuation is in-between G and OV intensity values. It can be (i) an undifferentiated mix of mineral grains, pores and organic matter whose dimensions are below the X-ray μCT resolution, but also (ii) the effects of the interface between objects, where voxels will suffer from partial volume effects. Figure 2 shows an example of segmentation where one can clearly observe, in a qualitative way, that the proportion of G, OV and M can be very different according to the aggregate treatment (*L. rubellus*, *A. chlorotica*, *L. terrestris* and control). These differences will be quantified in the section below that describes statistical analysis.

Figure 2 shows also clear variations in dimensions of particles in a single aggregate, but also between aggregates. Considering the X-ray μCT resolution, it was possible to individualize elements of about 30 μm (three times the voxel size) and above. For the mineral grains, this corresponds at highest resolution to silt fractions and for voids it essentially corresponds to the domain of capillary porosity (200 nm to 2 mm). The aggregate matrix, as identified by X-ray μCT, was supposed to be composed of a mix of very fine organic matter, fine silts or clay minerals and micro-, colloidal and/or reticular porosity.

3.2.1 | Quantification of the different components

For all analysed variables, the average value of the control was statistically different from the average value of the three earthworm treatments (P2 in Table 4). For all the analysed variables, the earthworm effect was significant (P1 in Table 4).

As shown by the multiple comparison tests at the significance level of 5% (Table 4), earthworm level (*L. rubellus*, *A. chlorotica*, *L. terrestris* and control) had a significant effect on GVol, MVol and MGRat. The effects of earthworm on VolAgg and MVolAgg were not statistically significant. In the case of GVol, the average value of *L. rubellus* was significantly higher than the average value of *A. chlorotica*. *L. terrestris* was not statistically different from *L. rubellus* and *A. chlorotica* (Figure 3; Table 4). In the case of MVol, the average value of *L. terrestris* was significantly lower than the average value of *L. rubellus* and *A. chlorotica*. *L. rubellus* and *A. chlorotica* were not statistically different. The observed difference between *L. rubellus* and *A. chlorotica* was not statistically different. For MGRat, the average value of *A. chlorotica* was statistically higher than that of *L. rubellus* and *L. terrestris*. There was no significant difference between *L. rubellus* and *L. terrestris*. The coefficient of determination, R^2, was the highest for GVol (0.835), showing that the goodness of fit for explaining the effect of EW is the best for this variable. By contrast, the weakest was given by MVol.

3.2.2 | RDA analysis

The first two axes of the biplot explained most of the variation (92% of the total inertia) and seem to represent the

TABLE 3 Average values of percentage of water-stable aggregate (WSA) in the four treatments: control without earthworm (K); mesocosms with *Lumbricus rubellus* (R); with *Lumbricus terrestris* (T); and with *Allolobophora chlorotica* (C)

	C ($n = 5$)	K ($n = 5$)	R ($n = 5$)	T ($n = 5$)	p
WSA (%)	14.9 (±2.28)[a]	29.2 (±1.36)[b]	16.3 (±0.85)[a]	14.7 (±1.46)[a]	<0.001

n, number of replicates. Standard errors are given in parentheses. Two treatments with different letters are significantly different at the 5% treatment.

TABLE 4 Average values for CT parameters (volume of aggregate grains (GVol), volume of the undifferentiated matrix (MVol), ratio matrix/grains (MGRat), aggregate volume (VolAgg) and volumetric mass of aggregate (MVolAgg)) for the four treatments: control without earthworm (K), mesocosm with *Lumbricus rubellus* (R), with *Lumbricus terrestris* (T) and with *Allolobophora chlorotica* (C)

	Average values of the four EW levels						
Variable	C ($n = 3$)	K ($n = 5$)	R ($n = 4$)	T ($n = 4$)	P1	R^2	P2
GVol (% of total volume)	18.9 (±0.95)[b]	13.5 (±0.99)[a]	23.2 (±0.79)[c]	20.9 (±0.51)[bc]	<0.0001	0.835	<0.0001
MVol (% of total volume)	65.2 (±0.23)[b]	52.6 (±1.83)[b]	63.4 (±1.42)[b]	53.6 (±3.85)[a]	0.0047	0.596	0.0079
MGRat (ratio)	3.50 (±0.16)[b]	4.00 (±0.21)[b]	2.74 (±0.06)[a]	2.58 (±0.13)[a]	<0.0001	0.777	<0.0001
VolAgg (mm^3)	527 (±136)[a]	1,376 (±104)[b]	503 (±182)[a]	729 (±151)[a]	0.0021	0.615	0.0003
MVolAgg (g/cm^3)	1.39 (±0.01)[a]	1.27 (±0.02)[b]	1.45 (±0.02)[a]	1.38 (±0.03)[a]	0.0002	0.737	<0.0001

n, number of replicates. Standard errors are given in parentheses. Two treatments with different letters are significantly different at the 5% treatment. P1, probability for testing the four treatments. R^2, coefficient of determination for the model. P2, probability when testing the contrasts "average of K versus average of the three other treatments".

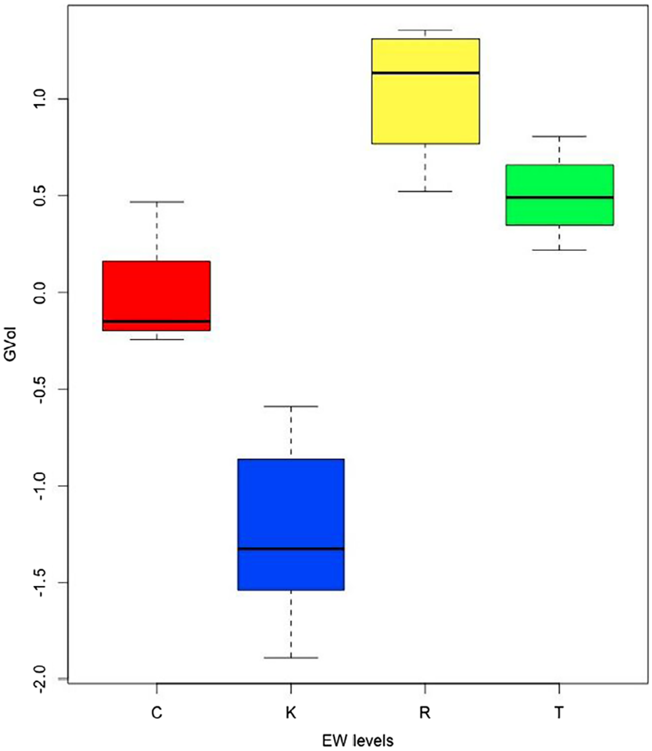

FIGURE 3 Boxplot for the volume of grains (GVol) within the aggregates as a function of earthworm (EW) levels (C = *Allolobophora chlorotica*, T = *Lumbricus terrestris*, R = *Lumbricus rubellus* and K = control sample) [Color figure can be viewed at wileyonlinelibrary.com]

data well. The biplot showed that the four earthworm levels were linked with four data groups, suggesting an EW effect (Figure 4). Variables MVolAgg and GVol were positively correlated ($r_{pearson} = 0.93$). They were also negatively correlated with VolAgg ($r_{pearson} = -0.77$ and -0.76, respectively). MGRat was negatively correlated with GVol and MVolAgg ($r_{pearson} = -0.88$ and -0.74, respectively), whereas MVol was only slightly correlated with the four other variables. Level K (control without earthworm) was mainly related to variable VolAgg, whereas level C (*A. chlorotica*) was mainly related to MVol and level R (*L. rubellus)* to MVolAgg and GVol. The link between level T (*L. terrestris*) and the variables was less clear than for the other earthworm levels.

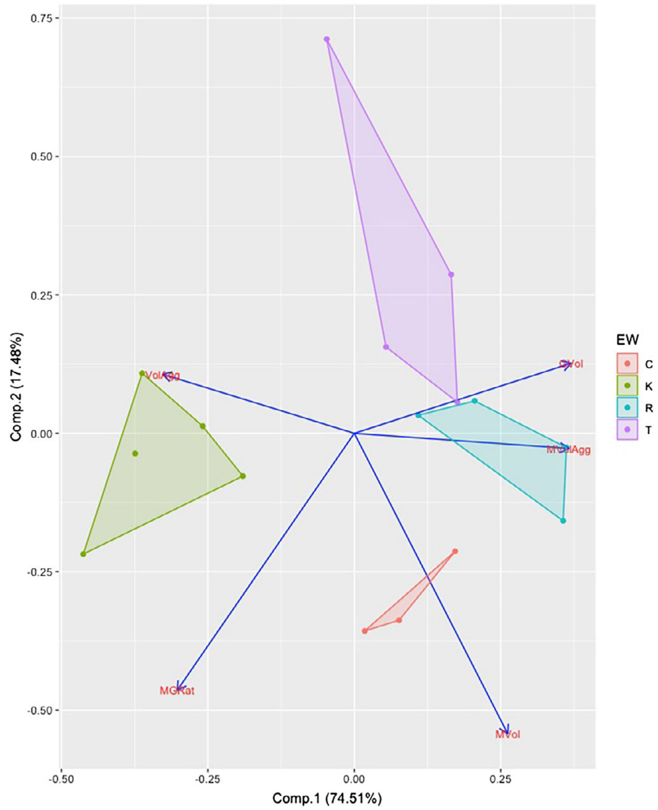

FIGURE 4 Principal component analysis biplot based on five variables (GVol, MVol, MVolAgg, VolAgg and MGRat; arrows in blue and name in red), for four earthworm (EW) levels (C = *Allolobophora chlorotica*, T = *Lumbricus terrestris*, R = *Lumbricus rubellus* and K = control sample) [Color figure can be viewed at wileyonlinelibrary.com]

4 | DISCUSSION

4.1 | Stability of soil aggregates

In our study, the percentage of water-stable aggregates (WSA) was very low, especially in the presence of earthworms. We also observed a significant difference between the stability of earthworm aggregates and non-earthworm aggregates, but no significant differences between the three species of earthworms, *L. rubellus*, *L. terrestris* and *A. chlorotica*.

Our results differ from those cited in the literature. According to Garvín et al. (2001), the tensile strength of aggregates, an index of stability in dry conditions, is generally higher for earthworm aggregates than for non-earthworm aggregates. In addition, tensile strength appears to be species dependent, being higher in casts of the epigeic *Dendrobaena octaedra* than in those of *L. terrestris* (Flegel, Schrader, & Zhang, 1998) and higher in casts of *L. terrestris* than in those of the endogeic *Aporrectodea caliginosa* (Schrader & Zhang, 1997). The contribution of earthworm activity to casts' stability depends also on the original composition of the parent material. Thus, both WSA and tensile strength have antagonist trends: whereas the first is negatively related to clay and carbonate contents of the parent soil, the second shows the opposite (Schrader & Zhang, 1997). In our study, the very low values of WSA could be partly due to an antagonist effect of a low clay content and a high amount of carbonate in the sediment. In addition, WSA is also often linked to the amount of organic matter that reinforces the clay–humus complex, thus enhancing the stability of aggregates.

Earthworms are known to have a selective feeding behaviour that usually leads to higher contents of organic matter in casts and burrow linings compared to bulk soil (Jégou, Cluzeau, Balesdent, & Tréhen, 1998; Le Bayon & Binet, 2006). This was not the case in our study, probably due to the soil's initial content of organic matter, which offered a sufficient food supply to earthworms. The original composition of the parent soil, as well as food quality, are known to largely influence physicochemical properties of casts (Flegel et al., 1998; Schrader & Zhang, 1997). As the soil ingestion provided enough resources for them, earthworms did not search for food and hence did not concentrate organic matter in their casts. Combined with a high amount of water, the similar proportion of organic matter in earthworm aggregates compared to non-earthworm ones is an additional explanation for the low resistance of earthworm casts. It is well recognized that, following egestion, fresh casts contain high amounts of water due to mucus addition during transit in the gut, enhancing both the dispersion of particles and the enzymatic activities leading to organic matter mineralization (Angst et al., 2019; Blouin et al., 2013; Curry & Schmidt, 2007; Le Bayon & Binet, 2006; Salmon, 2001). By grinding and mixing soil in their digestive tracts, earthworms completely destabilize the soil structure and contribute to the rearrangement of soil particles, thus modifying the tensile strength (Flegel et al., 1998; Schrader & Zhang, 1997; Shipitalo & Protz, 1988, 1989).

In the present study, the low stability of earthworm aggregates may be mainly attributed to the fact that we worked on belowground casts, whereas most other studies have focused on surface casts (Hauser, Norgrove, Asawalam, & Schulz, 2012; Jouquet et al., 2009; Jouquet, Bottinelli, Podwojewski, Hallaire, & Tran Duc, 2008; Le Bayon, Moreau, Gascuel-Odoux, & Binet, 2002). Being directly exposed to the atmosphere, these latter are quickly air-dried and then more subject to desiccation processes, which in turn reinforce the tensile strength (Lipiec, Turski, Hajnos, & Świeboda, 2015). Over time, sometimes several days, surface casts then become hard and arid, similar to small pebbles, and are very resistant to the impact of rainfall (Le Bayon & Binet, 1999, 2001).

To summarize, in our study, the WSA allows discrimination of the non-earthworm aggregates from earthworm aggregates, but is not efficient for discriminating the earthworm species. This could be explained by the combined effect of texture, organic matter content of the soil, and also aggregate characteristics (age and belowground location).

4.2 | Is X-ray µCT a relevant tool for studying and discriminating aggregates?

X-ray µCT showed significant differences between earthworm aggregates and non-earthworm ones. However, contrary to the WSA method, X-ray µCT allowed discrimination of aggregates produced by different earthworm species.

4.2.1 | Earthworm *versus* non-earthworm aggregates

Both the volume (VolAgg) and density (MVolAgg) allowed clear differentiation of non-earthworm aggregates from earthworm casts. Therefore, X-ray µCT provided new insights into casts, especially the fact that earthworms compact soil particles, resulting in small aggregates with a higher density compared to bulk soil. Moreover, X-ray µCT showed this trend for the three species, which is explained by the selective ingestion of the finest soil particles combined with the high pressure in earthworm guts (McKenzie & Dexter, 1987). Thus, the muscular contractions of the earthworm crop and gizzard, the peristalsis of the gut wall, and contractions of the body wall create a great range of pressures that can lead to the disruption of existing interparticle water and cation bridges in the aggregates (see the review of Shipitalo & Le Bayon, 2004). It usually takes 2 to 24 hours for soil to pass through the gut of lumbricid earthworms (Barley, 1959; Bolton & Phillipson, 1976; Piearce, 1972) and the average coelom pressure may reach 1.6 kPa in segment 28 and 0.8 kPa near the tail region of *L. terrestris* (Newell, 1950). Such pressures, concomitant with the addition of large amounts of watery mucus (Barois et al., 1993; Salmon, 2001), can lead to the complete reorganization of particles and the formation of new aggregates (Duarte, Melo, Brown, & Pauletti, 2014; Marinissen, Nijhuis, & Van Breemen, 1996; Shipitalo & Protz, 1988, 1989). Both belonging to the genus *Lumbricus*, *L. terrestris* and *L. rubellus* have similar musculature that is strong enough to exert high pressures, thus affecting the formation of casts in a more or less similar way.

In addition, our results showed that the endogeic *A. chlorotica* is also able to increase the bulk soil density, thus acting as a compacting agent. Several experiments have been conducted in microcosms to study compaction processes using different techniques. Milleret, Le Bayon, Lamy, Gobat, and Boivin (2009) and Milleret, Le Bayon, and Gobat (2009) used shrinkage analysis to show that *A. chlorotica* decreases the specific bulk volume and the hydrostructural stability by around 25%, but significantly increases the bulk soil density. They also demonstrated that *A. chlorotica* can decrease the pore volumes (Kohler-Milleret, Le Bayon, Chenu, Gobat, & Boivin, 2013). In our study, X-ray µCT provides additional information on earthworm aggregates and especially how endogeic and epi-anecic species can compact the soil through their belowground casts. This is essential because a large part of the burrows was probably backfilled with casts (Amossé et al., 2015; Capowiez et al., 2011).

4.2.2 | Composition of aggregates

Within the aggregates, the grain volume (GVol) reflects the mineral content and X-ray µCT easily identifies it because of the high contrast of GVol compared to the other soil components. The composition of aggregates showed that casts of different species could be clearly distinguished from each other, especially regarding the volumes of grains (GVol). This result identifies the selective feeding of earthworms, particularly of *L. terrestris* and *L. rubellus,* which are close to each other compared to *A. chlorotica*. This is in line with the findings of Hoeffner, Santonja, Cluzeau, and Monard (2019), who showed that these two earthworms behave as epi-anecic earthworms, and thus have similar trends in selecting organic matter compared to strictly anecic species. In our study, we demonstrated that not only organic matter (Hoeffner et al., 2019; Le Bayon & Binet, 2006), but also mineral particles, are selected by earthworms. Unfortunately, the low amount of aggregates did not allow us to go further into detail by performing granulometry, but earthworms usually tend to select fine particles such as clay and silt (see the review of Blouin et al., 2013 and Le Bayon et al., 2017). Those fine particles reinforce the formation of the clay–humus complex at the core of the formation of stable aggregates. However, in particular cases, earthworms may also select coarser sandy grains. Hence, Schulmann and Tiunov (1999) showed that individuals of *L. terrestris* actively select sand particles and show evident preference for the small grains (0.5–1 mm) over larger ones (1–2 mm and 2–3 mm). Such sandy particles may help the grinding of organic matter in the muscular gizzard (Edwards & Bohlen, 1996).

One of the main challenges cited in the literature is how to discriminate and quantify the components of aggregates, especially organic matter (Baveye et al., 2018). This is mainly due to the high variation of organic matter particles in terms of shape, size, origin and decomposition stage. Despite the development of new methods combining X-ray µCT and labelling (Arai et al., 2019; Peth et al., 2014), discriminating the presence of organic matter remains a controversial and unresolved issue (Kravchenko, Negassa, Guber, & Schmidt, 2014). In our study, we also encountered the problem of organic matter discrimination, mainly due to (a) the presence of two main types of organic matter inside aggregates (raw pieces of catgrass and humified organic matter already present in the initial sediment), and (b) different feeding behaviours of different ecological categories of earthworms. Moreover, at the end of the experiment, all earthworms survived but most of them had lost weight. This is probably due to the manner in which the food resource was provided: the mixing of raw residues of catgrass was done to homogenise the food supply for all three ecological categories. However, Frazão, de Goede, Capowiez, and Pulleman (2019) recently highlighted the importance of providing residues on the soil surface for *L. terrestris*. In addition, these authors showed that there was competition between *L. terrestris* and *L. rubellus,* mostly explained by their epi-anecic behaviour.

Finally, the matrix fraction (MVol) represents the remaining volume of the aggregate (with intermediate density) and is supposed to be representative of the agglomeration of the smallest particles of the soils (mineral and organic matter). It behaves in the same way as the OVVol component and does not appear to discriminate earthworm from non-earthworm aggregates, or among earthworm casts produced by different earthworm species. Despite the fact that only the GVol was discriminant in our study, we suggest that the relative proportions of all three components together need to be taken into account (grain volume, voids and organic matter fraction, and matrix fraction). Indeed, our results, especially the multivariate statistical analyses, demonstrate that these relative proportions are species specific and can thus provide typical signatures for characterizing non-earthworm from earthworm aggregation processes, these latter resulting from activities of different earthworm species. Consequently, to address the question of the composition of aggregates, we focus specifically on organic matter discrimination, but we propose instead three relevant bulk components to characterize aggregates: the grain volume (GVol), the voids and organic matter fraction (OVVol), and the matrix fraction (MVol).

5 | CONCLUSION

In our study, X-ray µCT clearly discriminated earthworm casts from non-earthworm aggregates. Moreover, this very high-resolution technique, used alone (i.e., not combined with other chemical methods), also allowed differentiation of aggregates produced by different earthworm species. The relative volume of grains within aggregates was the best proxy to identify the selective feeding of earthworms, which differs between ecological categories. Moreover, the voids and organic matter fraction in aggregates highlighted the aggregate compaction, which results from earthworm behaviour. X-ray μCT thus provides new insights into how earthworms may influence soil structure. It is also a relevant approach to better understanding of how soil aggregates are formed, even at the earthworm species level. Distinguishing voids from organic matter is a future objective, using ultra-high X-ray μCT resolution at a micron-scale resolution.

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CONFLICT OF INTEREST

The authors involved in this research confirm that they have no interest (relationship, financial or otherwise) that might be perceived as influencing their objectivity. The authors have no conflict of interest to declare in the framework of the pre-cited research.

AUTHOR CONTRIBUTIONS

Study concept and design: Renée-Claire Le Bayon, Andreas Schomburg. Aggregate analysis: Franziska Fischer, Alexandre Luiset, Pascal Turberg. Image and Avizo work: Alexandre Luiset, Pascal Turberg. Analysis and interpretation of data: Renée-Claire Le Bayon, Claire Guenat, Pascal Turberg, Rodolphe Schlaepfer. Statistical analysis: Rodolphe Schlaepfer. Drafting of the manuscript: Renée-Claire Le Bayon, Claire Guenat, Pascal Turberg, Rodolphe Schlaepfer. Obtained funding: Renée-Claire Le Bayon, Claire Guenat. Study supervision: Claire Guenat, Pascal Turberg.

DATA AVAILABILITY STATEMENT

Availability of dataTemplate for data availability statement Data available on request due to privacy/ethical restrictions The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

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