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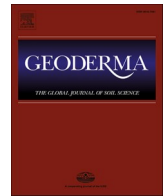
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Exploring the control of earthworm cast macro- and micro-scale features on soil organic carbon mineralization across species and ecological categories

Guillaume Le Mer^{a,*}, Nicolas Bottinelli^a, Marie-France Dignac^a, Yvan Capowiez^b,
Pascal Jouquet^a, Arnaud Mazurier^c, François Baudin^d, Laurent Caner^c, Cornelia Rumpel^a

^a Institute of Ecology and Environmental Sciences- Paris (iEES-Paris) UMR CNRS, INRAE, IRD, Sorbonne University, Paris 75005, France

^b INRAE, EMMAH, UMR 1114, Domaine Saint Paul, Cedex 09, Avignon 84914, France

^c Institut de Chimie des Milieux et Matériaux de Poitiers (IC2MP), Université de Poitiers, UFR SFA, Poitiers 86073, France

^d Institut des Sciences de la Terre de Paris (ISTeP), Sorbonne Université, Paris 75005, France

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ABSTRACT

The role of earthworms on biogeochemical carbon cycling is a major knowledge gap resulting from the difficulty of isolating and exploring the effects provided by the diversity of organisms. In this study, we investigated the effect of six earthworm species belonging to three ecological categories on soil organic carbon (SOC) mineralization. To this end, we produced casts in microcosms with the six species in the same soil and with the same litter material. The casts were subjected to laboratory ageing for 140 days. During this process, we monitored physicochemical parameters, CO₂ emissions and determined the micro-scale organization of the casts' particulate organic matter and pores using X-ray microtomography.

Our results showed contrasting properties of fresh casts of the three ecological categories, in accordance with the earthworm species' morphological or behavioral strategies, indicating that those were maintained in artificial environments. However, species-specific changes in cast properties throughout ageing increased intragroup variability among ecological categories. As a result we observed earthworm species-specific evolution of CO₂ mineralization rates during casts ageing. We found that at least half of the variability in CO₂ emissions was explained by cast microstructural changes, related to the spatial arrangement between particulate organic matter, porosity, and mineral particles. We conclude that earthworm species-specific traits may play a role in organic carbon protection through their impact on microstructural cast properties.

1. Introduction

Understanding the mechanisms involved in the mineralization and stabilization of soil organic carbon (SOC) is a major challenge to understand ecosystem functioning in the context of global changes. Microbial substrate access has been identified as a major control of soil organic matter (SOM) turnover (Dungait et al., 2012) and detritivore species may play an important role in the processes involved (Briones, 2018). For example, earthworms are detritivores and soil ecological engineers, which alter the accessibility of resources to soil (micro)organisms (Asshoff et al., 2010; Frouz, 2018; Jones et al., 1994). By regulation of microbial accessibility, the formation of microaggregates and organo-mineral associations at micro-scales (Negassa et al., 2015; Nunan et al., 2003; Six et al., 2004) may lead to the stabilization of SOC in earthworm casts for long timescales (Angst et al., 2019; Vidal et al.,

2019). These processes might change according to earthworm species, with different species having contrasting ecological strategies. While the biochemical and physicochemical processes leading to SOM protection are well studied (Cotrufo et al., 2015), the role of the detritivore diversity in the process of litter conversion into faeces and its consequences for organic matter (OM) turnover is poorly documented (Joly et al., 2020).

Earthworms are usually divided into three main ecological categories depending on behavioral and morphological adaptations to their environment (Bottinelli and Capowiez, 2021; Bouché, 1977). Epigeic species live in the litter layers and the upper centimeters of the soil and feed predominantly on fresh surface litter; endogeic species live deeper and feed on soil organic matter; anecic species built vertical permanent burrows from the surface into the soil profile and drag down the fresh surface and feed on both OM sources (Bouché, 1972; Edwards, 2004;

* Corresponding author.

E-mail addresses: lemer.guillaume@outlook.fr (G. Le Mer), cornelia.rumpel@inrae.fr (C. Rumpel).

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Lavelle, 1997). Therefore, the earthworms' degree of belonging to ecological categories could influence the regulation of microbial substrate access by modifying the quantity and quality of ingested OM (Bottinelli and Capowiez, 2021). The properties of casts produced by different species were shown to have different effects on soil functioning, and in particular on biogeochemical cycling (Hedde et al., 2005; Vos et al., 2019; Coq et al., 2022). Although several studies have shown that OM stabilization in earthworm casts may be more related to its physical protection than to its chemical changes (Angst et al., 2017; Le Mer et al., 2020), the effect of different earthworm species on the specific mechanisms leading to OM protection within their casts is still unknown.

Two opposite mechanisms have been identified to be involved in the control of SOC mineralization during the production of earthworm casts and their ageing in soil. On the one hand, fresh earthworm casts are microbial hotspots, where the mineralization is facilitated, leading to CO₂ release (Lavelle, 1988; Lavelle et al., 1995; Lubbers et al., 2013). On the other hand, incorporation of OM in casts increases SOC residence times by protecting it from microbial degradation (Bossuyt et al., 2006; Kuzakov and Blagodatskaya, 2015; Vidal et al., 2016a), as long as casts are not degraded (Bottinelli et al., 2021; Le Mer et al., 2021). During cast ageing, SOC destabilization kinetics was found to be controlled by (re-) arrangement of particulate organic matter (POM) and pores at micro-scale (Puche et al., 2022), which impacts the movement and availability of gases, substrate and water to soil (micro)organisms (Young et al., 2008). While this has been shown for one earthworm species belonging to the anecic ecological category, to the best of our knowledge, the controls of earthworm species-specific cast properties on the stability of OM occluded in earthworm casts has not previously been studied.

Here, we aimed to investigate how physicochemical characteristics, thermal stability and the micro-scale features of casts produced by earthworm species belonging to different ecological categories vary and how they relate to cast C dynamics. To this end, we produced casts with six earthworm species belonging to the three ecological categories under controlled conditions. We monitored CO₂ emission potential during laboratory cast ageing and characterized the evolution of their microstructural cast characteristics. We hypothesized that:

- (H1) the fresh casts' characteristics depend on the feeding habits of specific earthworm species, with ecological categories strongly affecting the quantity and quality of particulate organic matter (POM) incorporated in casts,
- (H2) the evolution of cast microstructures is species-specific and controls an important part of the C mineralization dynamics occurring during casts ageing.

2. Materials & methods

2.1. Sampling of earthworm species, soil and preparation for cast production

Five earthworm species were sampled in Avignon (Vaucluse, France) in two experimental orchards of INRAE (43°54'55.8" N, 4°52'42.3" E) free of pesticides for at least 10 years (with average annual rainfall and temperature for the last 20 years of 614 mm and 11 °C). The sampling was conducted by hand sorting in November 2018. We collected 50 to 100 individuals per species: two anecic species (*L. terrestris* and *A. nocturna*), two endogeic species (*A. icterica* and *A. caliginosa*) and one epigeic species (*L. castaneus*). In the field, the abundance of these five species was heterogeneously distributed among experimental plots. For example, *L. castaneus* was collected under apple trees in the center of the plot, while *A. icterica* and *A. nocturna* were the two dominant species at the edge of the orchard. Only adult earthworms were kept after identification and juveniles were released. Earthworms were put in boxes filled with soil collected in the same orchards. Moreover, individuals from another epigeic species *E. fetida* were purchased from La Ferme du

Moutta (Pyrénées-Atlantiques, France) and reproduced in the laboratory.

Before the beginning of cast production, we conducted a four-day fasting period with all individuals to empty earthworm guts. The worms were individually starved in a Petri dish, filled with a wet paper towel, which was changed twice a day to avoid re-ingestion. The Petri dishes were covered with perforated aluminum foil and placed in the refrigerator at 6 °C.

The soil used for the experiment was collected from another field under agricultural use at the INRAE "QualiAgro" experimental fields (Yvelines, France). We sampled the 50–65 cm layer of a Luvisol developed on decarbonated loess (pH 7.9, organic carbon 1.9 g kg⁻¹, organic nitrogen 0.4 g kg⁻¹, soil texture by weight: 3.5 % sand, 64.6 % silt, 31.9 % clay). This soil was dried (30 °C, 72 h) and sieved (<2 mm) before the incubation experiment.

2.2. Preparation of microcosms, cast production and incubation

Our experimental setup included two factors: the six earthworm species and three different incubation times for earthworm casts (7, 42 and 140 days) corresponding to progressive ageing stages. Cast production and ageing was carried out in microcosms consisting of plastic boxes (length and width: 10 cm, height: 10 cm), covered with perforated lids to prevent earthworms from escaping. To precisely control the quality and quantity of fresh OM, the soil was homogeneously mixed with crushed *Miscanthus giganteus* stem and leaf residues (<1 mm); at a rate of 3 g of dry plant residues per 97 g of dry soil (3 %). This quantity was selected during preliminary experiments to allow the earthworms to ingest fresh OM without limitation and thus minimize re-ingestion of casts. The microcosms were filled with this substrate (bulk density: 1 g cm⁻³) and the moisture levels were adjusted by adding distilled water. Cast production and ageing in earthworm free environment was carried out at 80 % (w/w) of the water holding capacity. Briefly, we introduced earthworms into the corresponding microcosms at a density of 2, 4, and 8 individuals for anecic, endogeic and epigeic species, respectively for a cast production period. The contrasting abundances were chosen due to differences in individual weight and to maximize the production of casts. In addition to microcosms with earthworms, we also prepared microcosms containing soil aggregates as control (three dates, five replicates).

Casts were produced for seven days in the microcosms (at 14 °C) and fresh casts present on the soil surface were collected on day 7. Thereafter, the earthworms were removed by placing microcosms on a warm plate (set at 20 °C), with about 0.5 cm of sand to buffer the heat and all individuals were recovered from the soil surface within three minutes. The remaining material containing the casts was left to age in the microcosms. Throughout the ageing period, the water content of the soil in the microcosms was kept constant and corrected twice a week if necessary. After 42 and 140 days of ageing we visually sampled earthworm casts from the first top centimeters of the microcosms.

For each species, we thus obtained fresh and aged casts. After collection, an aliquot of the casts were subjected to laboratory incubation in order to determine their potential CO₂ emissions. The rest of the cast material was dried (25 °C), weighed and stored prior to X-ray microtomography acquisitions and physicochemical analyses.

2.3. Physical characterization

Macro-scale characteristics included mass, which was determined by weighing the samples. In addition four macro-scale physical characteristics were assessed on earthworm casts and control aggregates using microtomography (volume, density, specific area and diameter).

2.3.1. X-ray microtomography

Microtomographic acquisitions of the earthworm casts were performed on an EasyTom XL Duo (RX-solutions, Annecy, France), at the

PLATINA platform of the Institut de Chimie des Milieux et Matériaux de Poitiers (IC2MP, UMR 7285, University of Poitiers). We used a sealed, microfocus X-ray source (L12161-07, Hamamatsu Photonics, Japan) coupled to a flat panel detector (PaxScan 2520DX, Varian, USA). The samples were scanned with a spatial resolution of 9.5 μm . The acquisition parameters were 80 kV (tube voltage), 125 μA (tube current), 10 W (power), 1440 projections (1840x1456 pixels; tif format, 16 bits), 4 frames per second (readout speed), average of 15 frames per projection, beam filtration by 1.2 mm of aluminum, source-to-detector distance and source-to-object distance of 40.98 mm and 547.95 mm, respectively. During the acquisitions, a procedure with random detector offsets was used to attenuate ring artifacts. At the end of the acquisition, a set of 32 reference images was recorded to correct for possible beam drift and/or sample motion. The reconstruction of the data was performed with the XAct® software (RX-solutions). The beam hardening artifacts were corrected by linearization with a polynomial function, during the reconstruction.

2.3.2. Micro-scale image analyses

Digital image processing and analyses were performed with Avizo® software (Thermo Fisher Scientific). The workflow, used to (i) reduce overall noise and to (ii) clearly separate fresh or degraded particulate organic matter (POM) from porosity and mineral matrix, is presented in supplementary materials (App. 1). The microstructure of earthworm casts was characterized through the proportions (%) and the mean volume (mm^3) of POM and pore space. These parameters were assessed on the (a) total porosity, (b) POM volume, (c) connected porosity, directly connected to the air outside the cast, and (d) connected POM, directly or indirectly (through the connected pores), to the outside of the cast (App. 2).

2.4. Chemical characterization

2.4.1. Potential CO_2 emissions

To evaluate the stability of SOC occluded in earthworm casts, we performed an incubation experiment with casts and control soil aggregates sampled after their production (7 days) and at two times (42 and 140 days) during their ageing. Briefly, between three and five g of moist earthworm casts and control aggregates were placed in 20 mL glass vials right after sampling from the microcosms and sealed with a septum to allow gas sampling. During preliminary experiments we evaluated the minimum time necessary to trap and detect the gases emitted by the two types of aggregates. The glass vials were placed in an incubator set at 14 °C in dark conditions. An empty vial was used as control, to correct for ambient CO_2 (ppm) concentrations. After accumulation of the gases in the vial for one hour, the CO_2 concentration (ppm) in the headspace was measured using a gas chromatograph (Agilent, 490 Micro GC System, California, USA). For each sample, two additional measurements were performed at exactly 30-minute intervals after the first acquisition. For all treatments (dates \times species) these steps were performed on five replicates in order to acquire the average CO_2 mineralization rates at 7, 42 and 140 days.

2.4.2. Determination of organic carbon (OC) content and thermal properties

The thermal stability of the OM occluded in earthworm casts was studied with a Rock-Eval® 6 turbo device (Vinci Technologies, Nanterre, France). For each sample, about 60 mg of finely crushed and ground ($\approx 200 \mu\text{m}$) casts were exposed to thermal treatments, which consisted of two stages: (i) pyrolysis (200–650 °C; thermal ramp of 30 °C min^{-1} ; under N_2 atmosphere) followed by (ii) combustion-oxidation (300–850 °C; thermal ramp of 20 °C min^{-1} ; under laboratory atmosphere). During both stages, CO and CO_2 emissions were measured by infrared detection. Hydrocarbon effluents were also measured during the pyrolysis step by a flame ionization detector (Disnar et al., 2003; Schomburg et al., 2018). The total OC of earthworm casts and control

soil aggregates was determined using the Rock Eval (RE6) signals. The RE6 R- and I-index were calculated following Sebag et al. (2016). The R-index corresponds to the relative contribution of thermally stable OM with refractory compounds released at higher temperatures; while the I-index provides information on the pool of thermally labile and immature OM with compounds released at lower temperatures (Cécillon et al., 2018; Sebag et al., 2016).

2.5. Statistical analyses

Prior to data analysis, normality and homogeneity of variances were checked using respectively the Shapiro-Wilk test ($p\text{-value} > 0.05$) and the Levene test ($p\text{-value} > 0.05$). When these two conditions were met, an ANOVA and a Tukey multiple comparison test were performed. For the same variable, the lower-case letters used in the tables and figures indicate significant differences between treatments ($p\text{-value} < 0.05$). The correlations between the variables were statistically tested with the Pearson correlation test, and only relevant and significant correlations ($p\text{-value} < 0.05$) were presented. The physicochemical, macro- and micro-scale features of the fresh earthworm casts, and their evolution at 7, 42 and 140 days after their production were compared using principal component analysis (PCA). The cast CO mineralization rates were followed for 140 days (at 7, 42 and 140 days) with, for each measurement, three technical replicates for each of the five replicates of the same treatment. Thus, raw concentrations obtained in ppm of $\text{CO}_2\text{-C}$ were reduced to mg of $\text{CO}_2\text{-C}$ emitted per hour and per g of dry cast (dry masses of casts were obtained after freeze-drying samples).

With the objective of predicting observed mineralization rates from physicochemical and microstructural cast features, we used machine learning algorithms via a generalized linear model (GLMnet). The logistic regression algorithm represents the conditional class probabilities by a linear function of the predictors. We performed a fast regularization algorithm, which fits a generalized linear model with elastic-net penalties. We performed a leave-one-out cross-validation (LOOCV) procedure to estimate the performance of the machine learning algorithm when it makes predictions on data that were not used during model training, which was our case. Thus, pseudo- R^2 were corrected by this LOOCV procedure to obtain LOOCV- R^2 .

All statistical calculations were performed using R software (R core team, 2005). We used the package 'agricolae' (version 1.3–5) for the Shapiro Wilk, Levene, ANOVA, Kruskal-Wallis and post hoc tests. The predictive machine learning model was performed with the 'glmnet' package (Friedman et al., 2010). The residual prediction deviation (RPD) and the root-mean-square error (RMSE) were assessed with the 'chillR' package (Luedeling, et al., 2013). The relationships between (i) cast features (produced by six species with three ageing dates) and (ii) morphological traits of earthworm species (from Bouché, 1972) were tested using a co-inertia analysis (CIA). PCA and CIA were performed with the 'ade4' package (Dray and Dufour, 2007). For CIA, the relative variance (RV) coefficient measured the correlation strength between two data sets.

3. Results

3.1. Macro-scale characteristics of earthworm casts

3.1.1. Organic carbon (OC) contents

The OC contents ranged from 12.1 mg g^{-1} for control aggregates at day 7, to 21.2 mg g^{-1} for *L. castaneus* casts at day 7 (Table 1). The control aggregates had always significantly lower OC contents (ranging from 12.1 mg g^{-1} to 15.4 mg g^{-1}) than the earthworm casts (Table 1). The OC contents of fresh casts produced by *L. castaneus* and *L. terrestris* (21.2 mg g^{-1} and 19.5 mg g^{-1} respectively) were significantly higher than those of *E. fetida* (17.3 mg g^{-1}), *A. caliginosa* (1.65 %) and *A. icterica* (13.5 mg g^{-1}) casts (Table 1). The casts produced by the anecic species *A. nocturna* showed intermediate OC contents (Table 1). Between 7 and 140 days,

Table 1

Chemical parameters related to the OM quantity and quality (Total OC content, OM thermal stability indices and C-CO₂ mineralisation output) of casts produced by several earthworm species and of control aggregates. Different letters represent significant differences, obtained by parametric ANOVA test (p-values: $p < 0.05$, $n = 5$), comparing the parameter average (and standard deviation) values between treatments for a same date (7, 42 or 140 days).

Parameters	Time (d)	Control	<i>L. terrestris</i>	<i>A. nocturna</i>	<i>A. icterica</i>	<i>A. caliginosa</i>	<i>L. castaneus</i>	<i>E. fetida</i>	p-value
Total OC (mg g ⁻¹)	7	12.1 (±1.9) d	19.5 (±1.7) ab	18.2 (±2.3) bc	13.5 (±1.4) d	16.5 (±2.6) c	21.2 (±2.8) a	17.3 (±0.8) c	0.0003
	42	15.4 (±1.5) bc	16.8 (±1.8) ab	17.3 (±2.2) ab	13.8 (±1.4) c	15.6 (±2.8) bc	19.6 (±1.8) a	16.9 (±2.2) b	0.02
	140	13.7 (±1.8) c	16.1 (±1.6) a	16.0 (±2.3) ab	12.8 (±0.8) c	13.6 (±1.4) c	17.1 (±2.3) a	13.9 (±0.3) bc	0.0071
R-index	7	0.45 (±0.02) a	0.31 (±0.02) c	0.32 (±0.03) c	0.41 (±0.03) ab	0.35 (±0.05) bc	0.29 (±0.05) c	0.34 (±0.02) bc	0.0005
	42	0.40 (±0.03) ab	0.34 (±0.03) bc	0.33 (±0.05) bc	0.41 (±0.02) a	0.39 (±0.04) ab	0.28 (±0.03) c	0.33 (±0.04) bc	0.001
	140	0.45 (±0.02)	0.38 (±0.02)	0.40 (±0.06)	0.44 (±0.03)	0.40 (±0.04)	0.36 (±0.05)	0.43 (±0.02)	n.s.
I-index	7	0.34 (±0.03) c	0.59 (±0.04) a	0.56 (±0.06) a	0.42 (±0.05) bc	0.52 (±0.08) ab	0.61 (±0.13) a	0.53 (±0.04) ab	0.001
	42	0.45 (±0.05) b	0.55 (±0.06) ab	0.56 (±0.09) ab	0.43 (±0.04) b	0.46 (±0.06) b	0.67 (±0.05) a	0.56 (±0.07) ab	0.001
	140	0.34 (±0.03) b	0.46 (±0.04) ab	0.43 (±0.11) ab	0.38 (±0.05) ab	0.42 (±0.06) ab	0.49 (±0.09) a	0.38 (±0.04) ab	0.04
Mineralisation Rates (mg CO ₂ -C h ⁻¹ g ⁻¹)	7	0.188 (±0.029) bc	0.255 (±0.078) abc	0.217 (±0.054) abc	0.199 (±0.052) bc	0.155 (±0.008) c	0.294 (±0.022) a	0.263 (±0.054) ab	0.005
	42	0.125 (±0.006) c	0.163 (±0.020) ab	0.192 (±0.023) a	0.143 (±0.014) abc	0.138 (±0.017) bc	0.168 (±0.037) ab	0.138 (±0.016) bc	0.004
	140	0.109 (±0.015) ab	0.117 (±0.017) ab	0.149 (±0.050) ab	0.114 (±0.044) ab	0.092 (±0.018) b	0.153 (±0.010) a	0.099 (±0.017) b	0.02

the OC decreased for all species: the largest decrease (20 %) was observed for *E. fetida* casts and the lowest (5 %) for *A. icterica* casts (Table 1). The significant differences among OC contents of fresh casts of different species persisted during cast ageing: 140 days after production, *L. castaneus* and *L. terrestris* casts presented higher OC content (17.1 mg g⁻¹ and 16.1 mg g⁻¹, respectively) compared to those of *E. fetida* (13.9 mg g⁻¹), *A. caliginosa* (13.6 mg g⁻¹) and *A. icterica* (12.8 mg g⁻¹) (Table 1).

3.1.2. Organic matter (OM) thermal characteristics

The R- and I-index are presented in Table 1. The R-index ranged from 0.28 to 0.45, and the I-index from 0.34 to 0.67 (Table 1). The fresh control aggregates and the fresh casts of *A. icterica* exhibited a significantly higher R-index and significantly lower I-index as compared to

fresh casts of the anecic species (*L. terrestris* & *A. nocturna*) and of the epigeic species *L. castaneus* (Table 1). For all casts except the epigeic ones, the R- index constantly increased between 7 and 140 days, while the I-index constantly decreased (Table 1). At the end of the incubation, the R-index was similar for all samples, while the I-index of *L. castaneus* casts was significantly higher than that of the control aggregates (Table 1).

3.1.3. Physical characteristics

Cast mass ranged from 131 and 469 g, volume varied between 101 and 254 mm³, specific area was between 242 and 428 mm² and the diameter between 5.5 and 8.2 mm. The casts of anecic species were heavier and had a higher volume than those of the epigeic species *L. castaneus* and *E. fetida* (Table 2). Endogeic species produced casts of

Table 2

Physical parameters related to the microstructure of casts produced by several earthworm species and of control aggregates. Different letters represent significant differences, obtained by parametric ANOVA test (p-values: $p < 0.05$, $n = 5$), comparing the parameter average (and standard deviation) values between treatments for a same date (7, 42 or 140 days). Parameters displayed in bold indicate statistical differences between species-specific casts over the whole 140 days of ageing.

Parameters	<i>L. terrestris</i>	<i>A. nocturna</i>	<i>A. icterica</i>	<i>A. caliginosa</i>	<i>L. castaneus</i>	<i>E. fetida</i>	p-value
Cast mass (mg)	469.7 a	347.8 ab	348.8 ab	299.3 ab	199.5 bc	131.8c	0.04
Cast volume (mm³)	290.3 a	218.7 ab	221.5 ab	179.1 bc	125.1 cd	80.0 d	0.02
Cast density (g cm ⁻³)	1.6	1.6	1.5	1.7	1.6	1.7	n.s.
Cast specific area (mm²)	428.3 a	313.0 abc	388.5 ab	283.1 bc	261.2c	242.2c	0.04
Cast diameter (mm)	8.3 a	6.1 abc	8.6 ab	6.0 bc	5.5c	5.9 bc	0.04
Porosity (%)	2.76	4.99	4.42	2.05	6.74	5.38	n.s.
Connected pores (%)	2.05	4.48	4.19	1.65	6.14	5.07	n.s.
Unconnected pores (%)	0.72 a	0.52 ab	0.23b	0.41 ab	0.60 a	0.32 ab	0.03
Mean pore volume (mm ³)	0.05	0.13	0.12	0.04	0.10	0.05	n.s.
Mean connected pore volume (mm ³)	0.08	0.20	0.13	0.13	0.13	0.06	n.s.
POM (%)	4.81	3.78	3.18	1.64	4.78	4.57	n.s.
Connected POM (%)	4.24	3.40	2.87	1.37	4.71	4.52	n.s.
Unconnected POM (%)	0.56 a	0.37 ab	0.30 ab	0.27 ab	0.07b	0.05b	0.04
Mean POM volume (mm ³)	0.12	0.10	0.11	0.04	0.16	0.07	n.s.
Mean connected POM volume (mm ³)	0.21	0.12	0.12	0.05	0.16	0.08	n.s.

intermediate size compared to the other ecological groups, with (i) significantly higher average volumes for the casts of *A. icterica* compared to the casts of epigeic species and (ii) significantly higher volumes for *A. caliginosa* casts compared to *E. fetida* ones (Table 2). The specific area (mm^2) and the thickness (mm) were significantly higher for *L. terrestris* casts as compared to those of *A. caliginosa* or epigeic species (Table 2).

3.2. Micro-scale characteristics of earthworm casts

3.2.1. Porosity

The micro-scale features of earthworm casts are presented in Table 2 and Fig. 1. On average over the whole incubation period (7 to 140 days), the cast porosity varied according to the ecological category. Casts of epigeic species showed the highest porosity followed by those of endogeic species, while casts of anecic species showed the lowest porosity (Table 2). While on average the cast porosity was characteristic for their ecological group, the evolution of cast porosity during ageing was species specific. Less than seven days after their production, *A. icterica*, *A. caliginosa* and *A. nocturna* casts were characterized by lower porosity (0.74 %) compared to *L. terrestris* (3.23 %) or *L. castaneus* casts (4.60 %) (Fig. 1). Except for the casts of the anecic species *L. terrestris*, which exhibited a decreasing porosity between 7 and 140 days, the casts of all other species showed an increase (Fig. 1). Indeed, while the porosity of *L. terrestris* casts decreased by 40 % over the incubation period, it increased by 239 % for *A. caliginosa* casts (Fig. 1). The porosity of casts produced by the two epigeic species evolved similarly during ageing. Indeed, among the six species, their fresh casts presented the highest porosity, which gradually increased until the end of the ageing period by 68 % for *L. castaneus* and by 73 % for *E. fetida* (Fig. 1). At the end of the cast ageing period, the porosity of *A. nocturna* and *A. icterica* casts were respectively 18 and 14 times higher compared to the casts of the other species (Fig. 1).

The proportion of connected porosity ranged from 0.38 to 10.80 % in the casts of all species (Fig. 1) and was closely related to the total porosity, with an average of 88 % of the porosity connected to air (Table 2). The mean pore volume, calculated as the mean of each individual pore volume of the same cast, was in general inferior to 0.10 mm^3 (ranging from 0.03 to 0.08 mm^3) (Fig. 1). Only the casts of *L. castaneus*, *A. icterica* and *A. nocturna* showed an increasing pore volume with time: 0.16, 0.29 and 0.32 mm^3 respectively (Fig. 2). Among all the porosity parameters, the proportion of unconnected pores is the only parameter that significantly discriminates casts produced by different species, even after 140 days (Table 2). The casts of *L. castaneus* contained 0.72 % of unconnected porosity and these average values were significantly higher, as compared to the proportion of unconnected pores of *A. icterica* casts (average: 0.23 %), while the other species showed intermediate values (Table 2).

3.2.2. Particulate organic matter

The fresh casts of *A. caliginosa* contained only half as much POM

(2.28 %) as the fresh casts produced by other species (6.01 % on average) (Fig. 2). The POM content decreased with time by 5.5 to 1.7 % in casts of all species. The casts of *L. terrestris* experienced the highest decrease over 140 days (Fig. 2), mainly during the second part of the incubation period, between 42 and 140 days (Fig. 2). The POM volume of casts produced by the two epigeic species showed a similar evolution as the casts produced by *L. terrestris*, with the highest POM contents observed during the second part of the incubation (Fig. 2). By contrast, in the casts of *A. caliginosa* (70 % in 140 days), strongest POM decrease was recorded in the early stages of incubation, between 7 and 42 days (Fig. 2). The POM content of *A. nocturna* and *A. icterica* casts gradually decreased by 68 and 77 % over the incubation period (Fig. 2). After 140 days of incubation, the casts produced by epigeic species displayed the highest POM contents and the casts of the endogeic species *A. caliginosa* the lowest (Table 2).

The proportion of POM connected to the atmosphere ranged from 0.82 % for the cast of *A. icterica* at 140 days to 6.32 % for the fresh cast of *L. terrestris* (Fig. 2). The mean volume of POM, calculated as the mean of each individual POM volume in the same cast, ranged from 0.04 to 0.24 mm^3 and tended to decrease over time in the casts of all earthworm species, except in the ones produced by the epigeic species *L. castaneus* (Fig. 2). Similar to the proportion of unconnected porosity (%), the contribution of unconnected POM (%) appeared to be a significant discriminating factor that varied among casts of several earthworm species (Table 2). The casts produced by the epigeic species *L. castaneus* and *E. fetida* showed significantly lower unconnected POM contributions (0.07 and 0.05 % respectively) as compared to the casts of *L. terrestris* (average: 0.56 %, Table 2).

3.3. CO_2 emission potential and relationships with cast characteristics

3.3.1. Carbon mineralization dynamics in earthworm casts

Over the whole incubation period, the mineralization rates ranged from 0.092 to $0.294 \text{ mg of CO}_2\text{-C h}^{-1} \text{ g}^{-1}$ of dry casts (Table 1). It decreased between 7 and 140 days for all species. The highest decrease of cast mineralization rates was observed for *E. fetida* (62 %) and the lowest for *A. nocturna* (31 %) (Table 1). After seven days, the casts produced by the epigeic species *L. castaneus* exhibited significantly higher mineralization rates ($0.294 \text{ mg CO}_2\text{-C h}^{-1} \text{ g}^{-1}$) than the casts of the endogeic species *A. caliginosa* ($0.155 \text{ mg CO}_2\text{-C h}^{-1} \text{ g}^{-1}$) and *A. icterica* ($0.199 \text{ mg CO}_2\text{-C h}^{-1} \text{ g}^{-1}$), as well as the control aggregates ($0.188 \text{ mg CO}_2\text{-C h}^{-1} \text{ g}^{-1}$) (Table 1). After 42 days of incubation, the mineralization rates of *E. fetida* casts strongly decreased to reach an average value of $0.138 \text{ mg CO}_2\text{-C h}^{-1} \text{ g}^{-1}$ (Table 1). Likewise, the casts of *A. caliginosa* and the control aggregates showed significantly lower mineralization rates (0.138 and $0.125 \text{ mg CO}_2\text{-C h}^{-1} \text{ g}^{-1}$ respectively) compared to *A. nocturna* casts ($0.192 \text{ mg CO}_2\text{-C h}^{-1} \text{ g}^{-1}$) (Table 1). At the end of the incubation, the differences among the mineralization rates were less pronounced: only the casts of *L. castaneus* displayed significantly higher rates compared to *E. fetida* and *A. caliginosa* casts (Table 1).

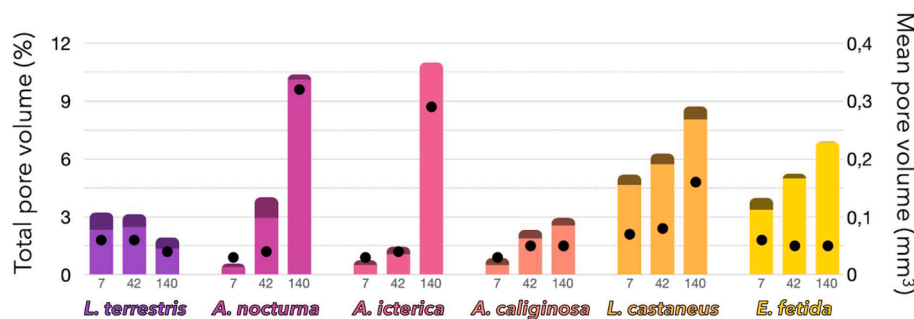


Fig. 1. Connected (light) and unconnected (dark) pore volume (%) of casts belonging to 6 earthworm species at 7, 42 and 140 days ($n = 1$). The mean volume of pore (mm^3) measured on each cast is displayed on the right-scale of the chart and is represented by black dots.

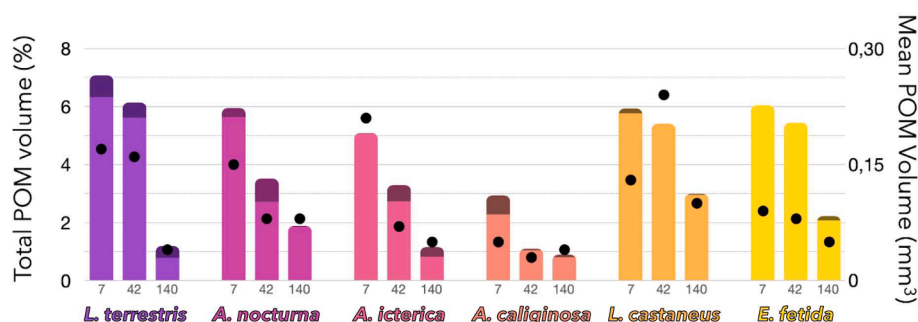


Fig. 2. Connected (light) and unconnected (dark) POM volume (%) of casts belonging to six earthworm species at 7, 42 and 140 days ($n = 1$). The mean volume of POM (mm^3) measured on each cast is displayed on the right-scale of the chart and is represented by black dots.

3.3.2. Earthworm specific-changes in cast features

The PCA of physicochemical and thermal parameters showed species-specific changes of cast features during ageing (Fig. 4). The first two dimensions of the PCA explained 56.3 % of the total dataset inertia (Fig. 4a). The first axis of the PCA was positively correlated with the proportion of POM, OC contents and I-index (Fig. 4b). It was negatively correlated to porosity and R-index (Fig. 4b). The casts characterized by a large size and high R-index (Table 2) were positively correlated with the second axis of the PCA (PC2), while the casts with higher porosity and/or POM were negatively correlated with PC2 (Fig. 4b).

After ageing, the casts of the epigeic species *L. castaneus* still emitted significantly more CO_2 than the casts of the other epigeic species *E. fetida* and endogeic species *A. icterica*. The casts produced by the two epigeic species and the endogeic species *A. caliginosa* presented a similar pattern of microstructure evolutions (Fig. 4a), with the difference that *A. caliginosa* fresh casts presented a lower POM content and smaller POM fragments (Fig. 2), resulting in a lower porosity shift (Fig. 3).

3.3.3. Factors determining cast CO_2 emissions

The Pearson correlation test showed that the mineralization rates of earthworm casts were significantly correlated with the total OC ($r = 0.73$, $p\text{-value} < 0.001$), and OM thermal indexes R and I ($r = -0.67$, $p\text{-value} < 0.01$; $r = 0.63$, $p\text{-value} < 0.01$) (Table 3). The mineralization rates also showed significant positive correlations with the spatial organization of POM at micro-scale (Table 3). Moreover, they were

correlated with the POM content ($r = 0.79$, $p\text{-value} < 0.001$), the proportion of connected POM ($r = 0.78$, $p\text{-value} < 0.001$), the mean POM volume ($r = 0.53$, $p\text{-value} < 0.05$) and the mean connected POM volume ($r = 0.56$, $p\text{-value} < 0.05$).

In order to explain the variability of the mineralization rates (Table 1), we performed a GLMnet machine learning algorithm using chemical and physical features (Tables 1 and 2). Out of the 18 parameters tested by the GLMnet model as possible predictors of the CO_2 mineralization rates, the best predictive model (Fig. 3) was a function of nine predictors driving the model outcomes: (i) the mean volume of connected pores, (ii) the POM content (iii) the mean volume of connected POM, (iv) the cast thickness, (v) the proportion of unconnected porosity, (vi) the cast specific area, (vii) the proportion of connected porosity, (viii) the mean volume of pores and (ix) the proportion of connected POM. As shown by the significant correlation between the predicted and measured mineralization rates (pseudo- $R^2 = 0.92$, $p\text{-value} = p < 0.001$), these nine indicators explained 52 % of the variability observed in earthworm casts during ageing (RMSE = 0.0403, LOOCV- $R^2 = 0.52$, RPD = 2.53).

4. Discussion

4.1. Do earthworms produce casts with species-specific properties?

As compared to the control aggregates, the fresh casts produced by

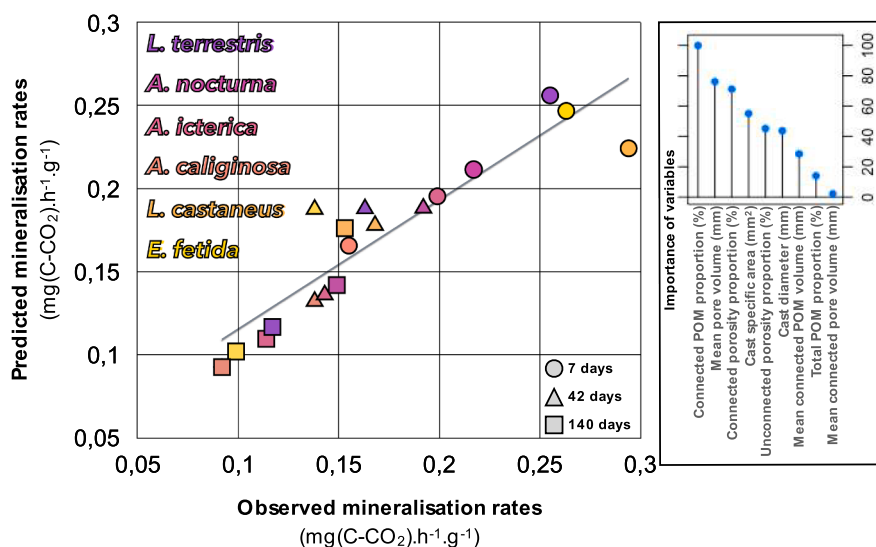


Fig. 3. Observed average values ($n = 5$) of the C-CO_2 emissions rates vs predicted values calculated from the best GLMnet models. Observed data were assessed on casts produced by six earthworm species (colors) and at different dates (round: 7 days, triangle: 42 days and square: 140 days). The explicative variables used in the predictive model and their respective contributions are presented on the right part of the chart.

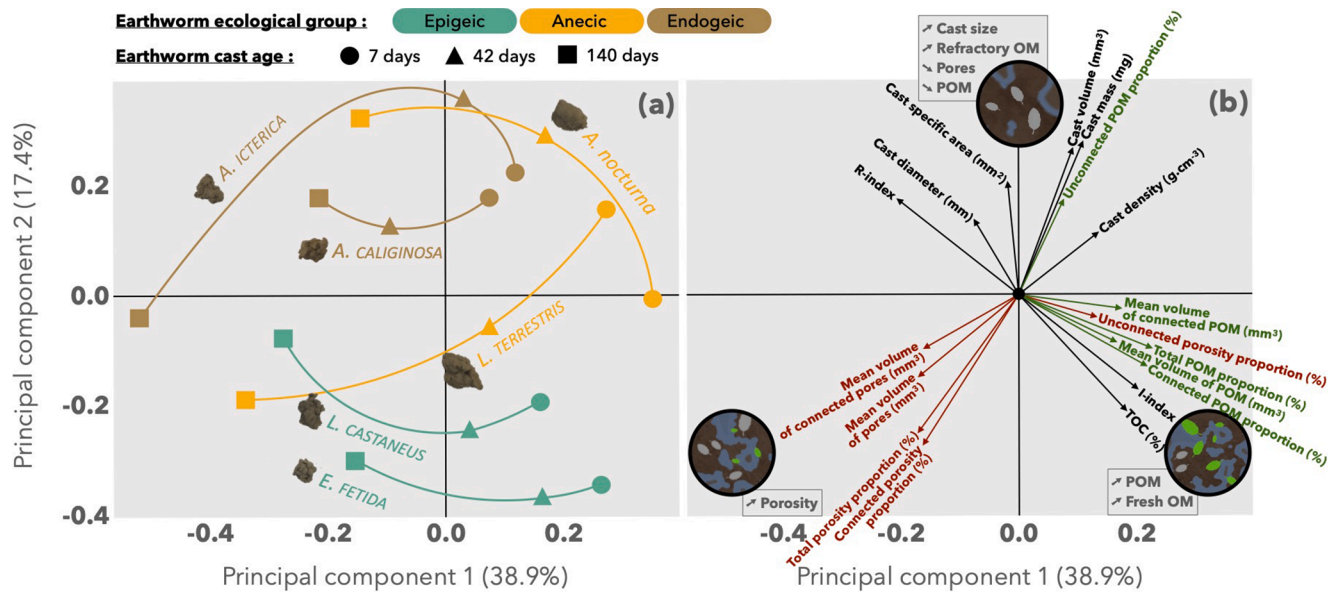


Fig. 4. Principal component analysis (PCA) of cast parameters of six earthworm species, from the three main ecological categories (colors), and at different dates (round: 7 days, triangle: 42 days and square: 140 days). On the left part of the chart, we displayed (a), the variability of earthworm cast microstructures throughout ageing, with PCA loadings of the general cast parameters (in black), of the porosity features (in red). POM features (in green) are presented on the right part of the chart (b).

Table 3
Pearson's correlation test between the 18 (chemical and physical) parameters assessed for earthworm casts (6 species, 3 dates, n = 18) and the average C-CO₂ mineralisation rates. Symbols for p-values: *** p < 0.001; ** p < 0.01; * p < 0.05. Parameters in bold were the 9 predictors used in the predictive GLMnet model.

	Mineralisation rates (mg CO ₂ -C h ⁻¹ g ⁻¹)
Chemical parameters	
Total OC (mg g ⁻¹)	0.73 ***
R-index	-0.67 **
I-index	0.63 **
Physical parameters	
Cast mass (mg)	0.18
Cast volume (mm ³)	0.18
Cast density (g cm ⁻³)	0.13
Cast specific area (mm ²)	-0.08
Cast diameter (mm)	-0.11
Porosity proportion (%)	-0.21
Connected porosity proportion (%)	-0.25
Unconnected porosity proportion (%)	0.45
Mean pore volume (mm ³)	-0.21
Mean connected pore volume (mm ³)	-0.30
Total POM proportion (%)	0.79 ***
Connected POM proportion (%)	0.78 ***
Unconnected POM proportion (%)	0.11
Mean POM volume (mm ³)	0.53 *
Mean connected POM volume (mm ³)	0.56 *

all earthworm species exhibited higher OC content and higher I indices, which could indicate a higher contribution of thermally labile compounds (Le Mer et al., 2020; Sebag et al., 2016). This is in agreement with previous studies indicating that earthworm casts are characterized by increased OC contents and higher contribution of fresh OM as compared to the surrounding soil aggregates (Bossuyt et al., 2004; Bottinelli et al., 2021; Guggenberger et al., 1995; Le Mer et al., 2021; Lee, 1985). The results of our study indicated that these specific cast properties are modulated by the earthworm species. Indeed, casts of the six earthworm species showed significantly different OC contents and I indices, although produced with similar soil and OM type.

PCA analyses of physicochemical and thermal properties separated

casts according to the earthworm species belonging to the three ecological categories. For example, fresh casts of epigeic species were separated from the other categories based on their high POM content and porosity (Fig. 4b), which is consistent with the ecological strategies of these species living in litter layers (Bouché, 1977; Edwards, 2004). Anecic casts were separated from endogeic casts along the first axis of the PCA (Fig. 4a), indicating a comparatively high proportion of fresh OM with larger sizes in anecic casts. This may be explained by the anecic species' feeding on plant debris at the soil surface (Judas, 1992; Scheu, 1987), while endogeic species living in soil ingest higher proportions of SOM. Our results thus indicate that the features of fresh casts are related to the earthworm ecological categories with specific feeding strategies (Andriuzzi, 2015; Blouin et al., 2013; McLaughlin, 1994), and that these are maintained even in artificial settings outside soil environment.

Are soil organic carbon (SOC) destabilization processes species-specific in earthworm casts during ageing?

We investigated the controls of SOC mineralization during ageing of earthworm casts and detected significant differences among casts produced by the six earthworm species (Table 1). The contrasting CO₂ emissions of casts suggest an earthworm-induced variation of SOC mineralization, which may derive from various mechanisms. In particular, the microbiome structure of casts may be earthworm species-specific (Sapkota et al., 2020). This may be a result of the guts' environments, which could act as selective filters for soil microorganisms (Byzov et al., 2015, 2007; Nechitaylo et al., 2010), thus providing different conditions for OC mineralization in the resulting casts. In addition, these conditions may depend on the evolution of physicochemical properties at macro- and micro-scale during cast ageing, which could be species-specific.

The cast microstructure evolution revealed a replacement of POM by porosity (Fig. 3, Fig. 4a and b), highlighting a positive feedback interaction: the more POM is decomposed, the more it will be connected to the air, providing oxygen for microbial decomposition of organic materials (Kravchenko et al., 2018; Kravchenko et al., 2015) and a greater accessibility for microorganisms (Rabbi et al., 2016; Rabot et al., 2018). These processes have been described as the main SOC destabilization mechanisms during cast ageing (Puche et al., 2022), which leads to a decrease of POM proportions and to a relative increase of mineral associated SOM (Vidal et al., 2019; Bottinelli, 2020). Our results indicate

a species-specific evolution of the porosity, which may control the casts' water content and POM accessibility (Puche et al., 2022), leading to differences in microbial and fungal communities (Vedere et al., 2020; Vidal et al., 2016b). Except for epigeic species, which mainly feed on litter, a species-specific effect seems to have led to the variation of the earthworm cast features during ageing (Fig. 4).

Our machine learning approach showed that 52 % of the CO₂ mineralization variability of earthworm casts was explained by the casts' physicochemical and thermal properties (Fig. 3). For instance, the fresh casts of *L. castaneus* emitted on average significantly more CO₂ compared to the ones of endogeic species showing lower OC contents (Table 1). These relationships were consistent with previous studies that demonstrated a stimulating effect of OM incorporation into earthworm casts on OC mineralisation (Barthod et al., 2020, 2021; Brown et al., 2000; Chotte et al., 1998; Hamer et al., 2004; Kuzyakov and Kuzyakov, 2010). On the other hand, the casts' microstructural features (Table 3) seemed to drive an important part of the OC mineralization dynamics during cast ageing (Fig. 3), in particular (connected) porosity, pores (Fig. 1) and (connected) POM contents (Fig. 2). This general shift observed throughout cast ageing highlighted (i) the important role of earthworm feeding strategies for the incorporation of OM (varying in quantity and quality between ecological categories) in fresh casts and (ii) how POM' interactions with the cast porosity seemed to control the OC mineralization, by regulating POM accessibility for microorganisms (Angst et al., 2017; Puche et al., 2022; Vidal et al., 2019). Briefly, our work shows that the species-specific morphological traits of earthworms may induce formation of casts with significantly different OC mineralization throughout their ageing. At the beginning of the experiment, we observed that the properties of anecic fresh casts differed from the ones of endogeic species while, after 140 days, the cast properties of *L. terrestris* and *A. icterica* exhibited less variability than those of *A. nocturna* and *A. caliginosa* (Fig. 4, Fig. 5). We argue that these differences vary between species belonging to the same earthworm ecological category, suggesting that these categories are not related to the earthworms' effects on OC mineralisation after cast deposition (Bottinelli and Capowiez, 2021).

4.2. Can earthworm morphological traits explain cast characteristics and CO₂ mineralization rates?

To further explore the relationship between characteristics of

earthworm casts (Table 3) and morphological earthworm traits (Bouché, 1972), we performed a co-inertia analysis (CIA) with these data (Fig. 5). Here, we hypothesized that earthworm traits such as (i) earthworm body size, (ii) epithelium rigidity, (iii) pigmentation, (iv) typhlosole and (v) muscle types might explain specific macro- and micro-scale cast features. We found that morphological earthworm traits were correlated to cast features (Fig. 5). For example, epigeic species with smaller body size produced smaller and lighter casts with lower specific area in contact with the air (Table 2) compared to species from the other ecological groups. This CIA also showed that some earthworm traits of endogeic species, negatively and positively correlated to the first and second axis respectively, were related to earthworm casts displaying higher R-index values (Fig. 5). We propose that this higher contribution of thermally recalcitrant OM (Albrecht et al., 2015; Sebag et al., 2016) can be explained by earthworm morphological adaptations to their specific environment. Living in deeper soil layers with lower access to fresh OM, a multi-lamellated typhlosole probably allows a more efficient absorption of digested nutrients (Bernier, 1998; Bouché, 1972). To some extent cast characteristics and CO₂ mineralization potential may thus be related to specific species traits (Coq et al., 2022). However, the RV coefficient decreased during casts ageing (Fig. 5). This demonstrates that the morphological traits, referring to the feeding strategy, cannot explain the whole variability of OC mineralization during cast ageing.

5. Conclusion

In this study, we explored the diversity of microstructures of casts produced by six earthworm species belonging to three ecological categories to assess their specific effects on OC mineralization. Our results, obtained in artificial environment, indicated that physicochemical and thermal properties of fresh casts may be related to ecological categories, while SOC dynamics, which was due to alterations of microstructural cast features during cast ageing could be species specific. We thus conclude that changes in cast properties during cast ageing may be earthworm species-specific, however, not directly related to earthworm morphological traits. The earthworm species' effect on micro-aggregation and/or organo-mineral interactions could be a key driver of SOC dynamics.

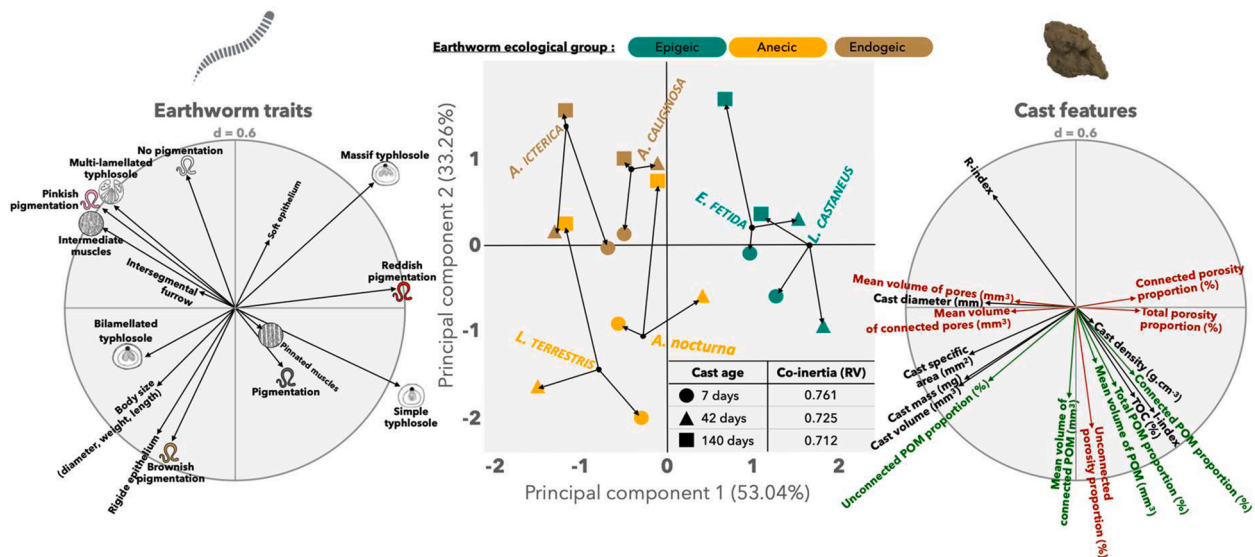


Fig. 5. Co-inertia analysis between (i) cast features at different dates (round: 7 days, triangle: 42 days and square: 140 days), with general cast parameters (in black), porosity features (in red) and POM features (in green), and (ii) ecological traits of earthworm species (from Bouché, 1972), with traits related to the earthworm body size and their epithelium rigidity, to their pigmentation, their type of muscles and typhlosole.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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