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N. Bottinelli[1,2]; P. Jouquet[2]; T.M. Tran[1], H. Aroui Boukbida[2]; C. Rumpel[2]

[1] Soils and Fertilizers Research Institute (SFRI), Duc Thang, Bac Tu Liem, Hanoi, Vietnam

[2]IRD, CNRS, Institut d'écologie et des sciences de l'environnement (IESS-Paris UMR Sorbonne Université, UPEC, CNRS, IRD, INRAe), FEST team, F-93143 Bondy, France

corresponding author : Nicolas Bottinelli (email : nicolas.bottinelli@ird.fr)

Abstract

Earthworm cast ageing controls soil ecosystem services such as carbon storage and nutrient availability. To test the hypothesis that biogeochemical changes during casts ageing can be traced by infrared spectroscopy, we collected topsoil aggregates and earthworm casts produced by the anecic earthworm *Amynthas khami* belonging to five distinct degradation stages in woodland in northern Vietnam. We analysed mid-infrared (MIR) spectra of both sample types using two approaches: (1) calculation of the proportion of aliphatic compounds and the humification index of organic matter (OM) using specific wavenumbers and (2) linear discriminant analysis (LDA) of the most important peaks and shoulders of the entire spectrum. Our results showed that aliphatic compound contribution to OM was similar and the humification index of OM lower (2-fold) in casts as compared to topsoil aggregates independent of their degradation stage. In contrast, LDA showed a continuous shift from fresh casts to aged casts and topsoil aggregates in agreement with biogeochemical changes of cast properties. We thus conclude that MIR analysis of bulk samples and LDA taking into account the absorbance intensity of most important wavenumbers is a promising tool to capture biogeochemical alterations of organic and mineral earthworm cast properties during ageing.

Key words: bioturbation; aggregation; macrofauna; FTIR spectroscopy

Introduction

Earthworms impact soil properties through their activity. In particular, they have a strong influence on soil organic matter (OM) storage and chemical composition through cast formation, which leads to enrichment of particulate OM occluded in aggregates (Bossuyt et al., 2004) and association of OM with minerals (Barthod et al., 2020). It was shown that the elemental content (Decaëns et al., 1999), and the composition of OM undergo changes after cast deposition. These changes reflect different cast ageing stages, which have distinct biogeochemical properties compared to surrounding soil aggregates without earthworm influence (Bottinelli et al., 2020). In the present study, we used mid infrared spectroscopy (MIR) to generate spectral fingerprints, which may allow in combination with chemometric approaches for the differentiation of cast degradation stages and topsoil aggregates. This technique has the advantage of being inexpensive and rapid. It has already been used to assess the biogenic origin of soil aggregates (Jouquet et al., 2009), and to identify biostructures produced by earthworms and their age (Dominguez-Haydar et al., 2020; Zangerlé et al., 2014). However, up to date, this method has never been used to follow biogeochemical changes of cast properties during field ageing. In this study we aimed to differentiate five different degradation stages of casts produced by the anecic earthworm *Amynthas khami* using mid-infrared (MIR) spectroscopy and two different approaches of data exploitation. We hypothesised that changes in organic compounds during the degradation of casts would be captured by MIR spectroscopy.

We sampled earthworm casts produced by the anecic earthworm species *A. khami* and topsoil aggregates in subtropical woodland in northern Vietnam (20° 57'N, 105° 29'E) in close proximity at sampling locations with similar microclimatic conditions. The soil type was Acrisol (WRB) (Podwojewski et al., 2008), with an organic carbon content of 2.5%, a clay content of 50% and a pH (H_2O) of 5. The mass of casts found at the time of sampling

represented 4400 g (oven dry weight) per m^2. We collected five replicates of casts with different degradation stages (A to E) differentiated by visual aspects and adjacent topsoil (2–7 cm depth) without visible earthworm activity. Cast A and B were fresh humid casts assumed to be 1 day and 1 week old. Cast C was in dry state. D casts showed signs of degradation (greenish colour due to the presence algae with cracks at the surface). The size of casts (A to D) and topsoil aggregates varied from 5 to 15 cm. Casts were produced through the accumulation of fecal pellets deposited the one on the other. After disintegration probably due to livestock trampling, human traffic, root growth and raindrop impact or natural aggregate turnover, individual pellet of 1 cm diameter were released: cast E of unknown age, consisting of small aggregates and representing the last stage of degradation recognizable by naked eye. MIR spectra of air-dry ground samples (< 200 µm) were collected with a Fourier Transform Spectrophotometer (FTIR 660 Agilent ex-Varian, Agilent Technologies, Inc., Santa Clara, USA), operating in diffuse reflectance mode. Measurements were made at 2 cm^{-1} intervals in the MIR range (4000-400 cm^{-1}) and converted to absorbance. The raw spectra were subjected to pre-processing, including Savitzky-Golay smoothing (first order polynomial filter with a 13-point window), baseline correction (polynomial fitting with degree 3) and normalisation by the sum of absorbance across 4,000-400 cm^{-1}.

The MIR spectra of earthworm casts and soil aggregates featured common peaks, with relatively similar intensities (**Fig. S1**). At first, we assessed OM changes during earthworm cast ageing through calculation of aliphatic compound contribution and the humification index (Demyan et al., 2012; Margenot and Odson, 2016). In fact, the digestion of fresh OM by anecic species and microbial activity in casts might result in degradation of easily decomposable OM compounds and concomitant increase of aliphatic and aromatic OM compounds and the humification index (Barthod et al., 2020). Aliphatic compound contribution was calculated as the absorbance intensity of C-H signals at 3010-2810 cm^{-1}

87 determined from the tangential baseline and normalized with the soils' organic carbon
88 content. In addition, the humification index was computed as the ratio of aliphatic absorbance
89 intensity with the absorbance intensity of aromatic C=C, ketone and quinone C=O, and/or
90 amide C=O at 1660-1580 cm^{-1} determined from the total baseline. One-way ANOVA was
91 used to compare these two indicators of OM changes in casts of different degradation stages
92 and topsoil aggregates (n = 5). Prior to running ANOVA, the data were tested for
93 homogeneity of variances and normality and log-transformed when required. LSD post hoc
94 multiple comparison tests were used when the effects were significant. Differences among
95 treatments were declared significant at the 0.05 probability level. ANOVA and LSD tests
96 were performed with "car" and "agricolae" packages of the R software. Our data showed that
97 the aliphatic peak intensity was greater in casts compared with topsoil aggregates without
98 difference among different degradation stages (**Fig. 1a**). The aliphatic peak intensity
99 normalized by soil organic carbon was similar in all samples (**Fig. 1b**). The humification
100 index of OM was smaller in casts than topsoil aggregates without difference among cast
101 degradation stages (**Fig. 1c**). Our results thus showed that direct interpretation of MIR signals
102 with general indicators assigned to OM compounds in an ANOVA did not distinguish cast
103 degradation stages. They were somehow useful to differentiate earthworm casts from topsoil
104 aggregates, containing less fresh OM (Guggenberger et al., 1996).
105 Our second strategy to differentiate earthworm cast degradation stages was based on
106 linear discriminant analysis (LDA) of important peaks and shoulders of the MIR spectra.
107 These signals were identified using the software OriginPro and the local maximum method
108 (local points equal 5): 2 peaks (3616 and 3427) from the region 4000-2800 cm^{-1} and 13 peaks
109 (1942, 1874, 1622, 1595, 1527, 1456, 1275, 1163, 1109, 1009, 949, 823, 708 cm^{-1}) from the
110 region 2000 to 600 cm^{-1} using the total baseline and (ii) 2 peaks (2920 and 2850 cm-1) from
111 the region 3010-2810 cm^{-1} using the tangential baseline (**Fig. S1**).

LDA is a multivariate supervised technique that maximizes the ratio of the between group variance to the within group variance. The Pillai test was used to verify the statistical significance of the discrimination. Our data showed that LDA was able to differentiate the five types of casts and the topsoil aggregates ($p < 0.05$, Pillai test) (**Fig. 2**). The first axis explaining 30% of the variability, differentiated cast A-E from topsoil aggregates reflecting their gradually changing properties (Bottinelli et al., 2020). The first axis was correlated to 3427, 2850, 2925, 1622, 1595, and 1456 cm^{-1} in the negative direction and 1942 cm^{-1} in the positive direction. Intensities of these wavenumbers may be related to OM as well as mineral soil constituents (Parikh et al., 2014). In particular, the band at 3400 cm^{-1} was found to be correlated with contribution of the light fraction to whole soil (Calderón et al., 2011). Light fractions are generally composed of fresh plant litter and were reported as early indicators of OM changes (Leifeld and Kögel-Knabner, 2005). The wavenumbers 1595 and 1456 cm^{-1} may be related to amide and carboxylate C, which are typical OM compounds subjected to change during OM degradation (Parikh et al., 2014). The wavenumber 1942 cm^{-1} corresponds to quartz and its gradual increase in intensity from cast A to topsoil aggregates might suggest the preferential loss of mineral constituents caused by rainfall (Nooren et al., 1995). Degradation of OM occurring during cast ageing and change in mineral composition thus explained the differentiation of the samples along axis 1. The second axis (explaining 25% of the variability) differentiated cast C, which showed lower absorbance intensity at 3616 cm^{-1} as compared to other casts and topsoil aggregates. Since this wavenumber corresponds to kaolinite, biogeochemical changes occurring at this stage may again involve mineral cast constituents. The heatmap (**Fig. S2)** based on the correlation matrix of significant peak intensities of MIR spectra (**Fig. 2**) with soil OM properties measured by Bottinelli et al. (2020) separated two groups of wavenumbers: (i) 3427, 2850, 1595 and 2925,1622 and 1456 cm^{-1} and (ii) 3616 and 1942 cm^{-1}. The first group was positively related to organic C

characteristics, while the second group may be related to involvement of mineral compounds in cast ageing because of its negative correlation with organic C. Our results therefore showed that MIR spectroscopy combined with multivariate analysis is capable of separating casts according to their degradation stage. Similar results were obtained by Zangerlé et al. (2014) and Dominguez-Haydar et al. (2020), who used near infrared spectroscopy to discrimate casts of different ages produced by lumbricid species in the laboratory. Contrary to Zangerlé et al. (2014), we found that field ageing affects not only organic but also mineral compounds possibly through selective loss of particles or soluble compounds.

We thus conclude that MIR analysis of bulk samples and LDA taking into account the absorbance intensity of the most important wavenumbers contributing to cast spectra is capable of capturing biogeochemical changes occurring during ageing and degradation of earthworm casts in the field.

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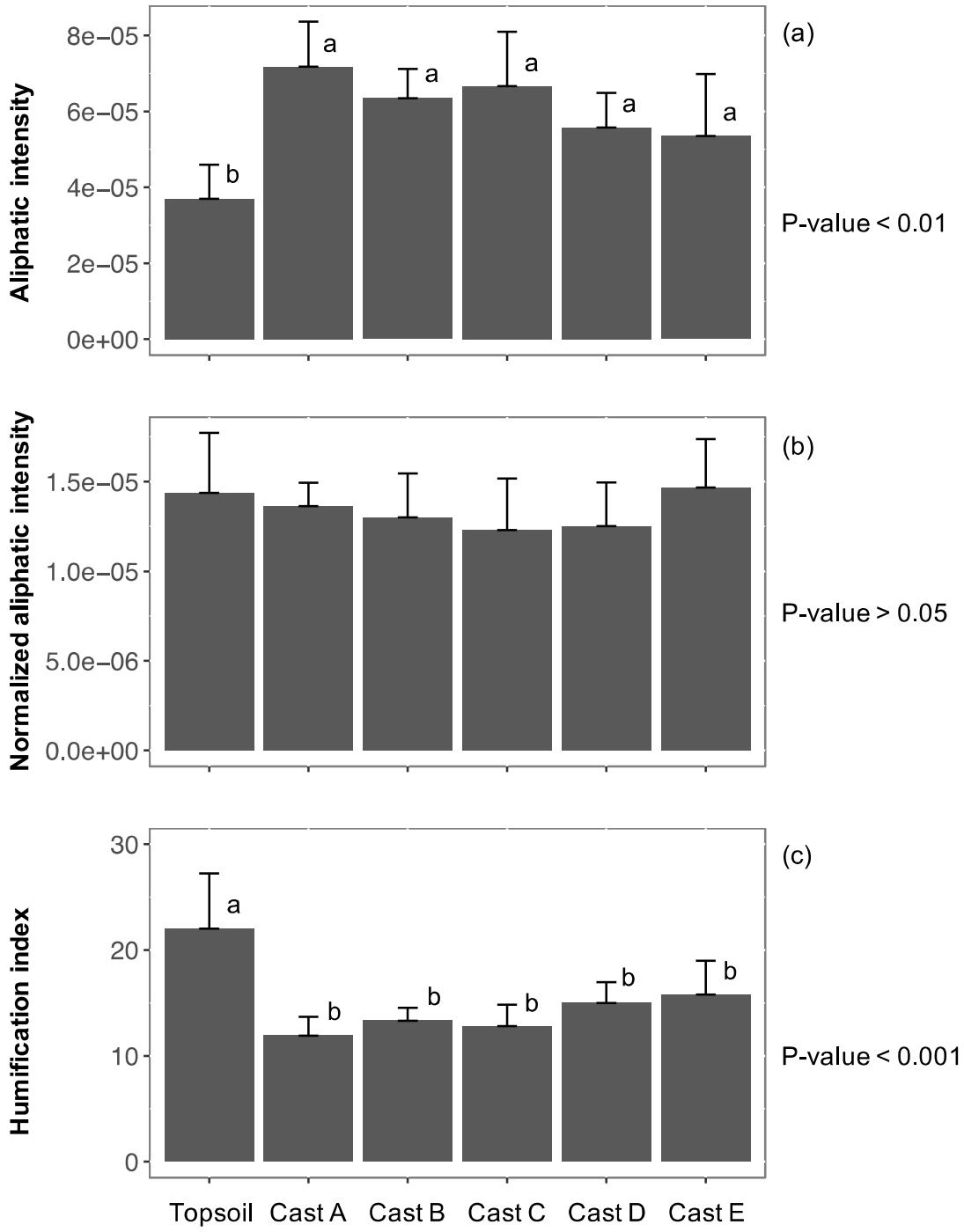
Figure captions

Figure S1: Average of MIR spectra showing mineral and organic peaks from earthworm casts presenting different degradation stages (A to E) and topsoil aggregates presenting no recent earthworm activity (n= 5). All spectra were smoothed, baseline corrected and normalized.

Figure 1: Average values (n = 5) of MIR peak intensity of C-H vibration from aliphatic carbon at 3010-2800 cm^{-1} (a), aliphatic carbon normalised by soil carbon (b) and humification index (c) for earthworm casts presenting different degradation stages and topsoil aggregates. Error bars indicate standard errors and letters denote statistical differences across samples.

Figure 2: LDA showing the differentiation of earthworm cast presenting different degradation stages (A to E) and topsoil aggregates based on the 17 peaks found on MIR spectra (**Fig. S1**). Ellipses correspond to 67 % confidence intervals around the species barycenter. The most important peaks contributing to the discrimination of the group samples (cosines between the wavenumbers and the canonical scores > 0.35 or < -0.35) are colored in red.

Figure S2: Heatmap based on the correlation matrix of significant peak intensities found on MIR spectra (Fig. 1) with soil OM properties measured in Bottinelli et al. (Bottinelli et al., 2020) for earthworm casts presenting different degradation stages (A to E) and topsoil aggregates presenting no recent earthworm activity. OM properties are percentage of lignin, N-containing, polysaccharides and unspecific compounds measured by pyrolysis; OC content and C/N ratio of the whole aggregate and OC the three physical fractions (macro-aggregate (mac.), micro-aggregates (mic.) and mineral-associated (min.)). Peaks are sorted according to a cluster analysis. Green color indicates positive correlations, red color indicates negative correlations and the strength of color indicates the magnitude of correlation coefficient, as shown on the correlation spectrum (inset top right).



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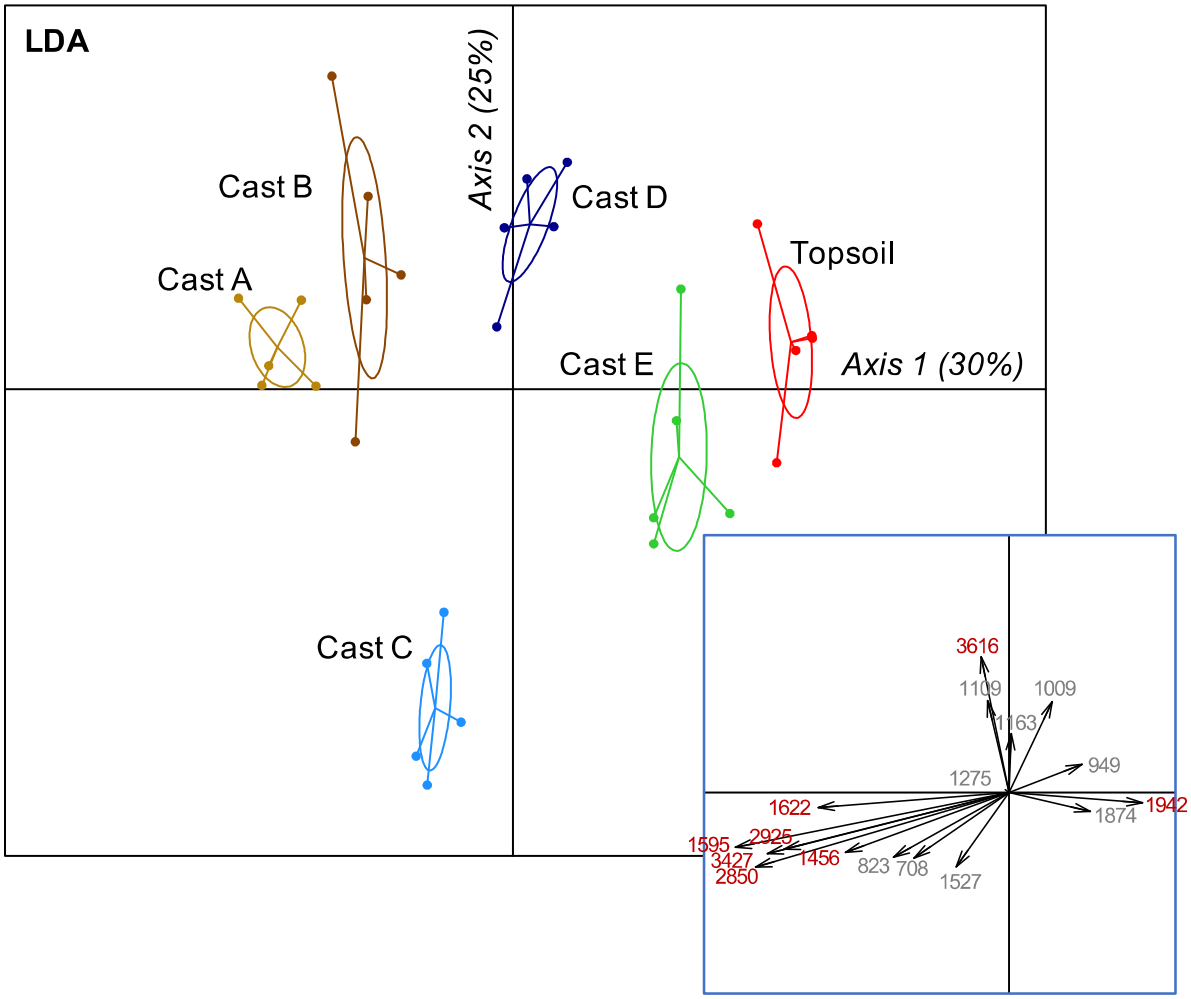

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