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1 **To what extent do ageing and soil properties influence *Amyntas khami* cast properties?**
2 **Evidence from a small watershed in northern Vietnam**

3

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26 **Abstract**

27 Understanding the variables explaining earthworm cast properties remains a key challenge in
28 soil ecology. For this purpose, this study aimed to determine the relationships between the
29 physical and chemical properties of earthworm casts and those of their surrounding soil
30 environment. Surface earthworm casts (recently emitted or degraded) produced by the anecic
31 earthworm *Amyntas khami* (Thai, 1984) and adjacent topsoil aggregates without traces of
32 earthworm activity were sampled in 50 different locations covering a small watershed in
33 northern Vietnam. We determined the organic carbon content, the spectral signatures of
34 aggregates and the humic index of organic matter *via* mid-infrared spectroscopy and analysis
35 of pH, hydrophobicity, wet aggregate stability and bulk density of soil aggregates.

36 While the physical properties of casts were not related to those of topsoil aggregates,
37 correlations were measured between cast and topsoil aggregate chemical properties. The
38 values of hydrophobicity, pH, bulk density and organic carbon were higher in casts than in
39 topsoil aggregates, whereas the humic index values were lower in casts than in topsoil
40 aggregates. No difference was measured between recent and degraded casts, with the
41 exception of their spectral signatures. Interestingly, the effect of *A. khami* was more important
42 in soils presenting a lower organic carbon content or higher humic index, whereas its
43 influence on soil pH was constant (i.e., the same regardless of the pH of the topsoil). In
44 conclusion, this study suggests a discrepancy between the impact of *A. khami* on soil physical
45 and chemical properties. The results also show that the spatial variability of cast physical
46 properties cannot be related with that of topsoil environment physical properties.

47

48 **Keywords:** Earthworm; aggregation; Vietnam; physical properties; chemical properties

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50

51 **1. Introduction**

52 With respect to soil turnover, earthworms are one of the most important soil bioturbators.
53 Earthworms ingest and egest large amounts of soil, so-called casts, which then significantly
54 impact the physical and chemical properties of soil (Lee, 1985). The properties of earthworm
55 casts vary among earthworm ecological categories (Clause et al., 2014; Hedde et al., 2013)
56 and among species belonging to the same ecological category (Vos et al., 2019). However, the
57 soil material ingested is probably the most important driver of cast properties (Van Groenigen
58 et al., 2019). To better estimate the impact of earthworms on soil functioning, the
59 relationships between soil and cast properties must be assessed. However, to the best of our
60 knowledge, only two studies have shown statistical relationships between casts and soil
61 properties. Hauser and Asawalam (1998) and Norgrove and Hauser (2000) showed positive
62 correlations between the nutrient contents in soils and in casts. Using negative power
63 relationships between the ratios in nutrients in cast and in soil and the soil concentration in
64 nutrients, they also showed that earthworms can modify the amounts of nutrients in casts as a
65 function of the nutrient contents in the soil. Conversely, other studies, not based on statistical
66 relationships, found that cast physical properties, e.g., wet aggregate stability or soil bulk
67 density, were independent of the properties of the soil environment (Barré et al., 2009;
68 Thomas et al., 2008), suggesting that earthworms may act as buffer agents on soil physical
69 properties. The abovementioned examples imply the need to better understand if and how the
70 physical and chemical properties of the soil environment impact the properties of casts. In
71 particular, a main gap lies in the fact that the influences of the soil environment on earthworm
72 casts' physical and chemical properties have seldom been compared in the same study. In
73 addition to the soil, the age of casts is another factor affecting the physical (Decaëns, 2000;
74 Marinissen and Dexter, 1990) and chemical properties of casts (Decaëns et al., 1999; Scullion

75 et al., 2003; Zangerlé et al., 2014). However, how the age or degradation stage of casts
76 influences the relationship between cast and soil properties is unknown.

77 This study was therefore designed to assess the relationships between soil and cast
78 properties. To do so, the chemical and physical properties of casts (recently emitted and
79 degraded) produced by the anecic species *Amyntas khami* (Thai, 1984) were compared to
80 those of topsoil aggregates within one small watershed in northern Vietnam. These casts
81 provide an interesting and unique model for understanding the influence of the environment
82 on earthworm cast properties because of their high resistance, abundance and large size (Hong
83 et al., 2011; Jouquet et al., 2007). Our hypotheses were (i) that the chemical properties of
84 casts were related to those of the surrounding topsoil, whereas the physical properties were
85 not, and (ii) that the relationships were affected by the age of the casts.

86

87 **2. Material and methods**

88 **2.1. Study area**

89 Soil and earthworm casts were sampled in a small watershed (50 ha) at the M-Tropics long-
90 term observatory located in Dong Cao village in northern Vietnam (20° 57'N, 105° 29'E). The
91 climate in the region is subtropical humid with an annual mean temperature of 20°C and 1800
92 mm of rainfall. The dominant soil type is Acrisol (WRB) (Podwojewski et al., 2008),
93 characterized by an average organic carbon content of 2.5% and a clay content of 50%. The
94 soils are acidic (pH-H₂O ~ 5). Woodlands, secondary forests, meadows and fallows are the
95 main land uses in the catchment. In this environment, the soil surface is often covered by
96 giant earthworm casts produced by the anecic earthworm *Amyntas khami* (Hong et al.,
97 2011). These casts can reach up to 20 cm in height but are often broken, probably by livestock
98 trampling, human traffic, root growth and raindrop impact (Jouquet et al., 2009, 2008).

99

100 **2.2. Sampling and laboratory analysis**

101 Sampling took place in February 2017 during the winter season. A total of 50 points were
102 sampled in the watershed (Fig. 1). At each location, 400 g of surface earthworm casts
103 produced by *A. khami* were collected. Cast age was visually determined from their shape and
104 casts were differentiated into recently emitted (humid) and degraded casts (greenish colour
105 with cracks at the surface) (Fig. 2). Topsoil aggregates (2–12 cm depth) with no traces of
106 earthworm activity were also sampled. In total, 150 samples were collected: (2 types of casts
107 + 1 topsoil aggregate) × 50 locations. Soil samples were then oven-dried at 40 °C for 2 days
108 and broken into smaller aggregates with a size between 10 and 13 mm.

109 Soil aggregate stability was assessed using the measured mean weight diameter
110 (MWD) after immersion of 5 g of soil aggregates in 200 mL of distilled water (10 minutes),
111 vertical sieving in the water through a column of sieves (5, 3.15 and 0.2 mm) and oven-drying
112 at 40 °C (Le Guillou et al., 2012). Because of the few recent casts collected, aggregate
113 stability was only measured for degraded casts and topsoil aggregates. The bulk density of
114 aggregates was determined from 4 g of soil by the kerosene saturation method of McIntyre
115 and Stirk (1954). The bulk density was measured only in 20 location points. The
116 hydrophobicity of aggregates was assessed by measuring the water drop penetration time
117 (WDPT) on four aggregates. It corresponded to the time required for a 13 µL drop of
118 deionized water to penetrate the aggregate (Chenu et al., 2000). The pH was measured in 0.01
119 M CaCl₂ at a soil:solution ratio of 1 to 2.5. The soil organic carbon (OC) content was
120 determined by potassium dichromate oxidation titration for aggregates previously crushed to a
121 size of 2 mm. An aliquot of these samples was grounded < 200 µm and scanned with a
122 spectrometer (FTIR 660, Agilent ex-Varian) in the mid-infrared spectral range from 4,000 to
123 400 cm⁻¹ with a KBr separator and a silicon detector. The diffuse reflectance measurements
124 were made at 2 cm⁻¹ intervals and was converted to absorbance. The raw spectra were

125 subjected to two different pre-processing steps. First, the spectral signature was measured
126 from the dataset after taking the second derivative to remove baseline shifts and separate
127 overlapping absorption. Absorbance measurements were selected each 20 cm interval in order
128 to reduce the number of independent variables (from 1766 to 176) and bands associated with
129 CO₂ interference (2500-2300 cm⁻¹) were removed. The data were then used for multivariate
130 statistical analysis. Second, the humification index (HI) was calculated as a measure of soil
131 organic matter aromaticity (Demyan et al., 2012; Margenot and Odson, 2016). The
132 absorbance intensity at each wavenumber was normalized to the sum of the absorbance across
133 4,000-600 cm⁻¹ and spectra were corrected for baseline shifts using a modified polynomial
134 fitting (degree 2). Finally, a Savitzky-Golay smoothing filter was applied (filter order = 1 and
135 filter length = 9) to remove noise from the spectra. HI was calculated as the ratio of the
136 absorbance areas of aromatic C=C, ketone and quinone C=O, and/or amide C=O at 1660-
137 1580 cm⁻¹ to aliphatic C-H at 3010-2810 cm⁻¹. Absorbance areas were calculated using the
138 tangential baseline method.

139

140 **2.3. Statistical analyses**

141 One-way ANOVAs were performed to assess differences in the soil physico-chemical
142 properties of recent and degraded casts and topsoil aggregates. Prior to the ANOVAs, data
143 were log-transformed (when requires) to achieve homogeneity of variances and normality,
144 which were confirmed using Levene test and Shapiro-Wilk test. Post hoc Tukey's tests were
145 performed to assess the statistical significance of differences between means. We also
146 performed between-class analysis (BCA) and Monte Carlo permutation tests (1000
147 permutations) to visually summarize the information of the second-derivative mid-infrared
148 spectra for recent and degraded casts and topsoil aggregates. Partial Mantel tests were
149 performed to evaluate the Spearman rank correlations between the dissimilarity of spectral

150 signatures of recent and degraded casts and topsoil aggregates while controlling the effect of
151 geographic distance. The dissimilarity of spectral signatures was calculated by the Euclidean
152 distance between samples. Spearman correlations were performed between physical and
153 chemical properties. Differences were declared significant at the 0.05 probability level. All
154 statistical calculations and plots were executed using R software (3.5.2 version) with the
155 following packages: “car”, “ade4”, “ggplot2”, “spectacles”, “signal”, “geosphere” and
156 “vegan”.

157

158 **3. Results**

159 **3.1. Properties of casts and topsoil aggregates**

160 Earthworm casts had, on average, higher values of OC (1.5-fold), pH (by 0.5 unit), bulk
161 density (1.03-fold), WDPT (14.2-fold), and MWD (1.1-fold) and lower values of HI (1.5-
162 fold) than did the topsoil aggregates (Table 1). Recent and degraded casts had similar values
163 of OC, pH, bulk density, WDPT and HI. The BCA carried out on the mid-infrared spectra of
164 soil aggregates clearly separated casts and topsoil aggregates on the first axis, which
165 explained 64% of the total variability (Fig. 3). Recent and degraded casts were differentiated
166 on the second axis, which represented 36% of the variability.

167 Bulk density was correlated with pH ($r = 0.43$, $p < 0.01$). MWD was correlated with
168 OC ($r = 0.56$, $p < 0.001$), pH ($r = 0.38$, $p < 0.05$) and HI ($r = -0.41$, $p < 0.05$). WDPT was
169 correlated with OC ($r = 0.80$, $p < 0.001$) and HI ($r = -0.45$, $p < 0.01$).

170

171 **3.2. Correlations between cast and topsoil aggregate properties**

172 The chemical properties (OC, HI and pH) of recent and degraded casts were positively and
173 linearly related to the topsoil aggregate properties (Fig. 4). The correlation coefficients were
174 the highest for OC and pH and the lowest for HI. The cast:topsoil ratios of OC and HI were

175 negatively related by a logarithmic function to the corresponding values of topsoil aggregates.
176 Conversely, no relationship was found for pH between the cast:topsoil ratio and topsoil
177 aggregate value. None of the physical properties (WDPT, MWD or bulk density) of casts
178 were related to those of topsoil aggregates. However, negative relationships were measured
179 for WDPT, MWD and bulk density between the cast:topsoil ratio and topsoil aggregate value
180 (logarithmic functions for WDPT and MWD vs. a linear function for the bulk density). Partial
181 Mantel tests showed a correlation between the dissimilarity of spectral signatures of degraded
182 casts and that of spectral signatures of topsoil, while no correlation was found with recent
183 casts (Fig. 5). The slope of the regression line (< 1) indicated that the variability between two
184 spectral signatures of degraded casts was lower than that between two spectral signatures of
185 topsoil aggregates.

186

187 **4. Discussion**

188 **4.1. Cast vs. topsoil chemical properties**

189 *A. khami* is an anecic species that ingests fresh organic matter and mixes it with soil (Hong et
190 al., 2011). This feeding strategy explains the increase in soil organic carbon content and pH
191 (1.5-fold and 0.5 unit, respectively), and the lower degree of aromaticity (1.5-fold) in the casts
192 than in the topsoil aggregates. Similar changes have been reported in the recent meta-analysis
193 conducted on earthworm cast properties (Van Groenigen et al., 2019). Therefore, casts can be
194 seen as nutrient patches in the soil, and *A. khami* can be seen as heterogeneity drivers.
195 Moreover, no difference could be observed between recent and degraded casts, confirming the
196 high stability of *A. khami* casts to degradation by rain (Jouquet et al., 2012, 2011), as well as
197 the persistence of these patches. At the scale of the watershed, the chemical properties of casts
198 and topsoil aggregates were highly variable. The relationships between the chemical
199 properties of casts and those of the topsoil aggregates confirmed that the soil properties

200 largely controlled the chemical properties of casts (Hauser and Asawalam, 1998; Norgrove
201 and Hauser, 2000). However, the negative relationships between the cast:topsoil ratio and the
202 surrounding topsoil aggregate values for organic carbon and humic index suggest that the
203 effect of *A. khami* was more important in soils presenting less organic carbon content and
204 where the organic matter had a higher aromaticity. These results are in line with Hauser and
205 Asawalam (1998) and Norgrove and Hauser (2000), who showed that the lower the soil
206 organic carbon content is, the more anecic earthworm species feed on litter and incorporate
207 organic matter into their casts.

208 The greater organic matter content in casts than in topsoil aggregates can partially
209 explain the higher pH of casts than topsoil aggregates. Indeed, the mineralization of organic
210 matter and the production of NH_4^+ during gut transit and afterwards in the soil are known to
211 increase alkalinity (Basker et al., 1994). Another explanation could be that earthworms feed
212 on plant materials enriched in oxalic acid and/or oxalate minerals, which are thereafter
213 metabolized by oxalotrophic bacteria, leading to increased soil alkalinity (Martin et al., 2012).
214 Finally, a last possibility could be that *A. khami* increased the pH of casts through the
215 secretion of calcium carbonate by calciferous glands, as observed with Lumbricidae species
216 (Robertson, 1936). The production of CaCO_3 granules is usually considered to result from an
217 adaptation of earthworms for the regulation of blood pH and tissue fluid (Briones et al., 2008;
218 Lambkin et al., 2011; Versteegh et al., 2014). However, more research is clearly needed to
219 determine whether *A. khami* has this organ and whether the production of CaCO_3 granules is
220 sufficient to significantly increase soil pH. Interestingly, our study also showed that *A. khami*
221 increased pH with the same amplitude regardless of the pH of the soil (i.e., no relationship
222 was found between the cast:topsoil ratio and the pH of the topsoil). This result was somewhat
223 surprising and highlights the need to better understand the complex relationship between
224 earthworm ecology and the regulation of soil pH.

225

226 **4.2. Cast vs. topsoil physical properties**

227 Our results confirmed previous results carried out at the same study site, showing that casts of
228 *A. khami* have a higher bulk density, wet aggregate stability and hydrophobicity than topsoil
229 aggregates (Jouquet et al., 2008; Bottinelli et al., 2010) and thus contribute positively to the
230 resistance of soil to erosion (Jouquet et al., 2012). Despite the fact that wet aggregate stability
231 and bulk density vary during cast ageing (Decaëns, 2000; Marinissen and Dexter, 1990), no
232 difference could be measured between recent and degraded casts. Again, this lack of variation
233 during ageing was probably due to the high resistance of casts to degradation. The wet
234 aggregate stability of casts was at its maximum in most of the locations, while the
235 hydrophobicity and bulk density were quite variable. Moreover, no relationship was found
236 between the physical properties of the casts and those of the topsoil aggregates. Since
237 physical properties were correlated with chemical properties (e.g., bulk density with pH, and
238 hydrophobicity and wet aggregate stability with organic carbon, humic index and pH), the
239 lack of a relationship between casts and topsoil aggregates suggests that during the formation
240 of casts, physical changes occurred (e.g., changes to the microstructure) that directly
241 influenced physical properties, obscuring the relationships with soil properties. Our results are
242 therefore in line with other studies showing that the soil aggregate stability of casts (Thomas
243 et al., 2008) and soil bulk density impacted by earthworms (Barré et al., 2009) were not
244 directly influenced by the soil. Finally, the negative relationships between the cast:topsoil and
245 topsoil aggregate values indicated that at high values of soil physical properties, the impact of
246 earthworms would become null (i.e., on hydrophobicity and wet aggregate stability) or would
247 even be reversed (i.e., on bulk density). This relationship suggests a high potential of
248 earthworm casts to improve soil physical properties in degraded environments but a limited
249 impact when topsoil already has a high potential to resist erosion. Because of the high risk of

250 erosion in the humid tropics, the beneficial effects of anecic species under these conditions
251 might be large.

252

253 **4.3. Spectral signature of aggregates**

254 While recent and degraded casts could not be differentiated by their elementary chemical and
255 physical properties, the utilization of infrared spectroscopy allowed discrimination between
256 casts and topsoil and between recent and degraded casts. This result confirms the relevance of
257 infrared-reflectance spectroscopy for differentiating earthworm casts from the surrounding
258 soil environment (Hedde et al., 2005). The results are also in line with the study of Scullion et
259 al. (2003) and Zangerlé et al. (2014), who showed the potential of this technology for
260 differentiating minor changes that occur during the degradation of organic matter within casts.
261 The results from the partial Mantel tests showed a positive relationship between the spectral
262 dissimilarity of topsoil and that of degraded casts and a lack of relationship with recently
263 emitted casts. These results showed that the spectral variability of recently emitted casts could
264 not be related to the spectral variability of the topsoil, suggesting important changes in the
265 organization of soil after the consumption and excretion of casts by earthworms. They also
266 showed that the degradation of casts reduced their spectral variability (regression slope < 1),
267 resulting in properties that were correlated to those of the topsoil aggregates. The spectral
268 signature measured with mid-infrared spectroscopy corresponds to soil organic and mineral
269 composition (Parikh et al., 2014). If we assume that the mineral composition was similar
270 between recent and degraded casts, it is likely that differences in the dissimilarity of spectral
271 signatures of recent casts compared to those of degraded casts were due to an evolution in the
272 quality of organic matter.

273

274 **5. Conclusions**

275 Although intertwined, cast physical and chemical properties are rarely compared while taking
276 into account the variability that can be observed in the field or the stage of degradation of
277 casts. Therefore, this study is the first to pinpoint a discrepancy between the impact of
278 earthworms on soil chemical and physical properties. If recent and degraded casts conserve
279 most of their properties, the environment appears to decisively influence the regulation of the
280 chemical properties of earthworm casts. Since the physical properties of casts are not directly
281 related to the topsoil environment, more studies are needed to understand the mechanisms
282 affecting the physical changes during cast formation and how their impact affects soil
283 functioning at larger scales (i.e., soil erosion at the watershed scale).

284

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291

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Figure captions

Fig. 1. Example of earthworm casts used in this study: recently emitted cast (humid) and degraded cast (greenish colour with cracks at the surface).

Fig. 2. Map of sampling locations in the watershed of Dong Cao in northern Vietnam.

Fig. 3. Biplot showing the between-class analysis (BCA) performed on the MIR spectra of recent and degraded casts and topsoil aggregates without recent earthworm activity. Group significance was assessed by Monte Carlo tests. The most important wavenumbers contributing to the discrimination of the group samples are depicted.

Fig. 4. Organic carbon (OC) content, humic index (HI), pH, bulk density, water drop penetration time (WDPT) and mean weight diameter (MWD) plotted for recent (black circles) and degraded (grey circles) casts and for cast/topsoil ratio as a function of the topsoil aggregate value without recent earthworm activity (n= 20 for bulk density and n= 50 for other properties). Fitting regression models are presented if they are statistically significant. The black line corresponds to the line $y = x$ or $y = 1$.

Fig. 5. Relationship between mid-infrared spectra of casts (recent in black circles and degraded in grey circles) and topsoil aggregates. Fitting regression models are presented if they are statistically significant. The black line corresponds to the line $y=x$.

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Table

Table 1. Organic carbon (OC) content, humic index *via* mid-infrared spectroscopy (HI), pH, bulk density, water drop penetration time (WDPT) and mean weight diameter of water-stable aggregates (MWD) in recent and degraded casts and topsoil aggregates. Data are presented as the means and standard deviations (n = 20 for bulk density and n = 50 for other properties). Values bearing different letters are significantly different.

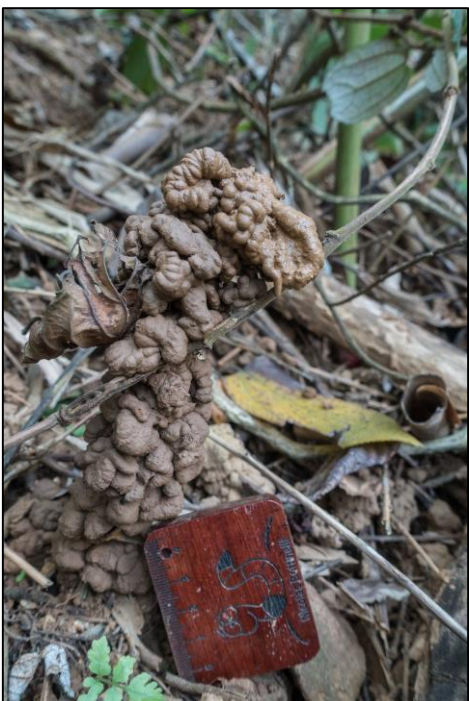
Properties	Recent cast	Degraded cast	Topsoil	F and p-values
OC (%)	4.4 (0.7) a	4.4 (0.7) a	3.0 (0.7) b	108.1 ***
HI	15.9 (3.9) b	14.9 (3.4) b	23.3 (19.8) a	19.3 ***
pH	4.2 (0.4) a	4.2 (0.4) a	3.7 (0.2) b	78.5 ***
Bulk density (g cm ⁻³)	1.40 (0.03) a	1.43 (0.06) a	1.37 (0.04) b	11.5 ***
WDPT (s)	26.1 (14.3) a	35.6 (28.8) a	2.1 (1.3) b	380.8 ***
MWD (mm)	-	7.9 (0.4) a	7.0 (0.9) b	32.1 ***

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*The number of stars for the significance level indicates the p-value range (***) p < 0.001).*

Figure 1

Recent cast



Degraded cast



Figure 2

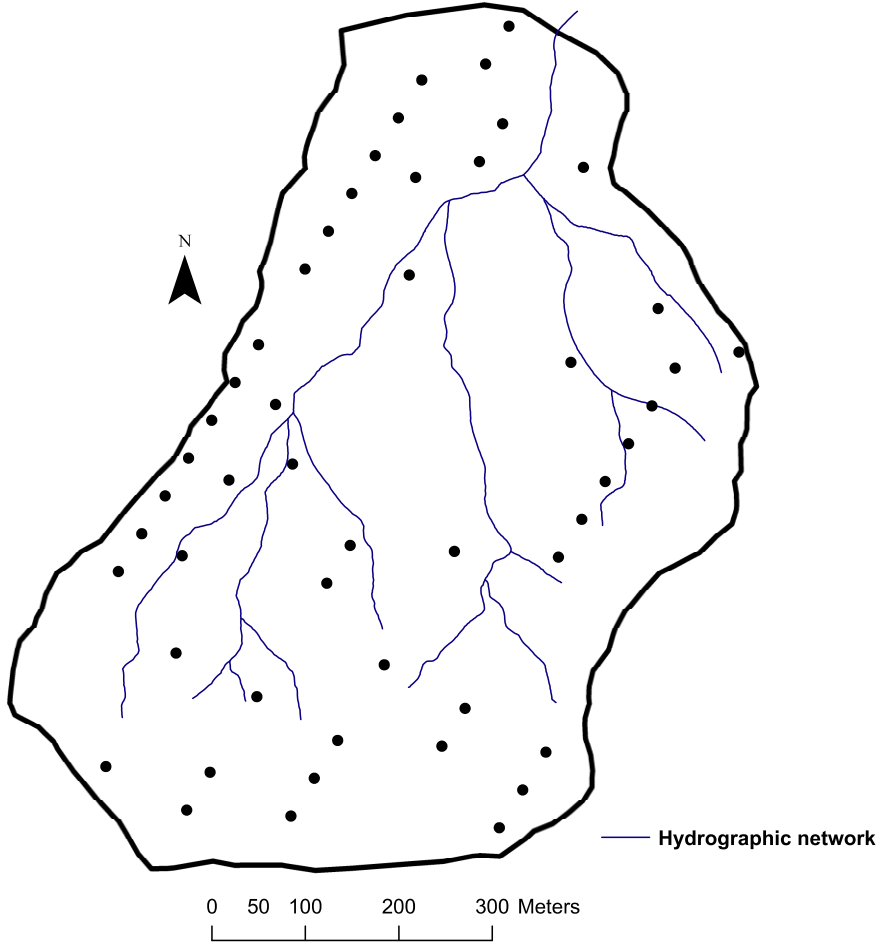
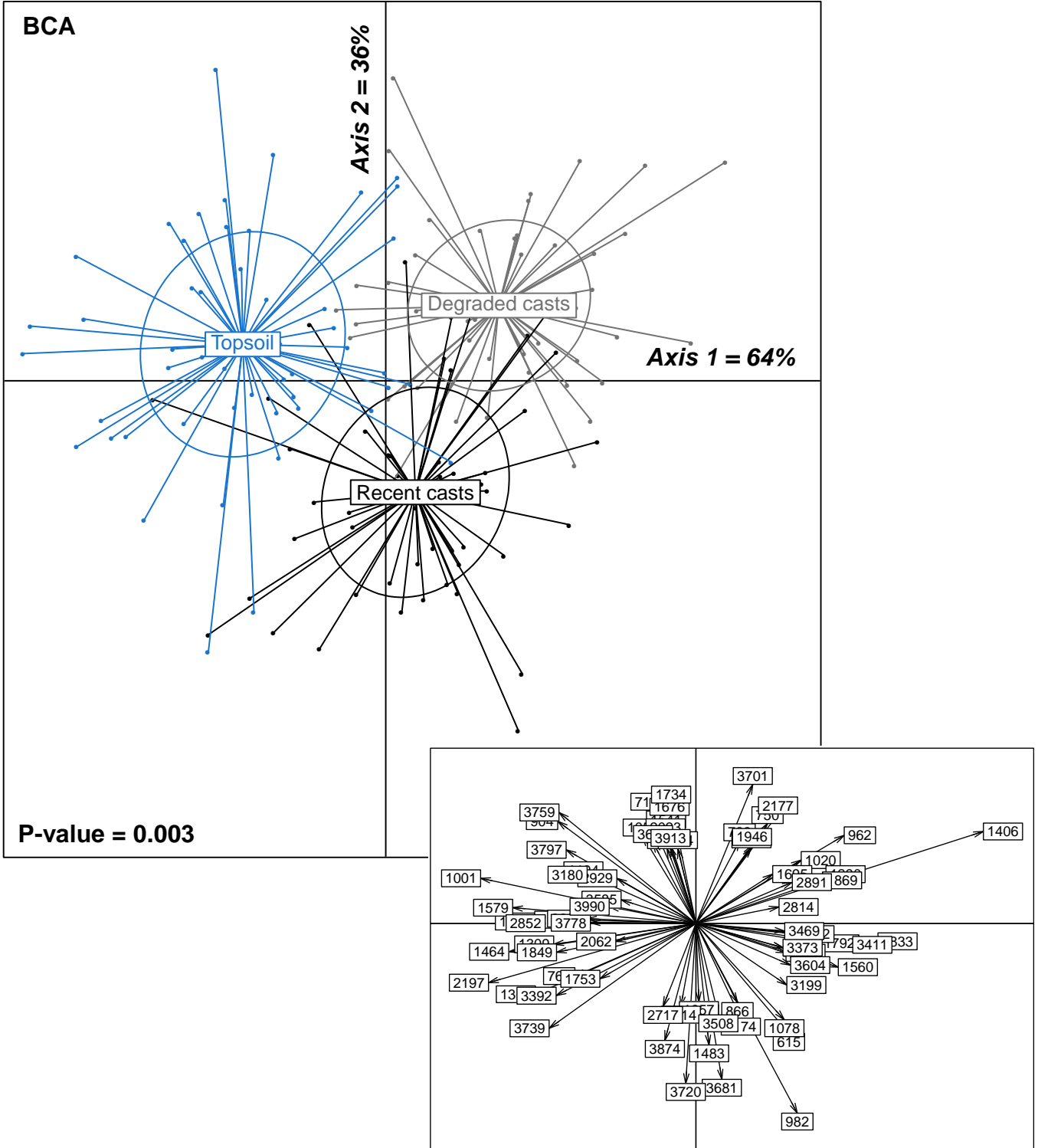
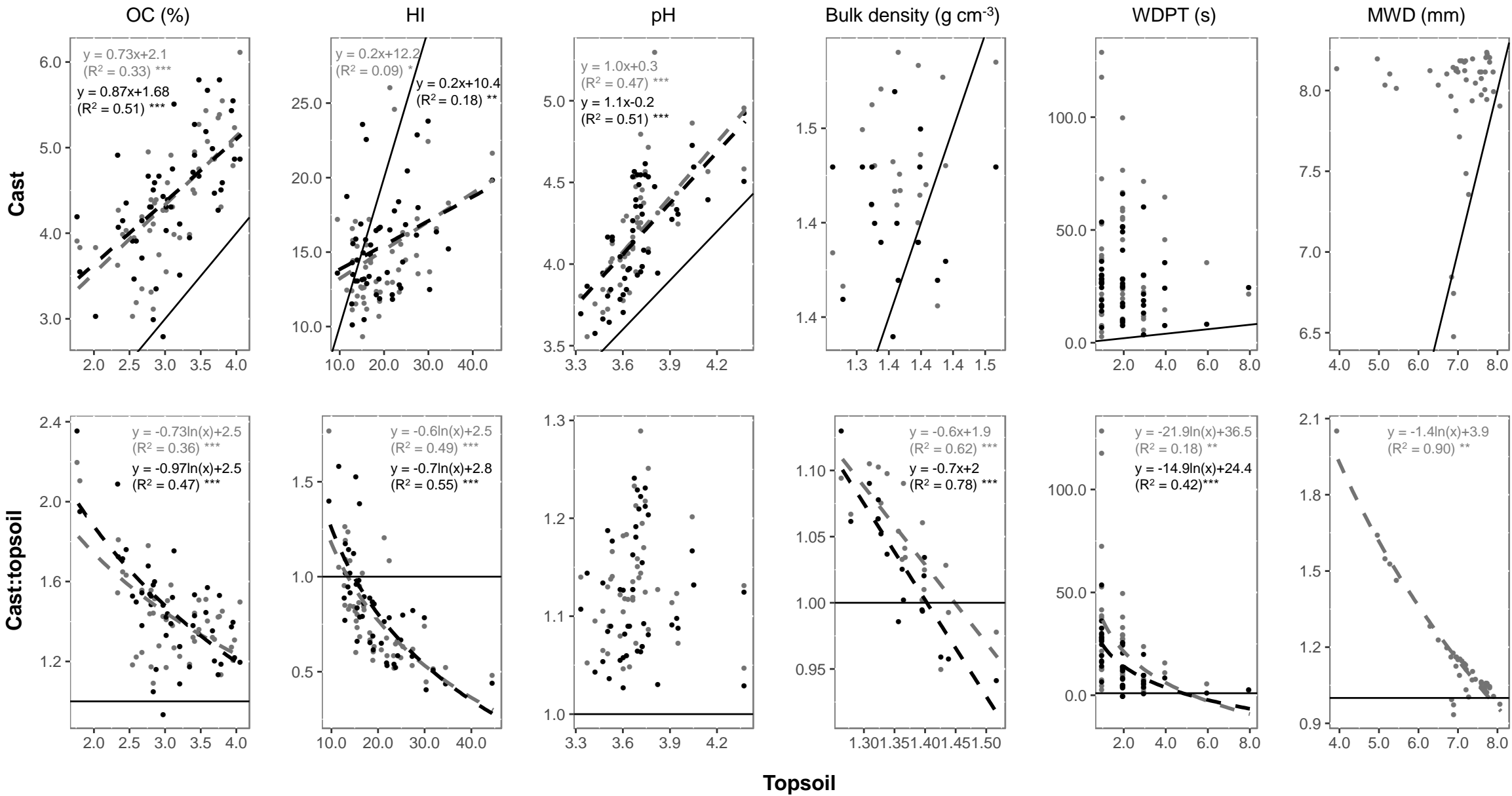


Figure 3





Dissimilarity in mid-infrared spectra signatures

