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1 From the importance of organic carbon in reproduction tests with *Eisenia fetida*

2

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9

## 10 Abstract

11 The standard *Eisenia fetida* chronic toxicity reproduction test is normalized and needs up to 8 weeks  
12 of experimentation. Many matrixes have shown toxic effects on the earthworms' survey, growth and  
13 reproduction. But the food factor may affect the organisms' responses to contaminated media in such  
14 tests. We try to evaluate the effect, or not, of the organic carbon content present in the environment,  
15 as spreadable organic wastes added in soil, on the reproduction test of *Eisenia fetida*. We confirm the  
16 influence of the organic matter content on earthworm reproduction. This "food" factor must be  
17 considered when studying environmental samples rich in organic matter, since that could mask  
18 potentially harmful effects. To minimize this potential "food" effect during reproduction tests, we  
19 recommend an inverse gradient of organic matter contribution by the "horse dung" food applied in all  
20 conditions according to the dose of agricultural amendments tested.

21

22 Key words : Carbon content; *Eisenia fetida*; reproduction test; toxicity

23

## 24 Highlights

- 25 • organic waste can impact the activity of earthworms during bioassays
- 26 • influence of the organic matter content on earthworm reproduction was confirm
- 27 • To minimize the "food" effect on earthworm reproduction carbon must be considered
- 28 • an inverse gradient of organic matter "horse dung" must be applied in bioassays

29

30

## 31 1. Introduction

32 The toxic effects of mixtures of pollutants in solid matrices have been subject to few studies  
33 although evaluation by ecotoxicological tests have shown themselves to be pertinent for  
34 assessing the environmental risks of them [1]. Of the various standardised ecotoxicity tests  
35 available, lethality tests [2] and avoidance tests [3] for earthworms are particularly quick and  
36 economical. But, several authors advise to use as much or more a chronic test consisting of  
37 the earthworm reproduction test during 56 days with the ISO 11268-2 [4]. Most of these  
38 studies focuses on the specie *Eisenia fetida*. Thus, to evaluate impact of spreadable organic  
39 waste, the laboratory bioassays often used earthworms as model [5, 6, 7, 8]. And in these  
40 amendments several pollutants are present and could contaminate the receptive soil with  
41 trace metals (TMEs) and persistent and non-persistent organic pollutants [9, 10].

42 Many studies have highlighted the toxic effects of several matrices on earthworm mortality,  
43 growth and reproduction [5, 6, 7, 8]. However, some studies have also showed that a  
44 stimulation of the growth and the reproduction of earthworms can be observed as a function  
45 of the type of amendment [11, 12] which can act as a bio-fertilizer in such matrixes [13]. Then,  
46 the ecotoxic impact of some pollutants or environmental samples can be masked by a lack or  
47 surplus of nutrients in the exposure medium. Indeed, the confounding factor of "food" can  
48 affect the responses of organisms to contaminated environments in chronic toxicity tests [14].  
49 Thus, if an increase in organic carbon content is present in the environment such element  
50 modifies the reproduction rate of terrestrial and aquatic organisms [15, 16]. And such  
51 spreadable organic wastes provide a high organic matter content in amended soils during  
52 ecotoxicological tests [17]. Some studies underline that in long term inoculation assays soil  
53 conditions are not suitable for earthworm establishment, especially because of insufficient  
54 organic matter (OM) quantity [18]. Thus, OM improves earthworm establishment and  
55 development not only by supplying nutrients but also by impacting the stability of  
56 temperature and moisture [19].

57 Thus, such OM quantity must be considered for further studies to minimize the effects of organic  
58 matter (i.e. food supply) on metal accumulation and, what is more important, in the assessment of  
59 toxic effects. It is therefore necessary to verify whether the characteristics of the organic waste (when  
60 tested), including their high organic matter content, can impact the activity of the organisms during  
61 our bioassays. To verify this hypothesis, we carried out on the effect of the organic carbon content  
62 present in the samples on the reproduction of the earthworm *Eisenia fetidae*.

## 63 **2. Materials and Methods**

### 64 **2.1 Materials: ISO soil and organic horse manure**

65 The reference soil called ISO substrate [20] was a mixture of : 10 % peat, 70 % silica [industrial  
66 quartz sand, more than 50 % particles must be between 50 and 200  $\mu\text{m}$  and all the particles  
67 must be less than 2 mm], 20 % clay (less than 30 % Kaolin) and  $\leq 1$  %  $\text{CaCO}_3$ . The ISO soil remains  
68 a poor matrix in terms of nitrogenous elements and Trace Metallic Elements (MTE) but with  
69 carbon due to the presence of 10% peat in its composition (Sup Mat 1). No pharmaceutical  
70 compounds or Polycyclic Aromatic Hydrocarbons (PAHs) were detected. Finally, only a few  
71 Polychlorobiphenyls (PCBs), in particular None Dioxin Like (NDL), were present, as well as  
72 other organic compounds (as PCDD/DF, BFRs) at low concentrations (Sup Mat 1).

73 The organic horse manure used comes from an organic farm (69; Rhône) guaranteeing the  
74 absence of phytosanitary products (confidential farm). For its use, the horse manure is ground  
75 to 2 mm, then dried at 70°C during one week, and then stored at room temperature. Organic  
76 horse dung is the feed used to maintain earthworm farms. It is also used as a base feed in  
77 earthworm breeding tests. Indeed, the ISO 11268-2: 2015 [4] standard for earthworm  
78 reproduction testing mentions horse dung as a suitable feed for rearing and performing  
79 ecotoxicology tests.

80 According to the analytical results detailed in supplementary material 2, this amendment  
81 “horse manure” has a C/N value close to ISO soil but with carbon and nitrogen and other  
82 elements such as phosphorus, calcium and magnesium (Mat sup 2). It also contains low levels  
83 of MTEs, but no drug residues. Finally, PAHs and other organic compounds are detected at  
84 relatively low concentrations (Mat Supp 2). A quantity of about 20 dry g of organic horse  
85 manure, *i.e.* 8.36 g of carbon, was reached for the controls.

## 86 2.2. Methods: Reproduction test with *Eisenia fetida*

87 The test organisms, *Eisenia fetida*, were supplied by an internal earthworm breeding unit of  
88 Provademse (Villeurbanne, France), where they were kept in a medium comprising horse  
89 manure from an organic horse breeding stable and coco peat (50/50) with a water content  
90 close to 70% of maximum water holding capacity and a temperature of  $20 \pm 2$  °C [17]. As  
91 described before in [17], only the adult earthworms between 350 mg and 600 mg in fresh  
92 matter with a visible and well-developed clitellum were selected for the toxicity tests. Before  
93 starting the tests, the earthworms were cleaned by rinsing with demineralized water, weighed  
94 and fasted for 24 h.

95 The *Eisenia fetida* earthworm reproduction test is carried out for 56 days by using the ISO  
96 11268-2: 2015 procedure [4]. All the assays were done in an air-conditioned room at  $20 \pm 2$  °C  
97 and a photoperiod of 16 h day/8 h night. Each mixture is prepared 5 days before the start of  
98 the test at 70% of the CRE of the mixture. Such delay allows recolonisation of the medium by  
99 the microorganisms and stabilisation of the water content. As described in [17] for the acute  
100 test with earthworms, approximately 600 g of moist artificial ISO soil or the moist mixture is  
101 introduced into each box with a volume of soil introduced that is adjusted to the same level  
102 between all the conditions tested. For this reproduction test, we used 1 L glass jars (Le  
103 Parfait®; 11 cm diameter, 16.5 cm height) and closed them with evenly pierced plastic lids to  
104 prevent earthworms from escaping and ensure oxygenation of the medium. One per each  
105 week, the water content is checked by weighing and adjusted if necessary with demineralised  
106 water. Then, ten earthworms were introduced into each of the conditions in four replicates  
107 and were exposed for 28 days to the four conditions tested (Table 1).

108 For this study, we therefore carried out (Table 1):

- 109 - The control treatment (T) where the food supply follows the conditions of the ISO standard,  
110 *i.e.* 5 g of dry dung added each week to the medium;
- 111 - Treatment A where 10 g of manure is added weekly to the medium
- 112 - Treatment B where 20 g (B) of manure is added weekly to the medium;
- 113 - Treatments C and D where, at the beginning of the test, 40 g and 80 g of manure are added,  
114 *i.e.* a manure content equivalent to 10 and 20% in the medium. And no more manure is  
115 added afterwards.

116

117 **Table 1**

118

119 The earthworms were weighted after fasted for 24 h and rinsed with demineralized water  
120 then placed on absorbent paper at each sampling date. After 28 days of exposure, adult  
121 earthworms are removed and counted to assess earthworm mortality (Equation 1) and  
122 weighed to assess earthworm growth (Equation 2). If no movement is observed when their  
123 rear end is punctured, they are considered dead. The percentage of dead earthworms was  
124 obtained for each replicate by the following formula:

125 **Equation 1:**  $z = \left(\frac{D}{T}\right) \times 100$

126 where z= percentage of dead earthworms, D = number of dead earthworms and T = initial  
127 number of earthworms.

128 The gain or loss of the earthworm biomass rate (noted g) was obtained for each replicate by  
129 the following formula:

130 **Equation 2:**  $g = \left(\frac{mf-mi}{mi}\right) \times 100$

131 Where mf = mass final at the end of the exposure and mi = mass initial at the beginning of the  
132 exposure. g expressed in %.

133

134 After 56 days of exposure, unhatched juveniles, as well as cocoons, were manually removed  
135 from the soil and counted. The test validity was obtained if in controls we observed a mortality  
136 of earthworm adults less than 10 %, and a loss of fresh biomass in adults not exceeding 20%,  
137 and also that the number of juveniles was more than 30.

138

### 139 **3. Results and discussion**

140

141 With regard to the measurements carried out on the initial jars, we note some differences in  
142 pH and relative humidity, but this can be explained by the quantity of manure added at the  
143 start of the experiments (Table 1).

144 In any case, the volume of water added takes this difference into account to adjust to an initial  
145 CRE equal to 70% (see Supplementary material 1 and 2; with the CRE of each matrix: Iso soil  
146 and manure). However, it should be noted that conditions A and C and the conditions B and  
147 D have a very similar final carbon, respectively around 6.8 and 10.6 %, and that the difference  
148 between the conditions A and C and the conditions B and D is therefore just an initial or further  
149 input (Table 1).

150

#### 151 **Table 1**

152

153 *Eisenia fetida* are monitored during the 56-day breeding test on ISO artificial soil under  
154 different feeding conditions, i.e. with varying manure doses and input conditions and by  
155 measuring growth and reproduction parameters (Figure 1). At the end of the first 28-day  
156 exposure, little or no mortality was observed of adult earthworms. Only condition C (10%  
157 manure added at the beginning of the experiment) leads to a positive mortality rate, equal to  
158  $5.00 \pm 10.00\%$  (data not shown).

159

#### 160 **Figure 1**

161

162 First of all, a gain in biomass of adult earthworms was observed in all conditions. The control  
163 worms had an average gain biomass of  $12.75 \pm 6.29\%$ . A more consistent weekly supply of  
164 manure resulted in a slightly higher, but not significant, biomass gain. Indeed, conditions A (10  
165 g per week) and B (20 g per week) lead to growth rates of  $20.67 \pm 3.34\%$  and  $29.19 \pm 5.16\%$   
166 respectively (Figure 1-A). In the media enriched at the beginning of the experiment, with 10%  
167 (condition C) and 20% (condition D) manure, only the biomass of adult earthworms in  
168 condition D increased significantly with an increase of biomass reaching  $41.33 \pm 12.97\%$ .

169 The data on the number of juveniles, Figure 1-B, show similar results between the control  
170 conditions (T) and conditions A and B. Earthworms in condition A and B produced  $211 \pm 9.20$   
171 and  $190 \pm 23.11$  cocoons respectively compared to  $162.75 \pm 34.71$  in the control conditions.  
172 On the contrary, earthworms exposed to manure-enriched media at the beginning of the test  
173 (conditions C and D) show a significantly higher reproduction than the controls. Indeed, the  
174 earthworms obtained a juvenile production of  $261.75 \pm 29.33$  in condition C and  $548.50 \pm$   
175  $88.93$  in condition D (Figure 1-B). The presence of food from the beginning of the experiment  
176 with a high organic carbon content therefore positively influences the growth and  
177 reproduction of earthworms. The study by Reinecke and Viljoen [20] shows that growth and  
178 reproduction are positively correlated and strongly dependent on the food source tested and  
179 the bioavailability of the organic matter. This amount of organic carbon should therefore be  
180 considered in our bioassays.

181 Some works used avoidance behaviour and reproduction but to assess effects of such organic  
182 wastes at much lower application rates with the limits due to toxic compounds presence  
183 potential. For example, earthworms show avoidance behaviour as low as  $8 \text{ g.kg}^{-1} \text{ DM}$  in  
184 sewage sludge [7, 22]. At the same level, a decrease in the growth and number of juveniles  
185 produced by exposed earthworms is also observed [22]. Reprotoxic effects are observed at  
186 doses close to  $10 \text{ g.kg}^{-1} \text{ DM}$  of composted urban sludge [23]. Among the few studies on the  
187 assessment of the ecotoxicity of agricultural effluents, the authors mention reprotoxic effects  
188 appearing at similar doses [1, 8]. The recent work [8] shows that exposure to poultry manure  
189 has a significant impact on earthworm mortality from  $60 \text{ g.kg}^{-1} \text{ DM}$ , a decrease in growth from  
190  $40 \text{ g.kg}^{-1} \text{ DM}$  and a decrease in reproduction from  $20 \text{ g.kg}^{-1} \text{ DM}$ . The work [1] reveals an  
191 attraction behaviour towards the amended soil at cow manure contents of up to  $300 \text{ g.kg}^{-1}$   
192  $\text{DM}$  and reproductive effects only above  $150 \text{ g.kg}^{-1} \text{ DM}$  of cow manure.

193 The previously mentioned works agree that the ecotoxic effects of organic waste  
194 amendment are largely due to the presence of pollutants or mixtures of pollutants leading  
195 to stresses that can lead to the death of organisms [8, 22, 24]. However, other studies also  
196 highlight other phenomena that are harmful to terrestrial organisms in these organic wastes,  
197 such as salinity, acidity, presence of ammonia, desiccation and/or lack of oxygen [23, 25, 26].  
198 These different ecotoxic impacts will depend mainly on the properties of the applied  
199 amendment, the application rate, the duration of exposure, the species and the exposure  
200 substrate [24].

#### 201 4. Conclusion

202 In this study, the results confirm the influence of the organic matter content on earthworm  
203 reproduction. Under these conditions of enrichment of the soil environment by spreading  
204 organic matter, the growth and reproduction parameters are higher than under the control  
205 conditions of the ISO standard. This "food" factor must be considered when studying

206 environmental samples rich in organic matter, since this factor could mask potentially harmful  
207 effects.

208 To minimize the "food" effect, the chronic toxicity tests for Mafors on earthworm  
209 reproduction must therefore take account of carbon, and thus an inverse gradient of organic  
210 matter "horse dung" must be applied under all conditions, depending on the Mafor dose.  
211

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226

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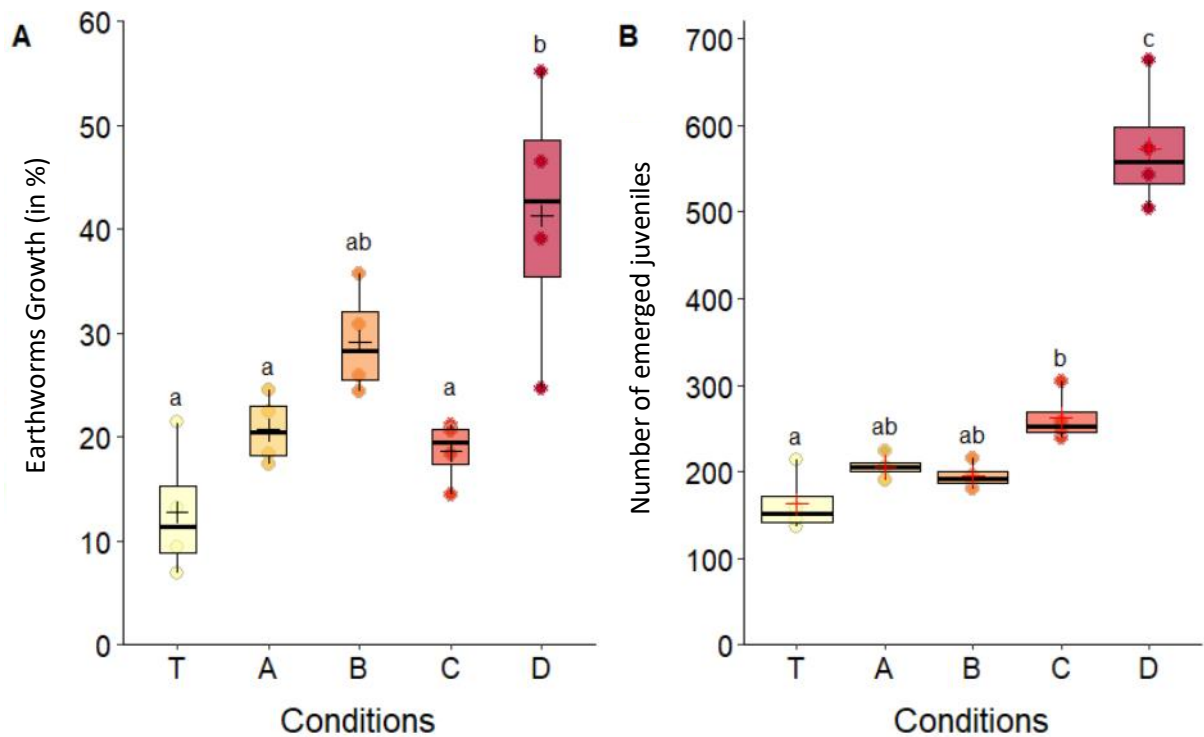
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314 **Figure 1.** Growth (in %) (A) and reproduction (measured as number of emerged juveniles) (B)  
315 of *Eisenia fetida* earthworms exposed to ISO artificial soil and different feeding conditions.  
316 Mean values (n = 4). The same letters indicate an identical statistical group (at 95%).  
317 Conditions are designated by different letters: T = addition of 5 g dry manure per week; A =  
318 addition of 10 g dry manure per week; B = addition of 20 g dry manure per week; C = 10%  
319 manure added at the beginning of the experiment; D = 20% manure added at the beginning  
320 of the experiment.

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Conditions tested	Initial content of Horse manure (%)	Initial Total Carbon (ISO soil) (%)	Addition of horse manure by week (g DW)	Total Carbon total final (%)	Initial Humidity (%)	Initial pH
T	0	3.12	5	4,94	24,36 ± 0,05	6,48 ± 0,01
A	0	3.12	10	6,76	24,36 ± 0,05	6,48 ± 0,01
B	0	3.12	20	10,40	24,36 ± 0,05	6,48 ± 0,01
C	10	6.96	0	6,96	35,54 ± 0,11	7,01 ± 0,01
D	20	10.80	0	10,80	56,17 ± 0,54	7,35 ± 0,03

334

335 **Table 1.** Different conditions tested to evaluate the effect of organic carbon content on the reproduction of *Eisenia fetida* earthworms. (n=3

336 ±SD for pH and humidity)

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