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1 **How to assess the feeding activity in ecotoxicological laboratory tests using enchytraeids?**

2

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25 **Abstract**

26 The risk assessment of pesticides on soil fauna is an issue to protect agroecosystem
27 sustainability. Enchytraeids are recognized as relevant soil bioindicators of chemical stress in
28 agroecosystems. In laboratory, the reproduction test was found to be sensitive to reveal
29 chemical impacts on enchytraeids. However, it does not allow to assess the impacts on
30 ecological functions in which enchytraeids are involved. The objective of this study was (i) to
31 explore the feasibility of the bait-lamina test with enchytraeids under laboratory conditions, and
32 (ii) to compare its sensitivity with the Enchytraeid Reproduction Test. We exposed individuals
33 of *Enchytraeus albidus* to two commercial formulations of fungicides widely used in Europe.
34 The EC50 reproduction for the Swing[®] Gold (50 g L⁻¹ epoxiconazole and 133 g L⁻¹
35 dimoxystrobin) and the Cuprafor micro[®] (50% copper oxychloride) were respectively estimated
36 at 1.66 ± 0.93 times the recommended dose and $> 496 \text{ mg kg}^{-1}$ of copper. However, no impact
37 was found on the feeding activity of enchytraeids. The bait-lamina test thus appeared less
38 sensitive than the Enchytraeid Reproduction Test to the tested fungicides. Despite that, this test
39 which is achievable under laboratory conditions and allows assessing indirect effects of
40 pesticides, is quick, cheap and easy to perform. It would deserve to be used to explore longer-
41 exposure effects through the repeated addition of bait-lamina sticks.

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48 **Keywords:** *Enchytraeus albidus*, Bait-lamina test, soil ecotoxicology, Pesticide, Risk

49 assessment

50

51 **Introduction**

52 Since many decades, the massive use of pesticides in agroecosystems has led to a
53 decrease in biodiversity and soil biological activity (Tilman et al. 2001; Hole et al. 2005).
54 Enchytraeids are soil annelid Oligochaeta involved in the soil structure dynamics, organic
55 matter decomposition and nutrient cycling (Didden, 1990; Maraldo et al. 2011). They are
56 recognized as relevant indicators of soil biological activity, anthropic activities and
57 physicochemical stresses in terrestrial ecosystems (Didden and Römcke 2001; Jänsch et al.
58 2005; Pelosi and Römcke 2016). Despite their ecological importance and their use in
59 standardized tests (ISO 16387 2014; OECD 220 2016), the toxicity of pesticides to enchytraeids
60 remains poorly documented (Römcke et al. 2017). The assessment of chemical impacts on
61 mortality and reproduction can provide key information on population dynamics that determine
62 the provision of ecological functions. For enchytraeids, reproduction is currently recognized as
63 the most sensitive endpoint (Römcke et al. 2017). In the same time, the effects of pesticides on
64 the ability of enchytraeids to degrade soil organic matter are currently unknown. The bait-
65 lamina test can be used to assess the impact of soil contaminants on the feeding activity of soil
66 dwelling organisms *in situ* (ISO 18311 2016). Kratz (1998) highlighted the potential of such
67 test in ecotoxicological laboratory experiments. The feeding activity could be indirectly linked
68 to an ecological function of soil organisms: the organic matter degradation.

69 Helling et al. (1998) assessed the feeding activity of enchytraeids, collembolans and
70 microorganisms with the bait-lamina test in a laboratory experiment and highlighted the high
71 feeding rate of enchytraeids and collembolans compared with the feeding rate of
72 microorganisms. However, as far as we know from the scientific literature, this method has
73 never been used to measure the consequences of pesticide use on the feeding activity by
74 enchytraeids. We thus tested the effects of two commercial formulations of fungicides on the
75 enchytraeid species *Enchytraeus albidus* (Henle, 1837) in a natural soil in order (i) to assess the

76 feasibility of the bait-lamina test under laboratory conditions as presented in Helling et al.
77 (1998) and (ii) to compare the sensitivity of this test with the Enchytraeid Reproduction Test
78 (ERT).

79

80 **Material and methods**

81 **Soil and pesticides**

82 For a better representativeness of field conditions, a natural soil was preferred over an
83 artificial soil for bait-lamina and reproduction tests. The soil was a Luvisol (FAO soil
84 classification) sampled in a permanent grassland in Versailles, France (48°48'31''N,
85 2°05'26''E). The grassland has not been treated with pesticides for more than 20 years. The
86 soil was collected from the soil layer 0–20 cm, air-dried, sieved at 2 mm and frozen at -80°C
87 for 48h to exterminate soil macro-meso invertebrates. The main soil physical and chemical
88 properties were as follows (n=7 ± SE): pH: 7.5 ± 0.2, soil bulk density: 1.29; organic matter
89 content: 32.6 ± 1.7 g kg⁻¹, C/N ratio: 12.7 ± 0.3, sand: 29% ± 1.3, silt: 48% ± 1.5, and clay:
90 23% ± 0.6 (see Bart et al. 2017 for more details).

91 We used two commercial formulations of fungicides. The first one was the Swing[®] Gold
92 (BASF Agro SAS, composed of dimoxystrobin at 133 g L⁻¹ and epoxiconazole at 50 g L⁻¹)
93 with the French RD at 1.5 L ha⁻¹ for wheat crop (E-phy 2017b). Taking into account the same
94 soil bulk density as earlier and the main concentration of this pesticide in the top 10 cm of the
95 soil (McDonald et al. 2013; Chabauty et al. 2016), the Swing[®] Gold at the RD was calculated
96 as 1.16 10⁻³ mL kg⁻¹ of the dry soil. As Bart et al. (2017) estimated a LC50 for this fungicide
97 of 11 times the RD for *E. albidus*, the following sublethal concentrations were used in both
98 experiments: 1, 3 and 9 times the RD, named as SG1, SG3 and SG9, respectively. The second
99 fungicide was the Cuprafor micro[®] (Quimicas del Valles, 50% copper oxychloride) with the
100 French Recommended Dose (RD) at 10 kg ha⁻¹ for potato crops and vineyards (E-phy 2017a).

101 Taking into account the soil bulk density (1.29) and the main concentration of copper in the top
102 5 cm of the soil (Couto et al. 2015), the Cuprafor micro[®] at the RD was calculated as 15.5 mg
103 kg⁻¹ of the dry soil which correspond to 7.75 mg kg⁻¹ of copper. In an OECD artificial soil,
104 Amorim et al. (2005) estimated the LC50 for *E. albidus* to be > 320 mg kg⁻¹ of copper. Based
105 on this study, the following sublethal concentrations were used for both bait-lamina and
106 reproduction experiments: 16, 32 and 64 times the RD named as C16, C32 and C64, which
107 correspond to 124, 248 and 496 mg kg⁻¹ of copper, respectively. The dry soil was spiked with
108 aqueous solutions of the fungicides, and the soil moisture was concomitantly adjusted to 75%
109 of the water holding capacity.

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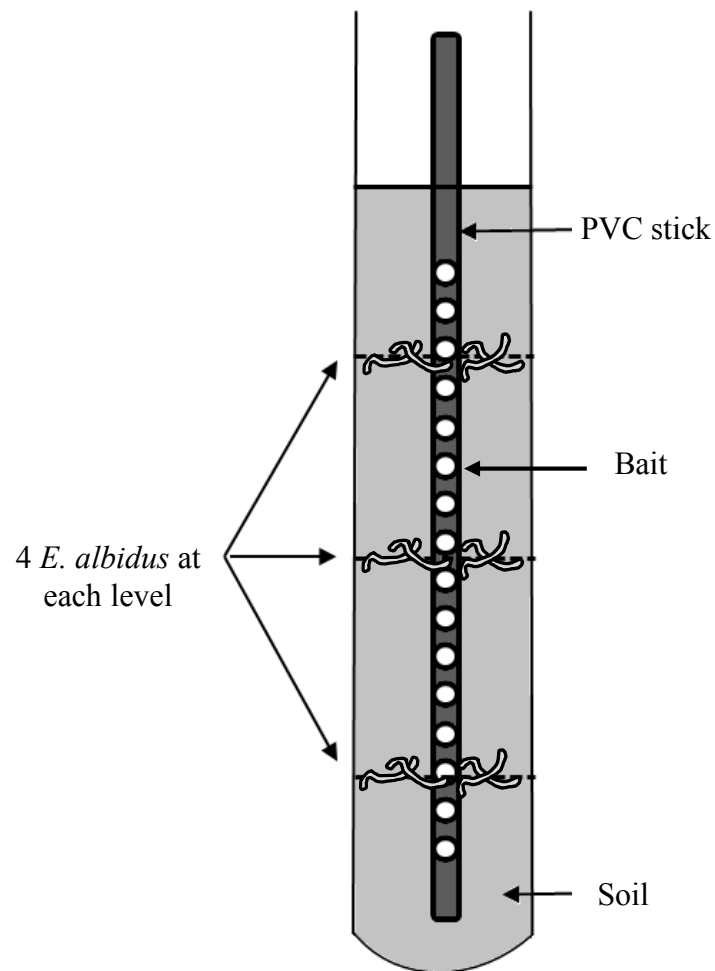
111 **Reproduction test**

112 The reproduction test was conducted according to the standard ISO 16387 (2014), but
113 by using the previously described natural soil. Ten mature individuals collected from a
114 laboratory culture (INRA Versailles-Grignon) were introduced in one vessel (75 mL)
115 containing 20 g of soil (dry mass equivalent). Soil moisture content was checked and adjusted
116 weekly with tap water. For food supply, 50 mg of finely rolled oats were mixed with the soil at
117 the beginning of the experiment and 25 mg were then added every week until the fourth week.
118 Five replicates per treatment were performed. The vessels were stored in a climate room (18 ±
119 0.5°C, 24h darkness). After three weeks, adults were removed from the soil using a binocular
120 microscope and the mortality was assessed. After six weeks, juveniles were fixed with ethanol
121 (99.8% v/v) and colored with 300 µL of Bengal red (1% solution in ethanol). This procedure
122 ensures the observation and counting of juvenile enchytraeids using a binocular microscope
123 (ISO 16387 2014).

124

125 **Bait-lamina test**

126 The feeding activity of enchytraeids was assessed with the bait-lamina test (ISO 18311
127 2016) adapted for laboratory conditions (Helling et al. 1998). We used PVC sticks (18 cm long,
128 6 mm width and 1.5 mm thick) purchased from Terra Protecta (GmbH, Berlin, Germany). A
129 stick contained 16 apertures which were 5 mm apart from each other (Fig. 1). As presented in
130 the ISO guideline 18311 (2016), apertures were filled with a mixture of cellulose (70%),
131 crushed wheat bran and sieved at 500 μm (25%) and activated charcoal carbon (5%).



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133

134 **Fig. 1** Illustration of the experimental device using a glass tube filled with soil, PVC bait-
135 lamina stick and 12 mature *Enchytraeus albidus*.

136

137 Twelve mature *E. albidus* from a laboratory culture (INRA Versailles-Grignon) were
138 inoculated (corresponding to a density of 21 000 individuals m^{-2}) in a glass tube (20 cm high, 3

139 cm in diameter) with 50 g of dry soil adjusted at 75% of the water holding capacity. The tubes
140 were filled with 4 layers of soil successively allowing a vertical distribution of enchytraeids in
141 the tube at 3 levels (4 individuals per level) as suggested by Helling et al. (1998) (Fig. 1). Five
142 replicates were used per treatment, including a control. One stick was added in each tube. After
143 five days of exposure in a climate room (18 ± 0.5 °C, 24 h darkness), the sticks were removed
144 from the soil and fed (i.e., disappearance of at least half of the organic bait material) and not
145 fed apertures were directly counted according to the ISO 18311 (2016). The number of fed
146 apertures was used to calculate the feeding rate expressed as bait consumed (%) per stick. The
147 mortality was assessed as in the ERT.

148 **Statistical analysis**

149 All data were analyzed with the statistical software R Core Team. The R package “ezec”
150 (Zhian N Kamvar 2016) was used to estimate the effective concentration that induced 50%
151 decrease of the reproduction (EC50 reproduction). Because normality and homoscedasticity
152 conditions were not satisfied (tested by the Shapiro–Wilk test), non-parametric Wilcoxon tests
153 were used to compare the control treatment to those treated with pesticides in both experiments.
154 A Kruskal-Wallis test was used to analyze the vertical distribution of bait consumption to test
155 that the feeding activity was homogeneous along the profile.

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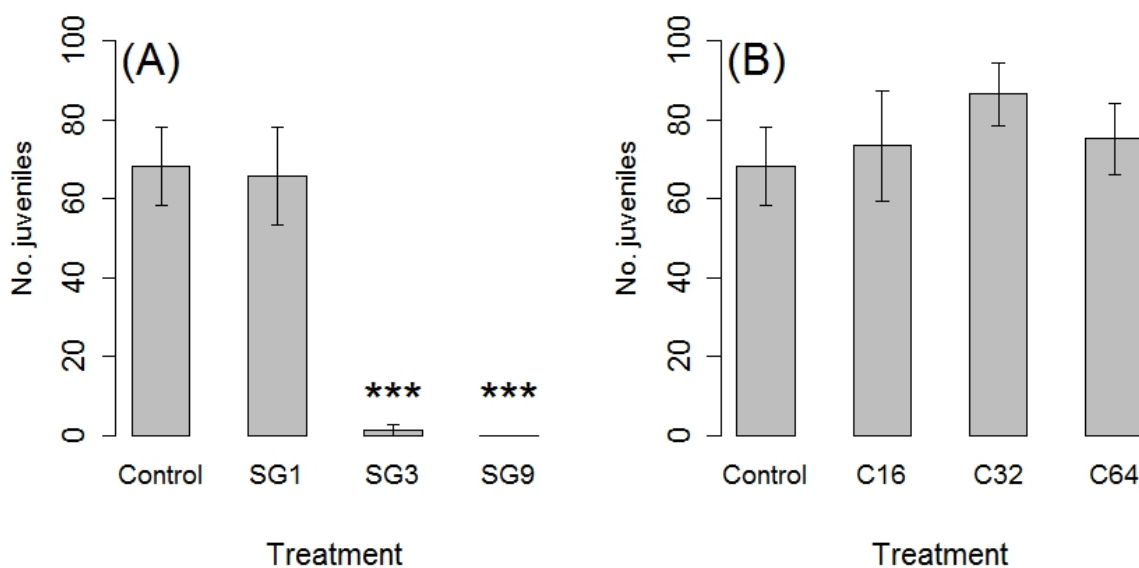
157 **Results and discussion**

158 **The reproduction test**

159 In the control treatment, no enchytraeid mortality was observed and we found on
160 average 68.2 juveniles (Fig. 2). In SG1 and SG3 treatments, corresponding to 1 and 3 times the
161 RD of Swing[®] Gold, no mortality was recorded. However, the number of juveniles was
162 significantly lower in the SG3 treatment (1.4 juveniles on average) than in control treatments
163 (Figure 2A). In the SG9 treatment, a mortality of 90% was recorded and no juveniles were

164 found (Fig. 2 A). This mortality rate is higher than expected as Bart et al. (2017) estimated a
 165 LC50 of 11 times the RD after 14 days in the same soil and with the same commercial
 166 formulation of fungicide. This could be explained by the incubation temperature which was
 167 higher in the present study (18°C, close to the 20 ± 2°C recommended by the ISO 16387, 2014)
 168 compared to the study of Bart et al. 2017 (i.e. 15°C). For instance, De Silva et al. (2009) showed
 169 that “the effects of pesticides (chlorpyrifos and carbofuran) on the earthworm *Eisenia andrei*
 170 survival, growth and reproduction may be higher at higher temperature”. Actually, a higher
 171 temperature may have increased the environmental bioavailability of pesticides (Harmsen,
 172 2007) and thus their toxicity towards animals. The high mortality rate could explain the absence
 173 of reproduction in the SG9 treatment. Finally, the EC50 reproduction was estimated at 1.66 ±
 174 0.93 times the RD. In the EFSA Scientific Report 46, 1-82 (2005), a NOEC value in a
 175 reproduction test with *Eisenia fetida* was estimated at <0.0887 mg kg⁻¹ for the dimoxystrobin
 176 in a formulation such as Swing® Gold, which would correspond to 0.6 times the RD in our
 177 experimental conditions. Our results on enchytraeids strongly support the conclusion that this
 178 fungicide is harmful to soil oligochaete reproduction.

179



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181

182 **Fig. 2** Juveniles produced by 10 mature *Enchytraeus albidus* exposed for 21 days in a control
183 soil and in soils contaminated with Swing Gold® (A) at 1, 3 and 9 times the recommended dose
184 (RD) (abbreviated SG1, SG3 and SG9 respectively), and in soils contaminated with Cuprafor
185 micro® (B) at 16, 32 and 64 times the RD (abbreviated C16, C32 and C64 respectively) which
186 corresponded to 124, 248 and 496 mg kg⁻¹ of copper. Results are expressed in number of
187 juveniles (n=5 ± SE). *** means significant differences between treatments and the control at
188 p-value < 0.001.

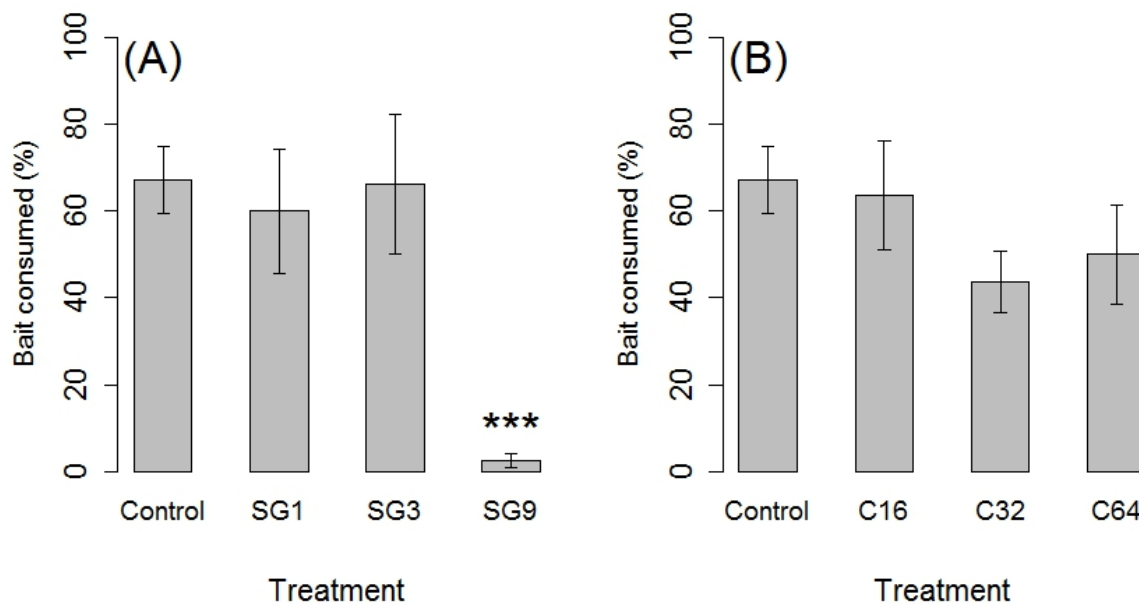
189

190 Whatever the concentration of the copper fungicide tested, no mortality was observed
191 and the reproduction of *E. albidus* was not significantly different between treatments (Fig. 2
192 B). The EC50 reproduction was > 496 mg kg⁻¹ of copper. This value is in accordance with
193 Amorim et al. (2005) who found for *Enchytraeus albidus* an EC50 reproduction value > 320
194 mg kg⁻¹ with copper chloride in an OECD standardized soil. At the same time, Amorim and
195 Scott-Fordsmand (2012) reported an EC50 reproduction value of 251 mg kg⁻¹ with copper-salt
196 (CuCl₂) in a natural sandy soil. Finally, Amorim et al. (2005) found an EC50 reproduction value
197 of 97 mg kg⁻¹ with copper chloride in a LUFA 2.2 standard sandy soil (Speyer, Germany). Thus,
198 copper ecotoxicity towards enchytraeid's reproduction appears to depend on the copper species
199 and the soil used in the experiments that once again drive the bioavailability of contaminants to
200 living organisms. The high soil organic matter content in the soil of our experiment (i.e. 32.6 g
201 kg⁻¹) may have decreased the copper bioavailability (Ma and Rao 1997) for enchytraeids and
202 thus alleviate its ecotoxicological effects.

203

204 **Bait-lamina test**

205 In the same line as the ERT performed with the Swing® Gold, a mortality rate of 92%
206 and no feeding activity was observed in the SG9 treatment (Fig. 3 A). However, no impact of
207 the Swing® Gold at 3 RD (SG3 treatment) was found on the feeding activity compared with the
208 control. The feeding activity of adult enchytraeids was thus not affected in a short-term by the
209 Swing® Gold fungicide.



210

211

212 **Fig. 3** Average feeding activity of 12 mature *Enchytraeus albidus* exposed for 5 days in a
 213 control soil and in soils contaminated with Swing Gold® (A) at 1, 3 and 9 times the
 214 recommended dose (RD) (abbreviate SG1, SG3 and SG9 respectively), and in soils
 215 contaminated with Cuprafor micro® (B) at 16, 32 and 64 times the RD, abbreviate C16, C32
 216 and C64 respectively which correspond to 124, 248 and 496 mg kg⁻¹ of copper. Results are
 217 expressed in bait consumed (%). (n=5 ± SE). *** means significant differences between
 218 treatments and the control at p-value < 0.001.

219

220 The Cuprafor micro® fungicide induced no mortality and no impact on the feeding
 221 activity was recorded whatever the concentration tested (i.e., 16, 32 and 64 times the RD
 222 corresponding to 124, 248 and 496 mg kg⁻¹ of copper respectively) (Fig. 3 B). As for the
 223 reproduction test, the relatively high soil organic matter content may have decreased the copper
 224 bioavailability to enchytraeids, thus explaining why the feeding activity was not affected.

225

226 **Feasibility, sensitivity and ecological relevance of the bait-lamina test under laboratory**
 227 **conditions**

228 Larink (1994) had proposed the bait lamina test as a tool for testing the feeding activity
 229 of animals in contaminated soils under natural conditions. After several other studies (e.g.,

230 Andre et al. 2009; Filzek et al. 2004; Kula and Römbke 1998), a guideline was published by
231 the International Standardization Organization (ISO) and recently updated (ISO 18311, 2016).
232 We tested this method under laboratory conditions and the statistical analysis performed
233 revealed that the feeding activity of enchytraeids, through the bait consumption, was
234 homogeneous along the soil profile in the glass tube. This result validated the experimental
235 design (i.e., vertical distribution of enchytraeids at 3 levels with 4 individuals per level)
236 proposed by Helling et al. (1998). This bait-lamina test adapted under laboratory conditions
237 was easy to perform, quick and cheap, making it relevant as a tool to be used in risk assessment.
238 However, our results highlighted that the bait-lamina test was less sensitive than the ERT on a
239 short time step (i.e. 5 days). The test could thus be adapted to properly assess pesticide impacts
240 on an ecological function provided by enchytraeids (i.e. organic matter consumption) in
241 laboratory. Considering the effects observed on the reproduction and thus the potential negative
242 impacts on the population dynamics, a longer period of exposure could be tested to increase the
243 sensitivity and the relevance of the bait-lamina test under laboratory conditions. However, given
244 that after 5 days, the sticks were consumed at $67 \pm 8\%$ in the control treatment, a longer
245 exposure will quickly lead to a 100% consumption. A longer exposure would thus imply the
246 successive addition of several sticks in the tube in order to assess the feeding activity over time,
247 thus allowing for a better assessment of the risk linked to the use of pesticides. Furthermore,
248 this test could be proposed for standardization and submitted to a ring test procedure. For this
249 purpose and for more repeatability, the soil could be an OECD soil.

250

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