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To cite this version:

Sylvain Bart, Sacha Roudine, Joël Amossé, Christian Mougin, Alexandre R.R. Pery, et al.. How to assess the feeding activity in ecotoxicological laboratory tests using enchytraeids?. Environmental Science and Pollution Research, 2018, 25 (34), pp.33844-33848. 10.1007/s11356-018-1701-3 . hal-01736375

HAL Id: hal-01736375

https://hal.science/hal-01736375v1

Submitted on 28 Feb 2020

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

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Abstract

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

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

Introduction

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

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

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

Material and methods

Soil and pesticides

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

We used two commercial formulations of fungicides. The first one was the Swing® Gold (BASF Agro SAS, composed of dimoxystrobin at 133 g L^{-1} and epoxiconazole at 50 g L^{-1}) with the French RD at 1.5 L ha−1 for wheat crop (E-phy 2017b). Taking into account the same soil bulk density as earlier and the main concentration of this pesticide in the top 10 cm of the soil (McDonald et al. 2013; Chabauty et al. 2016), the Swing® Gold at the RD was calculated as 1.16 10^{-3} mL kg^{-1} of the dry soil. As Bart et al. (2017) estimated a LC50 for this fungicide of 11 times the RD for *E. albidus*, the following sublethal concentrations were used in both experiments: 1, 3 and 9 times the RD, named as SG1, SG3 and SG9, respectively. The second fungicide was the Cuprafor micro® (Quimicas del Valles, 50% copper oxychloride) with the 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.
110

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**

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

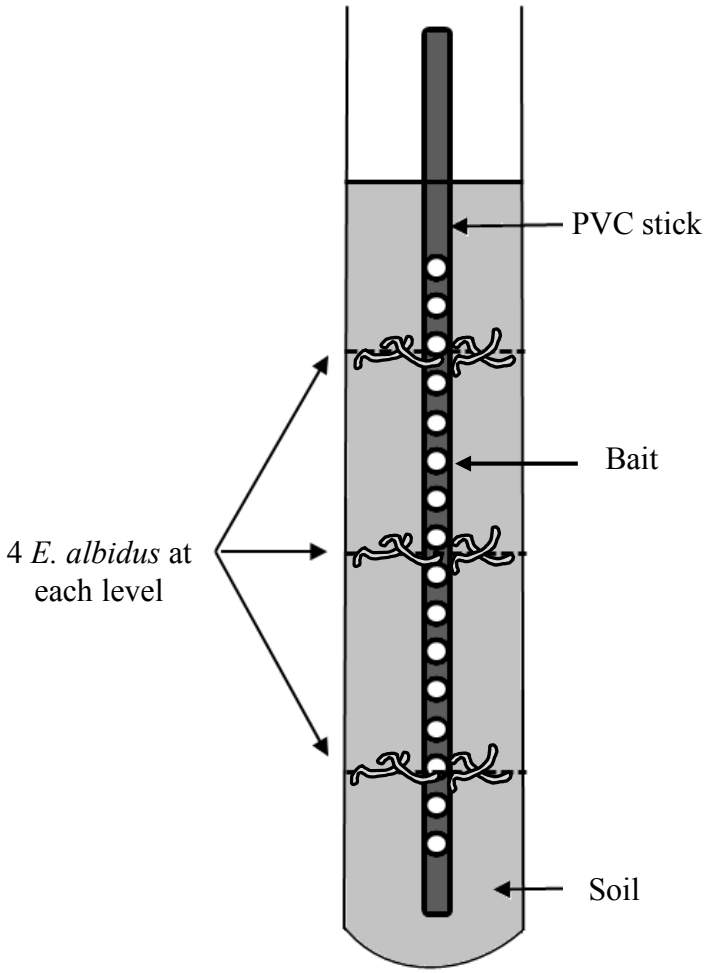


Fig. 1 Illustration of the experimental device using a glass tube filled with soil, PVC bait-lamina stick and 12 mature *Enchytraeus albidus*.

Twelve mature *E. albidus* from a laboratory culture (INRA Versailles-Grignon) were 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.

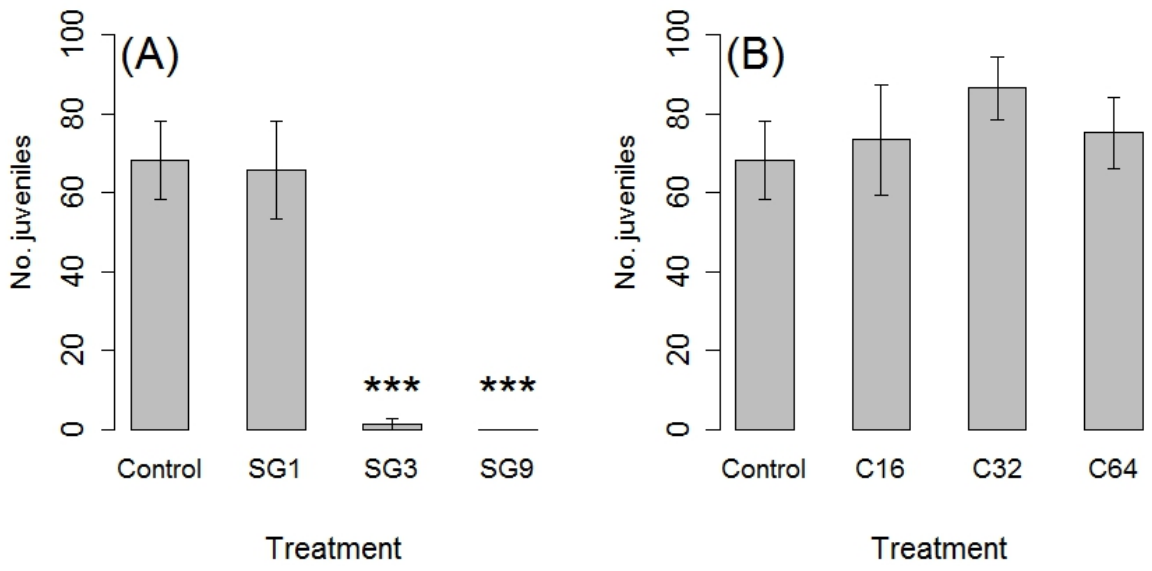
156

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

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



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 ($CuCl_2$) 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 bioavailabilty 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.

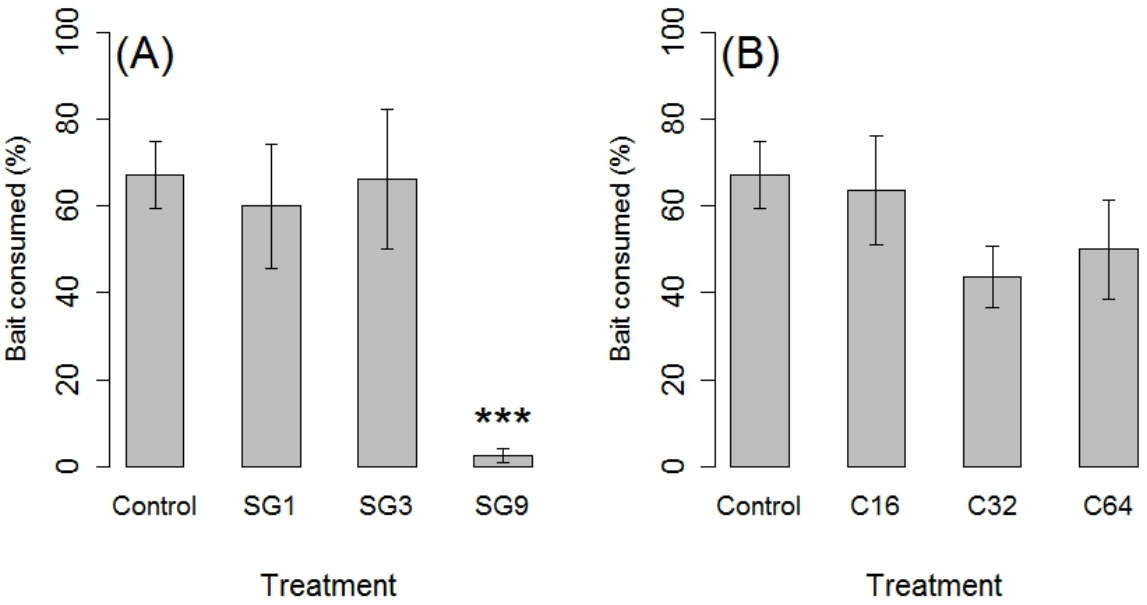


Fig. 3 Average feeding activity of 12 mature *Enchytraeus albidus* exposed for 5 days in a control soil and in soils contaminated with Swing Gold® (A) at 1, 3 and 9 times the recommended dose (RD) (abbreviate SG1, SG3 and SG9 respectively), and in soils contaminated with Cuprafor micro® (B) at 16, 32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

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

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

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

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

Acknowledgements

We would like to thank Jodie Thenard, Véronique Etiévant, Jean-Pierre Pétraud, Alexis Vincent and Sébastien Breuil for the maintenance of the enchytraeid culture. We thank the two

anonymous reviewers for the improvement of the manuscript. This work is supported by the "IDI 2015" project funded by the IDEX Paris-Saclay, ANR-11-IDEX-0003-02.

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