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1 Assessment of avoidance behaviour by earthworms (*Lumbricus rubellus* and *Octolasion cyaneum*) in
2 linear pollution gradients.

3

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5

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8

9 Abstract

10 Avoidance behaviour by earthworms is recognised as a valuable endpoint in soil quality assessment
11 and has resulted in the development of a standardised test (ISO 17512-1: 2008) providing epigeic
12 earthworms with a choice between test and control soils. This study sought to develop and evaluate
13 an avoidance test utilising soil-dwelling earthworms in linear pollution gradients with Visible Implant
14 Elastomer (VIE) tags used to identify individual organisms. Sequential experiments were established
15 in laboratory-based mesocosms (0.6 m x 0.13 m x 0.1 m) that determined the relative sensitivities (in
16 terms of associated avoidance behaviour) of *Octolasion cyaneum* and *Lumbricus rubellus* at varying
17 levels of polluted soil and also assessed the influence of introduction point on recorded movement
18 within gradients. In an initial gradient (0, 25, 50, 75, 100% polluted soil), both species exhibited a clear
19 avoidance response with all surviving earthworms retrieved (after 7 days) from the unpolluted soil. In
20 a less polluted gradient (0, 6.25, 12.5, 18.75, 25%) *L. rubellus* were retrieved throughout the gradient
21 while *O. cyaneum* were located within the 0 and 6.25% divisions, suggesting a species-specific
22 response to polluted soil. Results also showed that the use of a linear pollution gradient system has
23 the potential to assess earthworm avoidance behaviour and could provide a more ecologically
24 relevant alternative to the ISO 17512: 2008 avoidance test. However, further work is required to
25 establish the effectiveness of this procedure, specifically in initial chemical screening and assessment
26 of single contaminant bioavailability, where uptake of pollutants by earthworms could be measured
27 and directly related to the point of introduction and retrieval.

28

29 Keywords

30 Pollution gradient; avoidance test; earthworm tagging; visible implant elastomer

31 1.0 Introduction

32 Selection of organisms to assess pollution is based on critical factors including ecological relevance,
33 ecological importance and sensitivity of the species (Smith et al., 2006). In a soil context, earthworms
34 are considered particularly relevant test organisms as they form a significant component of the soil
35 biomass, are constantly in intimate contact with the soil medium and due to their influence on soil
36 physical, chemical and biological properties are considered ecosystem engineers (Jones et al., 1994;

37 Jouquet et al., 2006). In addition to the well-established role of earthworms in standardised acute
38 toxicity testing of chemicals, the relative resistance to pollutants of some species allows for sub-lethal
39 measures of exposure to pollution. Bioassays have been undertaken utilising ecologically relevant sub-
40 lethal endpoints including biomass, reproduction, casting and burrowing activity. For example,
41 Dittbrenner et al. (2010) demonstrated significant losses in biomass for *Lumbricus terrestris* and
42 *Aporrectodea caliginosa* at 1 and 3 times the recommended application dose (0.66 ppm) of
43 imidacloprid (insecticide) and Capowiez et al. (2010) recorded a significant decrease in casting activity
44 of *L. terrestris* at the recommended dose.

45 Avoidance has also been recognised as a valuable endpoint for pollution assessment (Hund-Rinke et
46 al., 2003) and for some chemicals, it may be as sensitive as reproduction (van Gestel, 2012). However,
47 the relationship between avoidance and metal uptake/toxicity is unclear and the question of whether
48 avoidance is triggered by a detrimental effect of pollutant uptake or by a sensory-based reaction
49 (earthworms possess chemical receptors in the prostomium) remains unanswered (Ma and Bonten,
50 2011). Field assessment of the potentially complex interactions involved in earthworm behavioural
51 responses to soil pollutants is difficult, due to their predominantly subterranean location and potential
52 absence of precise knowledge of pollutants and their concentrations in the environment. Therefore,
53 controlled laboratory-based experiments are recognised as a valuable step to assist understanding of
54 these processes. An ISO standard (ISO 17512-1: 2008) provides earthworms (*Eisenia fetida* or *Eisenia*
55 *andrei*) with a choice between a test soil and a control soil (Fründ et al., 2011). This standard details
56 methods for both a two-section and a six-section avoidance test. The former has been employed by a
57 number of researchers (e.g. Garcia et al., 2008; Owojori and Reinecke, 2009), but the latter has not
58 been widely adopted, which may be related to practical considerations in creation of the circular, 6-
59 chambered experimental vessels. In two-section avoidance tests, earthworms are introduced into the
60 centre of the vessel and after a fixed time period (48 h) the number of earthworms present in each
61 side is recorded and avoidance behaviour considered significant when more than 80% are located in
62 the reference substrate. Avoidance has also been determined by measuring the number of
63 earthworms which burrowed into a substrate after a fixed time period (Ma and Bonten, 2011).

64 Compared with other standardised sub-lethal tests (e.g. reproduction), avoidance tests are rapid,
65 inexpensive and simple to perform, but the behavioural response is species- and chemical-specific and
66 may be influenced by other soil properties (Naidu et al., 2008). For example, in two-section avoidance
67 tests of soil spiked with Cu and Zn, *Dendrobaena octaedra*, *Aporrectodea tuberculata* and *Lumbricus*
68 *rubellus* demonstrated a significant avoidance response in 48/80, 120/200 and 300/500 mg of Cu/Zn
69 per kg of soil respectively (Lukkari and Haimi, 2005). The influence of pollution on earthworm
70 movement has also been assessed using burrow length, topology and sinuosity in 2 dimensional
71 terraria (often referred to as Evans' Boxes (Evans, 1947)) with daily mapping of burrow creation
72 (Bolton and Phillipson, 1976) and 3 dimensional terraria with burrow measurements recorded at the
73 end of the experiment using X-ray tomography (Bastardie et al., 2003). However, these tests bear little
74 resemblance to the often heterogeneous nature of pollution and, with respect to 2D terraria, have
75 limited ecological relevance to field conditions. Fründ et al. (2011) suggested that 3D terraria should
76 be employed, containing a range of pollutant levels (gradients) as a proposed improvement to existing
77 laboratory-based avoidance tests. A further issue, in both laboratory and field-based studies, which
78 has restricted experimental design is the lack of a reliable method for identifying individual
79 earthworms. However, a method of semi-permanently marking individual earthworms with Visible
80 Implant Elastomer (VIE) tags has been demonstrated (Butt et al., 2009; Butt and Grigoropoulou, 2010;

81 Butt and Lowe, 2007; González et al., 2006). Under controlled laboratory conditions, Butt and Lowe
82 (2007) maintained tagged *L. terrestris* over a 12 month period and demonstrated that the tagging
83 process did not affect growth, reproduction or fecundity. Silicon-based VIE tags are injected as a liquid
84 under translucent tissue, before curing to a pliable solid, with tag colour selected to contrast
85 earthworm pigmentation. Tags are also fluorescent, so can be located under blue light.

86 It has been recognised that earthworm species selection can have a significant influence on the
87 outcome of ecotoxicological tests (reviewed by Lowe and Butt, 2007). The epigeic species *E. fetida* has
88 been widely used in acute toxicity tests (mainly due to its ease of culture) and has also been adopted
89 for use in chronic toxicity studies including the ISO 17512-1 avoidance test (ISO, 2008). However, the
90 ecological relevance of this species, especially in studies utilising sub-lethal end points, is becoming
91 more widely questioned (Lowe and Butt, 2007; Lukkari and Haimi, 2005; Svendsen et al., 2005). This
92 is particularly relevant in assessing soil quality, as *E. fetida* is not found within the soil profile, is
93 uncommon except in anthropogenic settings and is more tolerant to contaminants than many other
94 species (Lukkari et al., 2005). A number of ecotoxicological studies have employed *L. rubellus* as a test
95 species (e.g. Morgan and Morgan, 1999; Langdon et al., 2003; Spurgeon et al., 2004). Although *L.*
96 *rubellus* is considered to be an epigeic species, it does venture into mineral soil and is present in the
97 natural environment. Lowe and Butt (2007) have also suggested that mode of reproduction should be
98 considered in species selection and have advocated the use of parthenogenetic species (e.g.
99 *Octolasion cyaneum*) to reduce the influence of genetic drift.

100

101 The main aim of this research was to build on the recommendations of Fründ et al. (2011) by assessing
102 the viability of utilising linear pollution gradients in combination with VIE tagging and the use of soil
103 dwelling earthworm species in the development of a more ecologically relevant and practical
104 avoidance test of soil quality. Sequential experiments were established in laboratory-based
105 mesocosms to determine the relative sensitivities (in terms of associated avoidance behaviour) of *O.*
106 *cyaneum* (endoge) and *L. rubellus* (epige) to varying levels of field-collected, polluted soil and to
107 determine the influence of introduction point on recorded movement within gradients.

108 2.0 Methods

109 Polluted soil was collected from a derelict scrap metal depot in Preston, Lancashire (53° 45 'N 2° 43
110 'W; the soil is considered artificial "made ground", pH 7.6, organic matter 14%). The site is
111 contaminated with oil, PCBs and heavy metals. In a previous study (Glover, 2010) soil from the site
112 was analysed using a Thermo Electron X5 Inductively Coupled Plasma Mass Spectrometer (ICP-MS)
113 and recorded total metal soil concentrations for arsenic (75 ppm), copper (6617 ppm), chromium (403
114 ppm) and lead (33590 ppm); all above current UK Soil Guideline Value thresholds (Environment
115 Agency, 2008). A survey at the site of soil collection recorded no earthworms. Collected soil was
116 transferred to the laboratory where it was heated to 75 °C for 45 minutes (in a Camplex Soil Steriliser)
117 to kill living material, then homogenised by thorough mixing and passage through a 2 mm sieve.
118 Experimental soil gradients were formed by blending proportions of the polluted soil with a pre-
119 sterilised and sieved Kettering Loam soil (pH 7.2, organic content 5%) obtained from Boughton Loam
120 Ltd, Kettering, UK – a soil widely used in earthworm culture and laboratory-based studies (Lowe and
121 Butt, 2005). Dried horse manure (2 g per 100 g of soil) was incorporated into each soil blend as a feed
122 source and the substrate rewetted to a moisture content of approximately 25% (Lowe and Butt, 2005).

123 Mature (clitellate) individuals of *L. rubellus* and *O. cyaneum* were field-collected by digging and hand-
124 sorting soil in pasture at Cuerden Valley Park (53°42 'N 2° 39 'W; clayey loam soil) and in a tree
125 plantation at Bank Hall (53° 40 'N 2° 48 'W; silty-clay loam soil) respectively. Prior to experimental use,
126 these earthworms were maintained in laboratory cultures as described by Lowe and Butt (2005).

127 Three gradient experiments were conducted using opaque plastic containers (0.6 m x 0.13 m x 0.1 m)
128 filled to a depth of 0.075 m with soil. Each experiment employed five levels of polluted soil, initially
129 separated by plastic spacers (cut precisely to the dimensions of the containers section), resulting in
130 five, 0.12 m soil divisions in a prescribed pollution gradient. To encourage earthworm burrowing
131 activity within the soil profile, organic matter (horse manure) was not placed on the soil surface. After
132 earthworm introduction, the spacers were removed and the containers covered with plastic (cling
133 film), pierced with a mounted needle to allow ventilation and kept in 24 h darkness in a temperature-
134 controlled incubator at 15 °C. After 7 days, the spacers were re-inserted and earthworm positions
135 within gradients established by destructive sampling. Any earthworm cut by a spacer was recorded as
136 half retrieved from each section adjacent to the given spacer.

137

138 *2.1 Gradient 1: 0 to 100% (Introduction point at 100%)*

139 This initial experiment had a pollution gradient ranging from 0 to 100% of polluted soil (with divisions
140 of 0, 25, 50, 75 and 100%). The divisions were arranged in order of pollution level, from the least to
141 the most polluted. In each of five replicated containers, one mature *O. cyaneum* and one mature *L.*
142 *rubellus* were introduced on to the soil surface in the centre of the 100% polluted soil division.

143

144 *2.2 Gradient 2: 0 to 25% (Introduction point at 25%)*

145 In response to results obtained from Gradient 1, a subsequent, reduced pollution gradient was
146 established equating to sub-divisions of 0 to 25% of polluted soil (0, 6.25, 12.5, 18.75 and 25%). The
147 containers were constructed as for Gradient 1 and again, a single mature individual of each species
148 was introduced on to the soil surface, but here at the centre of the 25% polluted soil division. Once
149 again this set up was replicated 5 times and maintained under the same environmental conditions as
150 previously described.

151 *2.3 Gradient 3: 0 to 25% (introduction points at 0%, 12.5% and 25%)*

152 Here, a mature *L. rubellus* and a mature *O. cyaneum* were introduced at 3 selected points (0%, 12.5%
153 and 25% polluted soil) along a gradient ranging from 0 to 25% of polluted soil, set up as in Gradient 2
154 (n = 10 replicates). Individuals were VIE tagged (2 weeks prior to use), with a specific colour (blue (0%),
155 red (12.5%) or yellow (25%)) to distinguish introduction point into the gradient. For the first 24 h,
156 plastic spacers separating the soil divisions were left in place, to encourage earthworms to burrow
157 into the soil at point of introduction. The final location of each earthworm and colour of VIE tag was
158 recorded. Individual earthworm biomass was also recorded at both the outset and end of the
159 experiment.

160 *2.4 Statistical Analysis*

161 In gradient 3, differences in biomass associated with point of inoculation were statistically analysed
 162 using one-way ANOVA followed by a Tukey multiple comparison test. Each division (pollution level) in
 163 gradient 3 was coded (25% = 5, 18.75 = 4, 12.5% = 3, 6.25% = 2 and 0% = 1) and these used to record
 164 retrieval point. These data were then analysed using a non-parametric Kruskal Wallis significance test
 165 to establish the influence of introduction point on retrieval point across the pollution gradient. Where
 166 earthworms were cut by the spacer, an intermediate code was assigned (e.g. 3.5). All statistical
 167 analyses were undertaken using Minitab version 16.1 software.

168 3.0 Results

169 3.1 Gradient 1

170 On introduction on to the soil surface of the 100% polluted soil 2 *O. cyaneum* and 4 *L. rubellus*
 171 burrowed down and all others (3 *O. cyaneum* and 1 *L. rubellus*) burrowed into the adjacent 75%
 172 polluted soil. On destructive sampling after 1 week, all *O. cyaneum* (n=5) and 3 *L. rubellus* were located
 173 in the unpolluted soil (0%). Two *L. rubellus* were not located and were considered to have died during
 174 the experimental period. As all surviving earthworms were found in unpolluted soil, this suggested
 175 that all levels of polluted soils in the gradient were capable of eliciting an avoidance response and
 176 resulted in establishment of Gradient 2, ranging from 0-25% polluted soil.

177 3.2 Gradient 2

178 When initially exposed to 25% polluted soil, 3 *O. cyaneum* and 5 *L. rubellus* burrowed down directly
 179 and the remainder burrowed into the 18.75% polluted soil. At the end of the experiment, earthworms
 180 were recovered throughout the gradient (Table 1). A 100% recovery rate was achieved from this
 181 experiment with *L. rubellus* present across pollution levels ranging from 6.25 – 25%. In contrast, *O.*
 182 *cyaneum* were retrieved from unpolluted (0%) or 6.25% polluted soil only.

183

184 Table 1 *O. cyaneum* and *L. rubellus* numbers retrieved from Gradient 2 over a range of pollution levels
 185 derived from a contaminated field site in Preston

	Soil Pollution levels – earthworm retrieval				
	25%	18.75%	12.5%	6.25%	0%
<i>O. cyaneum</i>	0	0	0	1	4
<i>L. rubellus</i>	1	3	0	1	0

186

187

188 3.3 Gradient 3

189 After 7 days of exposure, the majority of earthworms were retrieved from a division of the gradient
 190 less polluted than their introduction point, but differences in response between the two species were
 191 noted (Table 2). A 100% earthworm recovery rate was recorded throughout the experiment and all
 192 VIE tags remained visible for identification. For earthworms introduced into 25% polluted soil, 60% of
 193 *L. rubellus* and 80% of *O. cyaneum* were retrieved from less polluted soil. For earthworms introduced
 194 into 12.5% polluted soil, 50% of *L. rubellus* and 100% of *O. cyaneum* were retrieved from less polluted

195 soil. However, some earthworms were retrieved from soil with higher pollution levels than their
 196 introduction point (Table 2). Introduction point had a significant influence on *L. rubellus* retrieval point
 197 ($p = 0.032$) but did not significantly ($p = 0.343$) influence retrieval point for *O. cyaneum* with individuals
 198 tending to move towards (or remain) in unpolluted soil (average movement = 2.65 and 1.65 divisions
 199 from 25 and 12.5% introduction points respectively). *L. rubellus* individuals tended to remain closer to
 200 25% and 12.5% introduction points than *O. cyaneum* with average movement away from these points
 201 recorded as 1.6 and 1.1 divisions respectively. Movement away from the 0% introduction point was
 202 low for both species (0.6 and 0.1 divisions for *L. rubellus* and *O. cyaneum* respectively).

203

204 Table 2 *O. cyaneum* and *L. rubellus* retrieval rates (%) from different pollution levels in Gradient 3 with
 205 three different introduction points (25, 12.5 and 0% polluted soil).

	Introduction point	Soil Pollution levels – earthworm retrieval (%)				
		25%	18.75%	12.5%	6.25%	0%
<i>O. cyaneum</i>	25%	20	15	5	0	60
	12.5%	0	0	0	35	65
	0%	0	0	0	10	90
<i>L. rubellus</i>	25%	40	10	20	10	20
	12.5%	0	10	40	0	50
	0%	0	0	20	10	70

206

207 Changes in earthworm biomass are shown in Table 3. *L. rubellus* introduced into 25% polluted soil
 208 exhibited decreased mean biomass which was significantly different ($p < 0.05$) from the increased mean
 209 biomass recorded for individuals of this species introduced into 12.5% and 0% polluted soil. Mean
 210 increase in biomass of *O. cyaneum* introduced into 0 and 25% polluted soil was positive, but was
 211 negative for individuals introduced into 12.5% polluted soil. However, there was a significant
 212 difference ($p < 0.05$) between biomass change for individuals of this species introduced into unpolluted
 213 soil compared with those introduced into 12.5 and 25% polluted soil.

214

215 Table 3 Mean change in % biomass for *L. rubellus* and *O. cyaneum* individuals introduced into 25%,
 216 12.5% and 0% polluted soil in Gradient 3. For each species, mean values not sharing the same letter
 217 were significantly different ($p < 0.05$).

	Inoculation point	Mean change in Biomass (%)	Standard Deviation (SD)
<i>O. cyaneum</i>	25%	4.12 (b)	15.45
	12.5%	-0.25 (b)	12.99
	0%	19.05 (a)	8.86
<i>L. rubellus</i>	25%	-1.06 (b)	8.92
	12.5%	17.83(a)	11.58
	0%	17.95 (a)	8.48

218

219

220 4.0 Discussion

221 Results from the first experimental gradient demonstrated a clear avoidance response to the polluted
222 soil with all surviving earthworms retrieved from unpolluted soil. This showed the ability of both
223 species to traverse the length of the experimental container (0.6 m) during the 7 day period. The
224 minimum percentage level of polluted soil that triggered the avoidance response was established as
225 less than 25%. This led to setting up of Gradient 2 (0-25%) that allowed for potential differences in
226 species avoidance response to be studied and to further assess the suitability of the linear gradient
227 design as a sub-lethal toxicity test. In Gradient 2, clear differences in retrieval location were recorded
228 after 7 days that suggested a species-specific response to the polluted soil. *L. rubellus* were retrieved
229 throughout the pollution gradient while *O. cyaneum* were located within to the 0 and 6.25% divisions.
230 This suggested that (in terms of an avoidance response) *O. cyaneum* is more sensitive to the polluted
231 soil than *L. rubellus*. The tolerance of *L. rubellus* to polluted soils is well documented (e.g. Nahmani et
232 al., 2007; Spurgeon and Hopkin, 1996). Observed differences in avoidance response between the
233 epigeic *L. rubellus* and endogeic *O. cyaneum* support the findings of Lukkari and Haimi (2005) using a
234 two-section avoidance test procedure to establish avoidance responses of three ecologically different
235 earthworm species (*L. rubellus*, *D. octaedra* and *A. tuberculata*) to Cu- and Zn-contaminated soil. *L.*
236 *rubellus* was found to be the least sensitive species and only responded to the highest metal
237 concentration.

238 A species-specific response to the pollution gradient was further supported by results from Gradient
239 3, where identification of individuals with VIE tags allowed earthworms to be simultaneously
240 introduced at 3 pollution levels within the gradient. Findings demonstrated the increased sensitivity
241 of *O. cyaneum* (when compared with *L. rubellus*) to all levels of polluted soil. Although differences
242 were recorded in retrieval point for *O. cyaneum* introduced at different points along the gradient,
243 these were not significant, with the majority of individuals recorded in 0% polluted soil. For *L. rubellus*,
244 significant differences in retrieval point were recorded with individuals generally remaining closer to
245 the point of introduction, which suggests a weaker, or even no, avoidance response. Although results
246 suggest that earthworm movement (dispersal/avoidance) is influenced by the level of pollutants
247 present in the soil, it also has to be recognised that other properties of the polluted soil may have
248 relevance.

249 Earthworm response to a fixed pollutant level may vary depending on the influence of a range of biotic
250 and abiotic factors and result in a hierarchical response. For example, Fründ et al., (2009)
251 demonstrated that the outcome of a soil preference test in 2D terraria could be reversed by increasing
252 the water holding capacity in one side of the terraria from 70 to 87%. Similarly, species-specific
253 pollutant avoidance behaviour associated within heterogeneous soils may be influenced by availability
254 of / foraging for organic matter (food) (Lowe and Butt, 2002) and the response may have a temporal
255 basis associated with changes in resource availability. Inter-specific and intra-specific interactions
256 (reviewed by Uvarov, 2009) may also influence earthworm position within the soil and so increasing
257 earthworm density in the Gradient 3 design may have influenced retrieval position results. However,
258 it is not possible to determine the influence of these factors on earthworm movement within the
259 experimental design without a method for tracking of individual movement throughout the
260 experimental period.

261 Significant differences in change of biomass over the 7 day experimental period were recorded and
262 these were associated with introduction point. However, as suggested by Lowe and Butt (2007) such
263 results should be interpreted with caution given the unknown age and history of the field-collected
264 earthworms used in the study. In contrast, and a significant advantage over other sub-lethal endpoints
265 such as reproduction, the use of field-collected, rather than laboratory-reared, individuals in assessing
266 avoidance is considered valid by the authors with the caveat that individuals for each species used are
267 collected from the same non-polluted location. This further enhances the proposed increased
268 practicality of avoidance tests.

269 Results have suggested that the use of a linear pollution gradient over a 7 day experimental period
270 may be used to assess earthworm avoidance behaviour in relation to field-collected polluted soil. It is
271 suggested that the gradient system can provide a more ecologically relevant (in terms of the
272 earthworm species used and heterogeneous nature of many polluted soils) alternative to the two-
273 section avoidance test and a more practical alternative to the six-section standardised avoidance test
274 (ISO 17512-1: 2008). However, further work is required to establish the effectiveness of this procedure
275 in initial chemical screening and assessment of single contaminant bioavailability. Therefore future
276 studies may seek to use a single laboratory-reared earthworm species and employ a standardised soil
277 (e.g. OECD artificial soil) with a pollutant gradient established by spiking soils with a single pollutant.
278 This would allow for the uptake of pollutants by earthworms to be measured and directly related to
279 the point of introduction and retrieval. Further research should also seek to compare avoidance
280 response over a range of time periods to establish if this modified avoidance test can provide reliable
281 results comparable with the 48 hours used in standardised tests.

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