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

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Abstract

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

Keywords

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

1.0 Introduction

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

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

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

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

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

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

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

2.0 Methods

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

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

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

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

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

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

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

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

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

2.4 Statistical Analysis

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

3.0 Results

3.1 Gradient 1

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

3.2 Gradient 2

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

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

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

3.3 Gradient 3

After 7 days of exposure, the majority of earthworms were retrieved from a division of the gradient less polluted than their introduction point, but differences in response between the two species were noted (Table 2). A 100% earthworm recovery rate was recorded throughout the experiment and all VIE tags remained visible for identification. For earthworms introduced into 25% polluted soil, 60% of *L. rubellus* and 80% of *O. cyaneum* were retrieved from less polluted soil. For earthworms introduced into 12.5% polluted soil, 50% of *L. rubellus* and 100% of *O. cyaneum* were retrieved from less polluted soil. However, some earthworms were retrieved from soil with higher pollution levels than their introduction point (Table 2). Introduction point had a significant influence on *L. rubellus* retrieval point ($p = 0.032$) but did not significantly ($p = 0.343$) influence retrieval point for *O. cyaneum* with individuals tending to move towards (or remain) in unpolluted soil (average movement = 2.65 and 1.65 divisions from 25 and 12.5% introduction points respectively). *L. rubellus* individuals tended to remain closer to 25% and 12.5% introduction points than *O. cyaneum* with average movement away from these points recorded as 1.6 and 1.1 divisions respectively. Movement away from the 0% introduction point was low for both species (0.6 and 0.1 divisions for *L. rubellus* and *O. cyaneum* respectively).

Table 2 *O. cyaneum* and *L.rubellus* retrieval rates (%) from different pollution levels in Gradient 3 with 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%
O. cyaneum	25%	20	15	5	0	60
	12.5%	0	0	0	35	65
	0%	0	0	0	10	90
L. rubellus	25%	40	10	20	10	20
	12.5%	0	10	40	0	50
	0%	0	0	20	10	70

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

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

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

4.0 Discussion

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

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

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

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

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

5.0 References

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