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DEVELOPMENT OF A FIELD-BASED EARTHWORM MARK-RELEASE-RECAPTURE TECHNIQUE FOR BIOMONITORING

Biomonitoring in soil systems presents difficulties in retrieval and differentiation of test organisms from local populations. The aim of this study was to develop a simple method of in situ containment and efficient retrieval of earthworms. Visible Implant Elastomer (VIE) tags were used to identify earthworms Lumbricus rubellus and Aporrectodea caliginosa introduced to unpolluted pasture or a polluted site. Containment PVC tubes of different height (inserted differently into the soil and covered or not covered by a mesh) were used. Highest recovery rates for both species came from use of 0.3 m tubes (100% for A. caliginosa and 71% for L. rubellus). This study confirmed the feasibility of using VIE tags to identify and aid recovery of introduced earthworms from field experiments. Results suggested that recovery rates were influenced by behavioural differences in ecological groupings and site-specific factors. There is scope for further improvement in the tagging procedure, mortality assessment and investigation of containment for earthworms.

Keywords: containment, earthworms, ecotoxicology, *in situ* experiment, VIE tagging

I. INTRODUCTION

In situ biomonitoring techniques have been successfully used in freshwater ecology since the early 1980s, to compare different sites in ecotoxicology experiments and decrease the influence of both laboratory conditioning and adaptation of test organisms to their local environment [Marquenie and Simmers 1988]. However, introduction of test organisms into the soil environment presents difficulties, particularly differentiating between introduced individuals and local populations. To address identification issues, authors such as Marinussen et al. [1997] have introduced earthworm species not naturally present in the locality, however, introduction of a potentially exotic species may disturb the local ecosystem [Blouin et al. 2013]. Furthermore, the natural absence of a species may be due to an irrelevance of the selected species to the site of interest, which would affect the toxicological results (e.g. use of *Eisenia fetida* in soils deficient in organic-matter [Lukkari and Haimi 2005]). To overcome such issues, use can be made of individually marked (tagged) earthworms. Different tagging methods have previously been attempted with earthworms for identification purposes (Butt and Lowe [2007] and references therein). However, each of these methods presents problems, such as short duration of marking, a necessity to kill earthworms before identification or use of relatively expensive materials.

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More recently, it has been shown that earthworms can be tagged with Visible Implant Elastomer (VIE) supplied by Northwest Marine Technology Inc. (NMT), Washington, USA, [Butt et al. 2009, Butt and Grigoropoulou 2010, Butt and Lowe 2007, González et al. 2006]. VIE tags are already widely used in aquatic ecology, for example with fish [Crook and White 1995] or crustaceans [Godin et al. 1996] and it is therefore suggested that this relatively inexpensive technique could be effectively used with earthworms [Blouin et al. 2013, Butt and Lowe 2007].

Mortality is an important endpoint in ecotoxicology. For example, it is used as validity criteria in OECD experiments, requiring less than 10% mortality of the original population under control conditions [OECD 2004, 2010]. For *in situ* experiments, potential recovery of VIE tags from the substrate could be used to discriminate between earthworm mortality and dispersal/avoidance at the end of the experimental period.

To maximise *in situ* earthworm retrieval rates, different containment methods have been trialled, for example using mesh cages [Hankard et al. 2004] or plastic fences [Grigoropolou and Butt 2010, Butt et al. 2015]. It has also been recognised that containment methods ought to allow the expression of "normal" earthworm behaviours (e.g. burrowing).

In contrast to aquatic environments, the physical structure of soil prevents the direct introduction of simple containment devices (such as cages) without disturbance. However, it has been proposed (by the authors) that tubes driven directly into the soil may minimise disturbance and act as effective containment. Stainless steel tubes (0.2 m height and 0.12 m diameter) have previously been used to take soil cores from sites to the laboratory and maintain earthworms in laboratory conditions by e.g. Reinecke and Reinecke [2007]. PVC tubes of different lengths have also been used to keep different earthworm species under laboratory conditions. For example, Butt et al. [2003] kept *L. terrestris* in 0.1 m inner diameter tubes of height 1.0 m and Pey used 0.15 m diameter tubes of height 0.1 m to keep *E. fetida* and 0.19 x 0.30 m to keep *L. terrestris* and *A. caliginosa* [Pey, 2010, Pey et al. 2013].

The main aim of this study was to develop a practical method for mark-release-recapture of earthworms leading towards the development of an *in situ* method of biomonitoring of soil contaminants. Two field-based trials, conducted at polluted and unpolluted sites, employed endogeic (*A. caliginosa*) and epigeic (*L. rubellus*) earthworm species. The first assessed the need for containment (utilising PVC tubes) and the second investigated issues associated with over surface movement of earthworms. The influence of earthworm ecological grouping and the use of VIE tagging to differentiate between introduced and resident individuals and to monitor earthworm mortality (via tag recovery from the soil) was also assessed in both trials.

II. MATERIAL AND METHODS

Experimental sites

To minimise disturbance of *in situ* experiments, sites with restricted public access were required. One metal-polluted and one unpolluted site were selected in Preston, UK. The unpolluted site, Wilson's Farm (U) (53°43'N 2°43'W) is a relatively undisturbed pasture (silt-clay loam, soil depth > 50 cm) occasionally used for grazing cattle. This site had not been exposed to any pesticide or artificial fertiliser for at least five years before this study. The resident earthworm community comprised *Allolobophora chlorotica, Aporrectodea longa*, *A. caliginosa*, *Lumbricus castaneus*, *L. rubellus* and *Octolasion cyaneum* (authors' unpublished data). The polluted site, Carlton Street (P) (an ex-engineering works), is in a residential area (53°45'N 2°43'W). Measured depth of soil ranged from 2 to 12 cm in the selected experimental part of the site, above a discontinuous layer of rocks. The site is covered with made ground of black ash (WYGE, 2004). No earthworms were found during site investigation and total metal soil concentrations for arsenic, copper, chromium and lead are above UK soil guideline threshold values (Lowe et al. 2016).

Earthworm species

Aporrectodea caliginosa and *Lumbricus rubellus* were selected for this study as both are common UK species [Sims and Gerard 1999]. Clitellate earthworms were collected by digging and hand-sorting soil from two unpolluted sites in Preston, UK: Bank Hall, a woodland site at 53°40’N 2°48’W, described by Grigoropoulou and Butt [2010] and Cuerden Valley Park, a pasture at 53°42’N 2°39’W. After collection (September 2011), earthworms were kept in the laboratory, based on maintenance guidelines provided by Lowe and Butt (2005, 2007), in a pre-sterilised loam soil (Boughton Loam, Kettering, UK) mixed with dried horse manure in 750 ml plastic vessels (Lakeland Plastics Ltd), with sealable lids pierced with a mounted needle to allow ventilation. Horse manure was added to the loam in the ratio of 4 g per 100 g of dry soil and the substrate rewetted to achieve a moisture content of 25%. The substrate was then added to the vessels, filling approximately 80% of the total volume. In vessels containing *L. rubellus*, re-wetted horse manure was also applied at the soil surface as a feed source. Four earthworms were maintained in each vessel for a period of up to 8 weeks until experimental use.

Earthworm Tagging

Earthworms were tagged with VIE following the procedure outlined by Butt and Lowe [2007]. To ensure ease of visibility, tag colour was chosen to contrast with earthworm colour (red for *A. caliginosa*; yellow for *L. rubellus*). For 48 h prior to tagging, earthworms were maintained at 4 ± 1°C to decrease metabolic activity [Phillipson and Bolton 1975] and reduce movement, making tagging simpler. Tagging took place a week in advance of Trial 1. However, as it appeared that some pieces of tags were egested by earthworms during Trial 1, earthworms were tagged two weeks before Trial 2 to permit visual assessment of a viable tag before experimental use.

Containment methods

PVC tubes (0.2 m inner diameter) were cut to lengths of either 0.3 m or 0.1 m. To facilitate insertion of each tube into the substrate, a tube of the same dimensions with a sharpened end was first driven into the soil (to a depth of 0.1 m), by hammering a block of wood placed across the upper surface. PVC tubes were selected for this study as they are easier to handle and cut to size and less expensive than equivalent sized steel tubes.

Trial 1: Containment

To assess the feasibility of introduction and recovery of tagged earthworms *in situ*, with and without containment, three treatments were established at Wilson’s Farm (U), an unpolluted site: (T) 0.3 m height PVC tubes, inserted to a depth of 0.1 m (UT1); (S) 0.1 m height PVC tubes inserted flush with the soil (*i.e.* to a depth of 0.1 m) (US1) and (0) no containment (U01) (Table 1).

In the (T) treatment, 2 holes were drilled approximately 0.05 m from the top of the 0.3 m tubes to allow insertion of a metal bar to assist removal of the tubes at the end of the experiment. Treatments (S) and (0) were repeated at the Carlton Street (polluted site) (PS1 and P01). However, treatment (T) was omitted, as prior to experimental set up, several 0.3 m tubes were deliberately left unattended at the site and within 24 h, these had been stolen, suggesting that any visible structure left above the soil surface would attract unwanted attention. Seven replicates of the given treatments were randomly positioned in rows, with the centre of each unit at least 2 m from its nearest neighbour. Flat-headed, plastic pegs (0.12 m in length), inserted flush with the ground were used to locate U01/P01 treatments. In establishing US1/PS1 and U01/P01 treatments, a 0.3 m tube was temporarily placed directly above the point of earthworm introduction to prevent initial over-surface movement. Once earthworms had burrowed into the soil, this tube was removed. Two *A. caliginosa* and two *L. rubellus* were introduced to each replicate (n=7). Trial 1 began in mid-October 2011 and ran for 14 days before destructive sampling, when 0.1 m soil cores were extracted for each treatment and taken intact to the laboratory. For treatments using tubes, soil cores were carried within each tube. In the (0) treatment, additional tubes were driven into the soil (to a depth of 0.1 m) centred on the pegs, to remove an equivalent volume of soil. Each soil sample was then hand-sorted for earthworms, dried for 24 h at 105 °C, and then searched for evidence of VIE tags using a blue Light Emitting Diode (LED) torch supplied by NMT.

Table 1 - *Tabela 1*

Treatments used in trials investigating effects of (1) containment and of (2) surface movement on recovery of *Aporrectodea caliginosa* or *Lumbricus rubellus* at an unpolluted (U) and polluted (P) site / *Działania stosowane w badaniu wpływu (1) ograniczenia ruchu i (2) migracji powierzchniowej Aporrectodea caliginosa lub Lumbricus rubellus w miejscu niezanieczyszczonym (U) i zanieczyszczonym (P)*

Treatment / *Działanie*	Location / *Lokalizacja*	
	Wilson's Farm (U)	Carlton Street (P)
Trial 1. Containment / *Próba 1. Ograniczenie ruchu*		
Tall tubes / *Długie rury* (0.3 m) / (T1)	UT1	Not used / *Nie użyto*
Short tubes / *Krótkie rury* (0.1 m) / (S1)	US1	PS1
No tube / *Brak ograniczenia rurą* (01)	U01	P01
Trial 2. Surface movement / *Próba 2. Migracja powierzchniowa*		
Short tube / *Krótkie rury* (0.1 m) no mesh / *bez siatki* (S2)	US2	PS2
Short tube / *Krótkie rury* (0.1 m) with mesh / *z siatką* (SM2)	USM2	PSM2

(U) unpolluted / *nie zanieczyszczony* (P) polluted / zanieczyszczony

Trial 2: Prevention of over-surface movement

Issues with the visibility of the 0.3 m tubes experienced in Trial 1 led here to a focus on 0.1 m tubes (S) inserted flush with the soil. Results from Trial 1 suggested that restriction of over-surface movement from 0.1 m tubes (S) might be required to maximise earthworm retrieval rates. Therefore, Trial 2, conducted at both Wilson's Farm (U) and Carlton Street (P), employed two treatments: 0.1 m tubes (S) covered with a 0.5 mm flexible plastic mesh (PSM2 and USM2) and 0.1 m tubes with no plastic mesh cover (PS2 or US2) (see Table 1). Both treatments were established, as described in Trial 1. Five replicates for each treatment were randomly assigned in two rows. Two tagged earthworms of *L. rubellus and A. caliginosa* were added to each replicate. Trial 2 began in mid-November 2011 and ran for 14 days, before destructive sampling as described in Trial 1.

Statistical analysis

Where appropriate (and viable) the median number of recovered earthworms and retrieved tags in the different treatments for each earthworm species were statistically compared using Minitab 17 Statistical Software. The Kruskal-Wallis test was employed for the 3 treatments at Wilson's Farm and the Mann-Whitney test for the 2 treatments at Carlton Street. $\alpha = 0.05$ was established as the level of significance.

III. RESULTS AND DISCUSSION

There was significant variation in earthworm recovery rate across experimental treatments (Table 2). At the unpolluted site (U) significantly fewer introduced *A. caliginosa* were recovered from U01 (median recovery = 0) compared with UT1 and US1 treatments (p=0.005), with a median recovery rate of 2 for both. Recovery of tagged *L. rubellus* in U01 and UC1 (median recovery = 0) was significantly lower than in the UT1 treatment (p=0.011) (median recovery = 2). The 0.3 m tubes employed in the non-polluted pasture (U) afforded the highest recovery rates for both species (100% for *A. caliginosa* and 71% for *L. rubellus*). The use of this exposed containment design (0.2 m tube extended above the soil surface) had proved problematic and could also attract the attention of livestock and wild animals increasing the risk of disturbance in rural locations.

Table 2 – *Tabela 2*

Earthworm recovery and the number of VIE tag pieces retrieved from collected soil / *Odzyskiwanie dżdżownic i liczba znaczników VIE odnajdywanych w glebie*

Treatment *Działanie*		Percentage of total recovered earthworms, median value of earthworm number in parentheses *Procent odzyskanych dżdżownic, mediana liczby dżdżownic w nawiasie*		Total number of tags retrieved from soil *Liczba znaczników odnalezionych w glebie*	
		A. caliginosa	*L. rubellus*	*A. caliginosa*	*L. rubellus*
Trial *Próba 1*	UT1	100 (2)	71 (2)	2	3
	US1	79 (2)	0 (0)	2	6
	U01	8 (0)	8 (0)	1	0
	PS1	21 (0)	29 (0	1	5
	P01	8 (0)	0 (0)	0	1
Trial *Próba 2*	US2	70 (1)	0 (0)	0	0
	USM2	80 (2)	70 (2)	1	4
	PS2	40 (1)	20 (0)	0	2
	PSM2	70 (2)	60 (2)	4	7

treatments employed at the non-polluted Wilsons Farm (U) and polluted Carlton Street (P) sites in Trial 1 and Trial 2 (T = 0.3 m tube, S = 0.1 m tube, 0 = no containment, M = mesh cover) / *zabiegi stosowano w nie zanieczyszczonej Wilsons Farm (U) i w zanieczyszczonych miejscach Carlton Street (P) w próbie 1 i próbie 2 (T = 0,3 m rury, S = 0,1 m rury, 0 = nie ma miejsca, M = pokrywa siatki) /*

When earthworms were introduced without containment (U01 and P01), maximum recovery rates in both species was only 8%, providing strong evidence that the development of a practical and effective mark-release-recapture methodology requires some form of earthworm containment. However, results also suggested that containment may hinder "natural" behaviours (e.g. pollutant avoidance) and may lead to artificially high mortality and pollutant uptake levels in introduced earthworms at polluted sites. Only 15.4% of tagged earthworms (both species combined) were recovered from treatments at the polluted site (P), where results showed no significant difference between P01 and PS1 treatments (e.g. median recovery = 0 for *A. caliginosa*; p=0.830).

Results have suggested that recovery rates were influenced by behavioural differences in ecological groupings [Bouché 1977] and site-specific factors (e.g. level of metal pollution). The insertion of 0.1 m tubes flush with the soil surface provided effective containment for *A. caliginosa* in the non-polluted site (79% and 70% recovery in US1 and US2 respectively). However, the 0.1 m tube was shown to be ineffective in containing *L. rubellus* at Wilson's Farm with 0% recovery of this species in US1 and US2 treatments. *A. caliginosa* is a predominantly soil dwelling species while *L. rubellus* is often located in/below surface organic matter (epigeic) and therefore the movement of this species is not constrained by the tube inserted flush to the soil surface.

In Trial 2 there was a major difference in recovery rates for tagged *L. rubellus* in the unpolluted site, with 70% of earthworms recovered when tubes were covered with mesh (USM2) and no earthworms recovered from the mesh-free treatment (US2). This result suggests that issues associated with over–surface movement of *L. rubellus* from 0.1 m tubes at the unpolluted site was effectively addressed by employment of a plastic mesh. However, there was no significant difference between the two treatments in recovery rates for tagged *L. rubellus* in the polluted site (p=0.350) suggesting that site-specific factors (e.g. pollution level) influenced earthworm recovery rates. The influence of pollution also reduced recovery rates for *A. caliginosa* from tubes without mesh, at the polluted site (21% in PS1 and 40% in PS2) compared with the unpolluted site (79% in US1 and 70% in US2). It is suggested that recorded differences in recovery rates are related to increased avoidance behaviour influenced by metal pollution at the Carlton Street site. In the tube and mesh (SM) treatments, the influence of site-specific factors on earthworm recovery was less clear because the mesh restricted potential over-surface dispersal. Although surface migration of introduced earthworms needs to be controlled and avoidance of the experimental area cannot yet be precisely measured, further work could be undertaken to assess avoidance, as already employed under controlled laboratory conditions [Hund-Rinke et al. 2003, Lowe et al. 2016].

The containment method using 0.1 m tubes and mesh was shown to be adequate for the selected epigeic and endogeic species, but use of deeper burrowing anecic earthworms may require use of tubes inserted at greater depth. More robust material (e.g. steel tubes) and mechanical insertion / extraction tools (e.g. hydraulic excavators, used for the extraction of the Terrestrial Model Ecosystem) [Knacker et al. 2004] may be required to achieve the depths of insertion (estimated at 0.5 - 0.75 m) to effectively contain anecic species.

The current study confirmed the viability of using VIE tags to identify and aid recovery of introduced earthworms in field experiments. Studies have shown that VIE tags can be used to identify earthworms at least 6 months after tagging in the laboratory [Butt and Lowe 2007] and after 5 to 12 months in the field [Butt et al. 2015, Grigoropoulou and Butt 2010], suggesting that longer field-based experiments could be conducted with relatively simple containment devices. A longer experimental period would also allow measurement of the influence of seasonal variations on earthworm survival and behaviour and for assessment of other sub-lethal endpoints such as reproduction.

In Trial 1 there was no significant difference between U01, US1 and UT1 treatments for the number of recovered pieces of VIE tag from the soil (see Table 2) corresponding to *A. caliginosa* (p=0.924) or *L. rubellus* (p= 0.194). There was also no significant difference between P01 and PC1 treatments for recovery of tags corresponding to *L. rubellus* (p= 0.6682). For *A. caliginosa* only one piece of tag was recovered from both P01 and PC1 treatments. However, measurement of earthworm mortality based on tag recovery was affected by the formation of multiple tags, also observed by Butt et al. [2009], and tag loss which artificially increasing tag recovery results and prevented accurate differentiation between mortality and dispersal rates.

IV. CONCLUSION

This study has investigated a number of methodological aspects in the expanding field of biomonitoring. It has suggested that effective mark-release-recapture of earthworms (*A. caliginosa* and *L. rubellus)* can be achieved using simple containment (with relatively shallow PVC tubes and a mesh covering) and identification (with VIE tags) in both polluted and unpolluted conditions. However, methodological improvements in tagging (to avoid formation of multiple tags), containment (to accommodate anecic species) and measurement of dispersal are still required to allow assessment of other sub-lethal endpoints such as reproduction and avoidance, often used in laboratory-based ecotoxicology experiments.

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BADANIA TECHNIKI ZE ZNAKOWANIEM DŻDŻOWNIC, WPROWADZANIEM DO GLEBY I ODNAJDYWANIEM OSOBNIKÓW DLA BIOMONITORINGU

Streszczenie

Biomonitoring systemów glebowych stwarza trudności w odnajdywaniu i odróżnianiu organizmów testowych od miejscowych populacji. Celem badań było opracowanie prostej metody zatrzymywania dżdżownic in situ oraz ich skutecznego odnajdywania. Oznaczone implantem VIE dżdżownice: epigeiczny Lumbricus rubellus i endogeiczny Aporrectodea caliginosa wprowadzono do nie zanieczyszczonego pastwiska lub terenu zanieczyszczonego. Stosowano ograniczenia z rury PVC o różnej wysokości i głębokości wprowadzenia w glebę, a także zamknięte lub nie. Najwyższe wskaźniki odzysku dla obu gatunków pochodzą z zastosowania rur 0,3 m (100% w przypadku A. caliginosa i 71% w przypadku L. rubellus). Zastosowanie pokrywy siatkowej (próba 2) nie poprawiło odzysku A. caliginosa, ale zwiększyło odzysk L. rubellus z 0% do 70% w nieskażonej glebie. Badanie potwierdziło możliwość polowego użycia znaczników VIE w celu identyfikacji i odzyskiwania dżdżownic. Wyniki sugerują, że na współczynniki odzyskiwania wpływ miały różnice zachowań w grupach ekologicznych i czynniki specyficzne dla danego obszaru. Stwierdzono konieczność dalszych badań nad poprawą procedury znakowania, oceny śmiertelności i możliwości zatrzymywania dżdżownic wprowadzanych do gleby.

Słowa kluczowe: izolacja, dżdżownice, ekotoksykologia, eksperyment in situ, znakowanie VIE