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Earthworm cast production as a new behavioural biomarker for toxicity testing

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Cast production of Lumbricus terrestris is affected by pesticides under laboratory conditions.

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ABSTRACT

There is currently a lack of ecotoxicity tests adapted to earthworm species of higher ecological relevance and whose endpoints could be directly related to their ecological role in the soil. We propose a new and relatively simple ecotoxicity test based on the estimation of cast production (CP) by *Lumbricus terrestris* under laboratory conditions. CP was found to be linearly correlated to earthworm biomass and to be greatly influenced by soil water content. Azinphos-methyl had no effect on CP at all the concentrations tested. Significant decreases were observed at the normal application rate for other pesticides with (imidacloprid, carbaryl, methomyl) or without (ethyl-parathion and chlorpyrifos-ethyl) a clear concentration–effect response. For the highest concentration tested, reduction in CP varied between 35 and 67%. CP is straightforward and rapidly measured and ecologically meaningful. We thus believe it to be of great use as an endpoint in ecotoxicity testing.

1. Introduction

Earthworms, as ecosystem engineers (Jones et al., 1994) have a great influence on many physical (transfer properties), chemical (biogeochemical cycles) and biological (interactions with other components of the soil ecosystem) processes that occur in the soil (McCoy et al., 1994; Görres et al., 2001). They are therefore important terrestrial model organisms requiring toxicity testing. To date, a number of normalized tests using *Eisenia fetida* and focusing on mortality, reproduction and behaviour (avoidance) are available. Changes in behaviour are promising targets for ecotoxicological studies because the results can be linked to effects at the ecosystem level (Little, 1990; Doving, 1991; Scherrer, 1992). In the case of earthworms, changes in behaviour such as modified or reduced burrowing activity are crucial factors because these could have drastic effects on soil functioning (Capowiez et al., 2006). Although significant modifications to earthworm burrow systems due to the presence of pollutants have been sometimes observed (Eijsackers et al., 2001; Capowiez et al., 2003), it is difficult to study earthworm behaviour because these animals are concealed by the soil in which they live. Previous attempts to study the effects of pollutants on earthworm burrowing behaviour were based on the use of X-ray tomography (Capowiez et al., 2006) or 2D terraria (Capowiez and Bérard, 2006) which are expensive and have very specific methods resulting in a limited number of observations. Furthermore both methods require either image analysis or complex mathematical analysis to translate the observations into measurements (endpoints). Overall, even if of scientific value, these techniques may prove difficult to justify.

Earthworms burrow in the soil either by ingesting soil particles or by pushing them aside (Lee and Foster, 1991). In addition, soil ingestion is necessary for alimentary reasons. After gut transfer, the soil is egested in a specific feature: the cast. It is deposited either on the soil surface or in the soil itself (Whalen et al., 2004). Cast production (CP) therefore contributes to soil bioturbation, i.e. the disruption and mixing of soils or sediments by organisms that live and/or feed in them and/or simply pass through them. Casts per se play an important ecological role in the soil (Lee and Foster, 1991; Blanchart, 1992; Le Bayon and Binet, 1999) and, equally, they can also be used as a proxy for earthworm activity. This latter approach was recently adopted by Loranger-Mercidis et al. (2008) to study potential interactions between earthworms and woodlice. Subsurface and/or surface cast production was examined in a great number of studies under controlled or natural conditions. Casting was shown to be influenced by biotic factors, such as the earthworm species under consideration (Scheu, 1987; Hindell et al., 1994), species association (Scullion and Ramshaw, 1988), but also

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by abiotic conditions, such as soil bulk density (Le Bayon and Binet, 1999), organic matter type and quantity (Shipitalo et al., 1988; Flegel et al., 1998; Buck et al., 1999), temperature, or water potential (Scheu, 1987; Hindell et al., 1994; Daniel et al., 1996). Overall, the results are difficult to compare directly since the methodologies used to sample earthworm casts were often very different (hand collecting, wet or dry sieving) and the results have been expressed in a variety of ways (number, area covered or weight of casts related to earthworm fresh or dry weight).

There is some limited evidence that pesticides can affect CP. It is well known that the application of some pesticides to golf courses (turf grasses) results in lower surface CP (Baker et al., 1998; Lal et al., 2001). However, we do not know in these cases if earthworms were casting more below ground (to avoid pesticides at the soil surface) or if earthworm abundance simply decreased. In studies of aquatic systems, measurements of the egestion rate of sediments by benthic invertebrates such as oligochaetes (*Lumbriculus variegatus*) or bivalves (*Hydrobia ulvae*) have been successfully used in sediment toxicity testing (Leppänen and Kukkonen, 1998; Shipp and Grant, 2006; Penttinen et al., 2008). To our knowledge, the effect of pollutants on earthworm CP has not been studied to date for ecotoxicology purposes. To set the foundations for using CP measurements in ecotoxicity testing, we initially conducted a series of experiments to investigate the influence of earthworm weight and soil moisture content on CP. We then studied the effectiveness of using CP as a biomarker for exposure of *Lumbricus terrestris* earthworms to 6 different pesticides. The aim of the study is to propose a new ecotoxicity test which produces reproducible and relevant results, is relatively easy to conduct and could be considered as a possible candidate for a standardized test in soil risk assessment protocols.

2. Materials and methods

2.1. Soil, earthworms and the sieving protocol

Soil (23.4% clay, 57% silt, 19.6% sand, 28.3 g kg^{-1} organic matter, pH = 8.3, CEC = 8.2 cmol kg^{-1}) was collected from an apple orchard abandoned in 1995 and located in Montfavet near Avignon, in south-eastern France. The water holding capacity (WHC) of the soil was 0.247 g g^{-1}. Total heavy metal concentrations were measured in the soil (Cu = 30.0, Zn = 76.8, Pb = 30.0 and Cd = 0.290 mg kg^{-1}). Adult and subadult earthworms (*L. terrestris*) were purchased from a local supplier (fisheries store). They were raised in Canadian farms and are therefore available all year round. The worms were acclimated in the orchard soil for 4 days prior to experiments.

In all experiments, the soil was primarily sieved at 3 mm. Soil moisture was measured and adjusted to the desired value by adding distilled water, mixing the soil and then setting it apart for 2 days in a dark chamber at 12 °C to reach equilibrium. Before each experiment, the soil was re-sieved and the moisture content was verified. At the beginning of each experiment, earthworms were washed in tap water, blotted dried on filter paper, weighed (without gut voiding) and individually placed for 7 days in 100 g moist soil in crystal transparent polystyrene round boxes (diameter = 10 cm; height = 3 cm; purchased at Caubère, Yebles, France) and called hereafter Petri dishes. Controls were set up in which no earthworms were added to the soil in the Petri dishes. Earthworms were weighed again (without gut voiding) at the end of the experiment.

In many previous studies (Scheu, 1987; Shipitalo et al., 1988; Buck et al., 1999), casts were collected manually after visual assessment (according to shape and size). For the sake of reproducibility and accuracy, in this study we separated out casts using a set of 4 sieves (diameter = 15 cm and mesh sizes = 5.6, 4, 3.15, 2.5 mm) since earthworm activity may modify soil granulometry leading to an increase in the amount of soil retained in some sieves (casting) and a decrease in others (soil consumption). All soil from each Petri dish, including the soil that adhered to the walls of the dishes which was removed with a knife, was sieved taking care not to break up the casts. The set of sieves was manually shaken for 10 s. The soil retained in each sieve was weighed. The effect of earthworm bioturbation was then examined by determining the changes in the particle size distribution (PSD), i.e. weight of fresh soil in each sieve minus the corresponding weight of soil for the control soil (without earthworm bioturbation).

2.2. Optimisation of the sieving protocol

To optimise the protocol, we independently assessed CP dependence on (i) earthworm weight, (ii) soil water content and (iii) drying the soil at the end of the experiment. Most authors (Scheu, 1987; Shipitalo et al., 1988; Curry and Baker, 1998) expressed CP on a weight basis (i.e. in g of soil per g of earthworm body mass) so we investigated the relationship between earthworm weight and CP using 60 Petri dishes. Adults or subadults of *L. terrestris* with a large range of body masses (from 1.13 to 6.38 g with a mean of 3.69 and a standard deviation of 1.26 g) were placed in fifty of them. Ten Petri dishes without earthworms were set up as a control.

Earthworm activity (burrowing and casting) is influenced by water potential and hence soil water content (Kretzschmar, 1991; Daniel et al., 1996). However, soils with high water contents are difficult to sieve because wet soil aggregates tend to combine. We therefore studied the relationship between CP and water content using 100 Petri dishes and 5 different soil water contents (15, 18, 21, 24 and 27% expressed on a weight/weight basis) ranging from 60 to 110% of the WHC. For each soil water content, 20 Petri dishes were fill with moist soil, 10 without earthworms (control) and 10 containing one *L. terrestris*. Because earthworm weights were variable and ranged from 2.47 to 5.62 g, a blocking procedure was used so that mean earthworm weight was similar in each treatment (McIndoe et al., 1998). This blocking procedure was used for the following experiments.

We hypothesised that waiting for the soil to dry at the end of the experiment may facilitate soil sieving. This in turn could influence the results of CP as a possible ecotoxicity test. To investigate this idea, a test was carried out using 160 Petri dishes and a carbamate insecticide (Lannate©, DuPont) the active ingredient of which is methomyl. Moist soil was prepared at initially 19.2% water content. Soil was contaminated by manually spraying each kg of soil with 40 ml of a solution containing increasing concentrations of methomyl. Control soil was sprayed with the same quantity of water. The spiking procedure, which generated good levels of pollution homogeneity, was described in detail and tested by Capowiez et al. (2005). The methomyl concentrations were chosen based on the usual application rate and calculation of the PEC (Predicted Environmental Concentration i.e. a single application of 750 g a.i. ha^{-1}, with a homogeneous distribution in the first 5 cm of soil, no crop interception and a soil density of 1.5 kg l^{-1}). This normal application rate (2.025 mg a.i. kg^{-1} of wet soil) is termed '1×' and we then used the following concentrations: '10×', '0.1×' accordingly. For each pesticide concentration, 40 Petri dishes were filled with moist soil and one *L. terrestris* (range weight was 1.69–5.97) was added in half of them. The final soil water content was 24%. After 7 days of exposure at 12 °C in a dark chamber, earthworms were weighed. The soil in half of the Petri dishes was sieved immediately. The other half was sieved after the Petri dishes were dried for 4 days at laboratory temperature (about 25 °C). The soil water content of 3 Petri dishes was measured in each treatment. Because casts were dried, this time CP was expressed in % of weight of the available soil g^{-1} fresh body mass day^{-1} to enable direct comparisons between fresh and dried soil (each worm was in 100 g of moist soil).

2.3. Effect of 6 pesticides on cast production

The sensitivity of CP as a behavioural biomarker was tested using 6 pesticides (Table 1). Four of these were long-standing and broad-spectrum insecticides belonging to the carbamate and organophosphorous families, which are currently still used in apple orchards in Provence (South-East of France) due to the increasing resistance of codling moths to other pesticides (Sauphanor et al., 2000). The fifth (ethyl-parathion) was banned by the EEC in 2001 but was used in the present study as a model pesticide so that comparisons with previously published data (Olvera-Velona et al., 2008) were possible. The last (imidacloprid) is a relatively new insecticide belonging to the family of neonicotinoids, which is currently used in peach orchards against aphids. This pesticide was previously shown to cause behavioural effects on earthworms even at low concentrations close to the PEC (Capowiez and Bérard, 2006). It was then used in the present study at lower concentrations than these other pesticides (Table 1). Four of these pesticides were classified as very toxic or extremely toxic to earthworms by Edwards and Bohlen (1996) whereas they found insufficient evidence to categorize azinphos-methyl. Imidacloprid was initially categorized as moderately toxic to earthworms by Elbert et al. (1990). The soil spiking procedure was as explained previously except that only 20 Petri dishes were filled with most soil for each pesticide concentration, 10 contained an earthworm and 10 did not. The final soil water content was 24%. The adults or subadults of *L. terrestris* used had an average weight of 4.01 g (the range of weight was 1.57–6.09 g). After 7 days of exposure in a dark chamber at 12 °C, the earthworms were re-weighed and the soil was sieved immediately by the same operator (wearing a mask when handling polluted).

2.4. Statistical analyses

Data were tested for normal distribution and homogeneity of variance and then were log-transformed before the ANOVA or regression analysis as necessary. The relationships between CP (in g of fresh soil day^{-1}) and earthworm fresh weight were assessed using linear regression. To study the effects of the soil water content on CP (in g g^{-1} day^{-1}) we performed a one-way ANOVA. The effect of drying the soil at the end of the experiment was assessed with a two-way ANOVA with soil drying and pesticide dose as factors. To study the effects of the 6 pesticides on CP or weight loss,