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Assessment of Lead Nitrate and Mancozeb Toxicity in Earthworms Using the Avoidance Response

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The presence of chemoreceptors in the prostomium of earthworms and the distribution of sensory tubercles on the body render them highly sensitive to chemicals in the environment. This sensitivity, coupled with their locomotory abilities, enable them to avoid environments that are chemically contaminated, provided that they can sense the specific chemicals. The question arises whether this sensitivity could be utilized as a tool to study environmental contamination.

Yeardley et al (1996) indicated that avoidance behavior can, in most cases, be a more sensitive and less time-consuming indicator of chemical contamination than acute and sublethal tests. The objective of a behavioral assay would therefore be to determine whether the animal can detect the toxicant and change it's behavior accordingly.

Pesticides and other chemicals introduced into the soil may alter the behavior of earthworms, modifying or minimizing their actual exposure (Tomlin 1992). Environmental risk is normally considered a function of probability of exposure and susceptibility to the toxicant. However, avoidance of exposure and possible emigration could lead to local disappearance of earthworms, which may have serious consequences for ecosystem functioning, since earthworms play an important role as soil engineers.

Keogh and Whitehead (1975) reported a behavioral response of earthworms to contamination by benomyl. They found that earthworm numbers were reduced as a result of increased emigration from contaminated areas, causing a localized reduction in surface castings and an increase in leaf litter accumulation. A more rapid sublethal earthworm-avoidance test as suggested by Stephenson et al (1997) has the potential to generate results able to predict the chronic responses to contaminants in the soil as well as population changes.

According to Mather and Christensen (1997) the consequence of pesticide application on the migratory behavior of earthworms has so far been overlooked in ecotoxicological studies. They found that migratory behavior resulting from detection of a toxicant can lead to alterations in population density, biomass and

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species diversity, thus affecting the structure and functioning of earthworm communities. These authors concluded that surface-migration by earthworms in the field appears to be a sensitive behavioral parameter for assessing side effects of chemicals. It can also provide a parameter with a high degree of ecological relevance. Edwards and Coulson (1992) also suggested that rapid and sensitive bioindication of adverse environmental quality could be provided by studying behavioral responses.

Soil in urban and agricultural areas represent a major sink for contaminants which may effect soil organisms (Van Gestel and Van Brummelen 1996). The ability to detect and avoid a toxicant may be beneficial for the survival of the individual but the reaction of a large number of individuals (by migration) could impact negatively on population density and community structure. If earthworms are, on the other hand, not able to avoid sublethal concentrations of heavy metals and metal-containing fungicides in the soil the chances of exposure to and bioaccumulation of these contaminants in body tissue are increased. The ability to detect a toxicant may also not be related to the organism's susceptibility to it.

The objective of this study was to test the hypothesis that avoidance response of earthworms can serve as a preceding range finder of toxicity. This was done by determining whether they could avoid a sublethal concentration of lead nitrate well above the expected environmental concentration as well as a range of concentrations of a metal containing fungicide, Mancozeb (zinc-manganese ethylene bis(dithiocarbamate)). Previous studies on the effects and accumulation of lead (Pb) (Reinecke et al 1997; Reinecke et al 2000) have shown it to occur fairly commonly in urban soils in South Africa where Pb containing fuel is still used extensively. It also tends to accumulate in earthworms (Reinecke et al 2000). The fungicide Mancozeb is used extensively in certain regions of the Western Cape in South Africa by deciduous fruit farmers. It is considered to have a relatively low toxicity for earthworm species such as *Eisenia fetida* (Roark and Dale 1979; Vermeulen et al 2001) although Cluzeau et al (1992) reported detrimental effects on *Lumbricus terrestris* under field conditions.

MATERIALS AND METHODS

Behavioral tests were conducted following the methods of Gunn and Sadd (1994). Plastic containers, (8 x 12 x 20cm) were divided into two sections by drawing a line on the outside and labeling the sections, A and B. For each species tested six containers were used as double controls and six for exposure to lead nitrate.

In a first series of tests sun dried cattle manure was used as substrate and was ground and sieved to a particle size of 500 - 1000 μm. The pH varied between 6.9 and 7.2. Lead nitrate at a nominal concentration of 2000 mg/kg was mixed directly into the substrate by dissolving the lead nitrate in the distilled water with which the dry substrate was wetted to obtain a moisture content of 76%. To ensure consistency the substrate was prepared in a large batch for all experiments and placed into the containers which was housed in an environmental chamber at a temperature of ≈25°C for 24 hours, to allow for equalibration and stabilization before the worms were added.

The control containers were filled with 200 g of uncontaminated substrate on both sides A and B. The exposure containers were filled with 200 g of uncontaminated substrate on side A and 200 g of contaminated substrate on side B, using a temporary plastic divider which was removed before the worms were added. A line of washed white sand was spread on the surface of the substrate to mark the division between sides A and B.

Ten clitellate worms of each of the species *Eisenia fetida*, *Eudrilus eugeniae* and *Perionyx excavatus* were tested seperately by placing them on the surface in each of the containers, five on each side, and allowed to penetrate the substrate. The containers were placed in an environmental chamber at 25 C and after four days a partition was placed slowly between the two sides of the containers and the number of worms in both sides were recorded for all the containers by handsorting. Six replicates were therefore done for each experiment as well as for the double control.

The moisture content in the substrates varied between 73 and 76% and the pH between 6.4 and 7.2 for all the containers between the start and the completion of the observations.

After four days substrate samples were collected from each experiment for atomic absorption spectrophotometric analysis to determine the lead content. The substrate samples from the contaminated as well as the control substrates were digested as described by Katz and Jennis (1983). One gram of substrate was dried for ≈ 48 hours at 106°C in a drying oven. These dried samples were ground and sieved to a particle size of 500 - 1000 μm to assist the digestion process. Samples were individually put in test tubes and 10 ml of nitric acid (HNO_3) was added. These mixtures were heated to a temperature of 40 - 60 °C for two hours and then 120 - 130°C for one hour, after which they were left to cool. Then 5 ml of perchloric acid ($HClO_4$) was added and this mixture was reheated to 120 - 130°C for one hour until brown fumes were emitted. The mixtures were allowed to cool again, before 5 ml of distilled water was added. They were then reheated to 120 - 130°C, until white fumes were emitted. For every batch of substrate that was digested a blank was prepared to detect and eliminate possible contamination during the digestion process.

After digestion the samples were centrifuged for five minutes at 3000 rpm. The solutions were decanted and the supernatant filtered into 20 ml volumetric flasks, using Whatman no 6 filter papers and glass funnels. Distilled water was used to make the filtrate up to a volume of 20 ml. These 20 ml solutions were microfiltered through 0.45 μm Whatman filter paper, into plastic containers, using filter holders and plastic syringes.

The Pb content of the samples was determined by using a Varian AA-1275 Atomic Absorption Spectrophotometer. Freshly made up 2-, 5-, 10- and 50 mg/kg standards of $Pb(NO_3)_2$ were used to calibrate the spectrophotometer. Concentrations of Pb are expressed on a wet weight basis. Spiked samples that were analyzed showed a recovery rate above 75%.

The data were statistically analyzed by using the SigmaStat® computer software package and all the values are presented as the mean ± SD (standard deviation). Two-tailed analysis of variance was used to compare results of the control and exposures in the avoidance-behavior tests.

In a second series of tests ground manure was treated in a similar way as with the first series and used in similar test containers to determine the responses of *Eisenia fetida* to the fungicide Mancozeb (zinc-manganese ethylene bis (dithiocarbamate)). The substrate on one side received nominal concentrations of 8, 44, 800 or 2000 mg/kg Mancozeb respectively, thoroughly mixed into the substrate. The other side of the container received untreated substrate. Ten worms (five on each side) or twenty worms (ten on each side) were placed on the surface. The number of worms in each side was counted after four days. Forty worms (two or four replicates) for each concentration were used. The substrate was not chemically analyzed for Mancozeb. The results were analyzed statistically as stated previously for Pb exposures.

RESULTS AND DISCUSSION

For *E. fetida* no significant differences ($p>0.05$) were found in the distribution of the worms between the two untreated sides of the control test (Figure 1A). The number of worms in the sides treated with lead nitrate however differed significantly ($p<0.05$) from those in the untreated side with significantly more worms aggregating in the contaminated side.

For *E. eugeniae* no statistically significant difference was found in the distribution of the earthworms between the two untreated sides as well as between the side treated with lead nitrate and the untreated side (Figure 1B). This occurred in spite of the fact that the analyzed concentration of Pb in the exposure tests was 48 times higher (1010 mg/kg vs 20.8 mg/kg) in the treated side than in the untreated side.

Also for *P. excavatus* there was no statistically significant ($p>0.05$) difference in the distribution of the earthworms between the two untreated control sides and between the untreated and lead nitrate treated sides of the exposure tests (Figure 1C). In this case the analyzed concentration of Pb in the exposure test was 83 times higher (1183 mg/kg vs 14.2 mg/kg) in the treated side than the untreated side.

E. fetida exhibited a consistent preference for the substrate that was contaminated with lead nitrate. The analysis of the substrate also showed that the worm activity

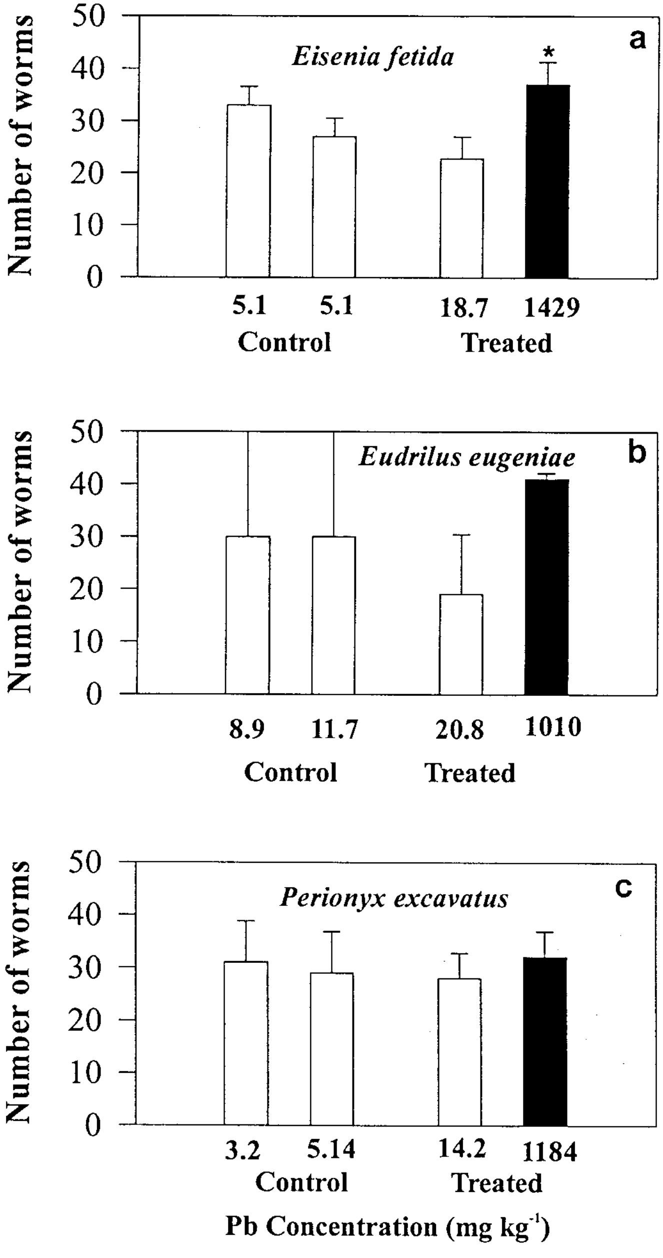

Figure 1. Spatial disribution of (a*) E. fetida* (b) *E. eugeniae* (c) *P. excavatus* in a double control and when given a choice between untreated and substrates treated with lead nitrate. (* significantly different, $p<0.05$).

influenced the distribution of lead slightly between the uncontaminated and contaminated sides of the substrate over the period of four days.

The fact that the worms were predominantly found in the contaminated sections of the substrates leads to the conclusion that they selected in favor of the substrate treated with lead nitrate at this sublethal level in spite of the fact that ample food was also available in the uncontaminated section. The possible nutrient enrichment of the contaminated substrate with nitrate anions may explain this phenomenon. The positive response exhibited by *E. fetida* correlates with the stimulating effects of low concentrations of lead nitrate found by Reinecke et al. (1997) for the same species. They found increased growth and cocoon production, indicating a higher food consumption rate, when the worms were exposed to low concentrations of lead nitrate.

E. eugeniae and *P. excavatus* showed no statistically significant response to the presence of lead nitrate. They did therefore not avoid the Pb in the substrate. This has important implications for the effects of Pb on these species. If earthworms cannot sense and avoid Pb in their environment they would continue feeding on the contaminated substrate. The Pb may as a consequence accumulate in the earthworm's body tissues with possible delayed physiological and toxicological implications. Their inability to avoid Pb when presented as the nitrate salt may make them a hazardous vehicle of Pb contamination for more sensitive predatory species higher up in the food chain as was indicated by Reinecke et al (2000).

E. fetida showed no statistically significant difference in its distribution between the two sides of the untreated controls (Figure 2). There were, however, significant differences ($p<0.05$) in the distribution of the worms between the treated and untreated sides of all the different treatments for Mancozeb. Most worms tended to avoid all the treated sides from the lowest (8 mg/kg) to the highest (2000 mg/kg) concentrations. All the worms avoided the substrate treated with 2000 mg/kg Mancozeb (not shown in Figure 2)

In contrast to its response in the Pb contaminated substrate, *E. fetida* was able to detect and avoid the fungicide Mancozeb, even at concentrations much lower than what earthworms will encounter in the field. This indicated a high sensitivity for this fungicide. Roark & Dale (1979) and Vermeulen et al (2001) however, showed that Mancozeb had little or no toxic effects on *E. fetida* when applied at recommended dosages. Our study provides evidence that behavioral changes could be expected to result from Mancozeb application which in turn could effect exposure and migratory patterns. Viewed against the background of the findings of Vermeulen et al (2001) that Mancozeb is of low toxicity to *E. fetida*, avoidance response will in this instance be a poor indicator of toxicity and cannot serve as a preceding range finder for acute or sub-acute lethal toxicity tests for Mancozeb. Species differences preclude extrapolating directly from a detritus feeding, epigeic species such as *E. fetida* to other endogeic or anecic earthworm species commonly

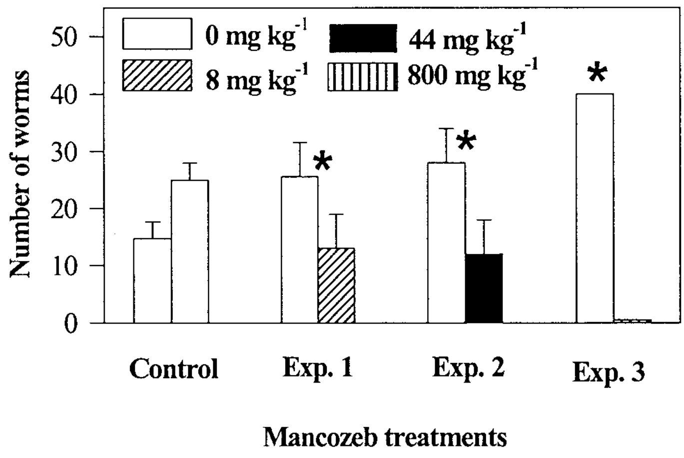

Figure 2. Spatial distribution of *E. fetida* in a double control and when given a choice between untreated and substrates treated with different concentrations of Mancozeb. (significantly different, $p<0.05$).

occurring in vineyards and orchards where this fungicide is normally used. Cluzeau et al (1992) found a reduction in mass gain, clitellum development and hatching success in *Lumbricus terrestris* when Mancozeb was applied at a rate of 2400 g ai per hectare. They concluded that disturbances in population dynamics induced by the repeated application of fungicides classed as of low or moderate toxicity (such as Mancozeb) must be taken into account when assessing effects in cultivated soils. The long term effects of Mancozeb on other species may still be more severe than what can be anticipated from acute toxicity data or behavioral reponses of *E. fetida* .

The ecotoxicological relevance of behavioral responses in earthworms requires further investigation. Species differences as well as variation in responses to different chemicals must be taken into account when considering the use of avoidance response as a tool for studying environmental contamination. Changes in migratory behavior may also reflect a change in habitat suitability caused by the toxicant. Changes in food quality and availability as well as effects on associated microorganisms resulting from the chemical exposure, could also induce behavioral responses and not the chemical *per se.*

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