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Assessment of the Effect of Varying Soil Organic Matter Content on the Bioavailability of Malathion to the Common Nightcrawler, *Lumbricus terrestris* L.

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Abstract This study investigated the effect of soil organic matter content on the bioavailability of malathion to the common nightcrawler, *Lumbricus terrestris*. Earthworms were exposed for 72 h to malathion on two soil types, 8% organic matter and 55% organic matter. Two different measures of bioavailability, malathion body burdens and tissue cholinesterase activities, were then measured in the malathion exposed animals. There were no significant differences in body burden or cholinesterase levels in *L. terrestris* exposed to malathion on soils with differing organic matter content. This suggests that absorption into organic matter is not a limiting factor of malathion bioavailability to earthworm species.

Keywords Earthworm · Malathion · Bioavailability · Soil organic matter

This study investigates the effect of soil organic matter content on the bioavailability of malathion to the earthworm, *Lumbricus terrestris*. Earthworms are often studied in toxicity assays because they represent an important subclass of invertebrates potentially susceptible to terrestrially applied pesticides (Edwards and Bohlen 1992). Soil organic matter is of interest because absorption into soil organic matter is an important factor in the bioavailability of many xenobiotics (Wauchope et al. 2002), including organophosphates, and *Lumbricus* are exposed to xenobiotics in soils with wide ranging organic matter content (Haines and Uren 1990). *L. terrestris* is an anecic earthworm that travels vertically through large amounts of soil to feed on debris at the soil surface (Hendrix 1995) and is exposed to bioavailable xenobiotics by cutaneous absorption and incidental ingestion (Kukkonen and Landrum 1996).

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Malathion, a commonly applied organophosphate, has been demonstrated to cause acute toxicity in earthworms as evident by LC_{50} values within the range of environmental application (Roberts and Dorough 1984; Panda and Sahu 2002) and cholinesterase suppression after malathion exposure (Booth et al. 2001). However, there appears to be a relatively wide range of sensitivity to organophosphate exposure in earthworms, even within a single genus (Stenersen et al. 1992), therefore, it cannot be assumed that *L. terrestris* will exhibit the same degree of toxicity as previously reported in other species.

The bioavailability of many xenobiotics appears to be dictated by their capacity to adsorb to soil components, absorb into soil organic matter, and the degradation half-life of the xenobiotic of interest (Wauchope et al. 2002). Malathion, with a log $K_{oc} = 3.2$ (Zambonin et al. 2002) is moderately adsorbed to soil and as a phophorothionothiolate organophosphate, soil organic matter content should be the most accurate predictor of its adsorption/absorption capacity (Sanchez-Martin and Sanchez-Camazano 1991). However, the degree of malathion's absorption into the organic matter compartment of soils is unresolved. Some studies demonstrate increased malathion absorption into soil with rising soil organic matter content (Zambonin et al. 2002) and other studies show no evidence of absorption to organic matter even in soils with high organic matter content (Rajukkannu et al. 1985). This study assesses malathion bioavailability in soils of differing organic matter content by measuring the malathion body burdens and cholinesterase suppression in experimentally exposed *L. terrestris*.

Materials and Methods

A commercial soil, Scott's® garden soil with 55% organic matter, was used for all experiments. Preparation of a reduced organic matter soil was accomplished by adding inert silica sand to the garden soil in a 1:1 (v:v) ratio, manually mixed until the soil appeared homogeneous. Subsamples of both test soils were analyzed for organic matter content by recording weight loss on ignition, and for particle size by screening, at North Carolina State's Department of Soil Science (Table 1). The pH of both soils was 6.8; determined with a soil pH meter (Kel instrumentation corporation, Wyckoff, NJ).

Lumbricus were procured from National Association of Supplies Bait and Tackle, Marblehead Ohio. The worms were maintained at 10°C in a polyethylene container, 32 cm by 13.5 cm, filled with 900 mL of Scott's® garden soil. Percent soil moisture saturation was measured at onset with a soil meter, Kel Instrumentation Corporation (Wyckoff, NJ) and deionized water was added to achieve 60% saturation. Prior to malathion exposures, a pilot study was run to establish that worms would survive for the 3-day exposure period in the amended low organic matter soil. Five worms were placed into the 8% organic matter soil and observed. No mortalities or morphological abnormalities were noted.

Table 1 Soil characteristics of Scott's garden soil

Sample ID	% OM	% Sand	% Silt	% Clay
Scott's garden soil	54.09	65.09	28.39	6.52
Amended with sand	7.9	96.44	2.75	0.81

Mean values n = 3

The exposure container was a 1,000 mL glass beaker filled with 600 mL of soil. Malathion, 96.5% purity American Cyanamid, was diluted in HPLC grade acetone to produce a surface exposure of 50 μg/cm^2 and applied by fine mist with a thin layer chromatograph spray. Acetone alone was applied to the soil in control containers. The soil in each container was thoroughly mixed by hand for 3 min to reduce the chance of behavioral avoidance of the malathion by the earthworms, and the acetone evaporated overnight in a fume hood. Exposures were run in triplicate with three worms per replicate. Deionized water was added to each prepared container to obtain 60% water saturation of the soil prior to adding the worms. The containers were covered with cheesecloth to reduce water evaporation, placed into an incubator at 10°C, and maintained on a 12-h day/night cycle. Treatments were: (1) low and high organic matter control (no malathion), 8%, 55% organic matter; (2) low organic matter exposure, 50 μg/cm^2 of malathion in 8% organic matter soil; and (3) high organic matter exposure, 50 μg/cm^2 in 55% organic matter soil. Exposures lasted 72 h, after which, worms were euthanized in scalding water, 40°C (Raty and Hunta 2003), and prepared for analysis by manually stripping any soil from the gastrointestinal tract. Soil samples were collected from each container and analyzed for malathion content.

All worms were placed on ice and immediately analyzed for cholinesterase inhibition by a modification of Ellman's assay (Ellman et al. 1961). Electric eel acetylcholinesterase (.411 U/μL, Sigma–Aldrich, St. Louis, MO) was used to generate linear standards curves between 43 and 144 s. One hundred milligram of the post-clitellum worm section was homogenized for 10 s with an electric homogenizer in 1 mL of 8.0 Tris buffer (Trizma Pre-set Crystals pH 8.0, Sigma–Aldrich). The homogenate was centrifuged and the supernatant removed. Twenty microliter of the supernatant was used in the assay. Absorbance was read at 405 nm on a spectrophotometer, Spectramax 190, Molecular Devices (Sunnydale, CA). The micro-BCA assay, Pierce Chemical (Rockford, IL), was used to standardize all results to total

protein content. Results are reported in nmol of cholinesterase hydrolyzed per minute per milligram of total protein.

The remainder of the post-clitellum *Lumbricus* samples, not used in the cholinesterase assay, and the soil samples were frozen at −80°C for no longer than 2 weeks before they were thawed at room temperature and extracted for the malathion assay. The extraction procedure was identical for the soil and the worm samples. Approximately 1 g of worm or soil was homogenized with a Dounce homogenizer in 1:4 acetone:hexane (v:v) for 30 s. The homogenate was pelleted by centrifugation and the supernatant removed. The supernatant was further extracted by a Folch wash (Folch et al. 1957) to remove interfering lipids and then evaporated under nitrogen. After evaporation, the sample was reconstituted in 200 µL of hexane and then eluted from a glass pipette filled with 1 g of Florisil®, Sigma–Aldrich. Sample elution occurred in 1:20 acetone:hexane (v:v) followed with 1:4 acetone:hexane (v:v). The elutes were combined prior to a second Folch wash, to further reduce lipid interference, and then re-centrifuged. The final supernatant was evaporated to dryness on a rotary evaporator under nitrogen. Earthworm and soil extraction efficiencies calculated from spiked samples were between 85% and 103%.

Each extracted sample was reconstituted in 40 µL of hexane and 4 µL was injected into a Hewlett Packard 5890 series II gas chromatograph with a DB-1 column, 30 m × 0.35 mm × 0.25 µm from J&W Scientific (Folson, CA). The FID detector and injector temperatures were set to 250°C. The column temperature was ramped from 60 to 220°C at a rate of 40°C/min and then at a rate of 2°C/min from 220 to 228°C. The total run time was 8.9 min. Malathion eluted at 6.5 min and malaoxon eluted at 6.2 min. No malaoxon was detected in the samples. The limit of detection for malathion with this method was 0.04 and 0.10 µg for malaoxon as determined by standard curves using an external standards, 100 µg/mL in methanol diluted into appropriate concentrations, Restek Corporation (Belafonte, PA).

All statistical analyses were performed using Prism®, Graphpad (San Diego, CA). Acetylcholinesterase activity and malathion body burdens were compared between exposure groups using the Kruskal–Wallis test, to compare all treatment groups, or the Mann–Whitney test, to compare between any two treatment groups. Significance was set to $\alpha = 0.05$.

Results and Discussion

The control exposures were run on soils with 8% and 55% organic matter content. There was no significant difference in cholinesterase levels between the controls (Mann–Whitney test, p-value 0.700), or malathion burdens (Mann–Whitney test, p-value 1.00). The control samples were condensed as a single unit for the rest of the analysis.

Exposed worms demonstrated cholinesterase inhibition of approximately 50% compared to unexposed controls (Fig. 1). There was no statistically significant difference between the treatments, Kruskal–Wallis (Mann–Whitney, p-value = 0.08). Similarly when the two exposed groups, 8% OM and 55% OM, were compared there was no significant difference in cholinesterase activity (p-value of 1.00).

Malathion body burdens were detected in all exposed worms and none of the unexposed worms (Fig. 2). There was no statistically significant difference in malathion burdens between the two exposed groups, 8% OM (median 0.70 ppm), and 55% OM (median 0.78 ppm) (Mann–Whitney test, p-value 0.70). The soil malathion burden was averaged across replicates: control-0 ppm, 8% OM-2.05 ± 0.29 ppm, 55% OM-2.34 ± 0.75 ppm. This resulted in a bioconcentration factor, earthworm burden divided by soil burden, of 0.34 when exposed to malathion in 8% OM and 0.33 when exposed to malathion in 55% OM.

This study compared two soils with identical pH, soil moisture, and exposure temperature but differing organic matter content and soil particle size. Soil adsorption of xenobiotics, including malathion, can be influenced by a multitude of other factors including the specific type of organic matter (Khan and Khan 1986) and the ion binding capacity of the soil (Cahill et al. 2003). The addition of inert silica sand to obtain test soils with differing organic matter content but similar organic matter composition was chosen to specifically exclude any other factors which affect this phenomena. Bioavailability was equivalent as measured by body burdens and degree of cholinesterase suppression in our report.

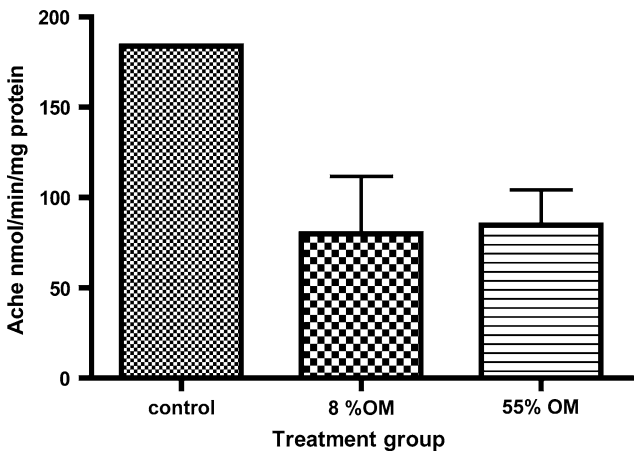

Fig. 1 Median levels of cholinesterase in *L. terrestris* when exposed in soils with differing organic matter content. Error bars represent the interquartile range

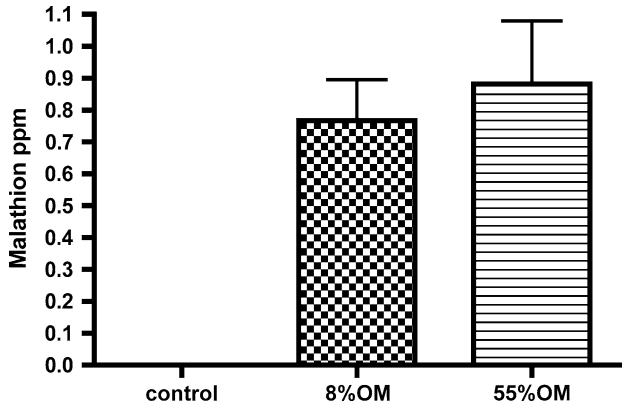

Fig. 2 Median xenobiotic burdens in *L. terrestris* when exposed in soils with differing organic matter content. Error bars represent the interquartile range

Though soil organic matter content did not appear to affect the bioavailability of malathion to *L. terrestris*, there were detectable levels of malathion in the earthworm, therefore, malathion was available for absorption. The bioconcentration factors calculated, 0.34 for 8% OM and 0.33 for 55% OM, do not suggest bioaccumulation of malathion in *L. terrestris.* The results suggest a plateau in earthworm body burden regardless of soil organic matter content. This in turn suggests that there may be a limitation of the amount of malathion that *L. terrestris* is capable of absorbing from the substrate regardless of bioavailability, possibly because of anatomical considerations.

There are two primary routes of xenobiotic absorption from soil into earthworms, percutaneous and oral (Lord et al. 1980). The percutaneous absorption of toxicants in most vertebrates is rate limited by transport through the epidermis (Rozman and Klaassen 2001). In earthworms, the epidermis is beneath a cuticle layer which is the first impediment to absorption (Jamieson 1981). The cuticle in *L. terrestris* composed of at least 24 layers of collagen (Coggeshall 1966), and is a substantial deterrent to chemical absorption. Malathion is moderately lipophilic, relatively small (M_w: 330.36 g/mol), and should passively diffuse through lipids (Rozman and Klaassen 2001) however, earthworm collagen is composed of 80% protein and 20% polysaccharides (Baccetti 1967). The paucity of lipids in the cuticle layer should substantially impede malathion transport through the cuticle to the epidermis limiting absorption.

The oral route of absorption of malathion faces fewer obstacles than the percutaneous route. *L. terrestris* gastrointestinal tract lacks the cuticular barrier present in the epidermis, and any incidentally consumed malathion in contaminated soil should be able to diffuse passively through epidermal lipids (Albro et al. 1992). However, this route of absorption is most likely limited in *L. terrestris* because as anecic earthworms, they do not regularly consume soil unless building new burrows. This would be expected to reduce their gastrointestinal exposure to terrestrially applied xenobiotics (Jager 1998).

Even if both percutaneous and oral absorption occur, the relatively low lipid content, 1.23% (Albro et al. 1992), of *Lumbricus* could further protect them from accumulations of malathion and resultant toxicity. The kinetics of absorption and elimination of many lipophilic xenobiotics are thought to be impacted by the lipid content of tissues, with slower absorption and faster elimination of lipophilic compounds occurring when tissue lipid content is low (Guthrie and Perry 1980).

This study demonstrates that soil organic matter content alone does not affect the bioavailability of malathion to *L. terrestris*. Anatomical and physiological concerns weigh heavily when considering the absorption of xenobiotics into earthworm species. This does not discredit the importance of understanding soil characteristics and their affect on chemical bioavailability to earthworms. Earthworm species are necessary for healthy fertile agricultural soils (Lavelle 1988), have been shown to contribute to the mineralization of organic contaminants in soils (Lydy and Linck 2003), and yet, these activities expose them to potentially harmful agricultural chemicals. Studying soil characteristics in isolation allows for a better understanding of the effects of soil adsorption on bioavailability on this important class of invertebrates.

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