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A Comparison of Two Exposure Systems to Apply Malathion to *Lumbricus terrestris* L

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Invertebrates are frequently used as biomarkers for the possible effect of xenobiotics applied to the environment (Weeks et al. 2004). Earthworms have been successfully used as sentinel organisms for nontarget toxicity of chemicals used in agricultural practices (Booth et al. 2001). Earthworms have also been used to assess the bioaccumulation potential of xenobiotics because they are preferred prey species of multiple classes of vertebrates (Stephenson et al. 1997). Their use in bioaccumulation studies makes it necessary to have practical and reliable methods for estimating body burdens in earthworm prey species.

The earthworm species most commonly used in toxicity testing include composting worms such as *Eisenia foetida* and the anecic worm, *Lumbricus terrestris* (Edwards and Bohlen 1992). Common tests used to study acute toxicity resulting from chemical exposure in earthworms include immersion tests, topical application, force-feeding, injection, filter paper contact, laboratory soil contact, and field soil tests (Edwards and Bohlen 1992; OECD 1984; Roberts and Dorough 1984). The laboratory soil exposure and the filter paper contact methods are considered particularly effective exposure regimes because of a perceived high degree of repeatability and increased likelihood of consistent results when compared with field testing (Edwards and Bohlen 1992). Xenobiotic exposure of earthworms by filter paper has been standardized by the Organization for Economic Cooperation and Development (OECD) (OECD 1984) and is often used as a screening tool for suspect chemicals (Edwards and Bohlen 1992). Although used extensively for toxicity assessments, the environmental relevance of the filter paper exposure method has been

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questioned because soil based factors (bioavailability; species/substrate interactions) cannot be assessed (Edwards and Bohlen 1992). Previously published comparisons of the OECD filter paper method and soil exposure methods for earthworms evaluated LC_{50} and EC_{50} values, but not earthworm body burdens (Edwards and Bohlen 1992; Roberts and Dorough 1984). The purpose of these experiments was to strengthen the hypothesis that different exposure methods will result in variable results by evaluating resultant body burdens in *L. terrestris* after malathion exposure by filter paper and soil.

Materials and Methods

Lumbricus terrestris (National Association of Supplies Bait and Tackle of Marblehead, Ohio) were maintained in polyethylene boxes (32 cm by 13.5 cm) at 10°C in a refrigerator on a commercial soil product substrate (Scott's® garden soil). The earthworms were stocked at a density of 60 worms/container in 900 g of soil moistened with 100 ml of deionized water. The boxes were covered with moistened cheesecloth and aluminum foil perforated 24 times with an 18-gauge needle for air circulation. Thirty grams of rabbit feces was added to each container every other week to feed the worms. Only adult worms with well-developed clitellum were used for the assays.

Malathion (96.5% purity) stock (American Cynamid, Wayne, New Jersey, obtained in 2002) was stored in a polyethylene bottle at 4°C. Dilutions were made using AR grade acetone (Mallinkroft Chemicals, Phillipsburg, New Jersey) the day of the experiment. Concentrations (0, 1, 2, 5, 50, and 100 μg/cm^2) were based upon surface area measurements of the experimental containers (filter paper, soil). This range of doses was determined by rolling EC_{50} trials using the filter paper exposure.

The filter paper exposure was modified to accommodate the husbandry requirements of the experimental animals, *L. terrestris*, by using larger culturette tubes (18 cm long and 1.8 cm in diameter) and a cooler holding temperature (10°C) than recommended by the OECD (OECD 1984). Whatman filter paper #1 (VWR) was cut to a rectangular shape, surface area of 108 cm^2, and placed inside the tubes. Malathion concentrations were diluted in 5 ml of acetone and applied to the filter paper. The acetone was passively evaporated over night and the filter paper remoistened with 5 ml of deionized water to provide a moist environment for the earthworms. A single worm was placed in each tube and the tubes were placed horizontally in a refrigerator at 10°C for 72 h.

A rolling EC_{50} trial (0–1,250 μg/cm^2), exposing one earthworm at a time to increasing malathion concentrations, was conducted to determine appropriate exposure concentrations. Earthworms were assessed for clinical signs of toxicity, which include coiling, body ulcerations, depression, and mortality (Rao and Kavitha 2004). There were no mortalities noted during the pilot EC_{50} trials.

Five worms were exposed by filter paper contact to each of six malathion concentrations: 0, 1, 2, 5, 50, and 100 μg/cm^2. The worms were assessed for the development of clinical signs after 72 h of exposure. The earthworms were then euthanized by immersion in hot water (40°C) and sampled for determination of malathion/malaoxon body burdens.

The laboratory based soil exposures were performed using five hundred ml of Scott's® garden soil (55% organic matter, 70% sand, 20% silt, 10% clay) in glass jars with a surface area of 84.90 cm^2. Malathion of the appropriate concentration, diluted into 10 ml acetone, was applied to the soil surface. The control exposures (n = 5 replicates) used only acetone. Each container was allowed to evaporate the solvent overnight, and then the soil was rehydrated with 50 ml of deionized water. One *L. terrestris* was placed in each exposure container, (n = 5 replicates per concentration). The containers were covered with moistened cheesecloth, and kept at 10°C for 72 h. After 72 h of exposure, the earthworms were examined for clinical signs, euthanized in hot water, and examined for malathion burdens.

Extraction of malathion from 400 mg of post-clitellum earthworm tissue was performed by homogenized each individual manually in a glass homogenizer in 3 ml of hexane: acetone (3:1) for 30 s. About 100 mg of anhydrous sodium sulfate (Tracepur®, Sulpelco) was added to each sample prior to transfer to partially dehydrate the homogenate. The samples were centrifuged and a Folch wash (Folch et al. 1957) was performed to remove interfering substances. The supernatant was evaporated to dryness, rehydrated in 200 μl of hexane, and then passed through a Pasteur pipette filled with 1 g of Florisil® PR (Sulpelco). The sample was eluted with hexane, hexane: acetone (19:1, v/v), then hexane: acetone (3:1, v/v). The required fraction was eluted in the second and third solvent wash. A second Folch wash was performed and the samples were evaporated. Malathion extraction efficiencies averaged 94% recovery with a standard deviation of 10.2%. Malaoxon recovery was similar with an efficiency of 95% and a standard deviation of 11.2%. The detection limits for malathion and malaoxon were 0.04 and 0.1 μg, respectively.

Malathion/malaoxon burdens were determined using a gas chromatograph with an FID (Hewlett Packard 5890 Series II) and a DB-1 capillary column (30 m by 0.32 mm by 0.25 μm from J&W Scientific). Hydrogen was used as the carrier gas and helium served as the makeup gas.

Samples were reconstituted in 40 µl hexane. The injector and detector were both set to 250°C. A splitless 4 µl injection was made onto the column. The initial purge was off and was turned on again at one minute. The oven was programmed to ramp 40°C/min to 220°C starting from 60°C followed by a 2°C/min climb to 228°C. The run time was 8.9 min with malathion and malaoxon eluting at 6.8 and 6.2 min, respectively.

All statistical analyses were performed using Prism® version 4 from Graphpad (www.graphpad.com). Data were tested for normality using the Kolmogorov–Smirnov test. Based on these results, nonparametric statistics were employed. To investigate the presence of a dose-response, the data were compared across the exposure concentrations within an individual exposure method, filter paper or soil, using the Kruskal–Wallis test. If the Kruskal–Wallis test was statistically significant, then a Dunn's multiple comparison test was employed to determine which of the exposure concentrations (1, 2, 5, 50, and 100 µg/cm^2) varied significantly from the control. To determine differences in earthworm malathion body burden as a result of exposure method, the data were compared at each concentration between the filter paper and soil exposures with the Mann–Whitney test. For all tests, a p value of 0.05 or less was deemed statistically significant.

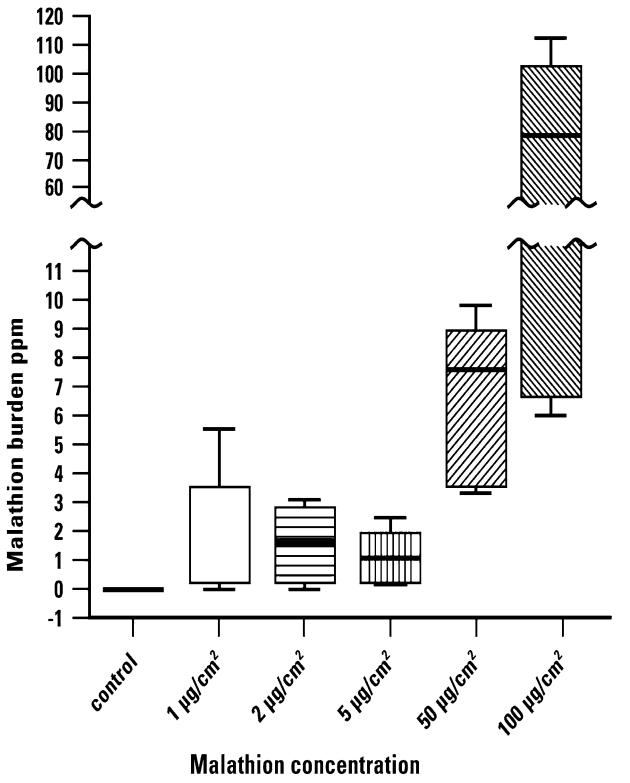

Fig. 1 Malathion body burdens in *L. terrestris* after exposure by filter paper. All values are reported in ppm of malathion. Reported are the median and interquartile ranges

Results and Discussion

Malathion was detectable in earthworm tissue; however, there was a lack of a demonstrable linear or sigmoidal dose-response for either the filter paper or soil exposures. There were statistically significant differences in the resultant malathion body burdens between exposure concentrations at an alpha of 0.05 (p value = 0.0003, K–W statistic = 23; filter paper, Fig. 3.1; p value = 0.0034, K–W statistic = 17.69; soil, Fig. 2). The post-test (Dunn's multiple comparison) was able to differentiate the control group from the 50 µg/cm^2 (p value <0.01) and 100 µg/cm^2 treatment groups (p value <0.001) after exposure to malathion by filter paper (Fig. 1) and the 50 µg/cm^2 concentration for the soil exposure (p value <0.01) (Fig. 2). There were no detectable levels of malaoxon in earthworm tissues after exposure by either method.

Lumbricus terrestris body burdens were compared for each application concentration between the two exposure methods. At the lower concentrations (1, 2, 5 µg/cm^2), there were no significant differences noted with the Mann–Whitney test between the soil and filter paper exposure. For 50 µg/cm^2, filter paper exposure resulted in statistically significant higher worm body burdens of malathion: Mann–Whitney (p value = 0.0079). At 100 µg/cm^2, the Mann–Whitney test demonstrated a significant difference between

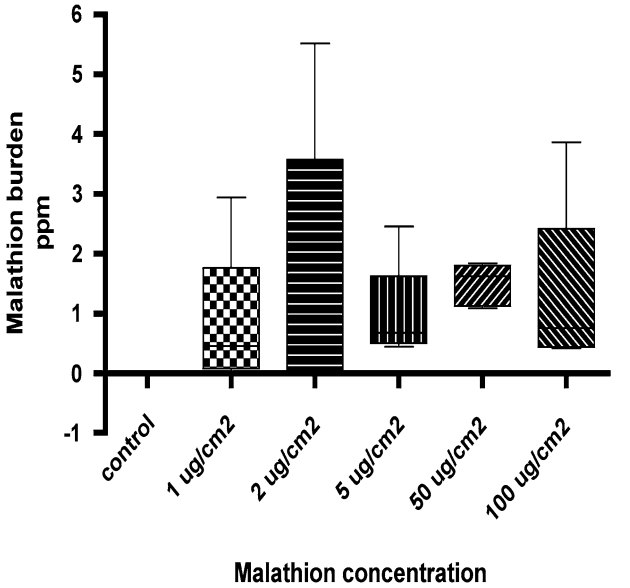

Fig. 2 Malathion body burdens in *L. terrestris* after exposure by soil. All values are reported in ppm of malathion. Reported are the median and interquartile ranges

the two exposures, again with the filter paper method resulting in the higher burden (p value = 0.0079). Overall, the filter paper application of malathion resulted in higher

earthworm body burdens than did soil exposure. Earthworms exposed to malathion by filter paper contact developed coiling in 50% of the animals at concentrations between 2 (40 % of the animals coiled) and 5 μg/cm^2 (80% of the animals coiled). After filter paper exposure to 50 μg/cm^2, all of the exposed *L. terrestris* exhibited coiling. Soil exposure did not result in coiling until 50 and 100 μg/cm^2 exposure concentrations and then less than half the animals exhibit organophosphate toxicosis.

The soil exposure technique is intuitively more likely to be an effective model for genuine environmental exposures than the filter paper method. It adds multiple dimensions of complexity to the exposure including the potential for adsorption of malathion to soil components, biological degradation of malathion within the contact medium, and increased potential for gastrointestinal absorption of malathion by the subject. The filter paper consistently resulted in higher malathion burdens and lower EC_{50} values. These may have resulted from the exclusion of additional biotic sources of variability in absorption of malathion. Decreased bioavailability due to binding to soil components (Connell and Markwell 1990), avoidance of the chemical by the earthworms (Slimak 1997), heterogenicity in the distribution of malathion within the soil (Schaefer 2004) and rapid biodegradation because of microbial activity could all contribute to the relatively low body burdens of worms exposed using the soil contact method. Filter paper exposure may overestimate malathion burdens in the earthworm, *L. terrestris*, when compared to soil exposures.

Bioavailability and adsorption to soil components should be considered when discussing soil exposures. The exposure soil has a high organic matter content, which would be anticipated to increase adsorption of malathion to the soil and a high concentration of sand which, inversely, should decrease soil adsorption (Sanchez-Martin and Sanchez-Camanzano 1991). A soil organic carbon partitioning coefficient (K_{oc}) for malathion of 1800 (Wauchope et al. 1992) has previously been reported. The percent organic carbon of the studied soil is 31.9%, which can be used to calculate a partition constant (Kd) of 575 (Wauchope et al. 2002). Partition constants greater than 100 are consistent with adsorbed pesticides and reduced bioavailability (Wauchope et al. 2002).

Lumbricus terrestris appears to be more resistant to malathion induced lethality than previously tested earthworm species. The LC_{50}'s reported for *E. foetida* and *L. rubellus* exposed to malathion by the filter paper method were 14.8 and 0.27 μg/cm^2, respectively (Roberts and Dorough 1984). The lack of lethality seen with *L. terrestris* could be due to ineffective conversion of malathion to its more active metabolite (malaoxon), efficient detoxification of malathion/malaoxon by carboxylesterases, competition for acetylcholinesterase by aliesterases, rapid excretion, or a lack of absorption. Malathion is activated to malaoxon in vivo by monooxygenases, specifically the cytochrome P-450 family (Eto 1974). This family of enzymes has been identified in *L. terrestris* (Stenersen 1984), therefore, it is unlikely that the earthworms' are incapable of metabolizing malathion to malaoxon. The lack of malaoxon detection is most likely due to the relatively high detection limit of malaoxon (0.10 μg).

Detoxification of malathion and malaoxon in mammals occurs by cleavage of the carboxyl groups by A-esterases and carboxylesterases resulting in mono- and di- acids, which are rapidly excreted in the urine (Eto 1974; Bouchard et al. 2003). The presence of carboxylesterases is considered to be a relevant factor in insect resistance to malathion (Devonshire et al. 2003) and may be an important factor in the apparent resistance in *L. terrestris*. A high concentration of carboxylesterases could make *L. terrestris* more efficient than other earthworm species at detoxification of malathion/malaoxon.

The apparent resistance to toxicity from malathion may also be a temperature dependent factor. Temperature has been demonstrated to have an affect on LC_{50} in earthworm species with the following trend: increasing LC_{50} values with increasing temperature (Spurgeon and Weeks 1998). The exposure temperature was not specified during the exposure of *E. foetida* and *L. rubellus* to malathion (Roberts and Dorough 1984), therefore, the effect of temperature cannot be ruled out as a cause of *L. terrestris*'s apparent malathion resistance.

This study has demonstrated that filter paper and soil exposure, acute exposure methods used to conduct studies with earthworms, do not produce the same results when considering clinical signs of toxicity and direct measures of resultant xenobiotic burdens. This work demonstrates a commonly held assumption that the two most frequently used earthworm toxicity exposure methods are not interchangeable. In addition, when evaluating a chemical for risk assessment, soil exposures may give more realistic results and they allow for the flexibility to manipulate and explore the effects of soil constituents on a xenobiotics behavior. Filter paper assays do not allow for this flexibility. As such, filter paper assays may be best used as a rapid screening method for highly toxic chemicals but should not be used when an environmentally realistic assessment is needed due to their tendency to overestimate body burdens in exposed earthworms.

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