

## ABSTRACT

HENSON-RAMSEY, HEATHER. Assessment of Malathion Environmental Kinetics Using Earthworm and Salamander Models. (Under the direction of Dr. Michael Stoskopf).

This research was designed to assess the toxicity, toxicokinetics, and bioaccumulation of malathion in an earthworm and amphibian laboratory model. Malathion toxicity was assessed by measuring cholinesterase activity with the Ellman assay in the earthworms, *Lumbricus terrestris* and *Eisenia foetida*, and in the tiger salamander, *Ambystoma tigrinum*. All exposed animals had decreased tissue cholinesterase activities compared to unexposed controls. Cholinesterase activities were suppressed approximately 50% in *Lumbricus* and 90% in *Eisenia*. Additionally, there were significantly different basal enzyme activities between the two species. *Ambystoma tigrinum* exposed to malathion by soil or by soil and food exhibited 50-90% suppression in cholinesterase depending on exposure concentration. Regardless of the degree of cholinesterase suppression, clinical signs did not correlate well with enzyme activity in any of the malathion exposed animals.

There were detectable xenobiotic burdens after exposure to malathion as measured by gas chromatography, in *L. terrestris*, *E. foetida*, and *A. tigrinum*. However, the tissue concentrations were not high enough when compared to soil concentrations to suggest bioaccumulation. Multiple earthworm exposure methods were compared and it was determined that exposing *L. terrestris* to malathion by filter paper contact significantly increased body burdens compared to exposure to similar concentrations by soil contact.

The toxicokinetics of malathion absorption and elimination in *L. terrestris* were studied and used to parameterize a mathematical model for the estimation of the bioconcentration of xenobiotics in earthworms. The model can be used to accurately predict the bioconcentration of multiple xenobiotics within 85-103% of experimentally determined values. Published literature was used to validate the model's effectiveness and the rate of absorption was determined to be the most sensitive parameter.

**ASSESSMENT OF MALATHION ENVIRONMENTAL KINETICS USING  
EARTHWORM AND SALAMANDER MODELS**

by  
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## **BIOGRAPHY**

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## **Chapter 1: Introduction**

### *1.0 Malathion overview*

Malathion, a phosphorothionothiolate pesticide, is used in agricultural and residential settings, and as a pharmaceutical to control a variety of insect pests including mosquitoes, boll-weevil, corn rootworms, and body lice in humans (Racke 1992; US 2000; Bouchard, Gosselin et al. 2003) (Fig 1.1). Historically, organophosphates, including malathion, have been considered to be safer than organochlorines and other pesticides because of their low environmental persistence (Racke, 1992). However, the frequent use and manner of application has raised concern over how organophosphates may impact non-target species. The most recent published estimates of usage are from 2000 and these probably understate current usage. Malathion is applied in the US at a rate of at least 17 million pounds per year, 4.1 million pounds of which are used for non-agricultural purposes, including mosquito control (Environmental Protection Agency 2000). Ultra low volume (ULV) spray, which is non-specific, is the most common application method for malathion (Environmental Protection Agency 2000) and this method often contaminates non-target areas.

Malathion's proven effectiveness as a mosquito adulticide has led to an increase in its public health application primarily for control of the West Nile virus vector (Miller 2001; Shapiro and Micucci 2003; De Guise, Maratea et al. 2004). This heavy use for preventing exposure to West Nile Virus has heightened concern over the potential environmental effects of malathion exposure (Thier 2001). Though malathion is not considered a persistent pesticide (Table 1.1), the use of ULV application has led to



**Table 1.1** Physiochemical properties of malathion (O,O-Dimethyl phosphorodithioate of diethyl mercaptosuccinate) (Environmental Protection Agency 2000)

Molecular formula	C <sub>10</sub> H <sub>19</sub> O <sub>6</sub> PS <sub>2</sub>
Molecular weight	330.36 g/mol
Physical state	Clear amber liquid
Melting point	2.85 <sup>0</sup> C
Boiling point (0.7 mm Hg)	156-157 <sup>0</sup> C
Specific gravity (25 <sup>0</sup> C)	1.23
Vapor pressure (30 <sup>0</sup> C)	4 x 10 <sup>-5</sup> mm Hg
Solubility (25 <sup>0</sup> C)	145 mg/l water
Log K <sub>ow</sub>	2.89
Log K <sub>oc</sub>	3.20

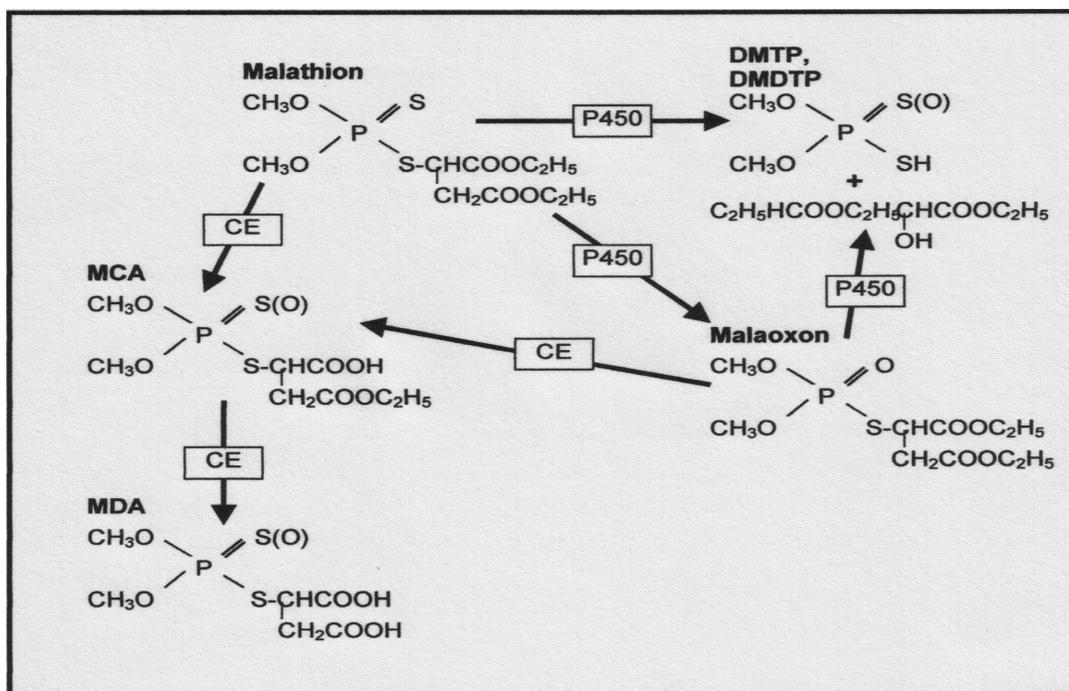
### 1.1 Metabolism

The metabolism of malathion is well characterized in mammals because of toxicity concerns in humans after occupational exposures. The metabolism of malathion is primarily regulated by the interaction of 3 enzyme systems: cytochrome P450 families (CYP450), carboxylesterases (CE), and glutathione-S-transferases (GST) (Malik and Summer 1982; Bouchard, Gosselin et al. 2003) (Fig 1.2). Metabolites that result from enzymatic reactions of malathion with cytochrome P450 and carboxylesterases are well characterized (Bouchard, Gosselin et al. 2003; Buratti, D'Aniello et al. 2005; Buratti and Testai 2005). Glutathione-S-transferase is suspected to be involved in the metabolism of malathion, however, the role and importance of GST activity in the metabolism of malathion is not well understood (Brocardo, Pandolfo et al. 2005). Studies have demonstrated a decrease in glutathione levels (Malik and Summer 1982; Brocardo,

Pandolfo et al. 2005) and an increase in a methylated glutathione product (Fukami 1984) after malathion exposure, which supports the hypothesis that GST is involved in malathion metabolism.

Cytochrome P450's and carboxylesterases (CE) compete for malathion as a substrate and are responsible for metabolizing malathion to its initial degradative products (Buratti and Testai 2005). Multiple cytochrome P450 isoforms, CYP1A2, CYP2B6, and CYP3A4, convert malathion by oxidation to malaoxon, a biologically active metabolite (Bouchard, Gosselin et al. 2003; Barata, Solayan et al. 2004; Buratti, D'Aniello et al. 2005), which is more acutely toxic than malathion itself (Eto 1974). Carboxylesterases are capable of hydrolyzing malathion and malaoxon to non-toxic metabolites, mono- and dicarboxylic acids (Fukoto 1990; Buratti and Testai 2005). The relatively high levels of CE in mammals and low levels in invertebrates is what allows for the selective toxicity of many organophosphates, including malathion (Ciesielski, Loomis et al. 1994).

All secondary metabolites (malaoxon and the mono- and dicarboxylic acids) can be converted to phosphoric derivatives, dimethylthiophosphate (DMTP) and dimethyldithiophosphate (DMDTP), by CYP450 isoforms (Buratti, D'Aniello et al. 2005). The carboxylic acids and phosphoric derivatives are then rapidly excreted in urine, 90%, and feces, 10% (Bouchard, Gosselin et al. 2003). The most common metabolite in human urine after malathion exposure is mono-carboxylic acid, followed by DMTP and DMDTP (Bradway and Shafik 1977). Studies in rats and humans have demonstrated that the bulk of malathion and its metabolites are excreted within 24 hours, with the remainder gone by 72 hours (Dary, Blancato et al. 1993).



**Fig 1.2** Metabolism of malathion excluding glutathione-S-transferase by-products. MCA- monocarboxylic acid, DCA- dicarboxylic acid, DMTP- dimethylthiophosphate, DMDTP- dimethyldithiophosphate, P450- cytochrome P450, CE- carboxylesterase (adapted from Bouchard, Gosselin et al 2003).

Few specifics are known about organophosphate metabolism in the earthworm species, *Lumbricus terrestris* and *Eisenia foetida*, though there is evidence for the presence of necessary metabolic enzymes in both species. The CYP450 family has been documented in *Lumbricus* and other earthworm species (Stenersen 1983). Therefore, oligochaeta should have the capacity to convert malathion to its -oxon analog, malaaxon. Glutathione-s-transferase metabolism of xenobiotics has been demonstrated in both *Lumbricus* and *Eisenia* (Stenersen, Guthenberg et al. 1979). However, as in mammals, the role of GST in organophosphate metabolism in earthworms is not fully understood. While malathion metabolism has not been studied in *Lumbricus* or *Eisenia*, the metabolism of another organophosphate, parathion, in exposed earthworms produces metabolites comparable to the mammalian metabolic pathway of malathion. Parathion is

metabolized to paraoxon and diethyl hydrogen phosphate is formed prior to excretion (Stenersen 1979). This suggests that the metabolism of malathion would proceed similarly.

More is known about malathion metabolism in amphibians, especially in anurans, than in earthworms. The toad, *Bufo arenarum*, converts malathion to malaaxon through induction of the cytochrome P450 family (Venturino, Gauna et al. 2001). Carboxylic acid metabolites have been reported, which suggests the presence of carboxylesterases (Venturino, Gauna et al. 2001). As in humans, indirect evidence suggests secondary conjugation of malathion by GST. Exposure to malathion leads to a decrease in GSH, a corresponding increase in glutathione-s-transferase, and the production of a potential GST metabolite, methyl glutathione (Anguiano, de Castro et al. 2001; Venturino, Anguiano et al. 2001; Venturino, Gauna et al. 2001). The evidence of glutathione, carboxylesterase, and CYP450 activities in the metabolism of malathion supports the hypothesis that amphibians will metabolize malathion in a manner similar to mammals.

There are, however, some differences in the efficiency of CYP450 system in amphibians in comparison to mammals. Basal levels of the cytochrome P450 family have been shown to be lower and possibly less active in amphibians, than in most mammals (Ertl and Winston 1998). As a result, induction of CYP450's is believed to be a less sensitive biomarker of xenobiotic exposure in amphibians than mammals (Venturino, Rosenbaum et al. 2003). Regardless of the apparent decreased sensitivity of this particular biomarker in amphibians, induction of the CYP450 family after pesticide exposure has been demonstrated in tiger salamanders (Johnson, Vodela et al. 2000) and toads (Venturino, Gauna et al. 2001).

## 1.2 *Malathion environmental fate*

The environmental fate of pesticides is dictated by a multitude of factors, including persistence, mobility, degradation, and the capacity for bioaccumulation (Guthrie and Perry 1980). Malathion is considered to be non-persistent, with reported half-lives varying from 1-10 days depending on the media of application. Malathion degrades more quickly in soil, 3 days (Rajukkannu, Basha et al. 1985) than in water, 1-11 days (Rajukkannu, Basha et al. 1985; Lacorte, Lartiges et al. 1995). The wide range in half-life estimates, 1-10 days, can be attributed to variation in test conditions in the multiple which performed the experimental situations. The exposures varied widely in temperature and pH. Warmer temperatures and alkalinity decrease malathion's half-life in soil and water by increasing the rate of degradation (Laveglia and Dahm 1977; Kaur, Mathur et al. 1997). Alkalinity speeds degradation of malathion by causing preferential cleavage at the C-S bond instead of the P-S bond (Fig 1.3) (Laveglia and Dahm 1977; Kaur, Mathur et al. 1997). In addition to temperature and pH, malathion's environmental fate depends upon the degree of adsorption to soil components (Zambonin, Lostito et al. 2002) and the particular processes that encompass the chemical (Racke 1992) and microbial degradation reactions (Levanon 1993).

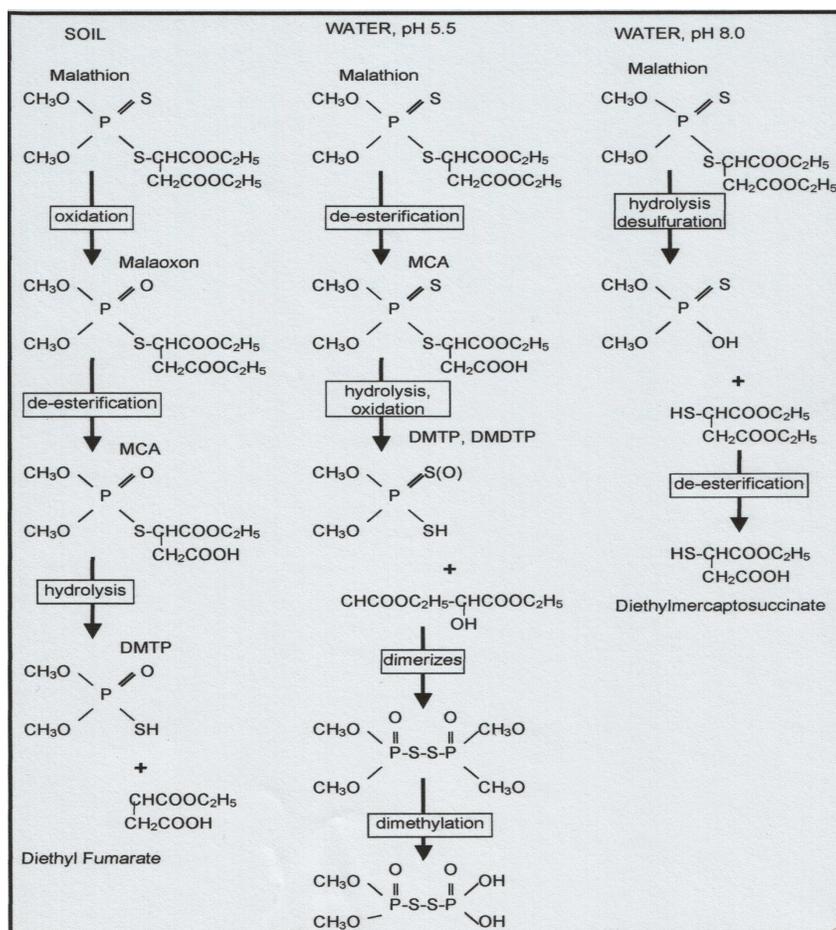
Soil adsorbed pesticides are less bioavailable (Wauchope, Yeh et al. 2002).

Therefore, any possible persistence has decreased ecological significance. Malathion has a log  $K_{oc}$  of 3.2, which suggests moderate soil adsorption (Environmental Protection Agency 2000). Particle size, organic matter concentration and type, and the capacity to complex to soil ions (Bailey and White 1964; Wauchope, Yeh et al. 2002) all affect soil adsorption and absorption to varying degrees. For most xenobiotics, adsorption is

increased in soils with high clay content and metals present for ion bonding, while high organic matter content increases absorption of xenobiotics to the soil (Bailey and White 1964). As a phosphorodithionate, the adsorption of malathion into soils is expected to be heavily influenced by adsorption to organic matter rather than sorption to particular surfaces because the parent compound does not ionize (Zambonin, Lostito et al. 2002).

In general, increasing soil organic matter content will result in amplified soil adsorption (Guthrie and Perry 1980; Sanchez-Martin and Sanchez-Camazano 1991); however, previous studies on the effect of organic matter on malathion soil absorption are contradictory. In one report, increasing organic matter content resulted in increased malathion adsorption into soil (Zambonin, Lostito et al. 2002). Other studies demonstrated no effect of organic matter content on malathion sorption into soils (Gibson and Burns 1977; Rajukkannu, Basha et al. 1985). The reason for these inconsistencies is not clear. The studies used different methods of measuring adsorption, wide-ranging analytical techniques with no extraction efficiencies noted, and lacked information on the specifics of the experimental designs such as exposure temperature (Gibson and Burns 1977; Rajukkannu, Basha et al. 1985; Zambonin, Lostito et al. 2002). As a result, the effect of organic matter content on malathion adsorption to soil particles is unresolved. Malathion degrades in soil primarily by hydrolysis, photo-oxidation, and microbial mineralization (Fig 1.3)(Gibson and Burns 1977; Brown, Petreas et al. 1993; Levanon 1993; Lacorte, Lartiges et al. 1995). Bacterial mineralization by carboxylesterases and phosphatases is believed to be responsible for the bulk of malathion's degradation in soil (Brown, Petreas et al. 1993; Levanon 1993), therefore, sterilizing soils substantially

increases the half-life of malathion (Levanon 1993). Compared to microbial degradation, chemical hydrolysis and photo-oxidation play a minor role in the degradation of malathion (Brown, Petreas et al. 1993). Photo-oxidation converts malathion to malaoxon (Brown, Petreas et al. 1993) which is then rapidly mineralized and hydrolyzed (Paschal and Neville 1976). Alkaline hydrolysis produces degradation products such as ethyl mercaptosuccinate and O, O-dimethyl phosphorodithioate (DMTP)(Brown, Petreas et al. 1993; Kaur, Mathur et al. 1997). Because the rate of degradation is decreased in acidic soils (Brown, Petreas et al. 1993), it is vital to measure the pH of soils when studying the environmental persistence of malathion.



**Fig 1.3.** Degradation pathway of malathion under different environmental conditions: in soil, water pH 5.5, and water pH 8.0.

It is not anticipated that malathion will distribute globally because of its short half-life (Rajukkannu, Basha et al. 1985), adsorption potential (Khan and Khan 1986), and low rate of volatilization (Environmental Protection Agency 2000). Adsorption of xenobiotics to sediments or soils will decrease distribution, while volatilization tends to increase the distribution of contaminants. Malathion tends to be relatively non-mobile in aqueous environments because it absorbs into sediments (Khan and Khan 1986) and once adsorbed it is typically degraded within 3 days (Bondarenko and Gan 2004). Volatilization of malathion (vapor pressure  $4 \times 10^{-5}$  mm Hg) could result in increased distribution, however, malathion also rapidly oxidizes and degrades in air (Racke 1992). The lack of global distribution of malathion may be a moot point, because it is used globally, which has led to quantifiable residues in air, water, fog, and soil samples (Schomburg, Glotfelty et al. 1991; Coupe, Manning et al. 2000; Hoffman, Capel et al. 2000). Therefore, the frequent and pervasive use of malathion provides the primary risk of exposure.

### *1.3 Bioaccumulative risk*

The risk of bioaccumulation and biomagnification of xenobiotics in wildlife are the primary reasons for the discontinued use of many organochlorine pesticides and PCB's and the substitution of organophosphates, which are less likely to accumulate (Racke 1992). Malathion is considered to have low bioaccumulative risk in most situations because of its octanol: water partitioning coefficient,  $\log K_{ow}=2.89$ , and short half-life, 1-10 days (Environmental Protection Agency 2000). However, malathion is often reapplied frequently, bi-weekly to monthly (Environmental Protection Agency 2000),

which makes bioaccumulation and chronic toxicity a potential concern in impacted habitats. The primary abiotic sources for malathion in the environment are soil, sediment, and water. The bioaccumulation of malathion from soil and sediments has been investigated in multiple species. Tissue levels of malathion were detected, but the tissue burdens were not high enough to provide evidence of bioaccumulation (Senapati, Biswal et al. 1991; Kuperman, Simini et al. 1999; Martinez-Tabche, Galar et al. 2002; Burgos-Hernandez, Zapien et al. 2006).

There have been demonstrated instances of bioaccumulation of organophosphates, including malathion, from water. Bioaccumulation of organophosphates from water have been reported in tadpoles (Hall and Kolbe 1980) and in fish (Jebakumar and Jayaraman 1988; Tsuda, Aoki et al. 1989; Dutta, Adhikari et al. 1994; Oster, Abd El Wahab et al. 1998; Amaraneni and Pillala 2001). The moderate polarity of malathion may be the reason for the documented instances of bioaccumulation from water. As a moderately lipophilic contaminant ( $\log K_{ow}=2.89$ ), malathion is 1000x more likely to be found in lipids than water (Environmental Protection Agency 2000). The possibility of bioaccumulation in surface waters suggests that exploration of malathion bioaccumulation at the aquatic-terrestrial interface is needed. These areas provide important resources for fisheries, recreation, and wildlife habitat.

#### *1.4 Mechanism of acute toxicity and resistance*

Acute toxicity resulting from organophosphate exposure is caused by irreversible inhibition of acetylcholinesterase (Eto 1974). The bound enzyme is inactivated resulting in an increase of acetylcholine at the synaptic cleft, which over-stimulates target

receptors on motor and sensory nerve endings, and cholinergic synapses (Fukoto 1990). Acetylcholine is produced at the nerve terminus by the synthesis of choline and acetyl Co-A and is then released from pre-synaptic vesicles into the nerve junction (Eto 1974). While in the synaptic cleft, acetylcholine is hydrolyzed by acetylcholinesterase into acetic acid and choline (Eto 1974). Hydrolysis of acetylcholine prevents over-stimulation of the cholinergic receptors.

The specific mechanism of acetylcholine and acetylcholinesterase binding has not been definitively determined. However, there is a commonly held hypothesis.

Acetylcholinesterase has a deep catalytic groove with a free anionic serine hydroxyl group that is capable of bonding to the cationic nitrogen on acetylcholine (Eto 1997). Once enzyme and substrate are bound, acetylcholine is hydrolyzed within milliseconds (Sussman, Harel et al. 1991).

Malathion/malaoxon phosphorylate acetylcholinesterase, which makes the enzyme unavailable to acetylcholine. The degree of enzyme inhibition that a particular organophosphate is capable of is determined by its affinity for and effectiveness at enzyme phosphorylation (Ecobichon 1970; Wang and Murphy 1982). Malathion and malaoxon are both inhibitors of acetylcholinesterase, however, malaoxon, as a more effective inhibitor, is more toxic. Phosphorylation of the serine hydroxyl group on acetylcholinesterase results in aging of the enzyme, after which, the enzyme is irreversibly inhibited (Fukoto 1990). Because phosphorylation of acetylcholinesterase is irreversible, recovery of the enzyme can only occur with oxime therapy (Eto 1974; George, Schule et al. 2003) or by de-novo synthesis (Barata, Solayan et al. 2004).

Organophosphates are capable of interacting with multiple esterases, including a-, b-, and c-esterases (Eto 1974). A-esterases, arylesterases, hydrolyze organophosphates and therefore are not inhibited (Chambers, Tangeng et al. 1994), b-esterases are inhibited by organophosphates, and c-esterases neither hydrolyze nor are inhibited by organophosphates (Eto 1974). Subclasses of the highly conserved b-esterases include acetylcholinesterase, butyrylcholinesterase, and carboxylesterases (Ecobichon 1970). Butyrylcholinesterases, sometimes referred to as pseudo-cholinesterases, are found in serum, pancreas, and liver tissues and to date, their function is still not understood (Cokugras 2003). Carboxylesterases are a mechanism of malathion/malaoxon resistance as they are capable of cleaving the carboxyl groups from organophosphates (Campbell, Trott et al. 1997). In this capacity, carboxylesterases can serve as an alternative binding site for organophosphates, thereby, reducing binding of organophosphates to acetylcholinesterase. If carboxylesterases are reduced experimentally, organophosphate toxicity increases by 2-4 fold (Barata, Solayan et al. 2004).

Organophosphates, including malathion, are more toxic to invertebrates than mammals primarily because of the relative lack of carboxylesterases in invertebrates (Ciesielski, Loomis et al. 1994). The decreased number of carboxylesterases in invertebrates results in a greater portion of malathion being converted to malaoxon by cytochrome P450s (Fig 1.2)(Fukoto 1990; Buratti and Testai 2005). Resistance to organophosphate toxicity can also be associated with the number and availability of arylesterases present to hydrolyze organophosphates (Chambers, Tangeng et al. 1994). There have been increasing reports of resistance to organophosphate toxicity in insects

since the 1960's, when the use of these pesticides increased (Hemingway and Karunaratne 1998). This suggests that target insect populations may be exhibiting genetic change as a result of frequent exposure to organophosphates.

### 1.5 *Chronic toxicity of Malathion*

While most of the research into toxicity from malathion exposure focuses on its capacity to acutely inhibit cholinesterases, there is evidence that malathion exposure can cause chronic toxicity as well, through the mechanisms of immunosuppression, genotoxicity, and the formation of reactive oxygen species. Doses of malathion, which suppress acetylcholinesterase, have been shown to decrease humoral immune responses in mice. It has been hypothesized that this may be a result of increased glucocorticoid production (Galloway and Handy 2003). Conversely, exposing rats to malathion doses that do not inhibit cholinesterases may benefit the immune system in the short term by increasing macrophage function, though, hypersensitivity reactions have also been reported (Galloway and Handy 2003). Some authors have speculated that immune suppression after malathion exposure could be a direct result of decreased T-cell esterase activity, reducing their antigen presenting capacity (Galloway and Handy 2003; Zabrodskii, Germanchuk et al. 2003).

Immune suppression after malathion exposure, regardless of the mechanism, has been reported in a variety of wildlife species. Decreased immune function after exposure to malathion has led to decreased disease resistance (Finch, Gardner et al. 1999; Taylor, Williams et al. 1999), a decreased ability to produce antibodies during a vaccine challenge (Galloway and Handy 2003; Soltani, Mikryakov et al. 2003), and decreased

phagocytosis (De Guise, Maratea et al. 2004). Most immunotoxicity studies have focused on adult animals, however, there is evidence that exposing mice during susceptible developmental stages may result in decreased T-cell response in adulthood (Navarro, Basta et al. 2001). This is an interesting because many amphibians would likely be exposed to malathion during larval stages, possibly increasing their susceptibility to pathogens in adulthood.

Malathion can be genotoxic for occupational workers who apply technical grade malathion. This toxicity is believed to be due to impurities such as isomalathion, which make up 5-10% of technical malathion (Flessel, Quintana et al. 1993). Studies in humans after incidental topical application of malathion have demonstrated multiple chromosomal abnormalities (Desi, Nagymajtenyi et al. 1998; Paz-y-Mino, Bustamante et al. 2002), however, the effect of these abnormalities in the individual and future offspring is not known. In-vivo studies in rats replicated these results and found additional abnormalities in sperm morphology (Giri, Prasad et al. 2002). Exposing *Eisenia foetida* to sub lethal concentrations of malathion in soil decreases sperm count and affects sperm morphology (Espinoza-Navarro and Bustos-Obregon 2004). The mechanism of action for genotoxic effects of malathion and related compounds is still not understood. Genotoxic effects of malathion exposure are suspected in amphibians but have not yet been positively identified.

There are two primary forms of chronic neurotoxicity from exposure to organophosphates, organophosphorus delayed ester-induced neurotoxicity (OPIDN), and organophosphorus ester-induced chronic neurotoxicity (OPICN) (Abou-Donia 2003). OPIDN is a result of a single or repeated exposure where symptoms such as muscle

fasciculations, coma, and ataxia are initially resolved but later recur. Malathion is thought to interfere with protein kinase regulation of cytoskeletal elements leading to an exacerbation of symptoms (Abou-Donia 2003). OPICN most commonly occurs in people who have been exposed to repetitive sub-clinical doses of organophosphates and results in chronic vague neurological symptoms such as depression and irritability. Research suggests that OPICN may be caused by malathion's capacity for lipid peroxidation and the formation of reactive oxygen species (Abou-Donia 2003). There has been recent progress in research designed to develop assays for lipid peroxidation which correlate with acetylcholinesterase suppression and could serve as biomarkers for OPICN (Akhgari, Abdollahi et al. 2003). Neither OPICN nor OPIDN has been reported in any wildlife species. However, renal toxicity in a brush-tailed rat has been associated with lipid peroxidation after malathion exposure (Bosco, Rodrigo et al. 1997).

#### 1.6 *Standardized earthworm toxicity assays*

Earthworms are frequent subjects of toxicology studies because they are important agriculturally (Haines and Uren 1990; Hendrix 1995) and are often inadvertently impacted by terrestrially applied pesticides (Fisher 1984). Earthworm species maintain soil structure through nutrient recycling (Lavelle 1988) and perform carbon mineralization of organic compounds (Scheu 1987), which can reduce the half-life of xenobiotics in soil (Edwards and Bohlen 1992; Lydy and Linck 2003). Therefore, a reduction of earthworm numbers from soil contamination affects not only the earthworm species itself, but can also increase toxicity from xenobiotics by increasing persistence.

Because of these concerns, standardized assays have been developed to allow for measurement of toxicity in earthworms.

There are multiple toxicity testing procedures for earthworms, however, most of the assays are designed to measure relatively few effects. The most common endpoints measured in acute and chronic tests are lethality ( $LC_{50}$ ,  $LD_{50}$ ) and decreased absolute earthworm numbers (Karnak and Hamelink 1982; Dean-Ross 1983; Fisher 1984; Heimbach 1984; Van Gestel and Wei-chun 1988; Giggelman, Fitzpatrick et al. 1998; Youn-Joo 2005). The investigation of sub lethal endpoints, which could be used as a more precise measure of toxicity in earthworm populations, is understudied and has been identified by an earthworm working group as a specific area of research need (Van Gestel and Weeks 2004). There has been some progress made as non-lethal endpoints of toxicity in earthworms have been identified, including behavioral changes (Youn-Joo 2005), weight loss (Karnak and Hamelink 1982; Rida and Bouche 1997), and decreased reproduction (Senapati, Biswal et al. 1991; Brown, Long et al. 2004). Standardized earthworm toxicity assays have been used to develop biochemical markers (Giggelman, Fitzpatrick et al. 1998; Ribera, Narbonne et al. 2001; Bundy, Lenz et al. 2002; Lydy and Linck 2003), which can be used to further understanding of toxicological mechanisms and the development of resistance to malathion.

Multiple methods can be used to expose earthworms to environmental contaminants. The simplest method, topical application, is very ineffective because earthworms have the capacity to slough the chemical by an overproduction of mucus (Fisher 1984; Edwards and Bohlen 1992), effectively reducing or even eliminating the exposure. Immersion assays were developed in an attempt to reduce the effect of mucus

production on absorption (Fisher 1984). However, this method underestimates toxicity when compared with other exposure methods (Dean-Ross 1983) and, as a result, is infrequently used. Injection of the chemical into the coelomic cavity has been used but this method of exposure (Nakatsugawa and Nelson 1972) makes it difficult to separate solvent effects from contaminant effects (Edwards and Bohlen 1992).

The most common methods of exposure are standardized by the Office of Economic Development (OECD). These are the filter paper assay and laboratory soil exposures which may use natural or artificial soil. Filter paper exposure involves saturating filter paper with the xenobiotic while in an inert glass tube. The earthworm is inserted into the glass tube and exposed to the contaminated filter paper for a specified contact time (Edwards and Bohlen 1992). Though this exposure is quick and efficient, it has been shown to overestimate lethality compared to soil exposure (Neuhauser, Durkin et al. 1986; Youn-Joo 2005). This is probably because filter paper exposure doesn't incorporate several key factors which can affect the bioavailability of contaminants, soil adsorption and microbial degradation (Edwards and Bohlen 1992). The major limitation and possible advantage, depending upon experimental need, of filter paper exposure is that it is incapable of assessing any form of absorption other than cutaneous (Heimbach 1984). However, its ease of use makes filter paper exposure ideal for the rapid evaluation of individual contaminants from a large pool of compounds that need to be reviewed (Neuhauser, Durkin et al. 1986), and in mechanistic studies that are designed to develop potential biomarkers of effect and exposure (Giggleman, Fitzpatrick et al. 1998; Bundy, Lenz et al. 2002).

The earthworm exposure method with the most environmental realism is laboratory soil exposure using artificial or natural soils, and field exposures. These exposure methods include the abiotic soil compartment, which has the capacity to affect the absorption of xenobiotics into earthworms by affecting bioavailability. Potential confounding factors such as adsorption to soil components and degradation by microbial organisms (Wauchope, Yeh et al. 2002) can be studied with these exposure methods. The major disadvantage to the laboratory soil exposure is variability, which results when researchers from different labs use different soils (Edwards and Bohlen 1992). Resolution of this conflict has occurred with the development of an artificial standardized soil by the OECD (Karnak and Hamelink 1982; Edwards and Bohlen 1992). Intuitively, field application of contaminants should provide the most environmental realism. However, field studies have an inherent disadvantage, uncontrollable circumstances that can affect results and reduce reproducibility. Because of these considerations, the laboratory soil exposure method is believed to be the most reliable and reproducible of the earthworm toxicity assays.

### *1.7 Acute toxicity of organophosphates in earthworms*

Clinical signs resulting from organophosphate poisoning of earthworms appear to be consistent across species and have been reported by multiple researchers. These signs include coiling, mucus secretion, and the development of ulcerations especially on the clitellum (Heimbach 1984; Edwards and Bohlen 1992; Rao and Kavitha 2004). There are moderate differences in sensitivity among earthworms to the main classes of cholinesterase-inhibiting insecticides, organophosphates and carbamates (Gilman and

Vardanis 1974; Stenersen, Brekke et al. 1992). Studies have not been done to assess differences in sensitivity to malathion exposure in *Lumbricus terrestris* and *Eisenia foetida* (Table 1.2). It has been documented, however, that *L. terrestris* are more sensitive to toxicity from carbamate exposure than *E. foetida* (Stenersen, Gilman et al. 1973; Gilman and Vardanis 1974). The variability in toxicity to carbamates even extends within a single genus; *E. andrei* and *E. foetida* are relatively resistant to the development of toxicity, while *E. veneta* exhibits increased toxicity as measured by cholinesterase suppression (Stenersen, Brekke et al. 1992). The differences in species sensitivity are believed to be either a direct result of the type and number of cholinesterases possessed by each species (Stenersen 1980; Stenersen, Brekke et al. 1992), or because of differences in xenobiotic elimination rates (Gilman and Vardanis 1974). Therefore, it is important to consider all earthworm species separately and not to extrapolate data between species when considering toxicity to cholinesterase-inhibiting pesticides.

It is assumed that earthworms will exhibit similar degrees of species variability in susceptibility to organophosphate exposure. There are multiple reports of toxicity after organophosphate exposure in earthworms, measured as lethality or cholinesterase suppression (Stenersen 1979; Stenersen 1979; Roberts and Dorough 1984; Neuhauser, Durkin et al. 1986; Senapati, Biswal et al. 1991; Panda and Sahu 2004). Earthworms appear to differ not only in the degree of cholinesterase suppression after organophosphate exposure, but also in their capacity to recover the enzyme after inhibition. Exposing *Aporrectodea caliginosa* to organophosphates resulted in 86% suppression of cholinesterase, compared to unexposed controls. There was less than 10% mortality in the exposed earthworms and no apparent recovery of enzyme activity

(Booth, Hodge et al. 2000). Organophosphate exposure in *Drawida calebi* resulted in comparatively less inhibition of cholinesterase, 40%, and recovery of enzyme activity occurred within 24 hours (Patnaik and Dash 1992). The length of exposure and differences in type and amount of metabolizing enzymes could have resulted in these species-specific differences. Recovery is most likely a result of an individual species capacity to de-novo manufacture enzyme, since organophosphates irreversibly inhibit acetylcholinesterase (Eto 1974). In general, cholinesterase suppression does not correlate well with clinical signs (Stenersen 1979; Booth, Hodge et al. 2001) and there appears to be wide species differences in sensitivity to this particular biomarker. Therefore, cholinesterase suppression is a good predictor of exposure but a poor measure of effect after a toxic exposure to organophosphates.

Malathion exposure and its toxic effects have been studied in multiple earthworm species. Unfortunately, these studies used multiple exposure regimens and measured different toxic effects (Table 1.2). This makes it difficult to compare the effects of malathion between different earthworm species, though a few trends can be identified (Table 1.2). Earthworms do exhibit cholinesterase suppression when exposed to malathion in soil at concentrations applied for pest control (Stenersen, Gilman et al. 1973; Panda and Sahu 2004). Exposing earthworms to malathion by filter paper rather than by soil appears to exaggerate toxic effect (Roberts and Dorough 1984; Kuperman, Simini et al. 1999). For example, when *E. foetida* were exposed to malathion by filter paper, rather than by soil contact, lethality occurred at exposure concentrations 3-4 fold lower (Roberts and Dorough 1984; Kuperman, Simini et al. 1999). Toxicity after malathion exposure has been previously documented in both modeled organisms, *L.*

*terrestris* and *E. foetida* (Stenersen, Gilman et al. 1973; Roberts and Dorough 1984; Kuperman, Simini et al. 1999).

**Table 1.2:** Toxicity of malathion to different earthworm species.

Author/year	Species	Exposure method	Dose/concentration	Effect
Stenersen/1973	<i>L. terrestris</i>	Soil (60 days)	1.3 ppm*	70% cholinesterase inhibition, 1 wk recovery
Roberts/1984	<i>E. foetida</i>	filter paper (2 days)	0.1-1000 $\mu\text{g}/\text{cm}^2$	LC <sub>50</sub> 13.5 $\mu\text{g}/\text{cm}^2$
Roberts/1984	<i>L. rubellus</i>	filter paper (2 days)	0.1-1000 $\mu\text{g}/\text{cm}^2$	LC <sub>50</sub> 0.27 $\mu\text{g}/\text{cm}^2$
Kuperman/1999	<i>E. foetida</i>	Soil (14 days)	6-150 $\mu\text{g}/\text{cm}^2$	LOEC 189-236 $\mu\text{g}/\text{cm}^2$
Kuperman/1999	<i>E. albidus</i>	Soil (14 days)	6-150 $\mu\text{g}/\text{cm}^2$	LOEC 21-73 $\mu\text{g}/\text{cm}^2$
Panda/ 2004	<i>D. willsi</i>	Soil (105 days)	2.2 ppm*	41% cholinesterase inhibition, 45 day recovery
Panda/2002	<i>D. willsi</i>	Soil (4 days)	not specified	LC <sub>50</sub> 15.1-18.1 mg/kg*

\*Unable to convert to  $\mu\text{g}/\text{cm}^2$  because surface area of exposure containers were not provided. (Stenersen, Gilman et al. 1973; Roberts and Dorough 1984; Kuperman, Simini et al. 1999; Panda and Sahu 2002; Panda and Sahu 2004)

### 1.8 Acute toxicity of malathion and organophosphates in amphibians

Amphibian population declines are frequently cited as a common concern of environmentalists and are considered a multifactorial problem. Possible causes of population declines include UV radiation, parasite pathogens, fungal pathogens including chytrid fungus, climatic change, impaired immune status, invasive species, and pesticide exposure (Kiesecker 1991; Alford and Richards 2001; Gillespie 2001; Kiesecker, Blaustein et al. 2001; Sparling, Fellers et al. 2001; Davidson, Shaffer et al. 2002; Blaustein, Romansic et al. 2003). Pesticide exposure is one of the most likely co-contributors to environmental stress in amphibians (Sparling, Fellers et al. 2001;

Blaustein, Romansic et al. 2003), yet there is a lack of ecotoxicologic data in amphibians (Hall and Henry 1992; Rattner, Eisenriech et al. 2005). Fewer than 0.6% of the studies in the Contaminant Exposure and Effects-Terrestrial Vertebrates (CEE-TV) database concern amphibians (Rattner, Eisenriech et al. 2005) and of these, there is only one report on xenobiotic exposure in salamanders.

Though there is a lack of toxicity data for amphibians in general, organophosphate exposure has been studied in some species of amphibians, especially in frogs and toads. Cholinesterase activity, an indirect measure of malathion exposure, can be used as a biomarker of organophosphate exposure and a measure of general population health in amphibians (Sparling, Fellers et al. 2001; Taylor, Williams et al. 1999). Clinical signs of organophosphate exposure in bullfrogs were first reported in the 1960's and included generalized paralysis, a loss of righting reflex, muscle fasciculations, and coma (Edery and Schatzberg-Porath 1960). Since then, suppression of acetylcholinesterase activity and similar clinical signs have been documented in multiple amphibian species (Hall and Kolbe 1980; Baker 1985; Rosenbaum, de Castro et al. 1988; Taylor, Williams et al. 1999). What is uncertain is the effect that cholinesterase suppression has on amphibians' biology because the degree of suppression does not correlate well with the development of clinical signs of toxicity (Baker 1985; Carlock, Chen et al. 1999). Greater than 30% suppression of cholinesterase activity has been reported in amphibians without apparent clinical effect, mortality or a disruption in feeding behavior (Baker 1985; Rosenbaum, de Castro et al. 1988).

The majority of studies that have assessed malathion exposure in amphibians have involved frog and toad larvae (Table 1.3) using mortality and tetratogenicity as measures

of exposure. There are differences in sensitivity to malathion toxicity between species of amphibians, as has been seen in earthworms. Toads are more resistant to mortality than frogs and can tolerate higher concentrations of malathion (Table 1.3) (Relyea, 2004; Willens 2005), which is most likely because of differential absorption of the contaminant (Willens 2005). In addition to species differences, malathion exposure during early developmental states, embryo or blastulae stage, is more likely to result in tetratogenicity, than exposure during later developmental stages (Rosenbaum, de Castro et al. 1988; Bonfanti, Colombo et al. 2004)(Table 1.3).

**Table 1.3.** A comparison of toxicity after malathion exposure across amphibian species.

Author/year	Species	Developmental stage	Effect Exposure concentration	Effect measured
Hall/1980	<i>Rana catesbeiana</i>	NR, tadpole	5 ppm	40% mortality
Rosenbaum/1988	<i>Bufo arenarum</i>	NR, embryo	44 ppm	67% mortality, deformities
Harris/2000	<i>Rana pipens</i> <i>Bufo americanus</i>	20, 46 20,46	0.001 ppm- 10 ppm	No mortality, no deformities
Fordham/2001	<i>Rana catesbeiana</i>	27	3 ppm	40% mortality, delayed development
Bonfanti/2004	<i>Xenopus laevis</i>	Blastulae to 47	2.4 ppm	No mortality, deformities
Relyea/2004	<i>Rana sylvatica</i> <i>Rana pipens</i> <i>Rana clamitans</i> <i>Rana catesbeiana</i> <i>Bufo americanus</i>	25 25 25 25 25 25	1.25 ppm 2.4 ppm 3.65 ppm 1.5 ppm 5.9 ppm 4.13 ppm	LC <sub>50</sub>

(Hall and Kolbe 1980; Rosenbaum, de Castro et al. 1988; Harris, Chora et al. 2000; Fordham, Tessari et al. 2001; Bonfanti, Colombo et al. 2004; Relyea 2004)

Very few studies have examined subacute effects of malathion exposure in amphibians. There is some suspicion that pesticide exposure may reduce immunity to parasites and other pathogens, such as the trematode, *Ribeiroia ondatrae*, resulting in amphibian declines and limb deformities (Kiesecker 2002; Blaustein, Romansic et al. 2003). Malathion has been shown to increase disease susceptibility in Woodhouse toads to *Aeromonas* infections though the mechanism of immune suppression is not fully understood (Taylor, Williams et al. 1999). Though cholinesterase inhibition does not correlate well with acute clinical signs of malathion toxicity, it is speculated that subacute effects of malathion toxicity, such as feeding and reproduction, may correlate better (Venturino, Rosenbaum et al. 2003). Lungless salamanders (Plethodontidae) exposed to environmental concentrations of malathion exhibited moderate cholinesterase suppression and a decreased feeding rate (Baker 1985). Subacute and chronic effects from malathion exposure in amphibians remains understudied. This makes it difficult to assess the effect of malathion exposure on amphibians at a population level.

### 1.9 Earthworm natural history and relevant anatomy

**Table 1.4:** Taxonomy of test earthworm species.

Class	Oligochaeta	Oligochaeta
Order	Haplotaxidae	Haplotaxidae
Suborder	Lumbricina	Lumbricina
Superfamily	Lumbricoidea	Lumbricoidea
Family	Lumbricidae	Lumbricidae
Genus	Lumbricus	Eisenia
Species	terrestris	foetida

(Edwards and Bohlen 1996)

Though *Lumbricus terrestris* and *Eisenia foetida* are closely related species (Table 1.4) (Edwards and Bohlen 1996), their reproductive strategies and environmental niche are very different. *Lumbricus terrestris* is considered a K selected species (Satchell 1980) because of their relatively long life span, up to 6 years in captivity, and slow reproduction (Blair, Parmelee et al. 1995; Kammenga, Spurgeon et al. 2003). They produce cocoons every 10 days, year around, and take 12-13 weeks to fully develop (Butt, Fredrickson et al. 1994), with one juvenile worm hatching from each cocoon (Edwards and Bohlen 1996) Comparatively, *Eisenia foetida* are an r selected species (Satchell 1980) with a rapid reproductive and metabolic rate and a possible life span in the laboratory of 4.5 years (Blair, Parmelee et al. 1995; Kammenga, Spurgeon et al. 2003). For every cocoon produced, *E. foetida* averages 3.3 hatchlings (Edwards and Bohlen 1996).

*Lumbricus terrestris* is classified as an anecic earthworm. It lives in deep, vertical burrows (Blair, Parmelee et al. 1995; Edwards and Bohlen 1996) and will exhibit territorial behavior (Capowiez and Belzunces 2001). The worm is capable of traveling long distances on land in search of food (Mather and Christensen 1988; Blair, Parmelee et al. 1995), but typically feeds by keeping its posterior end in the burrow with its setae and using the anterior end for food collection. For optimum growth and reproduction, *Lumbricus terrestris* prefer temperatures of 10<sup>0</sup> C and 40% soil moisture (Lakhani and Satchell 1970). They can achieve this, regardless of surface temperatures, by entering the depths of their burrows and as a result, they rarely undergo diapause (Edwards and Bohlen 1996).

Conversely, *Eisenia foetida* lives on the soil surface, feeds on organic matter and compost, and is classified as an epigenic worm (Edwards and Bohlen 1992; Blair, Parmelee et al. 1995; Kammenga, Spurgeon et al. 2003). They have different microhabitat requirements than *L. terrestris*, preferring warmer temperatures, 15-20<sup>0</sup> C, and higher soil moisture levels, 65-70% (Reinecke and Venter 1987). Because they live on the soil surface and have more stringent environmental requirements, *E. foetida* often undergo diapause in response to anhydrosis or low soil temperatures (Edwards and Bohlen 1996).

The skin is an important possible route of absorption in earthworms. The integument of *L. terrestris* and *E. foetida* is made of an external cuticular layer, an epidermal layer primarily made of columnar cells with extensions into the lower portion of the cuticular layer, and an inner muscular layer (Coggeshall 1966; Burke 1974). The cuticle layer in *Lumbricus* and *Eisenia* is made of collagen, 80% protein and 20% polysaccharides, which serves to reduce dehydration and absorption of lipophilic substances from the environment (Watson 1958; Baccetti 1967). Mucus discharged from epidermal glandular cells in response to noxious stimuli serves to further reduce toxicant exposure and water loss (Coonfield 1932). The primary difference in the integument between *L. terrestris* and *E. foetida* is the thickness of the cuticle layer. *Lumbricus terrestris* have greater than 24 layers of collagen in their cuticle (Coggeshall 1966) and *Eisenia foetida* have 10 layers (Burke 1974). The decreased comparative thickness of the cuticle layer in *E. foetida* could increase the earthworm's susceptibility to xenobiotic toxicity.

If xenobiotics are absorbed past the cuticular layer in earthworm integument, absorption should then occur relatively quickly because the body wall is heavily vascularized. This is designed to aid passive diffusion of oxygen and carbon dioxide through the epidermis since earthworms lack specialized respiratory organs (Edwards and Bohlen 1996). Once absorbed, xenobiotics enter the closed circulatory system and are transported through the blood stream prior to metabolism and excretion (Edwards and Bohlen 1996; Hickman, Roberts et al. 2004). The coelomic fluid may also contain absorbed xenobiotics and waste products, which are filtered from both the blood and coelomic fluid prior to excretion (Needham 1957). Waste products, primarily amino acids, urea, and ammonia (Needham 1957), are excreted by paired nephridia into a bladder and through nephridiopores, located in each segment, to the cuticular surface (Edwards and Bohlen 1996). It is believed that the overall process of urine production and excretion is very similar to mammals because nephridia are progenitors of mammalian nephrons (Clauss 2001). Though not specifically reported, earthworms should be capable of excreting polar metabolites of malathion from the blood or coelomic fluid through their nephridia.

Another port of entry of xenobiotics into earthworms is through oral ingestion. Oligochaetes have a very simple digestive tract which consists of a buccal cavity, a pharynx, an esophagus, a crop, a gizzard, and the intestine (Hickman, Roberts et al. 2004). A cuticle overlies the epithelium in all gastrointestinal segments except the site of absorption, the intestines (Edwards and Bohlen 1996). The intestines are made of an internal epithelial layer with microvilli and a typhlosole, two middle muscular layers, and on the serosal surface, chloragogen which is a modified peritoneum (Morowati 2000).

The typhosole, a specialization for absorption of nutrients, varies in size and structure based on the species' feeding strategy. Epigenic worms, which feed on nutrient rich substances, have a small typhosole with no invaginations. Anecic worms, which feed on less nutrient dense food sources, require a larger typhosole with multiple folds (Perel 1977). It is suspected, but unproven, that a larger typhosole may increase the likelihood of xenobiotic absorption in *L. terrestris* when compared to *E. foetida*. The chloragogen, an additional specialization in earthworms, functions as a liver, producing the nitrogen waste products urea and ammonia, converting food sources into fats and glycogen, and excreting products in the coelomic fluid or blood stream (Hickman, Roberts et al. 2004).

The neuromuscular junction of earthworms is believed to be physiologically very similar to mammals and is directed by the release of acetylcholine and cholinergic receptors (Gerschenfeld 1973; Walker, Holden-Dye et al. 1993; Volkov, Nurullin et al. 2003). Anatomically, the nervous system of earthworms is similar to vertebrates with rudimentary central and peripheral nervous systems, which consists of a central nerve cord and peripheral segmental nerves (Edwards and Bohlen 1996). Recently, research into the physiological functioning of the earthworm nervous system has been focused on identification and function of excitatory and inhibitory neurotransmitters at work along the gastrointestinal system (Lengvari, Csoknya et al. 1992; Barna, Csoknya et al. 2001). Because of the known presence of acetylcholine (Gerschenfeld 1973), in addition to research confirming its stimulatory effect at neuromuscular junctions (Volkov, Nurullin et al. 2003), it is anticipated that malathion will exert toxicity in *Lumbricus terrestris* and

*Eisenia foetida* through interference with acetylcholine re-cycling in a manner comparable to vertebrates.

#### 1.10 *Ambystoma tigrinum* natural history and relevant anatomy

**Table 1.5.** Taxonomy of *A. tigrinum*

Class	Amphibia
Subclass	Lissamphibia
Order	Caudata
Suborder	Salamandroidea
Family	Ambystomatidae
Genus	Ambystoma
Species	tigrinum

(Duellman and Trueb 1994)

*Ambystoma tigrinum*, (Hallowell 1856, Table 1.5), has the widest known distribution of any amphibian in the United States. However, it is a species of special concern and is threatened or endangered in most locales (Staniszewski 1995; Smith 2003). In North Carolina, tiger salamanders were declared threatened in 1990 (Friday 1994). Purported causes of their decline include clear-cutting and other forms of habitat destruction (Friday 1994; Petranka 1998).

Tiger salamanders are dependent upon both aquatic and terrestrial habitats for their life cycle. As a species, they exhibit a wide range of life-cycle strategies. They have 2 larval forms (normal morph and cannibal morph) and 3 adult forms (metamorphic, neotenic, cannibalistic neotenic) (Collins 1981). The eastern tiger salamander, *A. tigrinum tigrinum* has a type 1 life history (Sexton and Bizer 1978). They are primarily terrestrial adults who breed in semi-permanent ponds. In North Carolina, adult *A. tigrinum* enter ephemeral ponds in December with the majority of the larvae metamorphosed by summer when the ponds dry (Friday 1994). The majority of a

metamorphs' time is spent in burrows which they either make themselves or borrow from mammals (Bishop 1943; Duellman and Trueb 1986). Because they are rarely above ground, *A. tigrinum* are notoriously difficult to survey (Madison and Farrand 1998). Metamorphic tiger salamanders migrate from terrestrial over wintering areas to breeding sites to foraging sites within a single season (Phillips, Brandon et al. 1999). Tiger salamanders have a relatively small home range (300m) and don't migrate far from the breeding pond (Madison and Farrand 1998).

Tiger salamanders have an internal fertilization strategy (Bishop 1943). The males deposit spermatophores which the females pick up with their cloaca (Arnold 1976). The egg masses are then deposited onto aquatic vegetation and the larvae develop within 75-118 days (Bishop 1943), though this is highly dependent upon temperature.

*Abystoma tigrinum* are an r-selected species (Semlitsch 1983) defined by rapid development and a high reproductive rate. Environmental factors can greatly influence the success of r-selected species. Tiger salamanders can be negatively affected by changes in temperature, freezing of breeding ponds, and lack of rainfall leading to dehydration of breeding ponds (Anderson, Hassinger et al. 1971; Semlitsch 1983). Predation is a significant contributor to larval (Anderson, Hassinger et al. 1971) and metamorph mortality (Madison and Farrand 1998). Additional risks to *A. tigrinum* include acid rain (Kiesecker 1991), the aforementioned habitat destruction, and pesticide use (Larson, McDonald et al. 1998; Petranka 1998; Johnson, Franke et al. 1999). Acidification of water used for larval development has been shown to decrease survival and size at metamorphosis (Kiesecker 1991). Experimental exposure to the pesticides, TNT (Johnson, Franke et al. 1999) and atrazine (Larson, McDonald et al. 1998), has

resulted in toxicity in *A. tigrinum*, however, the tested concentrations were above those seen in the environment. Effects from organophosphate exposure have not been studied in *A. tigrinum*.

Aspects of the metamorphic tiger salamander anatomy, which may affect their susceptibility to disease from organophosphate exposure, include the neuromuscular junction, structure/function of the nephron, and the integument. Malathion exerts acute toxicity in most species by inhibition of cholinesterase at the neuromuscular junction (Eto 1974). Salamanders do produce acetylcholine at nerve end plates and the neurotransmitter has been shown to be responsible for neuroexcitability at the neuromuscular junction (Dennis and Ort 1977; Chevallir, Nagy et al. 2006). Plethodon salamanders demonstrate cholinesterase suppression after exposure to malathion (Baker 1985). Therefore, it is likely that the primary mechanism of acute toxicity after malathion exposure in *A. tigrinum* will be inhibition of cholinesterase at cholinergic synapses.

Malathion has been shown to be primarily excreted by the kidneys in mammals (Bouchard, Gosselin et al. 2003) and fish (Bosco, Rodrigo et al. 1997). Though tiger salamander kidneys are grossly different from mammalian kidneys, the microscopic anatomy of the *A. tigrinum* nephron has been shown to be structurally and functionally similar to mammalian nephrons (Hinton, Stoner et al.). Urine is formed from waste products in the blood and coelomic fluid by filtration in the glomerulus and reabsorptive and secretory processes in the tubules (Duellman and Trueb 1994). Damage to the glomerulus during chronic malathion exposure in fish (Bosco, Rodrigo et al. 1997) implies that malathion may enter the urine by filtration rather than by active secretion

into the tubules. There is evidence that tiger salamanders would convert malathion into polar metabolites that can be excreted renally, as they have many of the same detoxification enzymes as mammals (Johnson, Vodela et al. 2000). Additionally, other amphibians, i.e. frogs, have been shown to produce the polar metabolite, carboxylic acid, after exposure to malathion (Venturino, Gauna et al. 2001).

Amphibian skin is substantially thinner than mammalian integument, averaging 2 epidermal layers versus the 4-5 layers present in most mammals (Willens 2005), and it is more highly vascularized (Duellman and Trueb 1994). This adaptation allows amphibians to absorb water cutaneously to make up for high evaporative losses and respiration losses in lung-breathing amphibians (Duellman and Trueb 1994). The increased permeability of their skin makes them more susceptible to percutaneous absorption of toxicants. *Ambystoma tigrinum* integument is made of 2 epidermal layers, stratum corneum and stratum germinatum, and 2 dermal layers, stratum spongiosum and stratum densum (Dawson 1918; Rowe, Hopkins et al. 2003). Once metamorphosed, tiger salamanders have a thinner dermal layer with increased numbers of glands, used primarily for defense (Toledo and Jared 1995), and increased vascularization compared to larvae (Frolich and Schmid 1991; Rowe, Hopkins et al. 2003). Additionally, the adult integument is more water permeable compared to larval tiger salamanders (Bentley and Baldwin 1980). It is uncertain what effect a thinner dermis and increased skin permeability to hydrophilic substances would have when investigating the absorption of a moderately hydrophobic compound like malathion. Adult tiger salamanders may be less likely to absorb hydrophobic xenobiotics than larval salamanders.

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## Chapter 2: Analytical Methods

### 2.0 Extraction procedure

All samples (earthworm tissue, soil, and salamander tissues) were extracted for malathion analysis using the same procedure. The method was designed to optimally extract malathion/malaoxon while reducing interference from co-extractants. There are no published assays for the extraction of malathion from salamander or earthworm tissue. Solvent extraction of malathion from fish tissue was successful with a 1:2 acetone/hexane (v: v) solution with an extraction efficiency of 83% (Amaraneni and Pillala 2001). In the earthworm samples, this resulted in too much chromatographic interference from co-extractants. By increasing the amount of hexane in relation to acetone, 1:3 acetone/hexane (v: v) ratio, a cleaner chromatograph was achieved with decreased number of interfering peaks near malathion's retention time.

All samples were weighed on a microbalance to the nearest milligram. Typical sample sizes were 400 mg of post-clitellum earthworm, 500 mg of soil, and 500 mg of salamander tissue. Samples were placed in a Dounce glass homogenizer along with 0.1 gram of anhydrous sodium sulfate and manually mixed for 10 seconds in 3 ml of 1:3 acetone/hexane (v: v). After mixing, the samples were vortexed for 10 seconds and then transferred to a glass culturette tube. This was repeated 3 times with each sample and the homogenates for each sample were combined. The homogenate was then centrifuged for 8 minutes. After centrifugation, the supernatant was transferred to another culturette tube and 3 ml of 0.88% potassium chloride solution was added to the culturette for a Folch wash (Folch, Lees et al. 1957). The Folch wash removes interfering lipid compounds from the extractant. The sample was re-centrifuged for 8 minutes and the solvent layer

was removed. After transferring the solvent layer to another culturette, it was evaporated under nitrogen until dry.

A Florisil<sup>®</sup> column, made by filling a Pasteur pipette, was used for sample clean-up (Environmental Protection Agency 1994). Florisil<sup>®</sup>, magnesium silicate gel, is an adsorptive which is capable of binding to malathion until it is eluted with solvents. This allows for preferential release of malathion from the column while leaving behind co-extractants. The pipette was blocked at one end with glass wool to retain the Florisil<sup>®</sup>. After being filled with one gram of Florisil<sup>®</sup>, the pipette was topped with 0.1 gram of anhydrous sodium sulfate. The Florisil<sup>®</sup> column was prepared by the addition of 2 ml of hexane. After reconstitution in 200  $\mu$ l of hexane, the sample was transferred to the Pasteur pipette. 3 ml of hexane was passed through the Pasteur pipette, the hexane fraction was collected, and then discarded. The hexane fraction had been previously determined to be malathion free and reduces co-extractants. A second solvent wash of 6 ml of 1:19 acetone/hexane (v: v) eluted the majority of the malathion. An additional wash with 6 ml of 1:3 acetone/hexane (v: v) was used to ensure complete recovery. The second and third elutes were combined and a second Folch wash was performed. The solvent layer was removed and evaporated to dryness under nitrogen.

Extraction efficiencies were measured in earthworm and soil samples. Malathion, 0.1  $\mu$ g, was injected into earthworms (n=3) and added directly to soil (n=3) while in the Dounce homogenizer. The samples were extracted and ran on the gas chromatograph for quantification. Extraction efficiencies for both sets of samples were between 85-110%.

### *2.1 Gas Chromatographic Quantification*

Malathion burdens were determined using a gas chromatograph with an FID (Hewlett Packard 5890 Series II) and a DB-1 capillary column (30m by 0.32mm by  $\mu\text{m}$  from J&W Scientific) (Bavcon, Trebse et al. 2003). Hydrogen was used as the carrier gas and helium served as the makeup gas. Evaporated samples were reconstituted in 40  $\mu\text{l}$  hexane. Four  $\mu\text{l}$  of the reconstituted sample was injected in triplicate onto the column. The injector and detector were both set to 250° C. Initially, the purge valve was off and was turned on after one minute of run time. The oven was programmed to ramp from 60° C to 220° C at a rate of 40° C/min followed by a 2° C/min climb to 228° C. The oven temperature was held for thirty seconds at 220° C and 228° C. The run time was 8.9 minutes with malathion and malaoxon eluting at 6.5 and 6.2 minutes, respectively.

The injection of external standards confirmed the identity of the chromatographic peaks. Detection limits were calculated to be 0.04  $\mu\text{g}$  for malathion and 0.10  $\mu\text{g}$  for malaoxon using curves generated from standards provided by Restek Corporation (Bellafonte, Pennsylvania). Standard curves were made from malathion and malaoxon standards, 100  $\mu\text{g}/\text{ml}$  in methanol, in dilutions of 100  $\mu\text{g}/\text{ml}$ , 75  $\mu\text{g}/\text{ml}$ , 50  $\mu\text{g}/\text{ml}$ , 25  $\mu\text{g}/\text{ml}$ , and 10  $\mu\text{g}/\text{ml}$ . All standards were run weekly and in triplicate. All tissue burdens were calculated on a ppm or  $\mu\text{g}/\text{g}$  wet weight basis.

### *2.2 Acetylcholinesterase measurement*

A modified Ellman assay, a colorimetric procedure based on the reaction between acetylthiocholine (ASChI), 5,5'-Dithio-bis(2-nitrobenzoic acid) (DTNB), and any unbound cholinesterase in the sample was used to analyze salamander brain and

earthworm tissue cholinesterase activity (Ellman, Courtney et al. 1961; Willens 2005). Approximately 4 milligrams of salamander brain tissue or 100 milligram of earthworm tissue was homogenized in 80  $\mu\text{l}$  or 1000  $\mu\text{l}$ , respectively, of ice cold ( $4^{\circ}\text{C}$ ) Tris Buffer 8.0 with a hand-held electric homogenizer for 30 seconds. The samples were centrifuged for 8 minutes at 1000 G. The supernatant was removed with a glass pipette and kept on ice until assayed.

Electric eel acetylcholinesterase, 0.411 U/ $\mu\text{l}$ , Sigma-Aldrich (St. Louis, Mo) was used to generate standards curves by diluting the enzyme in deionized water to make concentrations of 0.411 U/ $\mu\text{l}$ , 0.2055 U/ $\mu\text{l}$ , 0.0822 U/ $\mu\text{l}$ , 0.02055 U/ $\mu\text{l}$ , and a blank. The standards and samples were prepared for analysis by combining 3 ml of DTNB (99 mg DTNB/liter of 0.05M Tris Buffer 7.4) and 20  $\mu\text{l}$  of sample/standard. This solution was kept on ice until addition of the substrate. Adding 100  $\mu\text{l}$  of acetylthiocholine (451 mg ASChI/ 10 ml deionized water) started the kinetic reaction. Immediately, the samples and standards were vortexed for 10 seconds and 100  $\mu\text{l}$  of the solution was loaded in triplicate onto a 96-well plate. A Spectramax 190® spectrophotometer, Molecular Devices (Sunnyvale, California) with Softmax Pro® software was used to quantify the resultant color change. The samples were run at 405 nm,  $25^{\circ}\text{C}$ , for 5 minutes with an absorbance measurement taken every 10 seconds. Linearity was achieved for the electric eel standards, salamander brain, and earthworm tissue samples between 43 and 144 seconds. Results are reported in  $\mu\text{mol}/\text{min}/\text{g}$  of brain for the salamander samples or  $\text{nmol}/\text{min}/\text{g}$  of protein for the earthworm samples. The protein content of the earthworms was measured using the micro-BCA assay.

### 2.3 *Micro-BCA™ Protein Assay*

The micro-BCA assay is designed to detect protein concentrations of dilute samples. It is a colorimetric assay, which measures the reaction of bicinchoninic acid, BCA, with copper,  $\text{Cu}^{+1}$ , which has been reduced from  $\text{Cu}^{+2}$  by the proteins in the sample. The reaction occurs in an alkaline environment and the chelation of BCA and copper results in a color change which can be read at 562 nm (Smith, Krohn et al. 1985).

Samples, earthworm tissue or salamander tissue, were prepared by homogenizing 0.1 gm of sample in 2 ml of protein extraction reagent with an electric tissue grinder for one minute. Homogenization occurred in a 5 ml cryovial. The cryovial was centrifuged for 8 minutes at 1000x G. The supernatant was removed and placed into a 2 ml cryovial. Disposables were used for the entire assay to reduce environmental protein contamination.

Bovine serum albumin, 2 mg/ml, was the protein standard for the assay. The standard was diluted into Tris Buffer 7.4 to make standards of 200  $\mu\text{g/ml}$ , 100  $\mu\text{g/ml}$ , 50  $\mu\text{g/ml}$ , 25  $\mu\text{g/ml}$ , and a blank. The Pierce kit provides the working reagent for the assay which, consists of an alkaline solution (sodium carbonate, sodium bicarbonate, and sodium tartate), 4% bicinchoninic acid, and 4% cupric sulfate. The working reagent is prepared by mixing 25 parts alkaline solution: 24 parts bicinchoninic acid: 1 part cupric sulfate. The assay was performed by adding 150  $\mu\text{l}$  of the working reagent and 150  $\mu\text{l}$  of the sample or standard into a 96-well microplate. All samples were run in triplicate. The plate was incubated at 38° C for 2 hours, cooled to room temperature, and then placed in the spectrophotometer, Spectramax 190® , Molecular Devices (Sunnyvale, California).

Absorbance was read at a single time point at 562 nm. All protein concentrations are reported in mg of protein per gram of sample.

#### 2.4 *Lipid assay*

The lipid content of various salamander tissues was determined in an attempt to reduce variability in malathion concentrations in specific tissues by providing an alternative method to sample weight for standardization. Prior to determination of lipid content, glass culturettes were dried in a dessicator for one month until their weight no longer changed. The tissue samples were prepared for lipid extraction by homogenizing approximately 0.1 gm of tissue in 2 ml of solvent, 2:1 dichloromethane: methanol (v: v) in a Dounce homogenizer (Auel and Hagen 2005). The homogenate was added to a culturette and a folch wash was performed with the addition of 1.5 ml of 0.88% potassium chloride in deionized water (Folch, Lees et al. 1957). The sample was centrifuged for 8 minutes at 1000 G. The hydrophilic phase was removed with a glass pipette and the lipophilic phase, solvent, was transferred into the pre-weighed, dehydrated culturette. The solvent was evaporated under nitrogen to dryness. The culturette was re-weighed and lipid content was calculated on a  $\mu\text{g/g}$  basis.

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### 3: A Comparison of Two Exposure Systems to Apply Malathion to

#### *Lumbricus terrestris* L.

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### 3.0 Introduction

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 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<sub>50</sub> and EC<sub>50</sub> 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.

### 3.1 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 grams 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 Cyanamid, 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 ( $0 \mu\text{g}/\text{cm}^2$ ,  $1 \mu\text{g}/\text{cm}^2$ ,  $2 \mu\text{g}/\text{cm}^2$ ,  $5 \mu\text{g}/\text{cm}^2$ ,  $50 \mu\text{g}/\text{cm}^2$ ,  $100 \mu\text{g}/\text{cm}^2$ ) were based upon surface area measurements of the experimental containers (filter paper, soil). This range of doses was determined by rolling  $\text{EC}_{50}$  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 culture tubes (18 cm long and 1.8 cm in diameter) and a cooler holding temperature ( $10^\circ\text{C}$ ) than recommended by the OECD (OECD, 1984). Whatman filter paper #1 (VWR) was cut to a rectangular shape, surface area of  $108 \text{ cm}^2$ , 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  $10^\circ\text{C}$  for 72 hours.

A rolling  $\text{EC}_{50}$  trial ( $0 \mu\text{g}/\text{cm}^2$  to  $1250 \mu\text{g}/\text{cm}^2$ ), 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  $\text{EC}_{50}$  trials.

5 worms were exposed by filter paper contact to each of 6 malathion concentrations: 0  $\mu\text{g}/\text{cm}^2$ , 1  $\mu\text{g}/\text{cm}^2$ , 2  $\mu\text{g}/\text{cm}^2$ , 5  $\mu\text{g}/\text{cm}^2$ , 50  $\mu\text{g}/\text{cm}^2$ , and 100  $\mu\text{g}/\text{cm}^2$ . The worms were assessed for the development of clinical signs after 72 hours of exposure. The earthworms were then euthanized by immersion in hot water (40<sup>0</sup> 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  $\text{cm}^2$ . 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<sup>0</sup> C for 72 hours. After 72 hours of exposure, the earthworms were examined for clinical signs, euthanized in hot water, and examined for malathion burdens.

Extraction of malathion from 400 milligram 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 seconds. 100 milligrams 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. The supernatant was evaporated to dryness, rehydrated in 200  $\mu\text{l}$  of hexane,

and then passed through a Pasteur pipette filled with 1 gram 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 ranged between 85-103%. The detection limits for malathion and malaoxon were 0.04 µg 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 (30m by 0.32mm 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 4µl 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 minutes with malathion and malaoxon eluting at 6.8 and 6.2 minutes, respectively.

All statistical analyses were performed using Prism® version 4 from Graphpad ([www.graphpad.com](http://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

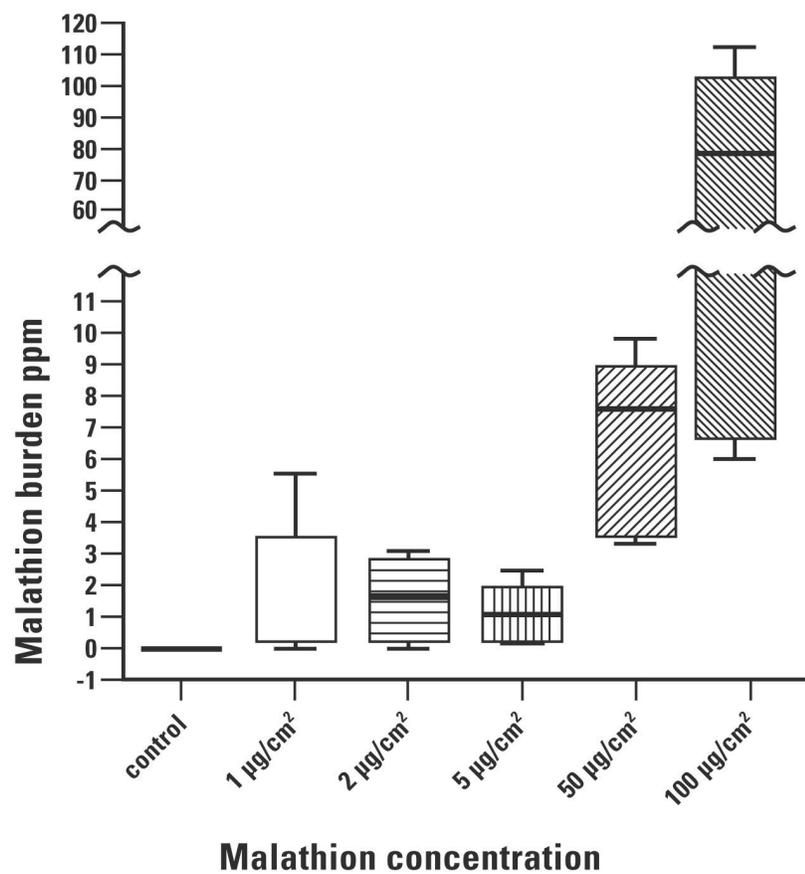
multiple comparison test was employed to determine which of the exposure concentrations ( $1 \mu\text{g}/\text{cm}^2$ ,  $2 \mu\text{g}/\text{cm}^2$ ,  $5 \mu\text{g}/\text{cm}^2$ ,  $50 \mu\text{g}/\text{cm}^2$ , and  $100 \mu\text{g}/\text{cm}^2$ ) varied significantly from the control. To determine 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.

### 3.2 Results and Discussion

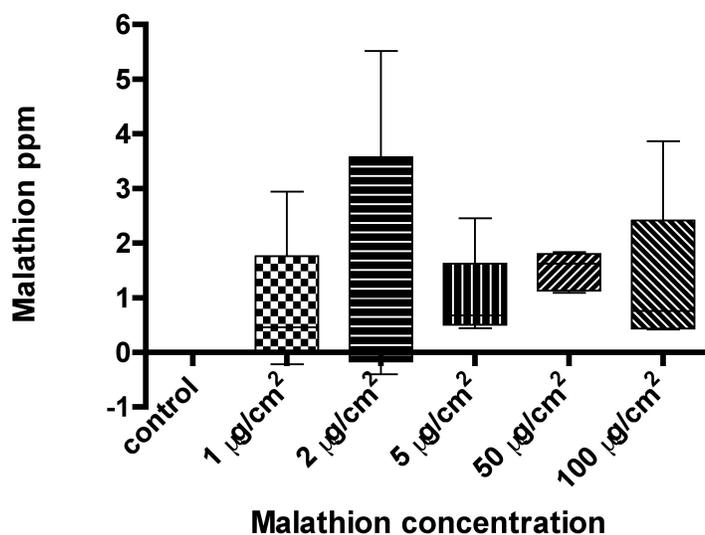
Malathion was detectable in earthworm tissue; however, there was a lack of a demonstrable linear or sigmoidal dose-response for either the filter paper or soil exposures. There were statistically significant differences in the resultant malathion body burdens between exposure concentrations at an alpha 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 3.2). The post-test (Dunn's multiple comparison) was able to differentiate the control group from the  $50 \mu\text{g}/\text{cm}^2$  (p-value <0.01) and  $100 \mu\text{g}/\text{cm}^2$  treatment groups (p-value < 0.001) after exposure to malathion by filter paper (Fig 3.1) and the  $50 \mu\text{g}/\text{cm}^2$  concentration for the soil exposure (p-value <0.01) (Fig 3.2). 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 ( $1 \mu\text{g}/\text{cm}^2$ ,  $2 \mu\text{g}/\text{cm}^2$ ,  $5 \mu\text{g}/\text{cm}^2$ ), there were no significant differences noted with the Mann-Whitney test between the soil and filter paper exposure. For  $50 \mu\text{g}/\text{cm}^2$ , filter paper exposure resulted

in statistically significant higher worm body burdens of malathion: Mann-Whitney (p-value=0.0079). At 100  $\mu\text{g}/\text{cm}^2$ , the Mann-Whitney test demonstrated a significant difference between 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 a concentrations between 2 and 5  $\mu\text{g}/\text{cm}^2$ . Soil exposure did not result in clinical signs in half the animals at the tested exposure concentrations.



**Figure 3.1.** 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



**Figure 3.2** 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 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<sub>50</sub> 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), heterogeneity 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 ( $K_d$ ) 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  $14.8 \mu\text{g}/\text{cm}^2$  and  $0.27 \mu\text{g}/\text{cm}^2$ , 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<sub>50</sub> in earthworm species with the following trend: increasing LC<sub>50</sub> 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.

### ***Acknowledgement***

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**Chapter 4: Assessment of the Effect of Varying Soil Organic Matter Content on the Bioavailability of Malathion to the Common Nightcrawler, *Lumbricus terrestris***

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#### 4.0 Introduction

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, Yeh et al. 2002), including organophosphates, and *Lumbricus* are exposed to xenobiotics in soils with wide ranging organic matter content (Haines and Uren 1990). *Lumbricus 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).

Malathion, a commonly applied organophosphate, has been demonstrated to cause acute toxicity in earthworms as evident by LC<sub>50</sub> values within the range of environmental application (Roberts and Dorough 1984; Panda and Sahu 2002) and cholinesterase suppression after malathion exposure (Booth, Hodge 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, Brekke 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, adsorb into soil organic matter, and the degradation half-life of the xenobiotic of interest (Wauchope, Yeh et al. 2002). Malathion, with a  $\log K_{oc} = 3.2$ , (Zambonin, Lostito et al. 2002) is moderately adsorbed to soil and as a phosphorothionothiolate 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, Lostito et al. 2002) and other studies show no evidence of absorption to organic matter even in soils with high organic matter content (Rajukkannu, Basha et al. 1985; Zambonin, Lostito et al. 2002). This study assesses malathion bioavailability in soils of differing organic matter content by measuring the malathion body burdens and cholinesterase suppression in experimentally exposed *Lumbricus terrestris*.

#### 4.1 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 and 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 4.1). The pH of both soils was 6.8, determined with a soil pH meter (Kel instrumentation corporation, Wyckoff, NJ.)

**Table 4.1.** Soil characteristics of Scott's garden soil. Mean values. n=3

Sample I.D.	% 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

*Lumbricus* were procured from National Association of Supplies Bait and Tackle, Marblehead Ohio. The worms were maintained at 10<sup>0</sup> 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 three-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.

The exposure container was a 1000 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<sup>2</sup> and applied by fine mist with a thin layer chromatograph spray. For the control containers, acetone alone was applied to the soil. The soil in each container was thoroughly mixed by hand for 3 minutes 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 3 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<sup>0</sup> C, and maintained on a 12-hour 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<sup>2</sup> of malathion in 8% organic matter soil, and 3) high organic matter exposure, 50 µg/cm<sup>2</sup> in 55 % organic matter soil. Exposures lasted 72 hours, after which, worms were euthanized in scalding water, 40<sup>0</sup> 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, Courtney 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 seconds. One hundred mg of the post-clitellum worm section was homogenized for 10 seconds 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. 20 µl of the supernatant was used in the assay. Absorbance was read at 405 nm on a spectrophotometer, Spectramax 190, Molecular Devices, (Sunnydale, California). The micro-BCA assay, Pierce Chemical (Rockford, Illinois), was used to standardize all results to total protein content. Results are reported in nmol of cholinesterase hydrolyzed per minute per mg of total protein.

The remainder of the post-clitellum *Lumbricus* samples, not used in the cholinesterase assay, and the soil samples were frozen at  $-80^{\circ}\text{C}$  for no longer than two 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 one gram of worm or soil was homogenized with a Dounce homogenizer in 1:4 Acetone: Hexane (v:v) for 30 seconds. The homogenate was pelleted by centrifugation and the supernatant removed. The supernatant was further extracted by a Folch wash (Folch, Lees et al. 1957) to remove interfering lipids and then evaporated under nitrogen. After evaporation, the sample was reconstituted in 200  $\mu\text{l}$  of hexane and then eluted from a glass pipette filled with one gram of Florisil  $\text{\textcircled{R}}$ , 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  $\mu\text{l}$  of hexane and 4  $\mu\text{l}$  was injected into a Hewlett Packard 5890 series II gas chromatograph with a DB-1 column, 30 m x 0.35 mm X 0.25  $\mu\text{m}$  from J&W Scientific (Folsom, California). The FID detector and injector temperatures were set to  $250^{\circ}\text{C}$ . The column temperature was ramped from  $60^{\circ}\text{C}$  to  $220^{\circ}\text{C}$  at a rate of  $40^{\circ}\text{C}/\text{min}$  and then at a rate of  $2^{\circ}\text{C}/\text{min}$  from  $220^{\circ}\text{C}$  to  $228^{\circ}\text{C}$ . The total run time was 8.9 minutes. Malathion eluted at 6.5 minutes and malaoxon eluted at 6.2 minutes. No malaoxon was detected in the samples. The limit of detection for

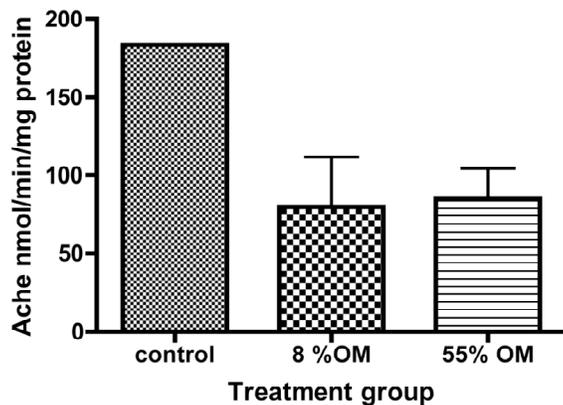
malathion with this method was 0.04  $\mu\text{g}$  and 0.10  $\mu\text{g}$  for malaoxon as determined by standard curves using an external standards, 100  $\mu\text{g}/\text{ml}$  in methanol diluted into appropriate concentrations, Restek Corporation (Belafonte, Pennsylvania).

All statistical analyses were performed using Prism®, Graphpad (San Diego, California). 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 2 treatment groups. Significance was set to an alpha of 0.05.

#### 4.2 Results and Discussion

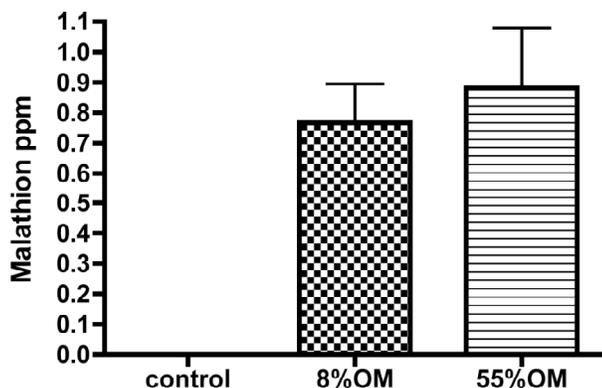
The control exposures were run on soils with 8% and 55% organic matter content. When analyzed with a Mann-Whitney test, there were no significant differences in cholinesterase levels between the controls, p-value 0.700, or malathion burdens, p-value 1.00. The control samples were condensed as a single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

Exposed worms demonstrated cholinesterase inhibition of approximately 50% compared to unexposed controls (Fig 4.1). There were no statistically significant differences between the treatments, Kruskal-Wallis, p-value = 0.08. A Mann-Whitney test was used to compare the two exposed groups, 8% OM and 55% OM, and showed that there were no significant differences in cholinesterase activity with a p-value of 1.00.



**Figure 4.1.** Median levels of cholinesterase in *L. terrestris* when exposed in soils with differing organic matter content. Error bars represent the interquartile range.

Malathion body burdens were detected in all exposed worms and none of the unexposed worms, Fig 4.2. There were no statistically significant differences in malathion burdens between the two exposed groups, 8% OM, median 0.70 ppm, and 55% OM, median 0.78 ppm, when using a 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.



**Figure 4.2** Median xenobiotic burdens in *L. terrestris* when exposed in soils with differing organic matter content. Error Bars represent the interquartile range.

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, Cousins 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 phenomenon. Bioavailability was equivalent as measured by body burdens and degree of cholinesterase suppression in our report.

Though soil organic matter content did not appear to affect the bioavailability of malathion to *Lumbricus 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, Briggs 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 *Lumbricus terrestris* is composed of at least 24 layers of collagen (Coggeshall 1966), and is a substantial deterrent to chemical absorption. Malathion is moderately lipophilic, relatively small, (molecular weight 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. *Lumbricus terrestris*'s 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, Schroeder 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, Schroeder 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 *Lumbricus 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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## **Chapter 5: A Comparison of Cholinesterase Levels and Xenobiotic Body Burdens in Two Species of Earthworms, *Lumbricus terrestris* and *Eisenia foetida*, exposed to Malathion by Filter Paper Contact**

### 5.0 Abstract

*Lumbricus terrestris* and *Eisenia foetida* were exposed to 5 µg/cm<sup>2</sup> of malathion by filter paper contact. Tissue cholinesterase activity, before and after exposure to malathion, was measured in both species. *L. terrestris* had significantly higher levels of basal enzyme activity and exhibited only 50% cholinesterase suppression after malathion exposure compared to *E. foetida*, which demonstrated 90% inhibition of cholinesterase. Both species had detectable levels of malathion in their tissues after 72 hours of exposure. There were no differences in malathion burdens between the two species.

### 5.1 Introduction

Earthworms are frequent subjects of toxicology studies because of their agricultural importance (Haines and Uren 1990; Hendrix 1995) and the risk of inadvertent toxicity from terrestrially applied pesticides (Fisher 1984). Earthworm species maintain soil structure (Lavelle 1988) and reduce the half-life of xenobiotics in soil (Edwards and Bohlen 1992; Lydy and Linck 2003) through carbon mineralization (Scheu 1987). The application of xenobiotics to agricultural fields for the control of pests can reduce earthworm numbers, thereby negating the beneficial effects of their presence.

Malathion, an organophosphate, is one of the most frequently applied pesticides in the United States and approximately 17 million pounds are applied annually (Environmental Protection Agency 2000). As a result, resident earthworm populations in agricultural settings can be exposed to malathion. Multiple researchers have reported

clinical signs of toxicity in earthworms after exposure to organophosphates. The symptoms of poisoning appear to be consistent across species and include coiling, mucus secretion, and the development of ulcerations especially on the clitellum (Heimbach 1984; Edwards and Bohlen 1992; Rao and Kavitha 2004). It is assumed that these symptoms are because of organophosphates' primary toxic mechanism, the irreversible inhibition of acetylcholinesterase (Eto 1974). However, organophosphates are capable of inhibiting multiple tissue cholinesterases, including acetylcholinesterase and butyrylcholinesterase, and it is often difficult to isolate the effects of exposure on these enzymes.

There appear to be moderate differences in sensitivity among earthworms to the cholinesterase-inhibiting activity of organophosphates (Gilman and Vardanis 1974; Stenersen, Brekke et al. 1992). The differences in species sensitivity are believed to be a direct effect of the type and number of cholinesterases possessed by each species (Stenersen 1980; Stenersen, Brekke et al. 1992), presence or absence of metabolizing enzymes (Chambers, Tangeng et al. 1994), or because of differences in xenobiotic absorption and elimination rates (Gilman and Vardanis 1974). Studies have not been done to assess whether *Lumbricus terrestris* and *Eisenia foetida* exhibit differences in sensitivity to malathion exposure, though differential sensitivities have been demonstrated between these two species when exposed to other cholinesterase-inhibiting pesticides (Gilman and Vardanis 1974).

## 5.2 Materials and methods

*Lumbricus terrestris*, National Association of Supplies Bait and Tackle (Marblehead, Ohio) and *Eisenia foetida*, culture stock (University of South Carolina,

Aiken) were maintained in polyethylene boxes (32 cm by 13.5 cm) at 10° C. The earthworms were kept on a substrate of Scott's ® garden soil (55% OM, 70% sand, 20% silt, 10% clay, pH 6.8). The boxes were filled with 900 g of soil, hydrated by the addition of 100 ml of deionized water, and covered with moistened cheesecloth and aluminum foil. The foil was 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 a well-developed clitellum were used for the assays.

Malathion (96.5% purity) stock, American Cyanamid (Wayne, New Jersey) was stored in a polyethylene bottle at 4° C. Dilutions were made in AR grade acetone, Mallinkroft Chemicals (Phillipsburg, New Jersey) to a concentration of 0 µg/cm<sup>2</sup> or 5 µg/cm<sup>2</sup> based upon surface area measurements of the experimental container. Both species were exposed to malathion in large culturette tubes (18 cm long and 1.8 cm in diameter). Whatman filter paper #1, VWR (West Chester, Pennsylvania) was cut to a rectangular shape with a surface area of 108 cm<sup>2</sup> 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 to prevent solvent effects. The filter paper was 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 hours. Nine worms, control and exposed, were assayed for the determination of malathion tissue burdens and five worms, control and exposed, were assayed for cholinesterase activity.

An identical procedure was used to extract malathion from both earthworm species. Four hundred milligram of each worm was independently homogenized in a Dounce glass homogenizer along with 0.1 gram of anhydrous sodium sulfate and manually mixed for 10 seconds in 3 ml of 1:3 acetone/hexane (v: v). This was repeated 3 times with each sample. The homogenates from each sample were combined and centrifuged for 8 minutes. After centrifugation, the supernatant was transferred and a Folch wash (Folch, Lees et al. 1957) was performed to remove interfering compounds from the extractant. The sample was re-centrifuged for 8 minutes and the solvent layer was removed, transferred, and evaporated under nitrogen until dry. Florisil<sup>®</sup>, in a Pasteur pipette, was used for sample clean-up (Environmental Protection Agency 1994). The evaporated sample was reconstitution in 200  $\mu$ l of hexane and transferred to the Pasteur pipette. Malathion was eluted with 6 ml of 1:19 acetone/hexane (v: v) and a second wash of 6 ml of 1:3 acetone/hexane (v: v) was used to ensure complete recovery. Both elutes were combined and a second Folch wash was performed. The solvent layer was removed and evaporated to dryness under nitrogen. Extraction efficiency for this method is between 85-110%.

Malathion burdens were determined using a gas chromatograph with an FID (Hewlett Packard 5890 Series II) and a DB-1 capillary column (30m by 0.32mm by 0.25 $\mu$ m from J&W Scientific) (Bavcon, Trebse et al. 2003). Dried samples were reconstituted in 40  $\mu$ l of hexane. Four  $\mu$ l of the reconstituted sample was injected in triplicate onto the column. The injector and detector were both set to 250<sup>o</sup> C. Initially, the purge was off and was turned on again after one minute of run time. The oven was programmed to ramp from 60<sup>o</sup> C to 220<sup>o</sup> C at a rate of 40<sup>o</sup> C/min followed by a 2<sup>o</sup> C/min

climb to 228° C with a thirty second hold at 220° C and 228° C. The run time was 8.9 minutes with malathion eluting at 6.5 minutes. External standards, Restek Corporation (Bellafonte, Pennsylvania), were used to confirm the identity of the chromatographic peaks. Detection limits were 0.04 µg for malathion. All tissue burdens were calculated on a ppm or µg/g wet weight basis.

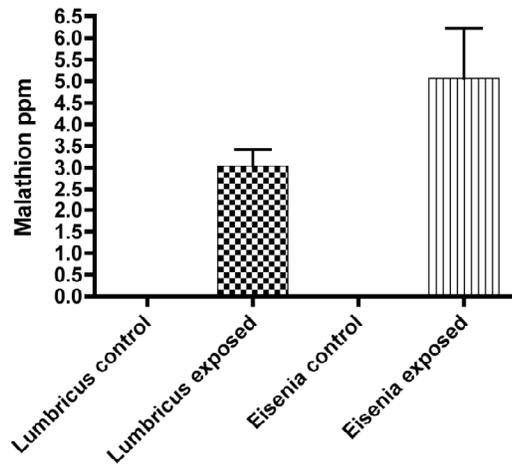
A modified Ellman assay, a colorimetric procedure based on the reaction between acetylthiocholine (ASChI), 5,5'-Dithio-bis(2-nitrobenzoic acid) (DTNB), and any unbound cholinesterase in the sample was used to analyze earthworm tissue cholinesterase activity (Ellman, Courtney et al. 1961; Willens 2005). Approximately 100 milligram of earthworm tissue was homogenized in 1000 µl of ice cold (4° C) Tris Buffer 8.0 with a hand-held electric homogenizer for 30 seconds. The samples were centrifuged for 8 minutes at 1000X G. The supernatant was removed with a glass pipette, and kept on ice until assayed. Electric eel acetylcholinesterase, 0.411 U/µl, Sigma-Aldrich (St. Louis, Mo) was used to generate standards curves. The standards and samples were prepared for analysis by combining 3 ml of DTNB (99 mg DTNB/liter of 0.05M Tris Buffer 7.4) and 20 µl of sample/standard. This solution was kept on ice until addition of the substrate. Adding 100 µl of acetylthiocholine (451 mg ASChI/ 10 ml deionized water) started the kinetic reaction. One hundred µl of the solution was loaded in triplicate onto a 96-well plate. A Spectramax 190® spectrophotometer, Molecular Devices (Sunnyvale, California) with Softmax Pro® software was used to quantify the resultant color change. The samples were run at 405 nm, 25° C for 5 minutes with an absorbance measurement taken every 10 seconds. Linearity was achieved for the electric eel standards and earthworm tissue samples between 43 and 144 seconds. Results are

reported in nmol/min/g of protein for the earthworm samples. The protein content of the earthworms was measured using the micro-BCA assay.

All statistical analyses were performed using Prism® version 4 from Graphpad ([www.graphpad.com](http://www.graphpad.com)). The Mann-Whitney test was used to compare earthworm malathion body burdens between exposed earthworms, *Lumbricus* and *Eisenia*, and activities of the cholinesterase enzyme between the following treatment groups: *Lumbricus* control versus exposed, *Eisenia* control versus exposed, and *Lumbricus* exposed versus *Eisenia* exposed. For all tests, a p-value of 0.05 or less was deemed statistically significant.

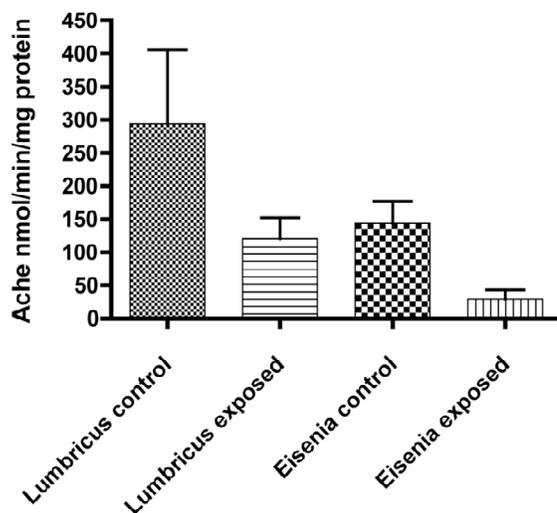
### 5.3 Results

The control worms had no detectable levels of malathion in their tissues. Exposed *Lumbricus terrestris* had median malathion body burdens of 3.31 ppm with a range of 1.27 to 4.42 ppm (Fig 5.1) and *Eisenia foetida*'s exposed to malathion had median body burdens of 3.41 ppm with a range of 1.46 to 12.33 ppm (Fig 5.1). When compared by the Mann-Whitney test, the malathion body burden between the two species was not significantly different with a p-value of 0.26 (Fig 5.1).



**Figure 5.1** A comparison of malathion body burden in *E. foetida* and *L. terrestris*. Results are shown as medians and interquartile ranges. n=9

There were significant differences in basal cholinesterase activity between *L. terrestris* and *E. foetida*, Mann-Whitney, p-value 0.0317 (Fig. 5.2). Both species exhibited significant cholinesterase inhibition, Mann-Whitney, p-value 0.02 (Fig 5.2). The degree of inhibition, 90%, in exposed *E. foetida* was significantly higher than in exposed *Lumbricus*, 50%, Mann-Whitney, 0.032 (Fig. 5.2).



**Figure 5.2** Cholinesterase levels in *L. terrestris* and *E. foetida* before and after malathion exposure. Results are represented by median values and interquartile ranges.

#### 5.4 Discussion

While there were no statistically significant differences in malathion body burdens between the two species, *Eisenia* had higher malathion body burdens than *Lumbricus* which suggests a greater degree of absorption. The primary route of absorption when exposed to malathion by filter paper contact is through the integument. The integument of *L. terrestris* and *E. foetida* is bordered by an external cuticular layer made of collagen, 80% protein and 20% polysaccharides (Coggeshall 1966; Burke 1974), which serves to reduce dehydration and absorption of lipophilic substances from the environment (Watson 1958; Baccetti 1967). *Lumbricus terrestris*' cuticle is composed of greater than 24 layers of collagen (Coggeshall 1966) where as *Eisenia foetida*'s cuticular layer has only 10 layers of collagen (Burke 1974). The decreased comparative thickness of the cuticle layer in *E. foetida* may increase that species susceptibility to xenobiotic absorption.

In addition to a potential faster rate of malathion absorption, *Eisenia* appears more sensitive to malathion toxicity than *Lumbricus* because of a greater degree of cholinesterase suppression after malathion exposure. There is also evidence for a higher innate cholinesterase activity in *L. terrestris* (Fig 5.2). The Ellman assay, which uses acetylthiocholine as a substrate is not specific for acetylcholinesterase (Ellman, Courtney et al. 1961; Sanchez-Hernandez and Moreno Sanchez 2002). Butyrylcholinesterase and acetylcholinesterase have similar amino acids in their active binding sites, resulting in both enzymes being able to hydrolyze acetylthiocholine (Cokugras 2003). When using the Ellman assay, the two esterases cannot be differentiated without the use of selective inhibitors (Kolf-Clauw, Jez et al. 2000; Strum, Wogram et al. 2000). In general,

butyrylcholinesterase has a lower binding affinity for acetylthiocholine than does acetylcholinesterase, however, in fish, it has been demonstrated that they hydrolyze acetylthiocholine equally well (Strum, Wogram et al. 2000). The relative contributions of acetylcholinesterase and butyrylcholinesterase to the overall cholinesterase activity demonstrated in *L. terrestris* and *E. foetida* were not determined. The higher cholinesterase activity documented in *Lumbricus* may be primarily due to levels of butyrylcholinesterase rather than acetylcholinesterase.

Regardless of the rate of absorption and binding capacity of malathion for specific esterases in *L. terrestris* and *E. foetida*, the apparent sensitivity of *Eisenia* to malathion toxicity (Fig 5.2) could be a direct result of the earthworms' metabolizing capacity. The presence of carboxylesterases (Ciesielski, Loomis et al 1994) and arylesterases (Chamber, Tangeng et al 1994) have been shown to affect toxicity after organophosphate exposure. Carboxylesterases competitively compete with acetylcholinesterase for the substrate, malathion, thereby reducing toxicity. Arylesterases are capable of directly hydrolyzing organophosphates. It is unknown whether *L. terrestris* or *E. foetida* have these metabolizing enzymes, however, their absence could result in increased sensitivity to malathion toxicity.

*Eisenia foetida* appears to be more sensitive to malathion exposure than *L. terrestris* as documented by a 90% reduction in total cholinesterase after exposure. This increase in sensitivity may be because of increased malathion absorption, the absence of protective metabolic enzymes, or differences in relative levels of butyrylcholinesterase and acetylcholinesterase. At this time, it is difficult to resolve the apparent sensitivity difference to malathion exposure in *Lumbricus terrestris* and *Eisenia foetida*.

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## **Chapter 6: Development of a Dynamic Pharmacokinetic Model to Estimate Bioconcentration of Xenobiotics in Earthworms**

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## 7.0 Abstract

A simple, dynamic pharmacokinetic model was developed to predict bioconcentration of organic contaminants in earthworms. The model was parameterized experimentally by placing *Lumbricus terrestris* in soil contaminated with 200  $\mu\text{g}/\text{cm}^2$  of malathion. The toxicokinetics of malathion uptake, depuration, and soil degradation were measured. After parameterization, the model was able to accurately predict the bioconcentration factor (BCF) of malathion at steady state. Sensitivity analyses were performed and the rate of absorption was determined to be the most sensitive parameter. Varying elimination, malathion degradation, and soil exposure by 25 fold did not result in the bioconcentration of malathion. An increase in absorption by 25 fold did result in the bioconcentration of malathion. Previously published pharmacokinetic studies on xenobiotics with  $\log k_{ow}$ s ranging up to 8.05 were used to test the predictive capacity of the model. The model was able to predict from 83-105% of the experimentally derived bioconcentration factors.

## 6.1 Introduction

Earthworms are often used as model organisms to study the effects of xenobiotics because of their ease of use in laboratory settings, their agricultural importance (Haines and Uren 1990; Hendrix 1995), and value in environmental quality assessment (Beyer and Cromartie 1987; Shipitalo, Edwards et al. 1994). Though rarely the target of pesticide use, earthworms can experience inadvertent toxicity from terrestrially applied xenobiotics (Edwards and Bohlen 1992). Their significance in toxicology and risk

assessment has led to the development of standardized toxicity assays to evaluate the effect of soil applied xenobiotics on earthworm mortality (Roberts and Dorough 1984; Panda and Sahu 2002, Dean-Ross 1983; Edwards and Bohlen 1992)), reproduction (Senapati, Biswal et al. 1991; Schaefer 2004), metabolism (Brown, Long et al. 2004), and the risk of bioaccumulation (Stephenson, Wren et al. 1997; Johnson, Franke et al. 1999). Knowledge of the toxicokinetics of terrestrially applied xenobiotics in earthworms is necessary to accurately predict the risks of bioconcentration and bioaccumulation (Van Gestel and Weeks 2004) on earthworm populations and ecological communities.

Most pharmacokinetic studies in earthworms assume that the movement of xenobiotics from soil into earthworms is a three-phase equilibrium process with equilibrium occurring first between soil and soil water and then absorption of the xenobiotic into earthworm tissues from the soil water (Connell and Markwell 1990). A non-dynamic, mechanistic approach has been used to model the three-phase equilibrium hypothesis, but it yielded a 5-6 fold over estimation of bioconcentration (Jager 1998). This is likely because the model was parameterized with hydrophobic chemicals that have long half-lives, therefore steady state was not reached in all cases, which is necessary to accurately estimate bioconcentration. A criticism of three-phase equilibrium based modeling is that it may lack sufficient precision to predict bioconcentration. It also includes a compartment that cannot be parameterized by data readily collected from standard experimental designs.

Linear and multiple regression models can accurately predict the bioconcentration of xenobiotics in earthworms (Sample, Suter et al. 1999). However, regression models are not easily adapted to organic compounds other than the ones used to parameterize the

model. Physiologically based dynamic pharmacokinetic models have been used to study the toxicokinetics of percutaneous absorption of hydrophobic organic compounds (Brown and Hattis 1989; Shatkin and Brown 1991). These models were not parameterized experimentally, relied heavily on previously published diffusion coefficients, and tended to under predict tissue xenobiotic burdens. Model theorists have suggested that prediction variability could be resolved by decreasing the number of model parameters (Woodruff, Bois et al. 1992) and by reaching steady state during parameterization. It should not be necessary to model all phases of the equilibrium process to predict bioconcentration potential based on reasonably obtainable experimental data. Determination of soil concentrations of xenobiotics by extraction should encompass both the soil and soil pore water compartments in controlled experiments, and therefore serve appropriately as the abiotic component for calculation of a bioconcentration factor.

We developed a two-compartment non-physiological based dynamic model to predict bioconcentration of xenobiotics from soil into earthworms. The model reduces the three-phase equilibrium hypothesis by combining the soil and soil-water into a single compartment. The earthworm is considered in its entirety, rather than as separate tissues. This decreased the number of parameters, and increased model precision, when compared to previous dynamic models designed to reduce variability and underestimation of bioconcentration.

Malathion, an organophosphate, was chosen as the parameterizing xenobiotic because it is not considered bioaccumulative;  $\log K_{ow} = 2.89$ , half-life of 1-10 days, and has successfully been used to parameterize models in humans (Bouchard, Gosselin et al.

2003). The low risk of bioconcentration makes malathion a good test chemical for the model because of the capacity to assess the model's limitations. Additionally, the short half-life of malathion eases parameterization of the mathematical model because steady state can be reached quickly allowing for more accurate measurement of absorption and elimination.

## 6.2 Methodology

### 6.2.1 Description of model

A dynamic minimalist model to predict xenobiotic accumulation of malathion in earthworms was established using STELLA® software (Isee systems, Lebanon, New Hampshire) (Figure 6.1). Mathematical relationships between the two compartments (xenobiotic soil exposure and earthworm), three kinetic parameters (degradation rate, absorption rate, elimination rate), and one result chamber (BCF) were defined using the following definitions and metrics (Figure 6.2): The xenobiotic soil exposure compartment was the amount of xenobiotic applied to the soil in ppm with a specific degradation rate or half-life. A surrogate for bioavailability, the xenobiotic soil compartment was used to represent the extractable portion of the applied chemical (Lanno, Wells et al. 2004). The rate of absorption ( $k_a$ ) and elimination ( $k_e$ ) were reported in ppm/hr. Absorption rate was considered dependent on the xenobiotic's octanol:water coefficient ( $\log k_{ow}$ ) and the absorption route, percutaneous and/or oral. Elimination rate included all forms of excretion and metabolism and was also contingent upon a chemical's  $\log k_{ow}$ .

The earthworm compartment was a measure of the total xenobiotic burden over time and based upon the soil input (Xenobiotic Soil Exposure\* $k_a$ ) minus the elimination (Earthworm\* $k_e$ ). This compartment distinguished between contaminants that were absorbed into earthworm tissues and contaminants that were in the digestive system because the gastrointestinal tract was stripped of all material prior to analysis. The result chamber, BCF (bioconcentration factor), was determined by dividing the concentration of the xenobiotic in the earthworm (Earthworm) by the concentration in soil (Xenobiotic Soil Exposure) at steady state.

#### 6.2.2 Model Input Data

*Lumbricus terrestris*, the parameterization species, were exposed to malathion through soil contact and the uptake, depuration, and malathion degradation in the soil were measured. All model parameters were calculated at steady state. The xenobiotic soil exposure was set to the initial malathion application, 200  $\mu\text{g}/\text{cm}^2$  or 36.18 ppm as determined by extraction and gas chromatography.

*Lumbricus terrestris* (common nightcrawler) the parameterization species, were obtained from National Association of Bait and Supply, Marblehead Ohio. The earthworms were maintained in polypropylene containers, 32 cm by 13.5 cm, on 900 ml of Scott's® garden soil at 10<sup>0</sup> C. Hydration was provided by the addition of 100 ml of deionized water as needed to maintain approximately 60% soil saturation. The exposure containers were 1000 ml beakers. The containers were filled with 600 ml of Scott's® garden soil (55% organic matter, 70% sand, 20% silt and 10% clay, pH= 6.8). All exposure containers were kept in an incubator at 10<sup>0</sup> C with a 12 hour light/dark cycle.

*Lumbricus terrestris*, were exposed to malathion through soil contact and the uptake, depuration, and malathion degradation in the soil were measured. All model parameters were calculated at steady state. The xenobiotic soil exposure was set to the initial malathion application,  $200 \mu\text{g}/\text{cm}^2$  or 36.18 ppm as determined by extraction and gas chromatography. The earthworms were exposed to  $200 \mu\text{g}/\text{cm}^2$  malathion (36.18 ppm). This concentration of malathion was used because it resulted in reliable and minimally variable earthworm body burdens in pilot studies. Five replicates of the exposure and control containers were prepared. Malathion was diluted in acetone and applied to the soil with a thin-layer chromatography sprayer. The control container's soil was sprayed with acetone only. All soil, control and exposed, was mixed by hand for 2 minutes to reduce chemical heterogeneity and the acetone was evaporated overnight prior to introduction of the earthworms. The soil was rehydrated with 50 ml of deionized water and 15 *Lumbricus* were added to each container. An earthworm was removed from the containers at the following time points: 0 hour, 2 hour, 6 hour, 12 hour, 24 hour, 48 hour, and 72 hour. After 72 hours, remaining worms were placed into uncontaminated soil. Worms were then removed at 6, 24, 48, and 72 hours after being placed onto clean soil. All worms were euthanized by placement in scalding water,  $50^\circ$  Celsius to induce rapid and complete neural dysfunction.

Soil samples were collected from the exposure and control containers for the determination of a half-life. A sample per container was removed at 0 hours, 36 hours, 72 hours, and 96 hours. The samples were then frozen at  $-80^\circ$  Celsius.

### 6.2.3 Extraction method

All worms for malathion analysis were frozen at  $-80^{\circ}$  Celsius for no more than 2 weeks prior to analysis. The earthworms were thawed on ice prior to analysis. Gut contents were stripped from the worms manually. Four hundred milligram of posterior segment of each worm was used for the malathion analysis. The homogenization procedure involved the addition of 100 milligram anhydrous sodium sulfate (Tracepur<sup>®</sup>) and then extraction of the organophosphate with 3ml of hexane: acetone (3:1, v/v) for 30 seconds. After centrifugation, the supernatant was removed and a Folch wash was performed (Folch, Lees et al.). The supernatant was evaporated to dryness under nitrogen and rehydrated with 200  $\mu$ l of hexane prior to cleaning through a Pasteur pipette filled with 1 gram of Florisil<sup>®</sup> PR (Sulpelco). Nine milliliter of Hexane: acetone (19:1, v/v) and hexane: acetone (3:1, v/v) were used to elute malathion from the Florisil<sup>®</sup>. The elutes were combined and a second Folch wash was performed to reduce interference from lipids prior to sample evaporation under nitrogen. The sample was rehydrated with 40  $\mu$ l of hexane for chromatographic injection. Extraction efficiencies for this method are between 85-103% with malathion detection limits of 0.04  $\mu$ g and malaoxon of 0.10  $\mu$ g.

Approximately 500 milligram of soil was extracted by the same method as used for the earthworms. Extraction efficiencies for soil ranged from 88-110%.

### 6.2.3 Analytical technique

Malathion body and soil burdens were determined using a gas chromatograph with an FID (Hewlett Packard 5890 Series II) and a DB-1 capillary column (30m by 0.32mm by

0.25µm, J&W Scientific). The carrier gas and make-up gas were hydrogen and helium, respectively. Four µl of sample was injected with the injector set to split mode with the initial purge off. The purge was turned on again after one minute of run time. The injector and FID were both set to 250° C. The oven was programmed as follows: starting at 60 ° C and ramping 40° C/min to 220° C followed by a 2 °C/min climb to 228 °C. The run time was 8.9 minutes. Malathion eluted at 6.5 and malaoxon eluted at 6.1 minutes as confirmed by the use of external standards.

### 6.3.0 Model Validation Procedures and Results

#### 6.3.1 Statistical analysis

Prism<sup>®</sup>, Graphpad, San Diego CA, was used to generate curves for the determination of half-life (linear, equation 1) and depuration rate (one phase exponential decay, equation 2) (Belfroid, Van Den Berg et al. 1995). The concentration of the xenobiotic in the soil is represented as  $C_s$ .  $C_{t=0}$  is the concentration of xenobiotic in the worms at the start of depuration,  $C_t$  is the concentration in the worms at time  $t$ , and the Plateau is the last xenobiotic burden, -72 hours during depuration, measured.

Equation (1)  $C_t = \text{slope}(t) + y\text{-intercept}$

Equation (2)  $C_t = C_{t=0} * e^{(-K_e * t)} + \text{Plateau}$

A bioconcentration factor, median burden in earthworm/median burden in soil at steady state, was generated and used to calculate a rate of absorption, equation 3. Steady state was determined by the Friedman test. Earthworm malathion burdens that were

significantly different from the control,  $p$ -value  $< 0.05$ , but were not different from each other,  $p$ -value  $> 0.05$ , determined steady state.

$$\text{Equation (3) } BCF = k_a/k_e = [\text{worm}]/[\text{soil}]$$

Prism<sup>®</sup> was used to generate upper and lower 95% confidence intervals and best fit values for the kinetic parameters, elimination rate and absorption rate, based upon the above equations.

The median soil concentration was measured at each of the following time points: 0 hours- 36.18 ppm, 39 hours- 21.84 ppm, 63 hours- 15.74 ppm, and 87 hours- 8.67 ppm. The malathion's half-life, which was calculated to be 59 hours (Figure 6.3). A half-life of 59 hours resulted in a xenobiotic degradation rate of 0.0123 ppm/hr. The concentration of malathion in the soil was 27.78 ppm at steady state.

There was no mortality of earthworms at the exposure concentration. Earthworms did exhibit clinical signs of cholinesterase suppression: coiling and decreased responsiveness (Roberts and Dorough 1984). Malathion was detectable in all exposed worms, however, no malaoxon was detected. None of the control worms had any detectable malathion or malaoxon.

There were detectable levels of malathion in *L. terrestris* at 2 hours post-exposure (Figure 6.4). The malathion burden increased to a maximum of 1.106 ppm at 48 hours. Depuration overcame absorption as of 72 hours post-exposure and the malathion burdens decline. Steady state was determined by the Friedman test and pairwise comparison to be between 24 and 48 hours. The bioconcentration factor at steady state was 0.034 (earthworm 0.889 ppm/ soil 27.78 ppm)

An elimination rate constant was calculated to be 0.084 ppm/hr (95% CI 0.0134-0.1549 ppm/hr) by use of equation 2 (Fig 6.5). Use of equation 3 allowed for back-calculation of an absorption rate from the BCF and the elimination rate at steady state. The absorption rate was 0.00294 (95% CI 0.000456-0.00527 ppm/hr).

### 6.3.2 Sensitivity analysis

Stella<sup>®</sup> has a built in sensitivity function that allows for varying the parameters. All kinetic parameters were initially set for the “best-fit” as determined above and the xenobiotic soil exposure was set to 36.18 ppm, derived experimentally. The effects of varying degradation rate, elimination rate, absorption rate, and xenobiotic soil exposure on BCF were assessed (Table 6.3). All parameters were varied by a factor of 100: degradation rate (0.0134-.155 ppm/hr), elimination rate (0.001-0.10 ppm/hr), absorption rate (0.47-0.0055 ppm/hr), and malathion soil input (3.62-362 ppm). Care was taken to make sure that the experimentally derived 95% confidence intervals for each parameter were contained within the ranges used during the sensitivity analysis. The relative degree of sensitivity was determined by calculating the change in BCF for every unit change in the above parameters by using the following equation.

$$\text{Equation (4) } \frac{\text{BCF}_{\text{final}} - \text{BCF}_{\text{initial}}}{\text{parameter}_{\text{final}} - \text{parameter}_{\text{initial}}}$$

The sensitivity analysis showed that absorption rate was the most sensitive parameter in the model. One unit increase in absorption resulted in an 11.67 fold increase in the BCF. Xenobiotic soil exposure was the least sensitive; every unit change in xenobiotic soil exposure resulted in  $5.5 \times 10^{-6}$  increase in BCF. Changing the

elimination rate and the degradation rate resulted in BCF alterations of  $-0.367$  and  $0.636$ , respectively.

### 6.3.3 Model Verification

The model was evaluated for its capacity to predict bioconcentration of malathion under a variety of conditions. All parameters were independently increased or decreased by 25 fold, and then all parameters were simultaneously increased/decreased from their best-fit values by a factor of five. For the purposes of model assessment, bioconcentration was defined as a BCF of  $> 1.0$ .

The ability of the model to predict the bioconcentration of other organic xenobiotics was evaluated by inputting parameters that were derived experimentally by other authors (Table 6.1). These studies were chosen because they present the toxicokinetics of a range of xenobiotics of varying lipophilicity ( $\log k_{ow}$  2.89-8.02) and half-lives (2.5 days- 100 months) in multiple earthworm species. Most of the studies provided absorption rates, elimination rates, experimentally determined BCF's at steady state, and initial chemical input into the soil. Half-life may or may not have been determined experimentally and if not, current literature was used to extrapolate values. In one paper, steady state was reported as a range, 10-100 days, rather than a specific time for each chemical (Belfroid, Van Den Berg et al. 1995). In this instance, the model was run initially until 100 days to allow the BCF to be calculated. If steady state was not reached, then the model run time was extended beyond the 100 days until steady state was reached. Steady state was determined in the model by a lack of change in earthworm xenobiotic concentration over time.

Of interest was whether or not malathion would bioconcentrate in earthworms under extreme circumstances. Increasing the rate of absorption by 25 fold resulted in bioconcentration,  $BCF > 1.0$ . There was no evidence of bioconcentration when the other parameters were altered by 25 fold. Increasing all parameters by 5 fold simultaneously did not result in bioconcentration of malathion.

Table 6.2 shows the % BCF that was predicted by the model when compared to the experimentally derived BCF. The model was able to predict 83-105% of the BCF accurately for xenobiotics that vary in  $\log k_{ow}$  from 2.89-8.04 and half-lives from 2.5 days to over 100 months.

## 6.4 Discussion

### 6.4.1 Toxicokinetics of malathion

Model parameterization necessitated measurements of earthworm and soil malathion burdens over time. The calculated half life of malathion, 59 hours, was within published half-lives for the compound (Environmental Protection Agency 2000). No published toxicokinetics parameters were found for malathion exposure in any earthworm species. However, the toxicokinetics of malathion has been reported in other species. Anuran larvae exposed to malathion in water had a comparable elimination rate, 0.0204/hr, but a substantially faster absorption rate, 0.1598/min than we found for *Lumbricus* (Venturino, Gauna et al. 2001). Malathion is moderately hydrophobic, which can result in an increased rate of absorption from an aqueous medium in comparison to absorption from soil and the cutaneous structure of amphibian larvae is distinctly different from that of *Lumbricus*. As anticipated, malathion reached steady state in *Lumbricus* quickly,

between 24-48 hours. Pharmacokinetic studies in humans report steady state in blood levels of malathion as early as 2 hours post exposure (Boutisiouki, Thompson et al. 2001).

#### 6.4.2 Sensitivity analysis

The sensitivity analysis determined that the rate of absorption is the most sensitive parameter in our model and therefore is most important to measure accurately. The absorption of toxicants in-vivo can be difficult to measure exactly through experimentation because absorption and depuration occur simultaneously (Rozman, 2001). This requires back calculation of the absorption rate from steady state bioconcentration factors and the elimination rate (Rozman, 2001). Multiple techniques have been developed to measure absorption directly. In-vitro methods for measuring absorption pharmacokinetics include the harvested perfused anuran limb (Willens 2005), diffusion cells (Sartorelli, Aprea et al. 1998; van der Merwe, Brooks et al. 2006), and the perfused porcine skin flap (Carver, Williams et al. 1989). An in-vivo system, microdialysis, has been used in humans to investigate the skin absorption of malathion (Boutisiouki, Thompson et al. 2001). Measuring absorption directly is necessary to get the most accurate estimate of risk.

#### 6.4.3 Model Verification

The model was used to examine scenarios that could result in bioconcentration of malathion from the soil to the earthworm. The concentration of malathion that was applied to the soil for parameterization of the model was 4 times the concentration used

for boll weevil control,  $50 \mu\text{g}/\text{cm}^2$  and 40 times the concentration used as a mosquitocide,  $5 \mu\text{g}/\text{cm}^2$  (Environmental Protection Agency 2000). Manipulation of the model by increasing the xenobiotic soil input by 25 fold and decreasing the modeled half-life, degradation rate, by 25 fold, did not result in bioconcentration of malathion. The only modeled scenario, which did result in bioconcentration, was an increase in absorption rate by 25-fold. Malathion absorption from the soil to the earthworm is likely limited under field conditions because of malathion's low  $k_{ow}$  (Eto 1974), the low lipid content of the *L. terrestris* (Albro, Schroeder et al. 1992), and the earthworms' thick cuticular layer (Jamieson 1981), all of which would tend to reduce absorption. Results from our model suggest that there is a low risk of bioconcentration of malathion from soil into earthworms.

This model was purposely designed to be simple in function, but sufficiently dynamic model to allow for prediction of bioconcentration while minimizing variability to prevent under or over estimation. Though parameterized experimentally with malathion, which has a short half-life and low  $k_{ow}$ , our model successfully predicted bioconcentration factors for multiple xenobiotics with wide-ranging characteristics,  $\log k_{ow}$ 's from 2.89- 8.05 and half-lives from 2.5 days to >100 months. The model predicted from 83% to 105% of the experimentally derived BCFs; an improvement over previously published models (Jager, 1998; Sample, Suter et al 1999). The compounds with the highest  $\log k_{ow}$  and longest half-lives, PCB 118, PCB 138, and PCB 180, resulted in the lowest success prediction, 83%, 92% and 84% respectively. If the model run time was extended beyond 100 days to allow the earthworm compartment to plateau, i.e. reach steady state, then the % BCF predicted increased from 92% to 97% for PCB 138 and

from 84% to 99% for PCB 180. The other compound with a low prediction value was PCB 118 with a prediction success of 83%. PCB 118 reached steady state within 100 days, therefore, extending the run time of the model did not improve prediction success. The reported earthworm burdens for this compound were highly variable,  $48 \pm 30$  ng/g (Belfroid, Van Den Berg et al. 1995). High variability in earthworm body burdens can lead to inaccurate calculation of the bioconcentration factor and by default, absorption rate, which would affect the model's predictive capacity.

## 6.5 Conclusion

This paper describes a simple, dynamic, pharmacokinetic model that can be used to predict bioconcentration of wide-ranging organic contaminants in earthworms. The model provides an accurate predictor of bioconcentration in earthworms, which is valuable for the risk assessment of environmental pollutants.

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## 6.6 Addendum:

Cholinesterase activity was measured in the exposed worms at each time point to assess the degree of enzyme inhibition and recovery. Basal cholinesterase activity in *L. terrestris* was determined by assaying the control worms. The Ellman's assay (see Section 2.2 and Section 2.3) was used to determine cholinesterase levels. Cholinesterase levels are reported in nmol/min/mg protein. Figure 6.6 demonstrates that exposing *L. terrestris* to 200  $\mu\text{g}/\text{cm}^2$  of malathion resulted in approximately 50% suppression of cholinesterase by 12 hours after exposure. While there was wide variability in the cholinesterase activities in individual earthworms, the degree of suppression remained at 50% during the exposure (Fig 6.6). Because malathion irreversibly inhibits acetylcholinesterase, recovery requires de-novo enzyme synthesis. There was no apparent recovery of cholinesterase activity by 72 hours post-exposure.

*Lumbricus terrestris* exposed to a lower concentration of malathion (1.3 ppm) exhibited a comparable degree of suppression, 70%, and recovered enzyme activity after one week (Stenersen, Gilman et al. 1973). However, this paper did not report the raw data nor demonstrate the information graphically. This makes it difficult to compare their data with our results. When exposing other earthworm species to organophosphates, there has been wide ranging estimates of enzyme recovery time from a complete lack of recovery (Booth, Hodge et al. 2000) to recovery within 45 days (Panda and Sahu 2002). Species differences in recovery rates may be because of the capacity of the individual earthworm to synthesize new enzyme (Barata, Solayan et al. 2004). Without a longer experimental recovery time, it is difficult to assess whether *L. terrestris* would be capable of de-novo synthesis of acetylcholinesterase.

**Table 6.1** Data used for verification of STELLA model. NP- not published. N/A- not available

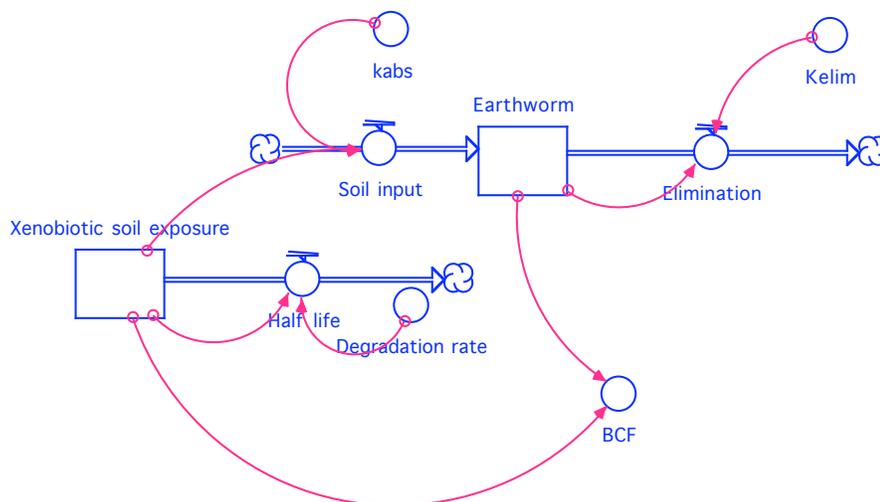
Study	Species	Chemical	Log K <sub>ow</sub>	Soil input (ppm)	Half-life (d)	K <sub>a</sub> ppm/hr	k <sub>e</sub> ppm/hr	Time at SS (d)
Henson-Ramsey et al. (NP)	<i>L. terrestris</i>	Malathion	2.89	36.18	2.4	0.00294	0.084	1
Belfroid, et al (1995)	<i>E. andrei</i>	Penta-CB	5.18	6.28	250	0.0011	0.048	10-100
	<i>E. andrei</i>	Hexa-CB	5.73	6.15	730	0.0018	0.0035	10-100
	<i>E. andrei</i>	PCB 101	6.92	0.278	4167	0.00052	0.00195	10-100
	<i>E. andrei</i>	PCB 118	7.07	0.128	4167	0.00042	0.0011	10-100
	<i>E. andrei</i>	PCB 138	7.30	0.589	7083	0.00032	0.0011	10-100
	<i>E. andrei</i>	PCB 153	7.53	0.571	22920	0.000532	0.0015	10-100
	<i>E. andrei</i>	PCB 180	8.06	0.629	41670	0.00019	0.0007	10-100
Barros, et al (2002)	<i>E. andrei</i>	Lindane	N/A	8.30	26	0.421	0.066	10
Hu, et al (2005)	<i>E. foetida</i>	Pentachlorophenol	5.12	0.500	45	0.0037	0.0062	20

**Table 6.2** Effectiveness of earthworm model at estimating bioconcentration

Study	Species	Chemical	% BCF predicted
Henson-Ramsey et al. (NP)	<i>L. terrestris</i>	Malathion	101%
Belfroid, et al (1995)	<i>E. andrei</i>	Penta-CB	100%
	<i>E. andrei</i>	Hexa-CB	104%
	<i>E. andrei</i>	PCB 101	103%
	<i>E. andrei</i>	PCB 118	83%
	<i>E. andrei</i>	PCB 138	92-99%
	<i>E. andrei</i>	PCB 153	95%
	<i>E. andrei</i>	PCB 180	84-97%
Barros, et al (2002)	<i>E. andrei</i>	Lindane	101%
Hu, et al (2005)	<i>E. foetida</i>	Pentachlorophenol	105%

Table 6.3 Sensitivity analysis

Parameter	Amount varied	$\Delta$ BCF/ unit $\Delta$ in parameter
degradation rate	0.0134-0.155 ppm/hr	0.636
elimination rate	0.001-0.10 ppm/hr	-0.367
absorption rate	0.47-0.0055 ppm/hr	11.67
malathion soil input	3.62-362 ppm	0.0000055



**Figure 6.1** STELLA earthworm model flow diagram

$$\text{Earthworm}(t) = \text{Earthworm}(t - dt) + (\text{Soil\_input} - \text{Elimination}) * dt$$

$$\text{INIT Earthworm} = \text{Soil\_input}$$

INFLOWS:

$$\text{Soil\_input} = \text{Malathion\_soil\_exposure} * \text{kabs}$$

OUTFLOWS:

$$\text{Elimination} = \text{Earthworm} * \text{Kelim}$$

$$\text{Malathion\_soil\_exposure}(t) = \text{Malathion\_soil\_exposure}(t - dt) + (- \text{Half\_life}) * dt$$

$$\text{INIT Malathion\_soil\_exposure} = 36.18$$

OUTFLOWS:

$$\text{Half\_life} = \text{Malathion\_soil\_exposure} * \text{Degradation\_rate}$$

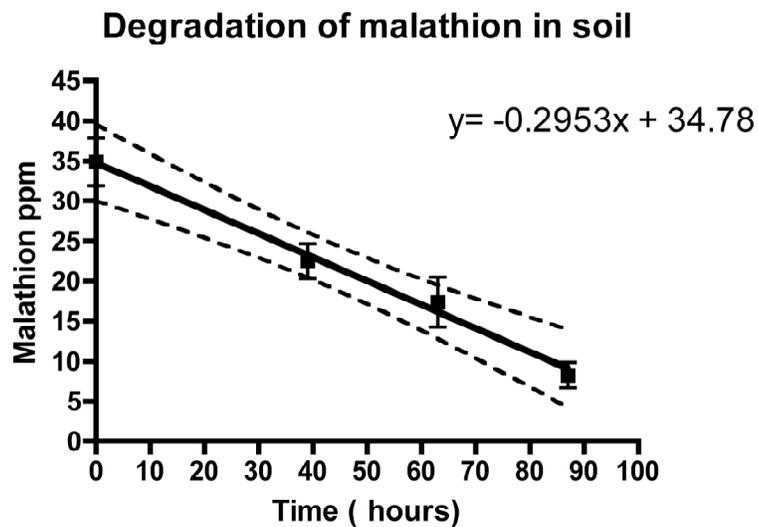
$$\text{BCF} = \text{Earthworm} / \text{Malathion\_soil\_exposure}$$

$$\text{Degradation\_rate} = .0123$$

$$\text{kabs} = .00294$$

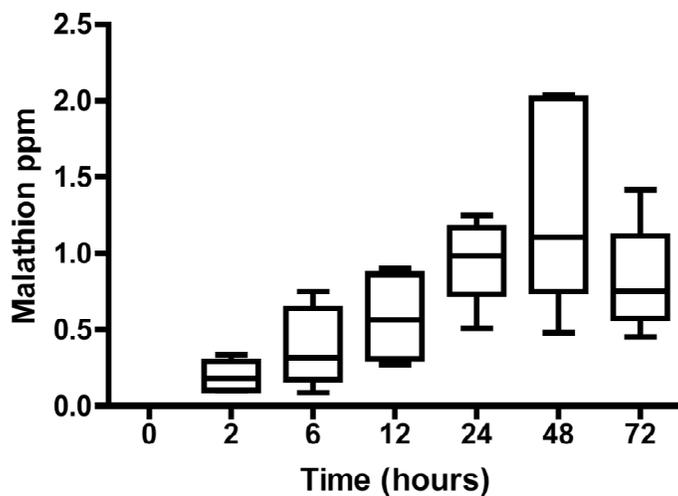
$$\text{Kelim} = .084$$

**Figure 6.2** Mathematical relationships between earthworm model parameters



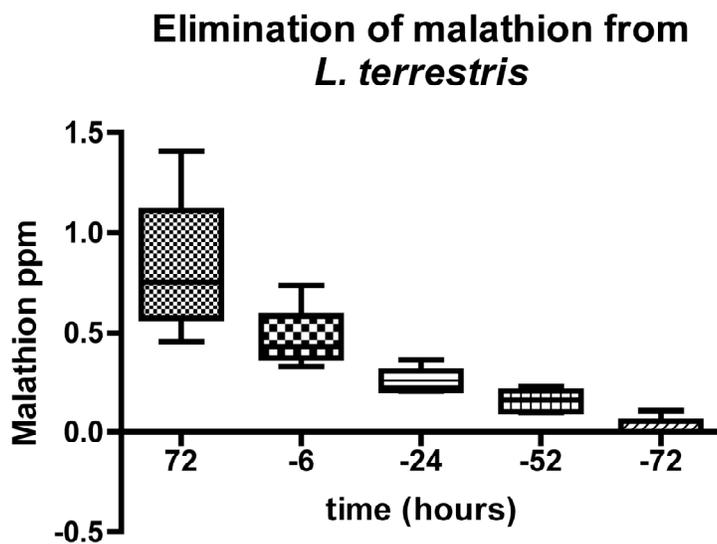
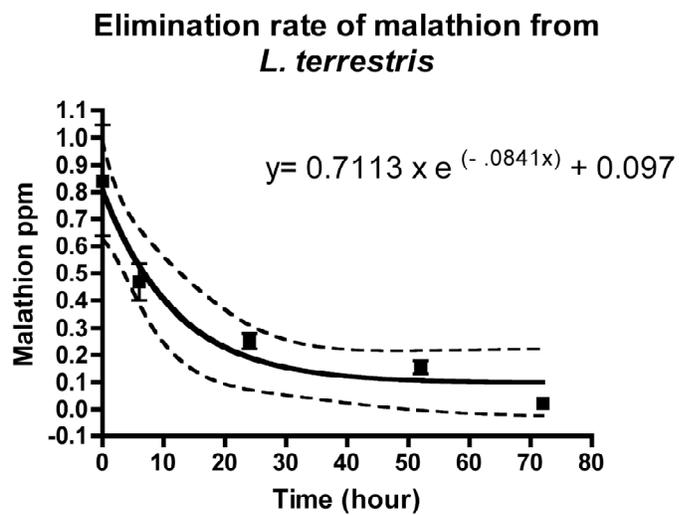
**Fig 6.3** Degradation of malathion in soil during earthworm exposures over time. Generated with linear regression. Resulted reported as median ppm with error bars representing the interquartile range.

***L. terrestris* absorption of malathion  
after soil exposure ( 200  $\mu\text{g}/\text{cm}^2$ )**



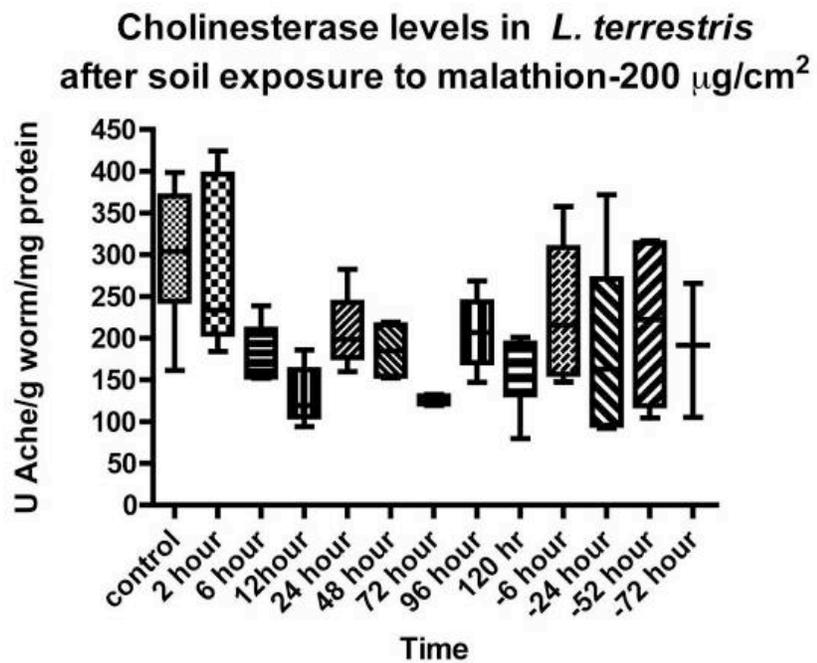
**Figure 6.4** The absorption of malathion in *L. terrestris* over time.

Box and Whiskers plot with error being represented by the interquartile range. Steady state was reached at 24 hours.



**Figure 6.5** The elimination of malathion in *L. terrestris* after being placed onto uncontaminated soil.

An elimination rate was calculated with a uni-phase exponential decay equation. The dotted lines represent the 95% confidence interval of the line.



**Fig 6.6** Cholinesterase levels in *L. terrestris* over time. Data represented in median values and interquartile ranges.

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**Chapter 7: Acute Toxicity and Tissue Distributions of Malathion in *Ambystoma******tigrinum***

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## 7.0 Abstract.

The kinetics of the bioaccumulation of malathion (O, O-Dimethyl phosphorodithioate of diethyl mercaptosuccinate) and the biological impact of exposure for tiger salamanders, *Ambystoma tigrinum*, were assessed through exposure to soil surface contaminated with 50  $\mu\text{g}/\text{cm}^2$  or 100  $\mu\text{g}/\text{cm}^2$  of malathion and ingestion of an earthworm exposed to soil contaminated with 200  $\mu\text{g}/\text{cm}^2$  of malathion. Malathion and malaoxon burdens in salamanders sampled at different times after exposure(s) were measured by gas chromatography in four tissue/organ subgroups: liver, epaxial muscle, pooled viscera (except the liver and brain), and pooled avisceral carcass (muscle, skin, and bone). The total tiger salamander xenobiotic burdens were calculated from these data. The malathion/malaoxon burden one day after exposure was greatest in the avisceral carcass and two days after exposure was greatest in the viscera. Bioconcentration and bioaccumulation factors remained less than unity throughout the experiment, and did not support the hypothesis of bioaccumulation of malathion in the tiger salamander.

Biological impact was assessed with a colorimetric brain cholinesterase microassay.

Brain cholinesterase activities in salamanders exposed to malathion contaminated soil (50  $\mu\text{g}/\text{cm}^2$  or 100  $\mu\text{g}/\text{cm}^2$  of malathion) were suppressed approximately 50-65% and 90%, respectively, compared to unexposed controls. The exposed animals did not exhibit overt clinical signs of malathion toxicosis.

## 7.1 Introduction

Exposure to xenobiotics has been suggested as one possible cause of amphibian population declines (Alford and Richards 2001; Blaustein, Romansic et al. 2003), yet the effects of many xenobiotics on amphibians are not well understood. This report evaluates the kinetics of malathion bioaccumulation in a food chain involving the fossorial salamander, *Ambystoma tigrinum* (tiger salamander), and an anecic earthworm, *Lumbricus terrestris*, by determining salamander body burdens and brain cholinesterase activities over time after exposure.

Though toxicity of malathion in mole salamanders (Ambystomidae) has not been well reported, acute toxicity and developmental effects after malathion exposure have been described in frogs (Baker 1985) and lungless salamanders (Plethodontidae) (Baker 1985; Venturino, Gauna et al. 2001). The acute toxicity of malathion and its active primary metabolic product malaaxon results from impediment of acetylcholine degradation at the neuromuscular junction through irreversible inhibition of acetylcholinesterase (Eto 1974). Increasing concentrations of acetylcholine can then cause clinical signs such as depression, and tremors, potentially leading to death, as well as affect complex behaviors such as feeding and reproduction (Taylor, Williams et al. 1999; Venturino, Rosenbaum et al. 2003).

Malathion, a commonly used organophosphate, is applied for mosquito control at concentrations designed to achieve a surface exposure of 5  $\mu\text{g}/\text{cm}^2$  (De Guise, Maretea et al. 2004) and is applied to combat agricultural pests at a rate ten times higher resulting in an exposure rate of 50  $\mu\text{g}/\text{cm}^2$  (EPA 2000). Malathion is not considered a persistent

pesticide (log Kow 2.89, half-life 1-10 days)(EPA 2000), however the annual application of 17 million pounds of malathion in the United States (EPA 2000) still fuels concern about potential bioaccumulation in highly impacted habitats, particularly wetlands adjacent to human population centers or those used for intense crop production.

## 7.2 Material and Methods

Adult tiger salamanders (n=17, weight 75-104 grams) were housed individually in terrariums (polyethylene, surface area of 1060 cm<sup>2</sup>) filled with Scott's garden soil<sup>®</sup> and sphagnum moss layered over pea gravel during acclimation. Sixty percent substrate moisture was maintained in half of each container by the addition of deionized water, and the soil in the other half of the container was left dry to allow the animals to self regulate their need for moisture. During exposures, the animals were housed in polyethylene cages (1060 cm<sup>2</sup>) on approximately 1200 grams of Scott's garden soil<sup>®</sup> hydrated with 500 ml of deionized water and incubated at 20° C. All animals purchased from Reptile City ([www.reptilecity.com](http://www.reptilecity.com)), were presumably wild caught, and were acclimated in the laboratory for at least one year prior to exposure.

*Lumbricus terrestris* were obtained from National Bait and Supply, Marblehead, Ohio and kept at 10° C in polystyrene boxes on a substrate of 900 grams Scott's<sup>®</sup> garden soil hydrated with 100 ml of deionized water for one month prior to exposure. Worms were exposed in a 1000 ml beaker filled with 600 ml of soil. The soil was hydrated with 100 ml of deionized water and the beaker was incubated at 10° C.

Ninety six point five % grade malathion (American Cyanamid, Wayne, New Jersey) was diluted into acetone to formulate the concentrations needed to achieve

substrate surface exposures of 50  $\mu\text{g}/\text{cm}^2$  and 100  $\mu\text{g}/\text{cm}^2$  for the tiger salamander exposures and 200  $\mu\text{g}/\text{cm}^2$  for the earthworm exposures. Acetone alone was used for the unexposed controls. The appropriate malathion concentration was applied in a fine mist to the soil surface using a thin-layer chromatography sprayer. The soil was manually mixed for 3 minutes to simulate soil disturbance and to reduce the risk of behavioral avoidance of exposure. The solvent was allowed to evaporate overnight under a chemical fume hood, and then, the soil was rehydrated with deionized water: 500 ml for tiger salamander habitats and 100 ml for the earthworm habitats. The study subjects were placed into the prepared containers and kept at the standardized temperatures for the species being exposed, 10<sup>0</sup>C for *Lumbricus* and 20<sup>0</sup>C for *A. tigrinum*.

*Lumbricus terrestris* were exposed to 200  $\mu\text{g}/\text{cm}^2$  of malathion on soil for 72 hours prior to being fed to the tiger salamanders. The 72-hour time frame and higher exposure concentration for earthworms were determined using pilot studies to establish a repeatable malathion concentration in the earthworms of 1 ppm.

The tiger salamanders were assigned to one of five treatment groups by generating F-statistics to identify groupings that minimized salamander weights as a confounding factor. The five treatment groups were (1) unexposed animals, (2) animals exposed to soil contaminated with 50  $\mu\text{g}/\text{cm}^2$  of malathion for one day, (3) animals exposed to soil contaminated with 50  $\mu\text{g}/\text{cm}^2$  of malathion for two days, (4) tiger salamanders exposed to contaminated soil, 50  $\mu\text{g}/\text{cm}^2$ , for two days and a fed a contaminated worm on the first day of exposure, and (5) tiger salamanders exposed to soil contaminated with 100  $\mu\text{g}/\text{cm}^2$  of malathion for two days and fed a contaminated worm on the first day of exposure. Two salamanders were used as unexposed controls

and handled identically to all of the exposed animals. Initially, three animals were placed into the four malathion exposed treatment groups. In an attempt to resolve variability issues, an additional replicate of 3 animals was exposed to group 3 conditions (soil contaminated with 50  $\mu\text{g}/\text{cm}^2$  of malathion for two days). All animals were observed twice daily for the development of clinical signs associated with malathion toxicity in amphibians (Rosenbaum, de Castro et al. 1988; Taylor, Williams et al. 1999).

Tiger salamanders were sacrificed by immersion in 10% MS-222 solution buffered with 10% sodium bicarbonate. Animals were weighed and the entire brain, liver, and approximately 0.05 grams of epaxial muscle were collected. The remaining carcass was subdivided into two groups: 1) pooled viscera with the exception of the liver and brain and 2) the avisceral carcass, which consisted of skin, remaining muscle, and bone. The brain was processed for acetylcholinesterase activity on the day of sampling. The other tissues were subsampled and frozen at  $-80^\circ\text{C}$  until assayed for malathion/malaoxon tissue burdens. For the calculation of bioaccumulation and bioconcentration factors, subsamples of the consumed earthworms ( $n=6$ ) and soil samples ( $n=17$ ) were frozen at  $-80^\circ\text{C}$  to verify malathion and malaoxon burdens. Malathion assays were performed within one month of sample collection.

An identical extraction procedure was used for all collected samples. Samples were first homogenized in 1:3 acetone/hexane (v: v) and then centrifuged for 8 minutes. The supernatant was removed and further processed by a Folch wash to remove interfering lipids (Folch, Lees et al. 1957). Passage through a Pasteur pipette filled with one gram of Florisil<sup>®</sup> served to further clean the sample. Elution from the Pasteur pipette transpired after washing the Florisil with 1:19 acetone/hexane (v: v). An additional wash

with 1:3 acetone/hexane (v: v) was used to ensure complete recovery. After a second Folch wash, the samples were evaporated to dryness under nitrogen.

The dried sample extracts were reconstituted with 40  $\mu$ l of hexane for analysis. 4  $\mu$ l of the reconstituted sample was injected in triplicate into a Hewlett Packard 5890 gas chromatograph with a flame ionization detector. The injector and detector were both set to 250° C. Initially, the oven temperature was 60° C, and then the temperature was ramped to 220° C at a rate of 2° C per minute. Malathion and malaoxon peaks were detected at 6.5 and 6.2 minutes, respectively, during the 11.4-minute run. The injection of external standards confirmed the identity of the chromatographic peaks. Detection limits were calculated to be 0.04  $\mu$ g for malathion and 0.10  $\mu$ g for malaoxon using curves generated from standards provided by Restek Corporation (Bellafonte, Pennsylvania). All tissue burdens and whole body burdens were reported in  $\mu$ g/g or ppm. Because malaoxon is a direct metabolite of malathion and biologically active, the detected tissue concentrations of both compounds were combined and a single contaminant burden was calculated for each assayed tissue.

A modified Ellman assay, a colorimetric procedure based on the reaction between acetylthiocholine, 5,5'-Dithio-bis(2-nitrobenzoic acid) (DTNB), and the unbound cholinesterase was used to analyze brain cholinesterase activity (Ellman, Courtney et al. 1961; Willens 2005). A Spectramax 190® spectrophotometer with Softmax Pro® software was used to quantify the resultant color change. Approximately 0.04 g of salamander brain tissue was homogenized in 80  $\mu$ l ice cold (4° C) Tris Buffer 8.0. The samples were centrifuged for 8 minutes at 1000X G and the supernatant was kept on ice until assayed. The samples and standard curves (electric eel acetylcholinesterase) were

run in triplicate. Results are reported in  $\mu\text{mol}/\text{min}/\text{g}$  of brain. All chemicals for the acetylcholinesterase assay were purchased from Sigma-Aldrich, St. Louis, MO.

Total tiger salamander burdens were compared between treatment groups graphically. Bioconcentration factors were determined by dividing the median *A. tigrinum* xenobiotic burden by the median malathion concentration in the soil. Bioaccumulation factors were calculated by dividing the median tiger salamander malathion burden by the summation of median xenobiotics burdens in *L. terrestris* and in the soil (Guthrie and Perry 1980; Tsuda, Aoki et al. 1989; Albanis, Hela et al. 1996). Inhibition of brain cholinesterase activity was calculated by comparing the results in groups of exposed and unexposed animals. Unexposed brain cholinesterase activities were set to 100%, and the percent suppression was determined for the exposed tiger salamanders.

### 7.3 Results and Discussion

Malathion and malaoxon were detected in all sampled tissues from exposed animals and were not detected in the unexposed animals. Total tiger salamander xenobiotic burdens from all exposed animals ranged from 0.3472-1.462 ppm with a median of 0.8596 ppm (Table 7.1). The tissue distribution of malathion after being exposed to contaminated soil for one or two days was visualized by comparing the relative concentrations of malathion/malaoxon in the four sampling groups--epaxial muscle, liver, viscera, and avisceral carcass (Fig 7.1). After the first day of soil exposure, the relative concentration of malathion/malaoxon was greater in the pooled avisceral carcass (median 0.84 ppm)

than in the other samples: epaxial muscle (median 0.12 ppm), liver (median 0.3 ppm), and pooled viscera (median 0 ppm). After two days of malathion exposure, the relative concentration of malathion/malaoxon was greatest in the pooled viscera (median 2.23 ppm) than in the other sampled tissues: epaxial muscle (median 1.1 ppm), liver (median 1.0 ppm), and pooled avisceral carcass (median 0.8155 ppm). There was no evidence of bioconcentration or bioaccumulation (Table 7.2).

Inhibition of brain cholinesterase activity was apparent after exposure to malathion (Fig 7.2). Exposing *A. tigrinum* to 50  $\mu\text{g}/\text{cm}^2$  of malathion by soil contact only or by contaminated soil and a contaminated earthworm resulted in 50-65% suppression of brain cholinesterase activity, when compared to unexposed controls. Animals exposed to 100  $\mu\text{g}/\text{cm}^2$  of malathion through soil contact exhibited 90% suppression of brain cholinesterase levels. No overt clinical signs of acetylcholinesterase suppression, lethargy, tremors, decreased responsiveness, anorexia, were seen in any the tiger salamanders.

Malathion concentrations were measured in multiple tissues to evaluate the xenobiotic tissue distribution over time and to determine the relative contributions of cutaneous and gastrointestinal absorption to the total malathion burden in *A. tigrinum*. The greatest concentration of malathion/malaoxon was found in the avisceral carcass initially, with increasing concentrations of the xenobiotic in the viscera over time (Fig 7.1) (Bouchard, Gosselin et al. 2003; Johnson, 1999). This suggests that cutaneous absorption was the primary method of malathion assimilation into salamander tissues.

Length of exposure (one or two days) and exposure concentration (50  $\mu\text{g}/\text{cm}^2$  or 100  $\mu\text{g}/\text{cm}^2$ ) did not appear to alter tiger salamander body burdens (Table 7.1).

However, the wide variability in the individual treatment groups may have obscured any small differences attributable to these factors. The greatest inconsistency in the samples was within the pooled viscera group, which included both the urinary and gastrointestinal systems. Doubling the sample size from 3 animals to 6 animals did not resolve this variability issue. In mammals, 90% of malathion is excreted renally and 10% is eliminated in the feces (Dary, Blancato et al. 1993; Boutisiouki, Thompson et al. 2001; Bouchard, Gosselin et al. 2003). If this is the case in salamanders then it would be expected that the viscera subgroup would be affected by individual variation in elimination efficiency. While the metabolism of malathion has not been previously described in Ambystomidae salamanders, it has been partially described in frogs and there is evidence for a similar metabolic and elimination pathway to that described in mammals (Anguiano, de Castro et al. 2001; Venturino, Anguiano et al. 2001). It is likely that the sample variability in the pooled viscera can be attributed to the impact of individual differences in excretion efficiency affecting malathion tissue burdens.

Tiger salamanders, which consumed an earthworm contaminated with malathion, did not exhibit increased xenobiotic body burdens compared to animals exposed only to contaminated soil. Literature searches did not reveal any estimates of malathion bioavailability after oral exposure in amphibians. However, there are estimates for human volunteers who consumed a gelatin capsule with malathion. Ninety-five percent of the consumed malathion was later recovered as metabolites in the volunteers (Krieger and Dinoff 2000). This suggests that in mammals, malathion is efficiently absorbed. However, malathion from a gelatin capsule and malathion in earthworm tissues are not equivalent. The lack of apparent increase in tissue burdens in tiger salamanders exposed

to contaminated earthworms and contaminated soil demonstrates the lack of malathion bioavailability in earthworm tissues.

Malathion burdens were assessed in soil, earthworms, and tiger salamanders for the calculation of bioconcentration factors (BCFs) and bioaccumulation factors (BAFs). The BCFs and BAFs were less than unity (Table 7.2), indicating that bioaccumulation did not occur in *A. tigrinum* exposed under these conditions (Guthrie and Perry 1980; Hall and Kolbe 1980; Geyer, Scheunert et al. 1986; Willens 2003). Bioconcentration of malathion from water into larval anurans has been demonstrated previously (Hall, 1980); however, the skin of terrestrial amphibians is less permeable to lipophilic compounds than aquatic larval amphibians (Bentley and Baldwin 1980). Malathion, log  $K_{ow}$  2.89, is approximately 1000 times more likely to be found in lipids than water (Bentley and Baldwin 1980; Environmental Protection Agency 2000). Exposing tiger salamanders to malathion by soil rather than an aqueous medium may reduce the partitioning of malathion into tissue lipids.

Brain cholinesterase levels documented in the exposed tiger salamanders suggest suppression of normal cholinesterase levels compared to unexposed controls. Exposing tiger salamanders to a surface contamination of 50  $\mu\text{g}/\text{cm}^2$  of malathion surface resulted in 50-65% suppression in brain cholinesterase activity, while exposure to a surface contaminated with 100  $\mu\text{g}/\text{cm}^2$  of malathion inhibited brain cholinesterase activities by 90%. There were no clinical signs observed as a result of either exposure, which would be attributable to cholinergic effects of acetylcholinesterase inhibition. While cholinesterase suppression has been shown to be an excellent marker of exposure to organophosphates (Sparling, Fellers et al. 2001), the degree of suppression does not

correspond predictably with resultant toxicity and clinical signs (Baker 1985; Carlock, Chen et al. 1999). A similar degree of cholinesterase inhibition without notable cholinergic effects has been documented in other species (Fulton and Key 2000). The lack of overt clinical signs makes it difficult to interpret the possible biological impact of this degree of cholinesterase suppression. Our studies do document that cholinesterase suppression can be used to document exposure to malathion in Ambystomidae salamanders.

Repetitive malathion applications are often necessary for efficient pest control. The frequency of use varies based on the mosquito and agricultural pest control regime. Most protocols recommend re-application every 7-10 days if needed (Environmental Protection Agency 2000). The lack of bioaccumulation in this report and malathion's short half-life in soil of 1-10 days (EPA 2000) suggest that a weekly application of malathion at 50  $\mu\text{g}/\text{cm}^2$  should not result in bioaccumulation in *A. tigrinum*. Additionally, the consumption of multiple malathion exposed prey would be unlikely to increase the risk of malathion bioaccumulation in tiger salamanders because ingestion does not appear to be an important source of malathion tissue concentrations in *A. tigrinum*. Though tiger salamanders experimentally exposed to current application rates for malathion had detectable burdens of the xenobiotic and measurable inhibition of brain cholinesterase activity, there was no evidence of bioaccumulation nor did the tiger salamanders exhibit anticipated clinical signs as a result of cholinesterase inhibition.

#### 7.4 Addendum:

Muscle and liver tissues were saved to assay total protein and lipid content (See Section 2.3 and 2.4). This was done to see if standardizing malathion body burden for protein or lipid content would reduce the variability seen when using weight as a method of standardization. The degree of variability was compared by assessing the coefficient of variation, COV (Table 7.3). Using protein to standardize contaminant levels in muscle tissue reduced the COV from 135.92% to 111.9%. Standardizing for protein content reduced variability in the liver samples from a COV of 57.29% to 36.50%. When assaying liver and muscle tissue for contaminant loads, protein content as a standardization technique may reduce variability.

**Table 7.1** Median values and ranges for salamander exposure groups.

Exposure	Median (ppm)	Range (ppm)
50 $\mu\text{g}/\text{cm}^2$ soil one day <sup>a</sup>	1.462	0.346-1.606
50 $\mu\text{g}/\text{cm}^2$ soil, two day <sup>b</sup>	1.424	0.182-3.730
50 $\mu\text{g}/\text{cm}^2$ soil/worm, two day <sup>a</sup>	0.347	0.275-0.4308
100 $\mu\text{g}/\text{cm}^2$ soil/worm, two day <sup>a</sup>	0.558	0.416-0.769

<sup>a</sup> n=3

<sup>b</sup> n=6

**Table 7.2** Bioconcentration and Bioaccumulation Factors in tiger salamanders

Exposure	Bioconcentration (BCF)	Bioaccumulation (BAF)
50 $\mu\text{g}/\text{cm}^2$ soil, one day <sup>a</sup>	0.077	NC
50 $\mu\text{g}/\text{cm}^2$ soil, two day <sup>b</sup>	0.133	NC
50 $\mu\text{g}/\text{cm}^2$ soil/worm, two day <sup>a</sup>	0.022	0.021
100 $\mu\text{g}/\text{cm}^2$ soil/worm, two day <sup>a</sup>	0.015	.0149

<sup>a</sup> n=3<sup>b</sup> n=6

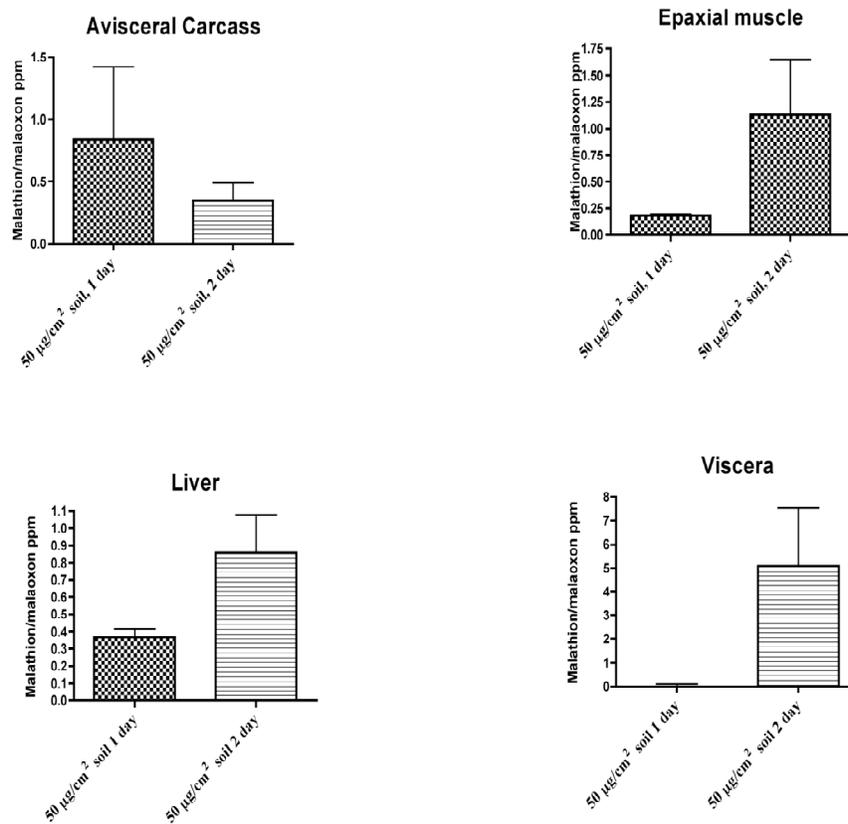
NC= not calculated

**Table 7.3** Comparison of variability in malathion body burdens with different methods of standardization.

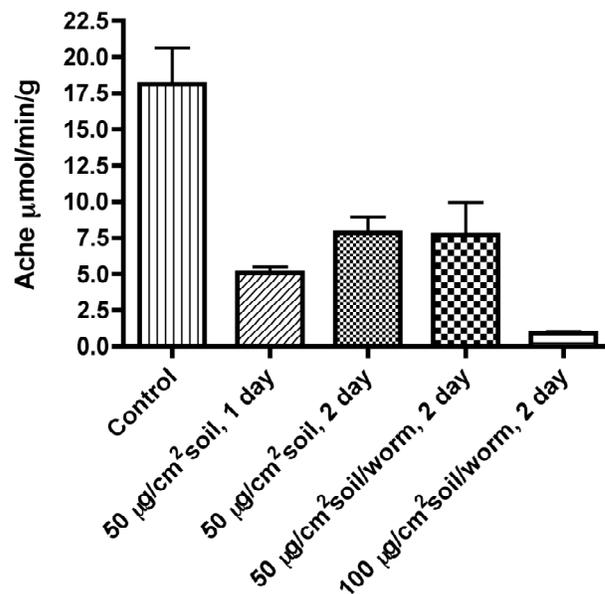
Sample	By weight	By lipid content	By protein content
Muscle <sup>a</sup>	135.92%	167.50%	111.92%
Liver <sup>b</sup>	57.29%	63.06%	36.50%

<sup>a</sup> n=4<sup>b</sup> n=5

Result reported as coefficient of variation



**Fig 7.1** Malathion tissue distributions in tiger salamanders after one or two days of soil exposure. Data is represented in median values with range.



**Fig 7.2** Cholinesterase activity in tiger salamanders exposed and unexposed to malathion. Data is represented in median values and ranges.

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## Chapter 8: Expanding the model

### 8.1 Introduction

This research has evaluated the effect of malathion exposure in two earthworm species, *Lumbricus terrestris* and *Eisenia foetida*, and one salamander species, *Ambystoma tigrinum*. Because malathion is frequently applied at the aquatic-terrestrial interface, there is the potential for simultaneous exposure of both earthworms and salamanders. Of primary interest is whether tiger salamanders exposed to contaminated soil and contaminated earthworms would exhibit an increased likelihood of accumulating malathion compared to exposure to contaminated soil only. Previous research, see Chapter 7, suggests that this is unlikely and that a large number of animals would be needed to evaluate the question experimentally. However, this is a situation where a mathematical model using experimental data already collected could be useful.

### 8.2 Description of model

The earthworm mathematical model (see Chapter 6) can be expanded to evaluate exposure of tiger salamanders to additional sources of contamination, in this case, malathion contamination from soil contact and from earthworm ingestion (Figure 8.1, Figure 8.2). Accomplishing this necessitates the addition of 2 additional compartments (Salamander xenobiotic soil exposure and Salamander body burden), 4 additional kinetic parameters (degradation rate 2, absorption rate 2, elimination rate 2, and rate of gastrointestinal absorption), and one more result chamber (bioaccumulation, BAF). The degradation rate ( $K_{deg2}$ ), elimination rate ( $K_{elim2}$ ), absorption rate ( $K_{abs2}$ ), and salamander

body burden compartment are defined in the same way they were defined in the earlier earthworm model (chapter 6).

A rate of digestive absorbance ( $K_{\text{dig}}$ ) was included to allow modeling of bioaccumulation. This rate is a direct input from the earthworm burden and can be modified by a parameter called, food per day, changing the frequency of earthworm consumption by the tiger salamanders. The additional result chamber, BAF, is determined by dividing the salamander burden by the concentration of the xenobiotic in the soil, xenobiotic soil exposure 2, summed with the earthworm burden, earthworm compartment.

The experimental data used to parameterize model is from the salamander exposures (Chapter 7) and the toxicokinetics earthworm exposures (Chapter 6). The salamander exposures were not designed to generate toxicokinetics data. This results in some limitations of the salamander burden data. Salamander body burdens and malathion concentration in the soil were measured 24 and 48 hours after exposure to 50  $\mu\text{g}/\text{cm}^2$  of malathion. Because only two time points were measures, the time at steady state could not be determined. Low replicate number led to wide variability in the data. Salamander body burdens at 24 hours ( $n=3$ ) were 1.46 ppm, median, with a range from 0.35 ppm to 1.61 ppm. After 48 hours of exposure ( $n=6$ ), the median value for the salamander body burdens was 1.42 ppm with a range of 0.18 ppm to 3.73 ppm. Malathion contamination of the soil was measured on the day of salamander euthanasia to allow for the calculation of a bioconcentration factor. Twenty-four hours after application, the soil burden of malathion ( $n=3$ ) was 19.20 ppm with a range of 17.01-20.55 ppm. Forty-eight hours after application, the soil burdens ( $n=6$ ) had decreased to a

median of 10.40 ppm with a range of 8.04-11.99 ppm. The methods for determining all of this data are fully detailed in Chapters 2 and 7.

### 8.3 Model Validation Procedures and Results

Prism ®, Graphpad (San Diego, CA) was used to generate a curve for the determination of a half-life of malathion degradation (linear, equation 1). The concentration of malathion in the soil is represented as  $C_s$  at a specific time  $t$ . The half-life was calculated to be 39 hours with a degradation rate of 0.017 ppm/hr. The equation was used to back calculate an initial malathion input into the soil, xenobiotic soil exposure 2, of 27.12 ppm.

$$\text{Equation (1) } C_s = \text{slope}(t) + y\text{-intercept}$$

Bioconcentration factors at 24 and 48 hours were generated for the salamanders using the median salamander burden and the median soil burden at the respective time points. The BCF at 24 hours was calculated to be 0.077 and at 48 hours it was 0.133. Because the salamander exposure were not designed to generate a depuration rate, it was not possible to calculate an exact estimate of absorption and elimination rates. However, the model can be used to generate estimates. The absorption rate 2 and elimination rate 2 were varied to achieve salamander burdens and bioconcentration factors, which matched the 24 hour and 48 hour time points. This resulted in an absorption rate of 0.0041 ppm/hr and a degradation rate of 0.04 ppm/hr.

There are no available estimates of gastrointestinal absorption in tiger salamanders and no reasonable way to generate this information from the salamander

exposures. The rate of gastrointestinal absorption will be varied from 0.00 ppm/hr, no absorption, to 1.00 ppm/hr, complete absorption. This will allow for the assessment of the contribution of consuming a contaminated worm to the salamander's xenobiotic burden. Additionally, the frequency of consumption will be varied from hourly, daily, and weekly.

### 8.3.1 Sensitivity analysis

The kinetic parameters for elimination, cutaneous absorption and degradation rates were set to best estimates. After running a 10 day exposure on the model to 27.12 ppm of malathion, there was no evidence for the bioconcentration of malathion from the soil to the salamander. The cutaneous absorption rate and elimination rate were then altered in the model until the salamander body burdens matched the upper range of experimentally achieved values at 24 and 48 hours. This increased the cutaneous absorption rate to 0.0045 ppm/hr and slowed the elimination rate to 0.02 ppm/hr. The model was again run for 10 days after malathion exposure with these altered parameters and there was still no evidence for bioconcentration.

A sensitivity analysis was performed on the new parameters, xenobiotic soil exposure 2, absorption rate 2, elimination rate 2, and degradation rate 2. The parameters were varied by a factor of 100 and the change in BCF was calculated (Equation 2). As in the earthworm model, the rate of absorption was the most sensitive factor. Altering the rate of absorption by one unit caused a 20 fold increase in the bioconcentration factor.

$$\text{Equation (2) } BCF_{\text{final}} - BCF_{\text{initial}} / \text{parameter}_{\text{final}} - \text{parameter}_{\text{initial}}$$

To evaluate the effect of consuming a contaminated earthworm, the gastrointestinal absorption rate,  $K_{\text{dig}}$ , was varied from 0 to 1 with all other parameters set to optimum ( $K_{\text{abs2}} = 0.0042$  ppm/hr and  $K_{\text{elim2}} = 0.04$  ppm/hr). The model was initially run for 10 days. If a salamander ate one contaminated worm in that period of time and absorbed all malathion from that worm, there was no bioaccumulation of malathion. Consumption was increased to the feeding rate that is necessary for weight gain and maintenance, 3 worms during the 10 days, and there was still no evidence of bioconcentration. According to the model, a salamander would have to consume an earthworm daily and efficiently absorb all the malathion in order to bioaccumulate the xenobiotic. The resultant bioaccumulation factor was still only 1.13.

## 8.4 Discussion

### 8.4.1 Toxicokinetics of malathion

Most of the toxicokinetics parameters in the salamander portion of the model were estimated from 2 time points, 24 hours and 48 hours post-exposure. For accurate estimation of kinetic parameters, steady state is typically required. Steady state was reached in *Lumbricus* within 24 hours (Chapter 6) and has been reported in humans as early as 2 hours post dermal exposure (Boutisiouki, Thompson et al 2001). Given that the median salamander burdens at 24 and 48 hours are equivalent, it is likely that steady state has been reached. However, salamander body burdens would be needed at earlier and later time points to statistically determine steady state.

Soil burdens at 24 and 48 hours were used to calculate an estimated half-life, 39 hours, that is within the range of half-lives reported by the EPA (Environmental Protection Agency 2000). The half-life in the salamander microcosm was shorter than in the earthworm exposures, 39 hours versus 59 hours. This is probably because of differences in exposure temperature. The tiger salamanders were exposed at 20<sup>0</sup> C, the temperature needed to best maintain the species in the laboratory (Maruska 1994), which is ten degrees higher than the earthworm exposures. The higher temperatures would be expected to increase the microbial mineralization of malathion which is the primary method of environmental degradation (Brown, Petreas et al. 1993; Introduction Section 3.2).

The cutaneous absorption rate and elimination rate for malathion in tiger salamanders was estimated from experimentally calculated BCF's. Tiger salamanders appear to absorb malathion more quickly than earthworms (Estimated  $K_{abs2} = 0.0042$  versus  $K_{abs} = 0.00294$ ) and eliminate the compound more slowly (Estimated  $K_{elim2} = 0.04$  versus  $K_{elim} = 0.084$ ). This is most the probable explanation for the comparatively higher body burdens in *A. tigrinum*, median 1.4 ppm, than *L. terrestris*, median 0.84 ppm, even though the earthworms were exposed to a higher concentration of malathion. Anatomically, the difference in xenobiotic absorption is expected. Tiger salamanders have a relatively thin integument (Duellman and Trueb 1994), conversely, *L. terrestris* have a thick cuticular designed to impede absorption (Coggeshall 1966). Even if *A. tigrinum* do absorb malathion at faster rate than *L. terrestris*, it does not appear to be enough to result in significant accumulation.

#### 8.4.2 Sensitivity analysis

As in the earthworm model, the sensitivity analysis determined that the rate of cutaneous absorption is the most sensitive parameter in the model. This parameter is difficult to measure directly as absorption and elimination occur simultaneously (Rozman and Klaassen 2001). There are methods that can be used to directly measure rates of cutaneous absorption in-vitro and two of these, diffusion cells and harvested perfused amphibian limb, have successfully been used in amphibians (Willens 2005). The diffusion cell assay is easy to use and should be able to provide a more exact estimate of malathion absorption in tiger salamanders. Additionally, a toxicokinetics experiment similar to that used in *L. terrestris* could be designed to complete data gaps by sacrificing exposed tiger salamanders at additional time points (2,6,12, 72, 96 hours) post-exposure. That data in combination with mirrored depuration exposures would allow for a more accurate estimate of kinetic parameters. However, the question is whether that information is necessary to assess the risk of malathion bioaccumulation in tiger salamanders?

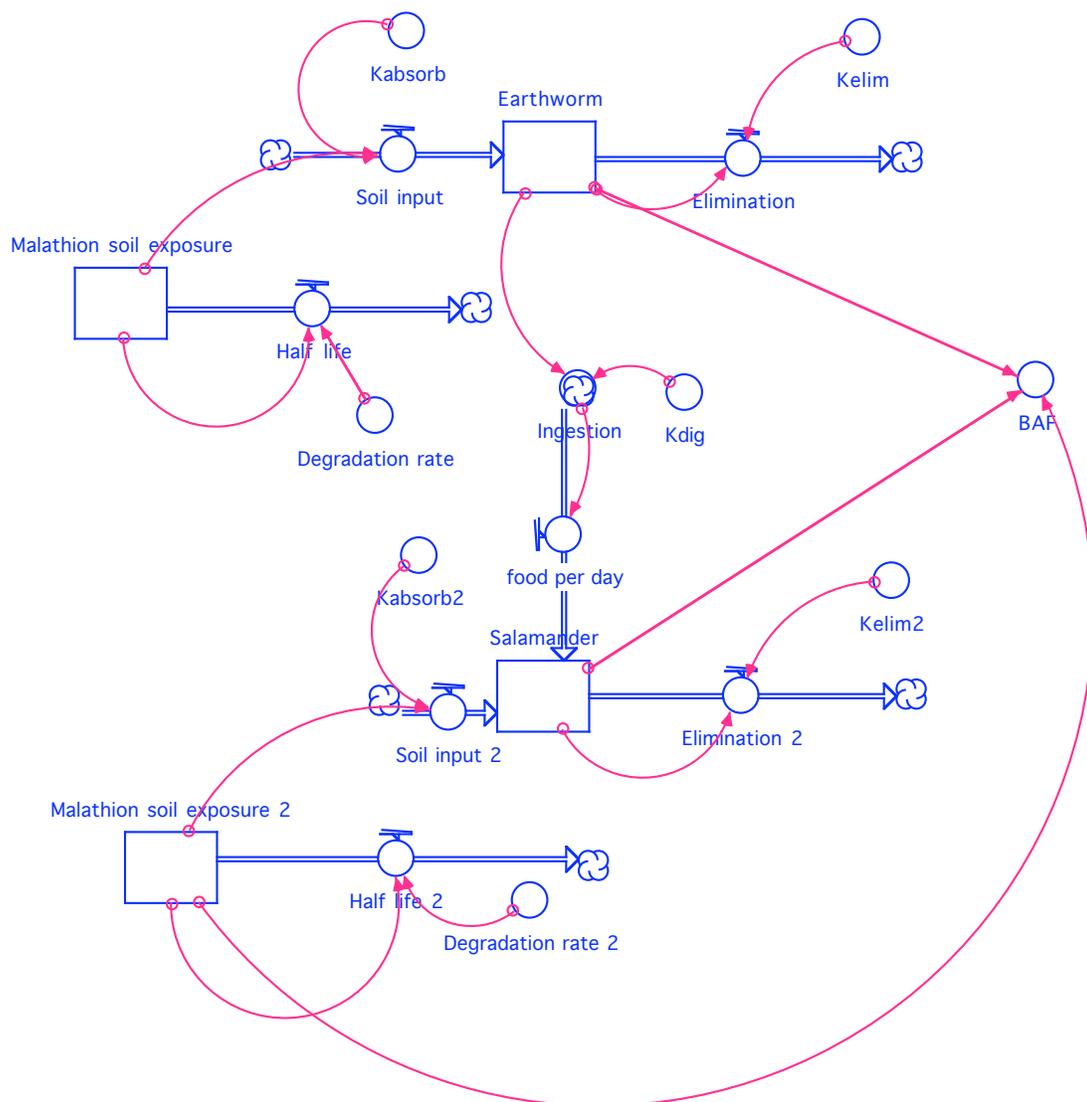
#### 8.4.3 Are more animals necessary?

While the salamander exposures were not designed to produce toxicokinetics data, they did provide enough information to parameterize the mathematical model. The kinetic parameters were varied to assess the probability of malathion bioaccumulation in *A. tigrinum*. When evaluating soil exposure alone, bioconcentration did not occur even if the cutaneous absorption rate was increased and the elimination rate was decreased to model the highest experimentally induced malathion body burdens at 24 and 48 hours. If

the exposure was extended to include the consumption of a contaminated earthworm, then bioaccumulation only occurred if the salamander ate an entire worm daily for 10 days and absorbed all of the malathion from the worm. This situation resulted in a BCF of 1.13, which is barely suggestive of malathion bioaccumulation. Unfortunately, there is a lack of data on feeding rates in wild Ambystomidae. This is because of their fossorial life-style, which limits observation of feeding. However, the consumption of prey every 3 days is recommended for maintenance (Maruska 1994). This has been observed with the experimental animals where feeding *A. tigrinum* one-half a *L. terrestris* every three days resulted in a 25% increase in body weight per week while growing and maintenance of weight when fully grown (Henson-Ramsey, unpublished). It is unlikely that wild salamanders would consume an entire earthworm on a daily basis. Additionally, the probability of salamanders having a gastrointestinal system so efficient to allow for 100% absorption is extremely low, therefore the risk of bioaccumulation from consuming contaminated earthworms is nil. Experimentally, tiger salamanders that consumed a contaminated earthworm in addition to cutaneous absorption of malathion (Chapter 7) did not exhibit an increase in tissue burdens. Based on the model's predictions, bioaccumulation of malathion in tiger salamanders exposed to environmentally relevant concentrations of malathion is extremely unlikely. Therefore, further experimentation to narrow kinetic parameters will not provide more information when calculating the risk of malathion bioaccumulation in *A. tigrinum*.

Mathematical models can be extremely useful when evaluating limited experimental data. They allow the researcher to explore "what-if" scenarios and evaluate which of those scenarios are worth additional experimentation. This allows for the

possible reduction in animal sacrifices, which is of value when working with threatened or endangered species.



**Fig 8.1** Stella salamander model diagram

$\text{Earthworm}(t) = \text{Earthworm}(t - dt) + (\text{Soil\_input} - \text{Elimination}) * dt$   
 INIT Earthworm = Soil\_input  
 INFLOWS:  
 $\text{Soil\_input} = \text{Malathion\_soil\_exposure} * \text{Kabsorb}$   
 OUTFLOWS:  
 $\text{Elimination} = \text{Earthworm} * \text{Kelim}$   
 $\text{Malathion\_soil\_exposure}(t) = \text{Malathion\_soil\_exposure}(t - dt) + (- \text{Half\_life}) * dt$   
 INIT Malathion\_soil\_exposure = 36.18  
 OUTFLOWS:  
 $\text{Half\_life} = \text{Malathion\_soil\_exposure} * \text{Degradation\_rate}$   
 $\text{Malathion\_soil\_exposure\_2}(t) = \text{Malathion\_soil\_exposure\_2}(t - dt) + (- \text{Half\_life\_2}) * dt$   
 INIT Malathion\_soil\_exposure\_2 = 27.12  
 OUTFLOWS:  
 $\text{Half\_life\_2} = \text{Malathion\_soil\_exposure\_2} * \text{Degradation\_rate\_2}$   
 $\text{Salamander}(t) = \text{Salamander}(t - dt) + (\text{food\_per\_day} + \text{Soil\_input\_2} - \text{Elimination\_2}) * dt$   
 INIT Salamander = Soil\_input\_2 + food\_per\_day  
 INFLOWS:  
 $\text{food\_per\_day} = \text{Ingestion} / 24$   
 $\text{Soil\_input\_2} = \text{Malathion\_soil\_exposure\_2} * \text{Kabsorb2}$   
 OUTFLOWS:  
 $\text{Elimination\_2} = \text{Salamander} * \text{Kelim2}$   
 $\text{BAF} = \text{Salamander} / (\text{Malathion\_soil\_exposure\_2} + \text{Earthworm})$   
 $\text{Degradation\_rate} = .0123$   
 $\text{Degradation\_rate\_2} = .017$   
 $\text{Ingestion} = \text{Earthworm} * \text{Kdig}$   
 $\text{Kabsorb} = .00294$   
 $\text{Kabsorb2} = .0042$   
 $\text{Kdig} = 1$   
 $\text{Kelim} = .084$   
 $\text{Kelim2} = .04$

**Fig 8.2** Mathematical relationships between parameters in salamander model

## 8.5 References

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## **Appendices**

## Appendix 1

*Raw data for Comparison of Filter Paper and Soil Exposure*

<i>Filter Paper</i>	control	1 $\mu\text{g}/\text{cm}^2$	2 $\mu\text{g}/\text{cm}^2$	5 $\mu\text{g}/\text{cm}^2$	50 $\mu\text{g}/\text{cm}^2$	100 $\mu\text{g}/\text{cm}^2$
Replicate #1	0.00	1.29	1.69	1.09	9.81	110.88
Replicate #2	0.00	0.00	0.80	1.36	7.61	7.67
Replicate #3	0.00	1.45	2.48	2.47	3.38	6.13
Replicate #4	0.00	0.62	3.07	0.34	3.84	91.48
Replicate #5	0.00	5.52	0.05	0.21	7.97	77.99
<i>Median</i>	<i>0.00</i>	<i>1.29</i>	<i>1.69</i>	<i>1.093</i>	<i>7.61</i>	<i>77.99</i>
<i>SD</i>	<i>0.00</i>	<i>2.17</i>	<i>1.23</i>	<i>0.91</i>	<i>2.79</i>	<i>48.83</i>
<i>Soil</i>	control	1 $\mu\text{g}/\text{cm}^2$	2 $\mu\text{g}/\text{cm}^2$	5 $\mu\text{g}/\text{cm}^2$	50 $\mu\text{g}/\text{cm}^2$	100 $\mu\text{g}/\text{cm}^2$
Replicate #1	0.00	2.94	0.00	0.79	1.62	0.45
Replicate #2	0.00	0.58	5.51	0.44	1.17	0.42
Replicate #3	0.00	0.46	0.06	0.56	1.84	0.96
Replicate #4	0.00	0.00	1.01	2.45	1.76	0.76
Replicate #5	0.00	0.16	1.62		1.09	3.87
<i>Median</i>	<i>0.00</i>	<i>0.46</i>	<i>1.012</i>	<i>0.67</i>	<i>1.62</i>	<i>0.76</i>
<i>SD</i>	<i>0.00</i>	<i>1.21</i>	<i>2.27</i>	<i>0.94</i>	<i>0.34</i>	<i>1.46</i>

## Appendix 2

### Raw data for comparison of effect of organic matter on bioavailability

	Control		8% Organic Matter			55% Organic Matter		
	Replicate #1	Replicate #2	Replicate #1	Replicate #2	Replicate #3	Replicate #1	Replicate #2	Replicate #3
<b>Protein content</b> (mg/g)	279.04	301.95	243.72	202.22	341.24	393.89	151.48	374.38
	214.32	202.23	291.75	262.61	313.90	284.75	253.85	211.77
	355.52	206.76		252.68	168.02	214.51	196.80	293.88
<b>Ache</b> (nmol/min/g) worm	46666.67	52134.15	25312.50	37136.75	16238.53	19419.35	24102.56	365.38
	78041.24	63170.73	16764.71	30731.71	3625.00	18820.22	28075.22	22192.31
	47220.00	48605.77		10392.86	15381.36	25938.14	20000.00	4385.06
<b>Ache</b> (nmol/min/g) protein	167.26	172.63	103.74	183.84	47.62	49.29	158.67	0.97
	364.68	312.73	57.41	116.85	11.54	48.89	110.53	104.68
	132.64	234.81		41.08	91.56	82.34	101.52	14.92
<i>Median Ache</i>	183.70		66.32			91.93		
<i>SD Ache</i>	93.71		27.57			23.86		
<b>Malathion</b> (ppm)	0.00	0.00	0.64	0.22	1.26	0.79	1.00	0.55
	0.00	0.00	1.49	0.44	1.10	0.59	0.66	0.55
	0.00	0.00	0.93	0.39	0.43	2.04	0.54	1.21
<i>Median Malathion</i>	0.00		0.71			0.78		
<i>SD Malathion</i>	0.00		0.22			0.34		

**Appendix 3**  
**Raw data for comparison of toxicity in two earthworm species**

	<b>Lumbricus control</b>	<b>Lumbricus exposed</b>	<b>Eisenia control</b>	<b>Eisenia exposed</b>
<b>Malathion</b> (ppm)	0.00	3.31	0.00	5.21
	0.00	2.71	0.00	1.46
	0.00	3.57	0.00	3.17
	0.00	1.27	0.00	4.63
	0.00	1.66	0.00	3.41
	0.00	4.08	0.00	3
	0.00	4.42	0.00	9.2
	0.00	4.15	0.00	12.33
	0.00	2.14	0.00	3.16
<i>Median</i>	<i>0.00</i>	<i>3.314</i>	<i>0.00</i>	<i>3.41</i>
<b>Cholinesterase</b> (nmol/min/mg protein)	478.45	38.49	144.04	8.39
	245.57	211.03	245.47	10.99
	216.01	79.53	86.22	67.1
	254.55	179.58	63.56	58.3
	332.72	91.44	181.98	4.45
<i>Median</i>	<i>293.6</i>	<i>91.44</i>	<i>144</i>	<i>10.99</i>

## Appendix 4

### Raw data for toxicokinetic study

#### Sample (time hours) Earthworm (0) Earthworm (2) Earthworm (6) Earthworm (12) Earthorm (24)

##### Malathion

ppm

Replicate #1	0	0.34	0.09	0.83	1.087
Replicate #2	0	0.11	0.75	0.56	1.245
Replicate #3	0	0.25	0.31	0.9	0.99
Replicate #4	0	0.1	0.26	0.35	0.95
Replicate #5	0		0.53	0.27	0.51

<i>Median</i>	<i>0</i>	<i>0.19</i>	<i>0.31</i>	<i>0.56</i>	<i>0.99</i>
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##### Cholinesterase

(nmol/min/g worm)

Replicate #1	58666.67	37170.54	62043.01	23260.87	
Replicate #2	59051.72	63649.19	30194.12	50346.15	33958.72
Replicate #3	54901.96	53349.51	46111.11	17605.26	49150.94
Replicate #4	56153.85	60963.3	57628.87	32464.79	54816.51
Replicate #5	70879.12	51666.67	60038.17	23539.86	31692.92

##### Protein

(mg/g worm)

Replicate #1	363	202	260	195	137
Replicate #2	258	150		271	167
Replicate #3	188	236	297	149	254
Replicate #4	141	261	379	234	194
Replicate #5	187	140	329	251	198

##### Cholinesterase

(nmol/min/g protein)

Replicate #1	161.62	184.02	238.63	119.29	203.35
Replicate #2	228.88	424.33		185.78	193.51
Replicate #3	292.03	226.06	155.26	118.16	282.56
Replicate #4	398.25	233.58	152.06	138.74	160.07
Replicate #5	379.03	369.05	182.49	95.78	

<i>Median</i>	<i>303.9</i>	<i>233.6</i>	<i>168.9</i>	<i>119.3</i>	<i>198.4</i>
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### Appendix 4 cont...

#### Sample (time hours) Earthworm (48) Earthworm (72) Earthworm (-6) Earthworm (-24) Malathion

ppm

Replicate #1	0.48	0.48	0.73	0.22
Replicate #2	2.03	1.41	0.43	0.36
Replicate #3	1.02	0.69	0.42	0.25
Replicate #4	1.11	0.45	0.43	0.22
Replicate #5	1.99		0.33	0.2

<i>Median</i>	<i>1.11</i>	<i>0.69</i>	<i>0.43</i>	<i>0.22</i>
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#### Cholinesterase

(nmol/min/g worm)

<b>Replicate #1</b>	38791.67	24402.17	47535.21	66572.58
<b>Replicate #2</b>	32686.57	30172.04	55175.44	52455.36
<b>Replicate #3</b>	36517.24	45230.77	464995.33	46848.74
<b>Replicate #4</b>	36269.23		41460.67	23046.88
<b>Replicate #5</b>	44101.12	35381.36	41949.44	36440.68

#### Protein

(mg/g worm)

<b>Replicate #1</b>	247	198	133	179
<b>Replicate #2</b>	149	253	256	322
<b>Replicate #3</b>	172	352	179	471
<b>Replicate #4</b>	238	299	250	250
<b>Replicate #5</b>	239	267	284	214

#### Cholinesterase

(nmol/min/g protein)

<b>Replicate #1</b>	157.05	123.24	357.41	371.91
<b>Replicate #2</b>	219.37	119.26	215.53	162.9
<b>Replicate #3</b>	212.31	128.5	259.75	99.47
<b>Replicate #4</b>	152.39		165.84	92.19
<b>Replicate #5</b>	184.52	132.51	147.71	170.28

<i>Median</i>	<i>184.5</i>	<i>125.9</i>	<i>215.5</i>	<i>162.9</i>
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## Appendix 4 cont.....

**Sample (time hours) Earthworm (-52) Earthworm (-72)  
Malathion**

ppm

Replicate #1	0.18	0.11
Replicate #2	0.23	0
Replicate #3	0.11	0
Replicate #4	0.09	0
Replicate #5	0.16	0

<i>Median</i>	0.16	0
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**Cholinesterase**

(nmol/min/g worm)

<b>Replicate #1</b>	58913.04	52908.17
<b>Replicate #2</b>	56009.17	40555.56
<b>Replicate #3</b>	31944.44	
<b>Replicate #4</b>	49280	44747.47
<b>Replicate #5</b>		49523.65

**Protein**

(mg/g worm)

<b>Replicate #1</b>	186	199
<b>Replicate #2</b>	180	212
<b>Replicate #3</b>	306	306
<b>Replicate #4</b>	369	425
<b>Replicate #5</b>	259	152

**Cholinesterase**

(nmol/min/g protein)

<b>Replicate #1</b>	316.74	265.87
<b>Replicate #2</b>	311.16	191.3
<b>Replicate #3</b>	104.4	266
<b>Replicate #4</b>	133.55	205.29
<b>Replicate #5</b>		

<i>Median</i>	222.4	235.6
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## Appendix 4 cont....

<b>Sample (time hours)</b>	<b>Soil (0)</b>	<b>Soil ( 39)</b>	<b>Soil (63)</b>	<b>Soil (87)</b>
<b>Malathion</b>				
ppm				
Replicate #1	36.18	15.39	29.48	8.67
Replicate #2	36.49	21.37	16.08	10.78
Replicate #3	31.07	21.84	12.92	5.29
Replicate #4	26.29	25.47	15.74	
Replicate #5	44.39	28.41	12.61	
<i>Median</i>	<i>36.18</i>	<i>21.84</i>	<i>15.74</i>	<i>8.67</i>

## Appendix 5

### Raw data for salamander exposures

	<b>50 mg/cm<sup>2</sup> soil, one day</b>			<b>50 mg/cm<sup>2</sup> soil, two day</b>					
	#9889	#9872	#9891	#9880	#9886	#9890	#9885	#9865	#9892
<b>Tissue malathion</b> (ppm)									
<i>Liver</i>	0.18	0.42	0.196	1.423	1.30	1.19	0.81	0.24	0.19
<i>Gastrointestinal</i>	11.62	21.47	0.149						
<i>Avisceral carcass</i>	0	0	0.113	0.291	0.89	10.8	3.57	0.39	0.61
<i>Visceral carcass</i>	0.84	1.42	0.464	14.34	0.29	0.27	1.02	0.00	0.2
<b>Sample weight</b> (g)									
<i>Liver</i>	3.65	3.47	2.77	3.38	3.18	3.04	3.82	4.66	3.34
<i>Gastrointestinal</i>	4.12	2.38	2.38						
<i>Avisceral carcass</i>	4.61	21.01	18.5	50.33	14.47	8.49	19.94	9.33	8.62
<i>Visceral carcass</i>	48.93	50.92	44.15	20.89	47.59	46.26	39.07	45.80	49.98
<b>Total tissue burden</b> (ppm)									
<i>Liver</i>	0.67	1.45	0.544	4.8	4.12	3.63	3.09	1.10	0.64
<i>Gastrointestinal</i>	47.89	51.1	0.354						
<i>Avisceral carcass</i>	0	0	2.081	14.64	2.75	91.78	71.27	3.65	5.23
<i>Visceral carcass</i>	41.05	72.36	20.49	299.56	13.84	12.69	39.89	0.00	10.17
<b>Total burden</b> (salamander ppm)	1.46	1.61	0.35	3.73	0.39	1.42	1.47	0.05	0.19
<i>Median</i>	1.46			1.42					
Brain cholinesterase (umol/min/g brain)	5.53	4.61	5.00	9.07	9.33	11.92	5.29	9.65	4.78
<i>Median</i>	5.00			9.2					

## Appendix 5 cont.....

	50 mg/cm <sup>2</sup> soil/worm, two day			100 mg/cm <sup>2</sup> soil/worm, two day			Control	
	#9877	#9884	#9875	#9870	#9871	#9879	#9864	#9874
<b>Tissue malathion</b> (ppm)								
<i>Liver</i>	1.61	1.18	0.69	0.33	0.52	1.42	0.00	0.00
<i>Gastrointestinal</i>								
<i>Avisceral carcass</i>	0.25	0.94	0.80	2.61	2.04	2.64	0.00	0.00
<i>Visceral carcass</i>	0.25	0.30	0.36	0.38	0.39	0.15	0.00	0.00
<b>Sample weight</b> (g)								
<i>Liver</i>	3.67	4.41	5.17	4.15	3.49	3.88	0.00	0.00
<i>Gastrointestinal</i>								
<i>Avisceral carcass</i>	21.09	9.55	8.53	9.33	24.1	6.50	0.00	0.00
<i>Visceral carcass</i>	57.41	60.29	60.29	55.2	57.2	51.2	0.00	0.00
<b>Total tissue burden</b> (ppm)								
<i>Liver</i>	5.93	5.22	3.57	1.36	1.81	5.49	0.00	0.00
<i>Gastrointestinal</i>								
<i>Avisceral carcass</i>	5.31	8.99	6.54	24.39	49.07	17.16	0.00	0.00
<i>Visceral carcass</i>	14.47	18.2	21.73	20.89	22.82	7.44	0.00	0.00
<b>Total burden</b> (salamander ppm)	0.28	0.43	0.35	0.56	0.77	0.42	0.00	0.00
<i>Median</i>	0.35			0.56			0.00	
Brain cholinesterase (umol/min/g brain)	3.63	8.12	11.38	1.17	0.85	0.72	20.61	15.59
<i>Median</i>	8.12			0.85			18.1	