

A Comparison of Multiple Esterases as Biomarkers of Organophosphate Exposure and Effect in Two Earthworm Species

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Abstract Two different earthworm species, *Eisenia fetida* and *Lumbricus terrestris*, were exposed to 5 µg/cm² of malathion to evaluate their usefulness as sentinels of organophosphate exposure and to assess three different esterases, as biomarkers of malathion exposure and effect. Tissue xenobiotic burdens and esterase activity were determined for each species and each esterase in order to assess variability. *E. fetida* exhibited 4-fold less variability in tissue burdens than did *L. terrestris* and had less variable basal esterase activities. An attempt was made to correlate malathion and malaoxon tissue burdens with esterase activity post-exposure. There was no malaoxon present in the earthworm tissues. No significant correlations were determined by comparing acetylcholinesterase, butyrylcholinesterase, nor carboxylesterase activities with malathion burdens.

Keywords Biomarker · Esterase · Earthworm · Organophosphate

Earthworms serve as sentinel species for toxicity associated with soil-applied xenobiotics (Edwards and Bohlen 1992) while simultaneously contributing to the degradation of the same xenobiotics through recycling of carbon based nutrients (Scheu 1987). Consequently, earthworm species are frequent subjects of toxicity studies that explore the

effects of exposure to commonly used pesticides, such as organophosphates and carbamates, by evaluating the effect of such pesticides on degradation and metabolizing enzymes. In this manner, enzyme up-regulation or inhibition can be used as a biomarker of pesticide exposure and effect (Booth et al. 2001; Chambers et al. 2002). Previous research has demonstrated that earthworm species vary considerably in their capacity to absorb and degrade xenobiotics (Gilman and Vardanis 1974; Stenersen et al. 1992). The prevailing hypothesis accounting for these differences is that earthworm species possess detoxification and metabolizing enzymes that diverge in their affinity for and activity towards a particular insecticide (Stenersen et al. 1992). This suggests that species differences need to be considered when developing biomarkers of insecticide exposure in earthworms.

Organophosphorous insecticides, such as malathion, interact with multiple esterases classified as a-, b-, and c-esterases (Augustinsson and Nachmansohn 1949; Eto 1974). Of these, the b-esterases have shown the most promise as useful biomarkers of organophosphate exposure. The b-esterases include acetylcholinesterase (AChE), butyrylcholinesterase (BChE), and carboxylesterase (CbE), all of which are inhibited by organophosphates (Eto 1974). Malathion results in toxicity by irreversibly binding AChE, thereby preventing degradation of the enzyme's natural substrate, acetylcholine (Fukoto 1990). This results in an increase in acetylcholine at visceral and neuromuscular synapses, leading to muscarinic and nicotinic toxicity. In earthworm species, AChE inhibition results in a clinical syndrome described as coiling (Henson-Ramsey et al. 2007), which is presumably a result of uncontrolled muscular contractions. In contrast, the binding of carboxylesterase by organophosphates has been shown to serve a protective function against toxicity in mammals because

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CbE hydrolyzes malathion to non-toxic metabolites, which are then rapidly excreted (Fukoto 1990). Additionally, carboxylesterases compete with AChE for malathion binding sites, increasing the relative amount of unbound AChE available for degradation of acetylcholine (Chanda et al. 1997). Butyrylcholinesterase is also inhibited by organophosphate exposure; however, the full consequences of BChE inhibition are unknown (Cokugras 2003). Acetylcholinesterase inhibition is the most widely investigated biomarker of organophosphate exposure because AChE inhibition exhibits significant chemical specificity. Inhibition of this enzyme implicates a relatively limited number of toxicants, namely organophosphates, pyrethroids, and carbamates. Additionally, acetylcholinesterase suppression can be linked directly to toxic effects in organisms and the assays are relatively sensitive (Chambers et al. 2002). However, there are disadvantages to using AChE as a biomarker. Of primary concern, acetylcholinesterase activity does not correlate well with xenobiotic burdens, nor does the degree of inhibition correlate with severity of toxicity (Booth et al. 2001; Chambers et al. 2002).

This project evaluated the use of three b-esterases, AChE, BChE, and CbE as biomarkers in two different earthworm species, *Lumbricus terrestris* and *Eisenia fetida* after exposure to a model organophosphate, malathion. The biomarkers are assessed based upon enzyme activity, variability both within and between the two earthworm species, and whether or not enzyme activities correlate with malathion body burdens. The overall goal of this project was to evaluate each esterase as a biomarker of malathion exposure and to compare two different earthworm species as sentinels for organophosphate exposure.

Materials and Methods

Lumbricus terrestris and *Eisenia fetida* were both obtained from Mountain Home Biological (White Salmon, WA). The worms were maintained at 10°C in separate polyethylene containers filled with Scott's garden soil. Both worm species were fed a commercial worm diet (obtained from www.wormman.com) every third day.

After a two-week acclimation period, the earthworms were exposed to 5 µg/cm² of malathion by filter paper contact for 72 h (Henson-Ramsey et al. 2007). Five worms of each species were exposed to malathion in individual contact chambers and five additional worms per species served as controls. The experiment was then repeated once. After the exposure period, all earthworms, both control and exposed, were assessed for the presence of coiling and then euthanized by placement into hot water. Each worm, exposed and control, was analyzed individually by the following methods.

A post-clitellum sample, 0.2 gram, was collected with half of the sample frozen at −80°C for later determination of malathion body burdens and the other half prepared for enzyme analysis by homogenization in 2 mL of 8.0 Tris buffer (Fisher Scientific) for one minute using an electric homogenizer. The homogenate was then centrifuged for 10 min at 100,000 g and 60 µL of the supernatant was removed and stored at −80°C for the enzyme assays. All enzyme assays were performed within one week of the euthanasia and all tissue burdens were determined within one month of the euthanasia.

The enzyme assays were performed using the following standards, electric eel acetylcholinesterase, butyrylcholinesterase from horse serum, and rabbit esterase as well as the substrates, acetylthiocholine 98%, butyrylthiocholine 98%, and 4-nitrophenylacetate 98%. All reagents for enzyme analysis were procured from Fisher Scientific. A uQuant microplate reader with Gen5 software (Biotek instruments) was used for all the enzyme assays. Measurement of acetylcholinesterase and butyrylcholinesterase activity was performed using a modified Ellman technique (Ellman et al. 1961). Sample size required for both the BChE and AChE assays was 20 µL. The sample was added to 3 mL of the chromophore, DTNB (5, 5'-Dithio-bis(2-nitrobenzoic acid)). After the addition of 100 µL of the appropriate substrate, enzyme activity was read at a wavelength of 405 nm. Carboxylesterase was assayed using the following technique (Leopold 1996; Park et al. 1961). A 20-µL sample was added to 278 µL of Tris Buffer 7.4. The addition of 2 µL of the substrate, 4-nitrophenylacetate, initiated the enzymatic reaction. The wells were read at 405 nm. All colorimetric samples were run in triplicate on the uQuant reader with appropriate blanks. Results were standardized to protein levels obtained with the Bradford protein assay (Pierce) (Bradford 1976).

The remaining worm samples were then prepared for determination of malathion tissue burdens by the following procedure. All reagents for body burden determination were obtained from Fisher Scientific. Each thawed sample was placed in a Dounce homogenizer with 100 mg of anhydrous sodium sulfate and thoroughly mixed until it appeared homogenous. After the addition of 3 mL of a 1:3 acetone/hexane solution, the homogenate was manually mixed and then vortexed for 10s. The homogenate mixture was then transferred to a borosilicate glass tube. This transfer was repeated twice more with 3 mL of the 1:3 acetone/hexane solution to ensure complete transfer. The combined transfer solutions were then centrifuged for 8 min, after which the supernatant was transferred to a clean borosilicate glass tube. A Folch wash was performed to reduce lipid contamination by adding 2 mL of 0.88% KCl solution (Folch et al. 1957) to the sample supernatants, which were then re-centrifuged for another 8 min. After the

second centrifugation, the solvent layer was then removed and evaporated to dryness in a clean borosilicate tube. The sample was then reconstituted in a minimal volume of 200 μL of hexane and eluted using a glass Pasteur pipette onto a column of 1 g of Florisil, 250 grain that had been previously prepared by rinsing with 1 mL of hexane. The first fraction was eluted with 3 mL of hexane and discarded. Second and third elutions were performed with 3 mL of 1:19 acetone/hexane and 3 mL of 1:3 acetone/hexane, respectively. The resulting combined fractions were collected and a second Folch wash performed. The solvent layer was removed to a clean borosilicate tube and evaporated to dryness with nitrogen. The samples were then refrigerated at 10°C. Samples were held no longer than one week prior to further processing.

The samples were then analyzed by gas chromatography with a mass spectrophotometer (Shimadzu) fitted with a DB-1 capillary column (30 m by 0.32 mm by 0.25 μm from J&W Scientific). The injector was set to 250°C. The column temperature was initially ramped from 60 to 220°C at a rate of 40°C/min and then ramped at a rate of 2°C/min from 220 to 228°C. The total run time was 8.9 min. Malathion eluted at 6.5 min and malaoxon eluted at 6.2 min. External standards (Restek Corporation, Belafonte, PA) were used to verify the identity of analytes based on elution times and were used to quantify the amount of xenobiotic based upon a standard curve. The limit of detection for malathion with this method was 0.04 and 0.10 μg for malaoxon with extraction efficiencies of 85–100%. Three replicates were used to determine the extraction efficiencies. All statistical analyses were performed using Prism[®], Graphpad (San Diego, CA).

Results and Discussion

One of the goals of this project was to evaluate two different earthworm species as sentinels of organophosphate exposure by determining xenobiotic tissue burdens after malathion exposure. None of the control earthworms from either species exhibited clinical signs of toxicosis, coiling, nor did they have malathion residues in their tissues. Malathion was detectable in all of the exposed earthworms (Fig. 1) and both species were observed to exhibit coiling. There was no malaoxon detected in any of the earthworm samples. There were no statistical differences in xenobiotic body burdens when comparing *L. terrestris* and *E. fetida*, Mann–Whitney test (p value 0.6905), however, there was a 4.4-fold higher variability from individual to individual in the amount of malathion present in *L. terrestris* when compared to malathion burdens in *E. fetida* (Fig. 1). Individual *Lumbricus* had malathion burdens that ranged from 0.2040 to 8.921 $\mu\text{g}/\text{mg}$ of worm (mean- 4.495 $\mu\text{g}/\text{mg}$,

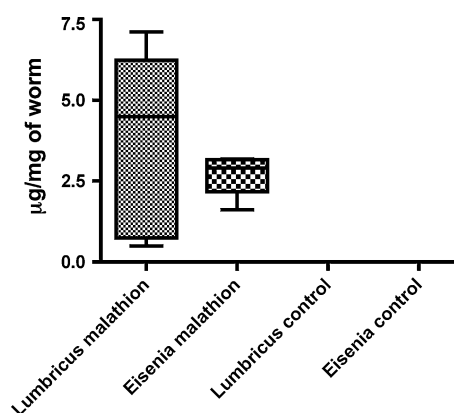


Fig. 1 Malathion body burdens in *Eisenia fetida* and *Lumbricus terrestris*. Box and whiskers plot illustrating the median body burden of malathion present in both control and exposed earthworm species

standard deviation- 2.855 $\mu\text{g}/\text{mg}$), while *Eisenia*'s malathion tissue burdens ranged from 1.158 to 4.367 $\mu\text{g}/\text{mg}$ of worm (mean 2.906 $\mu\text{g}/\text{mg}$, standard deviation 0.6437 $\mu\text{g}/\text{mg}$).

The relative lack of variability in tissue burdens may be innate to *Eisenia* species or may be an artifact of our sampling technique. Both species were sampled post-clitellum with a sample size of approximately 0.2 grams. However, because *E. fetida* is a small worm, this sample represented all digestive organs. In contrast, because of the larger size of *L. terrestris*, the sample included only the crop, gizzard, and the anterior section of the intestines. This may have contributed to the variability in tissue burdens noted in *Lumbricus*, because different organs have been hypothesized to absorb differential amounts of xenobiotics (Sanchez-Hernandez and Wheelock 2009).

The lower variability in malathion residues within *Eisenia* species increases the earthworm's usefulness as a sentinel. Additionally, *Eisenia* is more likely to come in frequent contact with malathion than *Lumbricus* due to their respective microhabitats. *Eisenia fetida* is epigenic, or a surface-dweller. *Lumbricus terrestris* is anecic and spends most of its time in burrows, surfacing only to feed or reproduce (Edwards and Bohlen 1996). Based on time spent on the soil surface, the risk of *E. fetida* being exposed to malathion is greater than for *L. terrestris*. Malathion is most often applied by surface spraying and has a relatively short half-life in soil of approximately 3 days (Environmental Protection Agency 2000). It is unlikely that *Lumbricus* would consistently come in contact with the xenobiotic unless the worm surfaced during a spray. Therefore, in addition to *Eisenia*'s lower variability in xenobiotic burdens, *E. fetida* may be the preferred sentinel earthworm species when examining xenobiotics that are applied to the soil surface and have a short window of bioavailability.

Malaoxon was not detected in the tissues of the exposed nor the control earthworms of either species. The –oxon metabolite of organophosphates is known to be more toxic than the parent compound (Eto 1974). Therefore, clinical signs are often attributed to malaoxon rather than malathion. Earthworms do possess the necessary metabolic enzymes, multifunction oxidase systems, for the production of the –oxon metabolite (Dhainaut and Scaps 2001). Since the earthworms exhibited cholinesterase inhibition and clinical signs of toxicity including coiling, it is assumed that malaoxon was produced. Little is known about the rate of malaoxon production in earthworm species or even if the –oxon metabolite is the more toxic compound in these species. The lack of malaoxon detected may be a result of rapid excretion of the –oxon, slow metabolism of malathion to malaoxon, or may be a lack of sensitivity of the GC–MS assay.

As well as determining xenobiotic burdens in the earthworms after organophosphate exposure, the activities of three different esterases were measured to evaluate each enzyme as a potential biomarker of malathion effect. For comparison purposes, basal activities of all three esterases were measured and are provided in Fig. 2. *Lumbricus terrestris* had significantly higher basal activities of all three enzymes than did *Eisenia fetida* (Mann–Whitney:

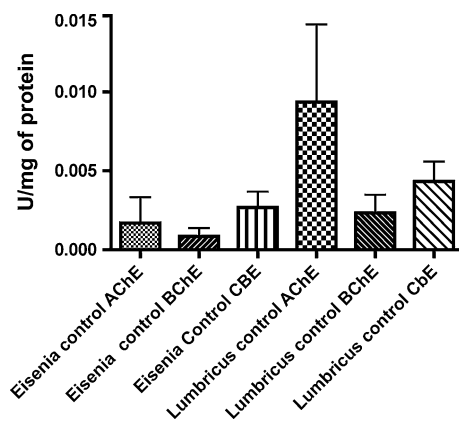


Fig. 2 Basal enzyme activities in *Lumbricus terrestris* and *Eisenia fetida*. Bar graph demonstrating basal levels of cholinesterase enzymes in both species. The graph shows median values and the error bars represent the interquartile range. Units are the amount of enzyme hydrolyzed per minute

AChE p value 0.0159, BChE p value 0.0317, and CbE p value 0.0317). In both species, butyrylcholinesterase had the lowest basal activity and in *Eisenia*, basal butyrylcholinesterase activity was sufficiently low that when inhibited by malathion, it was immeasurable. In general, there was greater enzyme variability between individual *Lumbricus terrestris* than between individual *E. fetida*. Overall, basal acetylcholinesterase activity was more variable than BChE and CbE between individuals of both species (Table 1). Figure 3 compares the basal esterase activities with the inhibited enzyme activity in both species after exposure to malathion. All esterases were suppressed in the exposed earthworms regardless of species. Acetylcholinesterase was inhibited approximately 60% in *E. fetida* and 76% in *L. terrestris* (Fig. 3a). BChE basal activity in *E. fetida* was low enough that after exposure, the amount of butyrylcholinesterase could not be consistently measured (Fig. 3b). In *L. terrestris*, BChE activity was inhibited 77% by malathion exposure. Carboxylesterase activity was suppressed to 67% of the basal activity in *E. fetida* and 75% in *L. terrestris* (Fig. 3c).

Spearman's r was calculated to assess correlation between malathion tissue burdens and esterase activity (Table 2). Because malathion inhibits enzyme activity, a negative r -value was anticipated. As Table 2 demonstrates, correlation of the individual cholinesterase enzymes to malathion loads was not statistically significant, however of the three esterases assayed, carboxylesterase correlated the most successfully. The correlations of CbE with malathion burdens in both species were weak with r -values of -0.45 and -0.37 in *Lumbricus terrestris* and *Eisenia foetida*, respectively. AChE and BChE activities did not correlate with malathion tissue loads. Improved correlations of CbE activities might be achieved by comparing metabolites of the carboxylesterase degradation pathway such as mono- and dicarboxylic acids (Fukoto 1990) with esterase levels instead of the parent organophosphate. CbE are also capable of interacting with multiple substrates, including alpha-naphthyl acetate, 4-nitrophenyl valerate, and 4-nitrophenyl acetate (Sanchez-Hernandez and Wheelock 2009). Determining carboxylesterase activity with one of these other substrates and then comparing to tissue burdens may improve correlations with body burdens. This would allow researchers to estimate the bioavailable

Table 1 List of earthworm species and basal enzyme activities with standard deviations

Species	AChE (U/mg protein)		BChE (U/mg protein)		CbE (U/mg protein)	
	Mean	SD	Mean	SD	Mean	SD
<i>Lumbricus terrestris</i>	0.0094	0.0041	0.0023	0.0009	0.0043	0.0010
<i>Eisenia fetida</i>	0.0022	0.0011	0.0008	0.0004	0.0027	0.0008

U- units of esterase hydrolyzed/minute

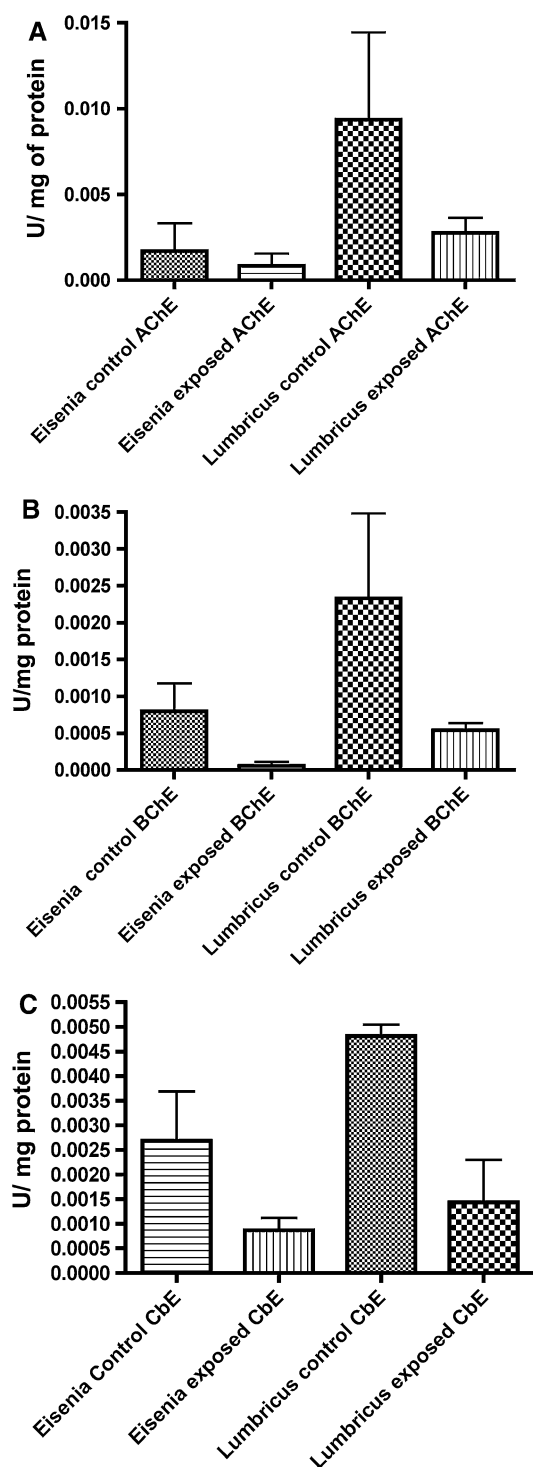


Fig. 3 Enzyme activities in exposed and unexposed earthworms. *Bar graph* illustrating the enzyme activities in each earthworm species both before and after exposure to malathion. The *graph* shows the median values and the error bars represent the interquartile range. Units are the amount of enzyme hydrolyzed per minute. **a** Acetylcholinesterase **b** Butyrylcholinesterase **c** Carboxylesterase

Table 2 List of earthworm species, enzyme assayed, and the correlation to malathion body burdens (Spearman's r)

Species	Enzyme	Spearman's r	p value
<i>Lumbricus terrestris</i>	AChE	0.376	0.3125
<i>Lumbricus terrestris</i>	BChE	0.033	0.9484
<i>Lumbricus terrestris</i>	CbE	−0.450	0.2298
<i>Eisenia foetida</i>	AChE	−0.260	0.5364
<i>Eisenia foetida</i>	BChE	N/D	N/D
<i>Eisenia foetida</i>	CbE	−0.370	0.3363

N/D- activities were too low to be reliably measured

portion of organophosphates in the environment more accurately and would provide information on organophosphate effect not just exposure.

Our study supports earlier research performed in other species using both CbE and AChE as biomarkers of organophosphate exposure (Bonacci et al. 2004; Sanchez-Hernandez and Moreno Sanchez 2002) by confirming that CbE, AChE, and BChE are present and quantifiable in both earthworm species. Additionally, all of the esterases were successfully inhibited by malathion exposure (Fig. 3) demonstrating the potential usefulness of all the esterases as a biomarker. Among the three studied esterases, we consider BChE the least desirable biomarker of malathion exposure because the basal activity of this enzyme in *E. fetida* was sufficiently low to prevent accurate measurement of enzyme activity after exposure to malathion. Though malathion exposure results in similar suppression of CbE and AChE activity (Fig. 3), the lower variance in CbE activity between individuals (Table 1) is an advantage when extrapolating from the individual to a larger population. With further research, carboxylesterases measured in earthworm tissue may be developed as a useful and practical biomarker of organophosphate loads in soil environments rather than simply as a measure of exposure as acetylcholinesterase is currently being used (Booth et al. 2001).

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References

- Augustinsson K, Nachmansohn D (1949) Distinction between acetylcholine-esterase and other choline esterase splitting enzymes. *Science* 22:98–99

- Bonacci S, Browne MA, Dissanayake A, Hagger J, Corsi I, Focardi S, Galloway T (2004) Esterase activities in the bivalve mollusc *Adamussium colbecki* as a biomarker for pollution monitoring in the Antarctic marine environment. *Mar Pollut Bull* 49:445–455
- Booth LH, Hodge S, O'Halloran K (2001) Use of biomarkers in earthworms to detect use and abuse of field applications of a model organophosphate pesticide. *Bull Environ Contam Toxicol* 67:633–640
- Bradford MM (1976) A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 72:248–254
- Chambers J, Boone JS, Carr R, Chambers H, Straus D (2002) Biomarkers as predictors in health and ecological risk assessment. *Hum Ecol Risk Assess* 8:165–176
- Chanda SM, Mortenson SR, Moser VC, Padilla S (1997) Tissue-specific effects of Chlorpyrifos on carboxylesterase and cholinesterase activity in adult rats: an in Vitro and in Vivo comparison. *Fund Appl Toxicol* 38:148–159
- Cokugras A (2003) Butyrylcholinesterase: structure and physiological importance. *Turkish J Biochem* 28(2):54–61
- Dhainaut A, Scaps P (2001) Immune defense and biological responses induced by toxins in Annelida. *Can J Zool* 79:233–253
- Edwards C, Bohlen P (1992) The effects of toxic chemicals on earthworms. *Rev Environ Contam Toxicol* 125:23–95
- Edwards C, Bohlen P (1996) Earthworm Biology. In: *Biology and ecology of earthworms*. Chapman and Hall, London, pp 46–70
- Ellman G, Courtney KD, Andres VJ, Featherstone RM (1961) A new and rapid colorimetric determination of acetylcholinesterase activity. *Biochem Pharmacol* 7:88–95
- Environmental Protection Agency (2000) Malathion: environmental fate and effects. In: EPA
- Eto M (1974) Organophosphorus pesticides: organic and biological chemistry. CRC Press, Cleveland, Ohio
- Folch J, Lees M, Stanley GHS (1957) A simple method for the isolation and purification of total lipids from animal tissues. *J Biol Chem* 226:497–509
- Fukoto TR (1990) Mechanism of action of organophosphorous and carbamate insecticides. *Environ Health Persp* 87:245–254
- Gilman A, Vardanis A (1974) Carbofuran: Comparative toxicity and metabolism in worms *Lumbricus terrestris* L and *Eisenia foetida* S. *J Agric Food Chem* 22(4):625–628
- Henson-Ramsey H, Kennedy-Stoskopf S, Levine J, Shea D, Taylor S, Stoskopf M (2007) A comparison of two exposure systems to apply malathion to *Lumbricus terrestris* L. *Bull Environ Contam Toxicol* 78:427–431
- Leopold V (1996) Esterase protection against diazinon toxicity in European starlings. In: *Clemson University*, Clemson, SC, p 104
- Park J, Meriwether B, Clodfelder P, Cunningham L (1961) The hydrolysis of p-nitrophenyl acetate catalyzed by 3-phosphoglycer-aldehyde dehydrogenase. *J of Biol Chem* 236:136–141
- Sanchez-Hernandez J, Moreno Sanchez B (2002) Lizard cholinesterases as biomarkers of pesticide exposure: enzymological characterization. *Environ Toxicol Chem* 21(11):2319–2325
- Sanchez-Hernandez J, Wheelock C (2009) Tissue distribution, isozyme abundance, and sensitivity to chlorpyrifos-oxon of carboxylesterases in the earthworm *Lumbricus terrestris*. *Environ Pollut* 157:264–272
- Scheu S (1987) Microbial activity and nutrient dynamics in earthworm casts (*Lumbricidae*). *Biol Fert Soils* 5(3):230–234
- Stenersen J, Brekke E, Engelstad F (1992) Earthworms for toxicity testing; Species differences in response towards cholinesterase inhibiting insecticides. *Soil Biol Biochem* 24(12):1761–1764