DISSERTATION

TOXICOLOGY OF MALATHION IN BULLFROGS AND LEOPARD FROGS

Submitted by

Carolyn L. Fordham

Department of Environmental Health

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WE HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER OUR SUPERVISION BY CAROLYN L. FORDHAM ENTITLED TOXICOLOGY OF MALATHION IN BULLFROGS AND LEOPARD FROGS BE ACCEPTED AS FULFILLING IN PART REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY.

Committee on Graduate Work

Adviser

Department Head/Director

ABSTRACT OF DISSERTATION

TOXICOLOGY OF MALATHION IN BULLFROGS AND LEOPARD FROGS

Malathion is an organophosphate pesticide commonly applied to agricultural and wetland areas. There is little information in the toxicological literature regarding the toxicity or residues of malathion in adult ranid frogs. A 96-h toxicity test was conducted with adult southern leopard frogs (*Rana utricularia*). The 96-h LC_{50} and 95% lower and upper confidence limits were 56.2 mg/L, 20.75, and 152.41, respectively. Behavior was monitored during the 96-h toxicity test, and frogs in the 10 mg/L malathion test concentration exhibited significantly fewer fright responses than the controls ($p=0.05$). Brain cholinesterase activity and malathion and malaoxon concentrations in liver and kidney were measured. Malathion was detected in liver and kidney of leopard frogs more frequently than malaoxon. The residues of malathion were investigated in the larger bullfrog *(R. catesbeiana)* by intravenous injection and monitoring of anesthetized frogs for a period as long as two hours following injection. Blood, liver, and kidney were analyzed for concentrations of malathion and malaoxon, and cholinesterase activity in brain was measured.

Because the larval stage is more sensitive than the adult, additional toxicity studies were conducted with bullfrog tadpoles. Effects on growth, development, and righting reflex

were determined in a 28-d static renewal test. Effects on learning behavior, gross motor activity, and brain cholinesterase were determined in tadpoles exposed for six days. Survival was significantly decreased ($p<0.001$) at malathion concentrations of 2500 μg/L and higher; development of tadpoles was delayed significantly ($p=0.003$) by malathion exposure as indicated by a dose-related decrease in developmental stage. In addition, righting reflex was impaired ($p<0.001$) in tadpoles in the malathion treatment groups. General activity levels were decreased ($p<0.05$) in the 3000 μg/L treatment group. Brain ChE activity was significantly less in all malathion treatment groups than that observed in the control group ($p<0.05$). Learning behavior was not significantly affected by malathion, although this could be confounded by the various physiological effects associated with poisoning. A lowest observed adverse effect level (LOAEL) for effects of malathion on bullfrog tadpoles was 500 μg/L; a no observed adverse effect level (NOAEL) was not identified.

Carolyn Louise Fordham
Department of Environmental Health
Colorado State University
Fort Collins, CO 80523
Spring 1999

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1.0 INTRODUCTION

This introduction describes the toxicology of malathion, as well as the available data regarding the toxicity of malathion to amphibians. Toxicological and pharmacokinetic data for malathion for ranid amphibians was lacking, and therefore much of the information in this introduction is based on data for mammals, birds, or insects.

1.1 TOXICOLOGY OF MALATHION

1.1.1 *Environmental Fate and Chemistry*

Malathion (diethyl mercaptosuccinate, S-ester with O,O -dimethyl phosphorodithioate) is an organophosphorus pesticide (Figure 1-1). Malathion has a vapor pressure of 1.25×10^{-4} mm Hg at 20°C, and a boiling point of 156 - 157°C at 7 mm Hg; malathion hydrolyzes at pH 5 or 8 (Matsumura, 1975). Malathion decomposes at high temperatures. It is slightly water soluble at room temperature (145 mg/L), and miscible with most organic solvents (>1 kg/L acetone, acetonitrile, dichloromethane, toluene)(Worthing, 1991).

Fate processes in surface water include volatilization, hydrolysis, and microbial degradation (Racke, 1992), and stability of malathion is greatest in natural waters at pH ranges of 5 to 7 (Brown et al., 1993). When aerially applied, malathion converts to

malaoxon and other products due to oxidation and hydrolysis (Montgomery, 1997; Brown et al., 1993); malaoxon and phosphoric acid are reported as breakdown products in drinking water; malathion monoacids are also reported in water (Montgomery, 1997).

Aerial drift and runoff are two mechanisms by which malathion could enter wetland ecosystems. Concentrations of organophosphates in surface water following direct application to water range from 1 to 10 μg/L, whereas concentrations in surface water due to aerial drift or soil runoff are typically in the ng/L range (Racke, 1992). Surface water concentrations due to forest spraying of organophosphates for pest control indicated small shallow ponds may contain a maximum of 2.5 mg/L (Berrill et al., 1994; Berrill et al., 1995). Malathion was reported in only 0.4% of surface water samples collected from agricultural watersheds (Racke, 1992). Brown et al. (1993) reported mean malathion deposition 24 hours after an aerial spray event onto ground-level surfaces to range from 1,100 to 2,200 $\mu g/ft^2$ (102.19 to 204.4 $\mu g/m^2$). Local conditions at each site (i.e., meteorological conditions, trees, houses) can influence spray deposition (Brown et al., 1993).

1.1.2 *Uses of Malathion*

Malathion is utilized in mosquito control applications in Colorado; however, malathion has been extensively used for control of other insects as well. Malathion has been used for control of coleopteran, dipteran, hemipteran, hymenopteran, and lepidopteran pests on crops including cotton, fruits, potatoes, and rice (Worthing, 1991). Other uses are as an

insecticide for the control of sucking and chewing insects and spider mites on fruits, vegetables, ornamentals, field and greenhouse crops, and in forestry (Montgomery, 1997). Malathion is one of the five most commonly used organophosphate pesticides that account for 60% of all organophosphate pesticides applied (EPA, 1998), and is registered for residential as well as agricultural purposes.

1.1.3 *Cholinesterase Inhibition*

Malathion acts as an irreversible cholinesterase (ChE) inhibitor and substitutes for the natural substrate for acetylcholinesterase (AChE), which is acetylcholine. There are two classes of cholinesterases that act on acetylcholine. True acetylcholinesterase (AChE) is the predominant cholinesterase found in brain, and also is associated with red blood cells (Munro et al., 1991). Nonspecific ChE (pseudocholinesterase or butyrylcholinesterase) is found in plasma.

The initial step in the reaction of an inhibitor with cholinesterase is the binding of a hydroxyl group of cholinesterase to the organophosphate acidic group of the organophosphate pesticide by the process of phosphorylation (Matsumura, 1975). The phosphorylated enzyme then rearranges, and with the presence of ambient water, forms a hydroxylated compound. With acetylcholine, the enzyme is regenerated by the process of deacylation; however, dephosphorylation occurs very slowly, according to the basic alkyl groups attached to the phosphorus atom. Because reactivation fails to occur at rates similar to the normal substrate, the enzyme is biologically inert for normal purposes. The

reaction can be expressed as (Matsumura, 1975; Wang and Murphy, 1982):

$$EH + PX \rightleftharpoons (EH \cdot PX) \rightarrow EP + XH \qquad (1)$$

where:

EH	=	free enzyme
PX	=	organophosphate inhibitor
EH·PX	=	reversible enzyme-inhibitor complex
EP	=	irreversibly phosphorylated enzyme
XH	=	acidic group

The substituents on the organophosphate molecule that lead to increasing electrophilicity (i.e., X is a stronger acid or more electronegative) increase insecticide potency.

There are several kinetic constants which determine the rate of enzyme inhibition. The rate of cholinesterase enzyme (ChE) inhibition depends on the affinity (K_a) of malathion for acetylcholinesterase, which is the ratio k_2/k_1, i.e.,

$$\frac{[EH][PX]}{[EH \cdot PX]} = \frac{k_2}{k_1} = K_a \qquad (2)$$

where:

k_1	=	the binding of [EH] with [PX] to form the unstable intermediate [EH•PX]
k_2	=	the dissociation of the intermediate complex

Another constant, K_p, is the rate constant for conversion of inhibited enzyme to a form that cannot be reactivated (i.e., EP); this constant is also termed the phosphorylation constant (Wang and Murphy, 1982). The ratio of K_p to K_a is the bimolecular rate constant (k_i).

A wide range of normal values for cholinesterase activities have been reported for any given species, and comparison of enzyme activities between different studies, laboratories, or by different test methodologies is questionable (Marden et al., 1994; Munro et al., 1991). A study comparing interlaboratory variability in cholinesterase measurements was conducted (Marden, 1994). The twelve laboratories that participated in the study all used the colorimetric assay (Ellman1 et al., 1961), but with varying temperature and time. Although the methodology between the laboratories was similar, the study indicated highly variable results of activity in eel and avian brain standards (Marden et al., 1994). The authors concluded that use of published "normal" ChE activity levels from different laboratories should be avoided (Marden et al., 1994).

Marden et al. (1994) recommend standardizing the modified Ellman procedure (Ellman et al., 1961) to include acetylthiocholine iodide (ACTI) of 5 μmol, 5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB) of 0.24 μmol, a temperature of 25°C, a reading time of 3 min, and wavelength of 405 nm. Ten laboratories that analyzed bovine ChE reported an average of 7.1% difference between duplicate samples (Munro et al., 1991). Mean interlaboratory variability ranged from 9 to 17%.

Age, gender, reproductive status, health, and season also influence baseline measurements of normal ChE activity. Variation in blood samples drawn from individual bulls over a 90 h period was 19 to 39% (Munro et al., 1991). Plasma ChE activity has been reported higher in calves than adult cattle, and higher in female rats and mice than in males (Munro et al., 1991).

1.1.4 *Inter-Taxonomic Differences in Sensitivity to Malathion*

Animals from different classes have widely differing sensitivities to organophosphorus compounds. The LD_{50} and other toxicity values for malathion for various species are summarized in Table 1-1. Studies have examined species related differences in pharmacokinetics (absorption, distribution, and elimination) as well as species related metabolic differences (Wang and Murphy, 1982; Kemp and Wallace 1990; Wallace and Kemp, 1991). Data collected for fish, birds, mammals, and amphibians (Wang and Murphy, 1982) indicated that the sensitivity is due in part to differences in K_a and K_p among species. Frogs (*Rana sp.*) tended to have the highest K_a and the lowest K_p of any of the species tested for several organophosphorus compounds. Variables associated with the esteratic site on the AChE enzyme, such as steric dimensions and nucleophilic strength, are also critical (Kemp and Wallace 1990; Wallace and Kemp, 1991).

Clinical signs of organophosphorus (OP) poisoning in mammals are associated with depression of ChE activity of 20 to 25% inhibition relative to normal control levels (Munro et al., 1991). A depression in ChE activity of 50% or more is diagnostic of OP

poisoning; however, lesser degrees of inhibition may be difficult to distinguish from the normal range due to both analytical variation and inherent biological variability (Munro et al., 1991).

Edery and Schatzberg-Porath (1960) reported that toads (*Bufo viridis*) and frogs (*Rana ridibunda*) exhibited ChE depression on exposure to organophosphates, although toad ChE was observed to be 100 times less sensitive than mouse ChE. True ChE (AChE) was the only ChE found in toad and frog blood, and the predominant ChE of nervous tissue as indicated by the fact that frog and toad blood ChE did not hydrolyze butyrylcholine, and the nervous tissue had a much lower response to butyrylcholine than to ACh (Edery and Schatzberg-Porath, 1960). Species differences or seasonal variation may be important in determining the types of ChE found in different studies, since ChE levels are lowest in the winter (Edery and Schatzberg-Porath, 1960). The ChE activity level in lizards was 39.4 ± 10.76 μmol/min/g (Hall and Clark, 1982).

1.2 METABOLISM OF MALATHION

Malathion is a phosphorus ester. Esterases hydrolyze or split ester compounds with the addition of water into alcohols and acids. The pH of the medium influences the hydrolytic process. There are A, B, and C-type esterases. Two esterases that are important in metabolizing malathion are the carboxylesterases, which act on the carboxylic acid site chains, and phosphatase, which acts at the P-S bond (Matsumura, 1975). Malathion mono- and dicarboxylic acids form as a result of hydrolytic degradation at its

carboxyesters as a result of carboxylesterase activity; insect species that exhibit malathion resistance typically have high carboxylesterase activity relative to sensitive taxa.

Malathion is metabolized by the hydrolysis of the carboxylate and phosphorodithioate esters, and oxidation of the phosphorothioate to malaoxon (Worthing, 1991), whereby the phosphorothioate (P=S) group in malathion is converted to the phosphate (P=O) in malaoxon in vivo (Figure 1-2). Malaoxon is a less stable compound, but a more potent cholinesterase inhibitor (Matsumura, 1975). The P=O analog is more susceptible to hydrolysis and is more polar than the P=S parent compound. Lack of carboxylesterases in insects allows more of the parent compound to be converted to malaoxon (Rodriguez et al., 1997). The carboxyl groups that compose the side chains are hydrolyzed readily by mammals, which makes malathion less toxic to mammalian species than to insects.

Malathion is considered a relatively safe insecticide with regards to mammalian toxicity due in part to metabolism to inactive products (Muan et al., 1989). The symptoms of organophosphorus poisoning in mammals include increased rates of defecation and urination, muscular twitching and weakness, anorexia, and lacrimation (Munro et al., 1991). Severe poisoning results in convulsions, although lower doses produce primarily parasympathetic symptoms (i.e., myosis, bradycardia). Insects exhibit restlessness, hyper-excitability, tremors and convulsions, and finally paralysis (Matsumura, 1975). Malathion is hydrolyzed by enzymes in many mammalian organs, although the highest activity is

found in the liver (Heath, 1961). Malaoxon is hydrolyzed rapidly by mammals, at a rate of approximately 1μg/mg tissue/min (Heath, 1961).

The major metabolites of malathion in mammals are malathion α-monoacid (MCA), and malathion diacid (DCA) (Muan et al., 1989), which result from hydrolysis by carboxylesterases. Other metabolites include O,O-dimethyl phosphorothioate (DPT) and O,O-dimethyl phosphorodithioate (DPDT), which result from hydrolysis at the P-S and S-C bonds, and desmethyl malathion (DMM) which results from demethylation (Muan et al., 1989).

Excretion studies with various species are consistent in that malathion is rapidly metabolized and excreted by all species studied (Table 1-2). When a 10 mg/kg dose was administered intravenously to sheep, over 80% was recovered in urine over the course of 96 h. Malathion could not be detected in plasma beyond 1 h following administration, and malaoxon was not detected in plasma (Muan et al., 1989). Recovery of MCA accounted for over 30%, and DCA about 25% of the administered dose. DPDT accounted for 16%, DPT about 10%, and DMM only 3% of the dose. Only 4% was recovered in feces; malaoxon was not detected in urine or feces (Muan et al., 1989). In cattle, plasma malathion also decreases rapidly during the first hour following administration (Muan et al., 1989).

1.3 PHARMACOKINETICS OF MALATHION

Pharmacokinetics is the application of the field of chemical kinetics to describe chemical behavior in biological systems (Barron et al., 1990; Tozer, 1981; Notari, 1980) whereby the processes of absorption, distribution, and elimination are described mathematically. By mathematically characterizing these fate processes, the amount and concentration of a chemical in various tissues over the course of time can be described (Rowland, 1986). Most of the available literature for the pharmacokinetics of malathion or other OP compounds describes the pharmacokinetic processes of chemicals in mammals (Chambers and Chambers, 1990; Sultatos et al., 1990) or birds (Table 1-2). Fewer pharmacokinetic studies have been performed with aquatic species (Bungay et al., 1976; Law et al., 1991; Nichols et al., 1990; Nichols et al., 1991; Zaharko et al., 1972), and often studies of this type with aquatic animals are focused on defining a steady state bioconcentration factor, in addition to identifying uptake or loss rates. Barron et al. (1990) state that parameters for biotransformation are rarely included in aquatic kinetic models.

1.4 TOXICITY OF MALATHION TO AMPHIBIANS

Malathion is an organophosphate pesticide commonly applied to agricultural and wetland areas (Mohanty-Hejmadi and Dutta, 1981). Because of its wide-spread use, there is the potential for exposure of amphibian populations to malathion. There is little information in the toxicological literature regarding the toxicity of malathion to amphibians, and none of the studies in the peer-reviewed literature have described the pharmacokinetics of malathion to adult ranid frogs.

Exposure to malathion in water could occur in wild populations of amphibians, for which a major exposure route would be absorption of malathion across dermal membranes. Absorption of compounds is influenced by molecular size and a balance of lipophilicity and water solubility (Welling, 1988). Drug metabolizing enzymes occur in the dermal membrane of mammals that are capable of both Phase I and Phase II reactions (Guy and Hadgraft, 1987); however, similar information regarding these types of reactions and their influence on malathion toxicity in amphibians is not currently available.

Malathion toxicity information was available for embryos and tadpoles of several species (Devillers and Exbrayat, 1992). Tadpoles of *Bufo woodhousei fowleri* and *Pseudacris triseriata*, exposed to static test conditions at a water hardness of 44 mg/L $CaCO_3$, temperature of 15°C, and a pH of 7.1, exhibited LC_{50} values that differed by a factor of 3.5 for a 24 h exposure, and by a factor of 2 for a 96 h exposure (Mayer and Ellersieck, 1986). The LC_{50} for *B. woodhousei* was 1.9 mg/L for a 24 hr exposure, and 0.42 mg/L for a 96 h exposure. The LC_{50} for *P. triseriata* was 0.56 mg/L for a 24 h exposure, and 0.20 mg/L for a 96 h exposure.

Data for toxicity of other organophosphates (e.g., Dimefox, Paraoxon) for toads (*Bufo viridis*) and frogs (*Rana ridibunda*) were reported by Edery and Schatzberg-Parath (1960). Chemicals were administered via the dorsal lymphatic sacs. The authors indicated that frogs were less resistant than toads.

Species-specific and chemical-specific differences in sensitivity have been reported in amphibians, indicating that responses by amphibians cannot be reliably predicted from investigations utilizing other classes of animal or different compounds (Hall and Henry, 1992). The toxicity of organophosphate compounds differs widely between and within different classes of animals. Amphibians are approximately 20,000 times less sensitive to some organophosphates than are birds (Hall and Clark, 1982). Oral toxicity data were unavailable for anurans; however, an oral LD_{50} of 2,324 mg/kg for malathion for lizards was reported by Hall and Clark (1982). Lizards were reportedly similar to birds and mammals in their responses to the chemicals tested (Hall and Clark, 1982). LD_{50} values for toads and frogs dosed by injection were 65 to over 1,000 times higher than those for mice (Edery and Schatzberg-Porath, 1960). Toads were more resistant to the adverse effects of organophosphates than were frogs (Edery and Schatzberg-Porath, 1960).

Anurans are resistant to certain cholinesterase inhibitors, which may be an adaptation to naturally occurring cholinesterase inhibitors that occur in *Anabaena flos-aquae*, a species of freshwater algae (Hall and Henry, 1992). The resistance appears to arise from an inability of the inhibitor to bind with the cholinesterase enzyme (Wang and Murphy, 1982; Hall and Henry, 1992).

1.5 DEVELOPMENTAL AND BEHAVIORAL EFFECTS IN TADPOLES

A study utilizing the frog *Rana tigerina*, where all animals exposed to concentrations of technical grade malathion during the egg or feeding stages failed to complete

metamorphosis, was reported by Mohanty-Hejmadi and Dutta (1981). Embryos of *Bufo arenarum* exhibited 100% mortality at test concentrations of 47.3 mg/L malathion in a semi-static test over a 5 day test duration (De Llamas et al., 1985). There are several systems commonly used by which to gauge the development of tadpoles (Taylor and Kollros, 1946; Gosner, 1960; Feder and Burggren, 1992), a process known as "staging".

Tadpoles are capable of learning behavior (Hoyer et al., 1971; Hoyer, 1973). Chemical exposure can adversely affect tadpole learning under laboratory conditions. Strickler-Shaw and Taylor (1991) reported that lead inhibits learning behavior in bullfrog tadpoles. Tadpoles in that study were given a conditioned stimulus of a strong light, followed 15 seconds later by an intermittent shock of 25 V for 20 seconds. An avoidance behavior was defined as a movement during the time that the light was on; an escape was a movement during the shock. Tadpoles that moved from one 10 cm square on a grid to another were considered to have responded. The number of avoidance behaviors increased over a 6-Day test period, with lead-exposed tadpoles responding less than controls. Fish exhibited inhibition of learning and avoidance response following exposure to parathion (Sun and Taylor, 1983).

Tadpoles exhibit other measurable behavioral responses, such as avoidance of acidic water with pH concentrations in the lethal and sublethal ranges (Freda and Taylor, 1992). Tadpoles in this experiment were tested in a flow-through tank where they were able to select different levels of acidity. The time that tadpoles spent in each pH and the times

that they entered a different compartment were recorded. Tadpoles failed, however, to avoid lead enriched water (Steele *et al.*, 1991) under fairly similar test conditions.

Tadpoles also respond to other chemical stimuli in their environment. Experiments documenting an avoidance response by tadpoles indicate that tadpoles will avoid areas of a tank with extracts of predatory fish or other novel chemical stimuli (Manteifel, 1995). Larval spade-foot toads (*Scaphiopus intermontanus*) were tested for their ability to discriminate kin and diet cues under laboratory conditions (Hall et al., 1995).

Changes in activity levels of tadpoles have been quantified by recording the distance traveled in a given period of time (Freda and Taylor, 1992). Distance was measured as the number of times a subject crossed a line that partitioned one section of the container from another.

1.6 PURPOSE AND OBJECTIVES

The purpose of this investigation was to explore the toxicity and pharmacokinetics of malathion in ranid (family Ranidae) frogs. The specific objectives were to determine:

1. Acute toxicity for adult leopard frogs *(Rana utricularia)* exposed to malathion in water;
2. Uptake and excretion of malathion in adult bullfrogs *(Rana catesbeiana)* exposed intravenously; and
3. Cholinesterase inhibition in adult frogs due to malathion exposure.

The investigation into the toxicity of malathion to adult leopard frogs indicated that adult leopard frogs were not particularly sensitive to malathion. Also, literature review indicated lower toxicity for larval amphibians; therefore, additional experiments were conducted with tadpoles. Bullfrog tadpoles are relatively large, averaging 8 cm in length and weighing 7.3 g (Strickler-Shaw and Taylor, 1991). Leopard frog tadpoles are much smaller, ranging from 2.5 to 3.5 cm in length (Hoyer, 1973). Because analysis of neural tissue for cholinesterase activity is of interest following exposure to malathion, bullfrog tadpoles were a better choice as an experimental animal. The objectives of the investigations with tadpoles were to determine the effects of malathion on:

4. Survival, growth, and development of bullfrog tadpoles;
5. Righting reflex, learning behavior and gross motor activity in bullfrog tadpoles; and
6. Cholinesterase activity in tadpoles.

Table 1-1. Summary of Malathion Acute Toxicity Values for Various Species

Species	Test	Route	Value	Units	Reference
Rat	LD_{50}	oral	2800	mg/kg	Worthing, 1991
Rabbit	LD_{50}	percutaneous	4100	mg/kg	Worthing, 1991
Mallard	LD_{50}	oral	1,485	mg/kg	Hudson et al., 1984
Ring-necked pheasant	LD_{50}	oral	167	mg/kg	Hudson et al., 1984
Horned lark	LD_{50}	oral	403	mg/kg	Hudson et al., 1984
Fruit fly	No effect (cytogenetic toxicity)	immersion	2	µg/L	Kumar et al., 1995
Bluegill	96-h LC_{50}	immersion	103	µg/L	Worthing, 1991
Largemouth bass	96-h LC_{50}	immersion	285	µg/L	Worthing, 1991
Mosquitofish	24-h LC_{50}	immersion	12.68	mg/L	Tietze et al., 1991
Snail *(Helisoma trivolvis)*	24-h LD_{100}	immersion	500	mg/L	Tchounwou et al., 1991
Snail *(Biomphalaria havanensis)*	24-h LD_{100}	immersion	1200	mg/L	Tchounwou et al., 1991
Daphnia pulex	24-h EC_{50} (immobilization)	immersion	6.6	µg/L	Lilius et al., 1995
Daphnia magna	24-h EC_{50} (immobilization)	immersion	353	µg/L	Lilius et al., 1995
Onion (Root)	No effect (cytogenetic toxicity)	immersion	7.0	mg/L	Kumar et al., 1995

Table 1-2. Excretion Time and Cumulative Recovery (%) of Malathion in Various Species

Species	Time after Administration (t)	Cumulative Recovery (%) of Dose at Time = t	Reference
Sheep	2 h	44%	Muan et al., 1989
	24 h	81%	
	96 h	85%	
Cow	96 h	69%	O'Brien et al., 1961
Hen	24 h	90%	March et al., 1956; Gupta and Paul, 1977
Rat	8 h	45%	Bourke et al., 1968
	24 h	83%	

Figure 1-1. Chemical Structure of Malathion

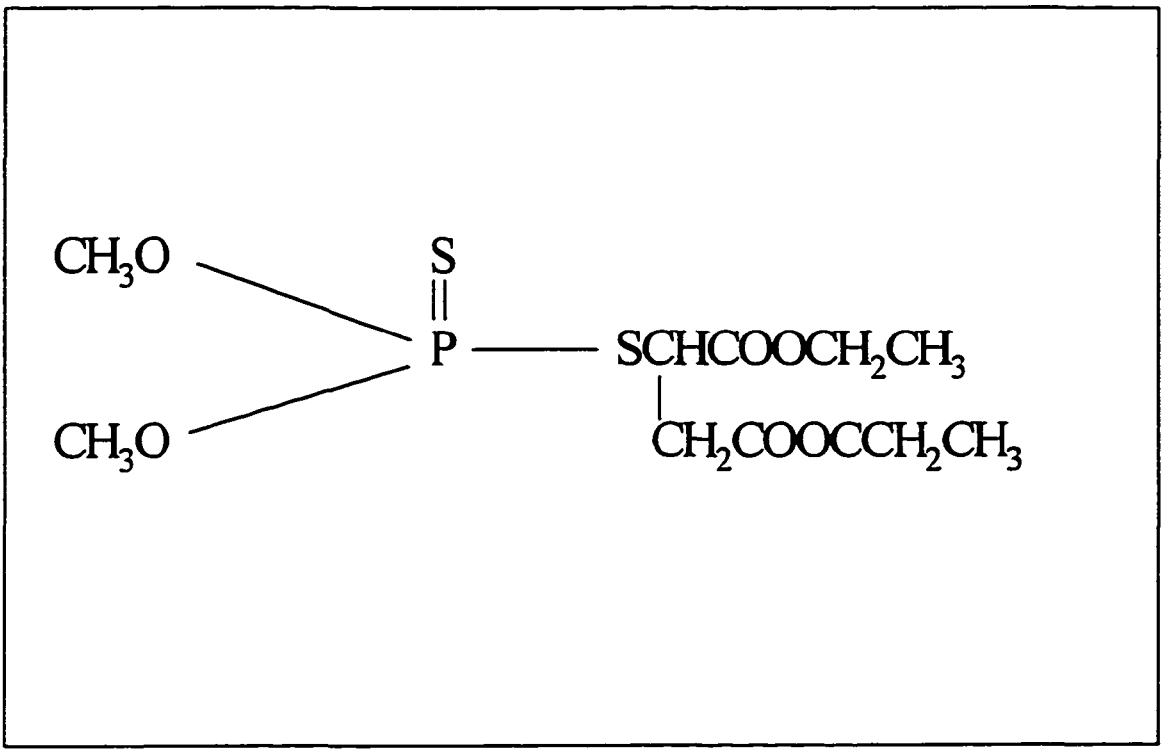
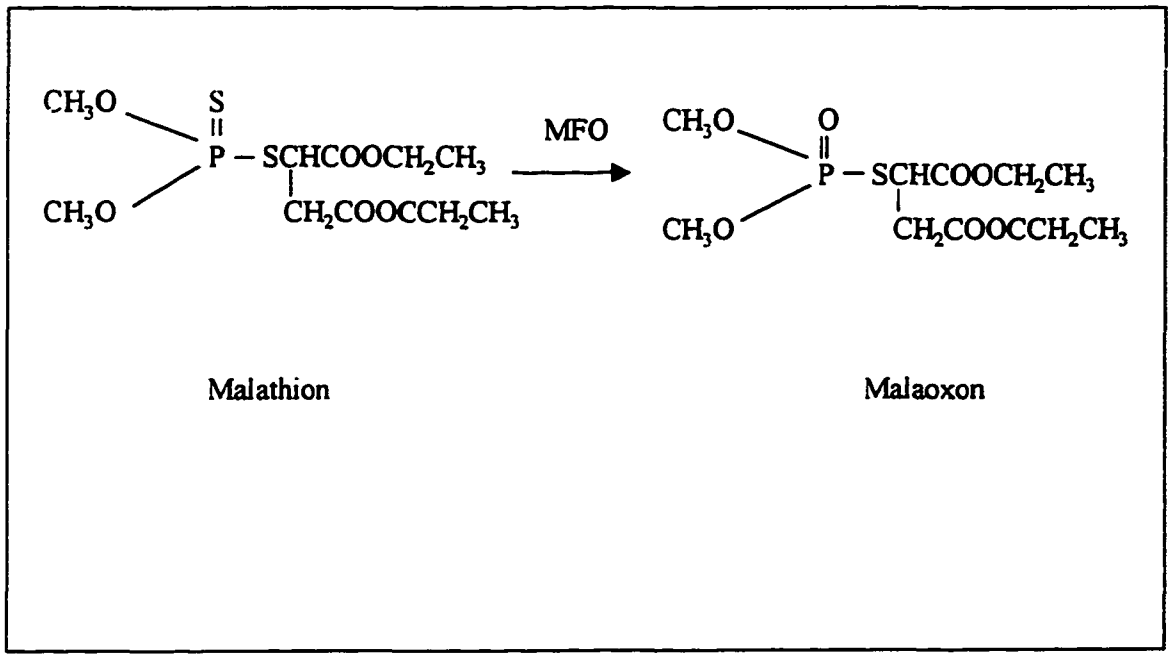


Figure 1-2. Activation of Malathion to Malaoxon by Mixed Function Oxidases (MFO)



2.0 METHODS

2.1 96-HOUR TOXICITY TEST WITH LEOPARD FROGS

2.1.1 *Animal Care*

Twenty-eight southern leopard frogs (*Rana utricularia*) were purchased from Carolina Biological Supply, North Carolina and housed at the Laboratory Animal Resources Center (LAR), Colorado State University, Fort Collins, Colorado. Animals arrived on December 19, 1993. There was extensive mortality during the first 2 weeks following shipping. The frogs were maintained in reconstituted soft water (ASTM, 1992) until December 29, 1993; then the salt concentrations were increased to decrease the incidence of bacterial infection. Aged deionized water was used in conjunction with reagent grade salts at concentrations of 96 mg/l $NaHCO_3$, 60 mg/l $CaSO_4*2H_2O$, 60 mg/l $MgSO_4$, and 4 mg/l KCl for the remainder of the study. Minnows were provided *ad libitum*, and earthworms were provided three times weekly. Water was changed three times weekly. Water pH ranged from 7.0 to 7.2. Room temperature was maintained at 70°F. Lights were kept on for 12 hours daily.

Frogs were dosed on December 30, 1993 with Ivomec (1% solution, MSD-AGVET) to reduce parasitism; dosing was by injection into the dorsal lymph sac of a 1:100 dilution.

An antibiotic, Baytril (2.27% enrofloxacin, Haver) was administered by injection into the dorsal lymph sac January 3, 1994. The toxicity experiment began January 25, 1994 with the surviving 20 frogs.

2.1.2 *Test Solutions*

Technical malathion (93% diethyl mercaptosuccinate, *S*-ester with *O,O*-dimethyl phosphorodithioate; American Cyanamid Company, Princeton, New Jersey) was used as the test chemical. A 10,000 mg/L stock solution was made by weighing 5 g malathion (4.065 ml at a density of 1.23 g/ml) into a 500 ml volumetric flask, and bringing the solution to volume with acetone (HPLC grade, Spectrum). The stock solution was stored by refrigerating in an amber glass bottle. All standards and test solutions were diluted from this stock solution.

Standards (10, 100, and 1000 μg/L) were made by diluting the stock solution with HPLC grade hexane (Baker, Phillipsburg, New Jersey). Standards were verified by gas chromatography. Figure 2-1 shows a representative chromatogram of a malathion and malaoxon standard.

There were four test solutions used in the adult leopard frog toxicity test. The nominal test solution concentrations and their formulations were as follows:

Treatment	
Control	0 ml stock solution: 1,600 ml reconstituted water
10 mg/L	1.6 ml stock solution: 1,600 ml reconstituted water
100 mg/L	16.0 ml stock solution: 1,600 ml reconstituted water
1000 mg/L	160.0 ml stock solution: 1,600 ml reconstituted water

Test solutions were made daily by diluting the stock solution directly with reconstituted water. The measured concentrations of malathion and malaoxon in the test solutions are presented in Table 2-1. The reconstituted water used in the test solutions was the same formulation as that used to maintain the frogs prior to testing. All glassware was washed in soap and water, rinsed three times with deionized water, and then rinsed with acetone and hexane prior to use.

2.1.3 *Toxicity Test*

The experiment began at 1350 h on January 25, 1994. Test containers were 3.8-L glass fish bowls with a diameter of 22 cm and depth of 13 cm. Containers were washed with soap and water and were rinsed with deionized water prior to using in the test. Aluminum pans were attached to the top of each container with tape to serve as lids. Approximately 250 ml of test solution was poured into each container; solution depth was 3.5 to 4 cm..

There were four treatment groups, each containing four frogs. Sixteen animals were selected for the experiment from the population of 20 animals. One holding container had two frogs apparently affected by redleg; these animals were excluded from the toxicity

test. A holding container with three or four frogs was randomly selected from the top shelf of the holding rack, and each frog in the holding container was randomly allocated to a different treatment level. This assured that a given holding container did not provide more than one frog to each treatment level in order to limit a potential source of bias. The next holding container was selected from a different position on the next lower rack, and the frogs were allocated to each treatment level as described above. The frogs were then assigned numbers on the treatment containers. The lids were attached, and frogs were checked approximately every two hours throughout the test for morbidity and mortality to minimize post-mortem changes in cholinesterase levels.

Test solutions were replaced with fresh solution every 24 hours. The old solution was gently poured out of the container without removing the animal so as to minimize disturbance. New solution was made and was then added to each test container. A sample of each test solution was collected for chemical analysis. In addition, a beaker of test solution was placed on the rack next to the test containers to determine chemical half-life for the test conditions. Reconstituted water was made fresh prior to starting the test and on days 3 and 4 of the test in order to minimize potential contamination from the ambient surrounding air in the room. Test solutions were made daily immediately prior to changing solution.

When an animal was observed dead, a necropsy was immediately performed. The frog was rinsed under tap water and then dried with a paper towel. Body weight was obtained.

Blood, liver, and kidney were collected for malathion and malaoxon residue analysis. Organ weights for liver and kidney were obtained. The skull was opened and the brain removed for cholinesterase determination. All tissues were immediately frozen. The animal carcass was disposed of by the Laboratory Animal Resources (LAR), Colorado State University, Fort Collins, Colorado. Blood samples were collected into a red-top tube (i.e., no additives). Blood was centrifuged, and the serum was removed by pipetting with a Pasteur pipet, placed into glass tubes, and frozen for later analysis. Necropsy instruments were washed with soap and water, rinsed with acetone, and finally rinsed with hexane.

Behavioral measurements were made several times daily. Frogs were monitored for a fright or startle response for the initial two minutes following entry of the investigator into the room. Each movement was counted. If a frog ceased moving for 1 or more seconds, and then initiated movement again, this was counted as a new movement. In order to avoid potential bias by the position of the investigator relative to the frogs, the test containers were shifted to different shelves at the time the solutions were changed. Thus, the controls would be on the first shelf one day, the second shelf on the next day, and the third shelf the third day.

Two animals were exposed to 100 mg/L malathion in water for 1 hour. Frogs were euthanized with 1.5 mg/100 g body weight with MS222, also known as tricaine sulfate or Finquel (Argent Chemical Laboratories, Redmond, Washington) by subcutaneous

injection. Liver, kidney, and brain tissues were collected from these two frogs. Two animals that were not used in the toxicity test were used to test procedures for anesthesia.

2.2 RESIDUE STUDY WITH ADULT BULLFROGS

Eighteen bullfrogs (*Rana catesbeiana)* were purchased from Amphibians of North America, Nashville, Tennessee, and shipped to the LAR the first week of November, 1994. Bullfrogs were used in additional studies in lieu of leopard frogs, because the larger organ size and blood volume of bullfrogs improved analytical capabilities.

2.2.1 *Animal Care*

Frogs were maintained on a diet of minnows provided *ad libitum.* Reconstituted water was used as described above for the leopard frogs. Frogs were allowed to acclimate to laboratory conditions for 1 month prior to testing. One frog died during the acclimation period. Nine frogs were used to evaluate surgical techniques and proposed dose levels. Test dosages indicated that 10 mg/kg was lethal to frogs. The remaining eight frogs were used in residue studies.

2.2.2 *Exposure and Tissue Collection*

Frogs were anesthetized by placing in a solution of 2.5 g MS 222 in one liter of water for approximately 15 minutes until all buccal movements stopped. Three frogs were used in the range finding study; each frog was injected with 10, 8, and 6.5 mg/kg, respectively. Only the frog at the lowest dose survived, indicating that this was an appropriate test

concentration to maximize potential tissue residues while minimizing lethality. Frogs were weighed to determine the appropriate volume of 10,000 mg/L malathion to administer to each frog in order to obtain a dose of 6.2 to 6.5 mg/kg (Table 2-2).

A ventral incision was made to expose the renal vein and the dorsal aorta without exposing the pericardial area. Malathion was administered into the renal vein over a period of approximately 1 to 2 minutes with a 1 cc syringe with a 25 gauge needle.

After a recorded length of time (Table 2-2), blood was withdrawn from the dorsal aorta, also with a 1 cc syringe equipped with a 25 gauge needle. The time between dosing and termination by blood collection differed for each frog in order to derive a time course of malathion *in vivo*. Blood was placed into tubes with no additives, and centrifuged for 20 minutes to obtain serum. The liver and kidneys were removed, weighed, and frozen for chemical analysis. The brain was removed and frozen for cholinesterase activity determination. Tissues were extracted by Global Environmental Systems, Fort Collins, Colorado, as described under analytical procedures. Blood plasma extraction was performed at the Center for Environmental Toxicology, Colorado State University, Fort Collins, Colorado. Brain cholinesterase activity was determined by the Veterinary Diagnostics Laboratory (VDL), Colorado State University, Fort Collins, Colorado as described below.

2.3 TWENTY-EIGHT DAY TOXICITY TEST WITH BULLFROG TADPOLES

2.3.1 *Animal Care*

Two hundred and ten (210) bullfrog tadpoles were obtained from Charles Sullivan Co. (Nashville, TN) on May 12, 1998. Tadpoles had barely visible limb buds, and were in Stage 26-28 of development (Gosner, 1960). Tadpoles were held in moderately hard reconstituted water in community aquaria at the LAR for 10 days prior to testing. There were 10 to 15 tadpoles in each community aquarium, of which each contained approximately 10 liters of reconstituted water. Tanks were opaque plastic containers 42 x 25 cm and had a stainless steel mesh lid to prevent escape.

To make the reconstituted water, deionized water was aged at least 24 hours to remove residual chlorine. A mixture of salts was added to the aged deionized water. The mixture consisted of 96 mg/L $NaHCO_3$ (Fisher, Fair Lawn, NJ), 60 mg/L $CaSO_4 \cdot 2H_2O$ (Mallinckrodt, Paris, KY), 60 mg/L $MgSO_4 \cdot 7H_2O$ (Fisher, Fair Lawn, NJ), and 4 mg/L KCL (Fisher, Fair Lawn, NJ) (ASTM, 1993). The reconstituted water was used in all of the tadpole studies, as well as for maintaining tadpoles not on test.

Hardness, conductivity, and pH of the reconstituted water were measured daily. Water hardness was measured with Sofchek test strips (Baxter Scientific, McGaw Park, IL). Conductivity was measured with an Oakton hand-held conductivity meter (Forestry Suppliers, Jackson, MS). The pH was measured with a Chek-Mite PH-30 sensor (Corning, Horseheads, NY). Measured hardness ranged between 50 to 80 mg/L as

$CaCO_3$, conductivity ranged from 247 to 377μS, and pH ranged between 6.8 to 8.6 pH units.

Tadpoles were fed boiled spinach, commercial trout chow (Purina), and algae disks (Hikari Algae Wafers, Kyorin Co., LTD 9 Minami-machi, Himeji, Japan) provided *ad libitum*, on a 12:12 light cycle, at a room temperature of 22 - 25°C according to test protocols specified by ASTM (1993). Water was changed three times weekly during the pretest period. Water hardness and pH were verified each time the water was changed.

2.3.2 *Range Finding Test*

A range finding test was performed with 12 tadpoles (stages 26 to 27) to determine appropriate dosing concentrations for the growth test. Tadpoles were exposed for 24 hours to 0 μg/L, 100 μg/L and 1000 μg/L malathion in water; there were four tadpoles in each treatment. The tadpoles were monitored for mortality every four hours for 24 hours. The brains were pooled by treatment and analyzed for ChE activity levels. The ChE activity was 1, 0.8 and 0.7 μmol/min/g in the 0, 100, and 1000 μg/L treatments, respectively. Mortality is typically associated with ChE inhibition of 50% relative to controls, indicating that test concentrations higher than 1000 μg/L malathion in water could be tolerated. Based on these results, the following treatment levels or dosing solutions were chosen for the 28-day growth study:

- 0 μg/L malathion in water (Control)
- 0 μg/L malathion; 1 ml/L acetone in water (Acetone Control)

- 500 μg/L malathion in water
- 1000 μg/L malathion in water
- 2000 μg/L malathion in water
- 2500 μg/L malathion in water
- 3000 μg/L malathion in water

2.3.3 *Survival, Growth, and Development of 28-Day Study Bullfrog Tadpoles*

The effect of malathion on tadpole survival, growth and development was examined by using a total of 70 tadpoles allotted in groups of 10 to each of the seven treatments (control, acetone control, and 500, 1000, 2000, 2500 and 3000 μg malathion/L water). Stock solutions were made by weighing 96% pure malathion (ICN Biomedical, Costa Mesa, CA) into volumetric flasks with a hexane-rinsed glass Pasteur pipet, and bringing to volume with acetone (Fisher GC Resolv, Fairlawn, NJ) as described in Table 2-3.

The acetone control was formulated to represent the acetone concentration in each of the treatment solutions, which was 1 ml acetone/liter water or 0.1%. Stock solutions were kept refrigerated in amber glass bottles with teflon-lined caps. The concentrations in the stock solutions were verified by GC, and were within 10% of nominal concentrations.

To formulate the dosing solutions, 1 ml of each stock solution was added with a 1 ml glass Hamilton syringe to reconstituted water in 1-liter glass volumetric flasks to formulate the

appropriate test concentrations. The flasks were inverted 10 times to mix the stock solution with water. The dosing solutions were made fresh daily.

Seventy tadpoles were selected from the 210 tadpoles in the community aquaria by capturing with a net. Tadpoles were weighed to 0.01 g by placing them in a beaker of clean reconstituted water with a tare weight of zero on a precision balance. Tadpoles were then assigned to a treatment by weight. The goal was to balance the initial body weights of tadpoles in each treatment in order to avoid biasing the study. Initial body length and developmental stage were also recorded. After assigning to a treatment, the tadpole was maintained individually in a seamless, round glass bowl 20 cm in diameter and 6 cm deep (Corning, Loveland, CO) containing approximately 200 ml of dosing solution to a depth of 3 to 3.5 cm. Stainless steel racks were placed over the bowls to prevent escape.

In order to make accurate measurements of developmental stage (Gosner, 1960) on live tadpoles, a magnifying glass with a built-in light was used (Radio Shack, Fort Collins, CO). Latex or nitrile gloves were worn when handling tadpoles to avoid injuring their skin.

The dosing solutions were replaced once daily with fresh solution. The tadpoles were given algae disks four times weekly and boiled spinach three times weekly, *ad libitum*. Three times a week the bowls were wiped clean with clean paper towels. If during the

study, tadpoles were not responsive to gentle prodding, they were humanely euthanized with MS222.

The developmental stage, body length, and body weight of each tadpole was measured once a week during the study. Brain weight was measured on a precision balance to the nearest mg at the end of the 28-day toxicity test. At the end of the 28-day exposure period, the surviving tadpoles were humanely euthanized by immersion in MS222, weighed, and the brains removed and weighed.

2.3.4 *Righting Reflex in 28-Day Study Bullfrog Tadpoles*

Loss of the righting reflex was monitored once daily in all 28-day toxicity test tadpoles. Each tadpole test container was placed onto a stainless steel cart, and the tadpole observed for the initial 15 sec following movement of the test container to the cart. The movement of the test container triggered swimming movements in tadpoles, possibly due to the inherent water movement or visual cues. Tadpoles that failed to maintain equilibrium position (i.e., presented the ventral surface upwards) at any time during the 15 sec were considered to have an impaired or lost righting reflex.

2.4 SIX-DAY BEHAVIORAL STUDY WITH BULLFROG TADPOLES

After the 10 day acclimation period, 35 tadpoles were selected from remaining tadpoles in the community aquaria for the first of three replicates of the behavior test. Tadpoles were stratified by body weight and were allocated to one of the five treatments or two controls

described above so that there were 5 tadpoles in each group. The goal of stratification was to balance the body weights of tadpoles in each treatment. Each tadpole was housed individually throughout the behavior study in a round glass bowl (20 by 6 cm) containing test solution to a depth of 3 to 3.5 cm (approximately 200 ml). This depth is similar to that used by Hoyer (1973), Hoyer et al. (1971), and Strickler-Shaw and Taylor (1991). Tadpoles were fed *ad libitum* with algae disks and boiled spinach while in the test containers, but not when in the learning test chamber.

Tadpoles were exposed for 24 hours prior to measuring behavioral response, and exposure and behavior measurements continued for an additional five days for a total of six days for each behavior test. The behavior test was replicated three times.

2.4.1 *Learning Behavior in 6-Day Behavior Study Bullfrog Tadpoles*

The learning test apparatus was constructed as described by Hoyer (1973), Hoyer et al. (1971) and Strickler-Shaw and Taylor (1991). Clean reconstituted water was placed in the behavior test chamber to a depth of 3.5 to 4 cm (Hoyer, 1973, Hoyer *et al.*, 1971 and Strickler-Shaw and Taylor, 1991).

Tadpoles were placed individually in the behavior test chamber, after rinsing briefly by immersion in clean reconstituted water to avoid cross contamination. The behavior test chamber was drained, rinsed, and refilled between each test group. The test container was a clear, solid glass tank 33 cm x 24 cm x 4 cm, with rounded corners and a clear bottom,

so that a grid of 10 cm squares was visible through the bottom. After an acclimation period of 2 minutes, the tadpole received a conditioned stimulus (CS) consisting of two high intensity 15 W lights placed on either side of the test container at a height of 25 cm. Total luminescence for the two lights was 1184 lux After 10 sec, an unconditioned stimulus (US) that was generated by a Grass Stimulator Model S4K, Serial No. K734X2 (Grass Instruments, Quincy, MA) was applied for a period of up to 20 sec. This was a mild, discontinuous shock of 25 V, pulsed once per second, 0.5 sec per pulse, supplied by electrodes located on opposite sides of the container. The current was 11.5 mA. A multi-meter was used to verify the voltage throughout the behavior measurements.

A dim room light was left on so that observations could be recorded. The test container was screened on the sides to shield the observer from the test animals, and a mirror was placed above the test apparatus so that tadpoles could be observed without introducing potential bias attributable to the presence of the investigator.

A response was recorded if the tadpole moved in any direction from one grid completely into another. If the animal responded during the first 10 sec of light (CS), it was considered an avoidance. A response during the following 20 sec of mild shock (US) was considered an escape. A response at any time terminated the trial, and a response during the CS prevented the US from occurring. The US was terminated after 20 sec if the tadpole failed to respond. A rest period of 30 sec elapsed between trials. A total of 15 trials were conducted each day on each animal for five consecutive days. The time to

respond (latency) and the distance traveled (cm) were recorded. All measurements occurred in the afternoon between 1200 and 2000 h except for the first day, when equipment set-up occurred. After testing, the animal was returned to its individual treatment container.

At the end of the fifth day of behavior measurements (day 6 of exposure), tadpoles were humanely euthanized with MS222, and the brains removed for analysis of cholinesterase activity. The learning experiment was replicated two more times with 35 tadpoles in each test. The brains were removed for ChE measurement.

2.4.2 *Righting Reflex in 6-Day Behavior Study Bullfrog Tadpoles*

Loss of the righting reflex was monitored once daily in all behavior study tadpoles. Each tadpole test container was placed on a stainless steel lab cart, and the tadpole was observed for 15 sec while the water moved in the container.

2.4.3 *Gross Motor Activity in 6-Day Behavior Study Bullfrog Tadpoles*

Activity levels were measured once daily in the tadpoles used in the learning study in order to predict the amount of general movement in its environment exhibited by a tadpole. Each tadpole test container was placed on a stainless steel cart. A two minute period was allotted to allow tadpoles to acclimate prior to initiating observations. The investigator left the room and performed observations through a window in the door. Each tadpole

was monitored for three minutes between 0745 and 1300 h each day. The data recorded were the total seconds of movement in a 3- min observation period.

2.4.4 *Brain Cholinesterase Activity in 6-Day Behavior Study Bullfrog Tadpoles*

Brain cholinesterase activity was measured in each of the tadpoles used in the behavior study. Following euthanasia with MS222, a shallow dorsal incision was made from the snout to the back of the head with a scalpel blade. A cross-section was made behind the eyes, and the brain tissue was removed with the scalpel. Brains were weighed to the nearest mg directly into 2 ml amber plastic vials containing 1 ml of cold 0.1 M phosphate buffer solution (pH of 8) to prevent dessication. Vials were frozen until analysis. Cholinesterase activity was measured by the VDL as described below.

2.5 ANALYSIS OF WATER AND TISSUE SAMPLES AND STOCK SOLUTIONS

2.5.1 *Analysis of Acute Toxicity Test Water Samples*

Water samples from the 96-h toxicity test were transported from the LAR to the Center for Environmental Toxicology (CET), Colorado State University, Fort Collins, Colorado for analysis immediately following changing of the test solutions (Table 2-1). Solutions made at 1300 hours were extracted prior to 1700 hours that same day, which is well within the holding period of 7 days for organophosphates specified by EPA (EPA, 1994). The extraction of malathion and malaoxon from water followed standard methods for organophosphates (EPA, 1980; EPA, 1994). A volume of 250 ml test solution was measured into a graduated cylinder and then poured into a separatory funnel. Forty

milliliters of pesticide grade dichloromethane (Burdick Jackson, Muskegon, MI) was added to the funnel. The mixture was shaken for two minutes. The phases were allowed to separate, and the bottom layer (dichloromethane) drained into a 250 ml boiling flask. The procedure was repeated two more times with 30 ml of dichloromethane for each extraction. The extract in the boiling flask was reduced to 5 ml volume by concentrating on a rotovap flash evaporator for approximately 10 minutes. Anhydrous sodium sulfate was added if necessary to remove water in the sample from the separation phase.

The sample was transferred to a 13 ml volumetric test tube with a disposable pipet. The boiling flask was rinsed with approximately 2 ml of hexane to obtain any residual sample in the flask, and this was also added to the 13 ml test tube. The test tube was vortexed for 5 seconds, and the sample was reduced to 0.2 ml by drying with nitrogen to remove dichloromethane. An additional 2 ml of hexane was added, the sample again vortexed, and the drying step repeated to give a volume of 0.5 ml. Hexane was then added to bring the sample to a final volume of 10 ml. Samples were stored in a refrigerator prior to analysis by gas chromatograph (GC), as described below.

Standards were checked each day prior to analyzing samples on a gas chromatograph with a flame photometric detector (GC-FPD), as described below. In addition, a reagent blank was verified each time new reagents were used in the extraction procedures. Glassware cleanliness was verified by vortexing 10 ml hexane in glassware in order to extract residues attached to glassware surface; the hexane extract was dried to 0.5 ml and then

analyzed by GC-FPD. To determine the percent recovery for the water analysis, 250 μg/L malathion was added to three 250 ml water samples. The average percent recovery and standard deviation (SD) was 109 ± 49.2%, which is similar to percent recoveries cited by EPA (1994) of 97%.

2.5.2 *Gas Chromatography Procedures*

Samples were analyzed on a Hewlett Packard 5890A gas chromatograph with a flame photometric detector (GC-FPD) equipped with a J.W. Scientific DB1 capillary column (0.53 mm in diameter (id), film thickness of 1.5 μm, 30 m in length). The FPD had a phosphorus filter of 526 μ. The inlet and detector temperatures were 220 and 200°C, respectively. The oven temperature was programmed from 175°C for two minutes, then increased to 225°C over 8 minutes at a rate of 8°C/min, and then held at 225°C for 18 min. The column head pressure was 28 psi, and the purge and carrier gas flow rate was 75 ml/min. An injection volume of 5 μl was used for all samples and standards. An FPD was necessary due to the low response of the electron capture detector (ECD) for malathion and malaoxon. In order to quantify malathion and malaoxon, standard solutions in hexane were prepared.

2.5.3 *Analysis of Environmental Water Samples*

During aerial spraying at a height of 5 m, droplets can travel 50 to 400 m in a wind of 1 m/sec (Joyce et al., 1977). The downwind deposit of droplets depends on the height of the release, wind speed, terminal velocity of the droplets considering their size range, and

air turbulence (Joyce et al., 1977). Field samples were collected to determine what concentrations of malathion could be expected in the environment following a typical agricultural or pesticide control spraying event. Water samples were collected from four locations near Alamosa, Colorado, on July 15, 1994 approximately 7 hours after an aerial ultra-low volume spray event at an application rate of 3 oz/ac (85.05 g/ac) for mosquito control. Two water samples were collected from near Adams Ditch (a control area outside the flight pattern), and one sample from each of three sites underneath the flight pattern (Figure 2-2). Samples were placed on ice for transport to the laboratory. Samples were refrigerated and extracted on July 18, 1994, as described in Section 2.5.1, which was within standard holding times for organophosphate pesticide analysis (EPA, 1994). Samples were analyzed by GC-FPD as described in Section 2.5.2.

2.5.4 *Analysis of Residues in Blood Serum*

Each blood serum sample was weighed to the nearest mg into a 10 mm x 125 mm culture tube with a hexane-rinsed Pasteur pipet. Because the samples were small, the entire blood sample was used. Extraction of samples and spikes proceeded by adding 0.5 ml of methanol in order to deprotonate the serum; samples were shaken for 10 minutes vigorously. To each tube, 5 ml of a 1:1 hexane:ethyl ether mixture was added. The tubes were capped and then rocked gently to mix sample. The sample was mixed on a Roto-rack for 15 minutes at 45-50 rotations per minute (RPM). The samples were centrifuged for 10 minutes at 1500 RPM. An emulsion was present, which was broken by adding ethanol (2 ml), followed by shaking for 3 minutes. Tubes were centrifuged again for

10 minutes. A disposable pipet was used to transfer the upper (organic solvent) clear layer to a 13 ml volumetric glass test tube. The extraction procedure was repeated two more times with 2 ml 1:1 ethyl ether/hexane added to each sample, followed by mixing, centrifuging, disruption of emulsion when present, and transfer to the volumetric test tube. A "keeper" was prepared by mixing 1 ml of light mineral oil with 99 ml hexane to make a 1% solution. One ml of keeper was added to each sample. The sample was concentrated to 0.2 ml by gently evaporating on an N-Evap, without disturbing the sample surface. Samples were then brought to a 5 ml final volume and analyzed by GC-FPD as described in Section 2.5.2.

"Spikes" were made by adding a known quantity of malathion to a tube containing bovine or rat serum in order to document percent recovery for the analytical methodology. Serum spikes were made by adding 1 ml of 1 μg/ml malaoxon and 0.1μg/ml malathion to 3 ml serum, and following the procedure detailed above. Two spike samples were analyzed for malathion and malaoxon in blood serum. Spike recoveries for blood serum ranged from 34% to 134% for malaoxon and 90.6% to 116% for malathion. Unknown peaks were observed in the tissue samples; these peaks were not included in the estimate of percent recovery. The method and reagent blanks did not contain measurable concentrations of malathion or malaoxon. The detection limits were calculated with the following equation:

$$Detection\ Limit(\mu g / g) = \frac{lowest\ \ standard\ used(\mu g / ml) \times injection\ volume\ (ml)}{amount\ of\ sample\ injected\ (g)} \qquad (3)$$

The lowest standards that were used were 0.01 μg/ml malaoxon and 0.005 μg/ml malathion, resulting in instrument detection limits of 0.05 ng and 0.025 ng for malaoxon and malathion, respectively (Figure 2-1). A volume of 5 μl was injected on the GC-FPD. The amount of sample injected on the GC-FPD was calculated as the sample weight (mg) divided by the final volume (μl) multiplied by the volume injected (μl).

2.5.5 *Analysis of Residues in Liver and Kidney*

Liver and kidney samples were extracted by Global Environmental Systems, Fort Collins, Colorado. The entire liver or kidney was weighed into a weighing dish and then transferred to a 250 ml beaker. One gram of sodium sulfate (Na_2SO_4) was added and mixed with a wooden stirrer. Samples were sonicated with 100 ml of extraction solvent, dichloromethane (CH_2Cl_2). The samples were filtered through sodium sulfate and collected in a RapEvap tube. Samples were then concentrated to 0.5 - 1 ml volume and transferred to a 1 ml amber autosampler vial. Samples were then adjusted to 1 ml final volume with hexane and transferred to the Center for Environmental Toxicology, Colorado State University, Fort Collins, Colorado. A volumetric 0.5 ml glass pipet was used to quantitatively measure 0.5 ml of sample to a 5 ml glass volumetric flask. All samples were brought to 5 ml final volume with hexane and analyzed by GC-FPD as described in section 2.5.2. A chromatogram of liver tissue extract is presented in Figure 2-3. GC-MS was used by Global Environmental Systems to tentatively identify a peak that eluted immediately after malathion.

There was one spiked sample of liver tissue analyzed for percent recovery. The spike recovery for malathion was 85% from frog liver; the recovery for malaoxon (66%) was lower than that for malathion. Recovery of malathion from a solid matrix (soil) was 67% (EPA, 1994), indicating that the extraction and analytical methods were satisfactory. The method and reagent blanks did not contain measurable concentrations of malathion. Detection limits were calculated as described above in Section 2.5.4.

2.5.6 *Brain Cholinesterase Analysis*

Brain cholinesterase activity (μmol/min/gm) was measured in whole brains from animals from the leopard frog acute toxicity test, the bullfrog residues study, and the tadpole behavior test. The analysis was performed by CSU VDL according to Richardson (1989). The whole brain was homogenized in cold 0.1 M phosphate buffer (pH of 8.0) at a buffer to brain ratio of 1 ml to each 100 mg of brain. Tadpole brains were homogenized in the same buffer at a ratio of 1 brain per 1.0 ml of buffer.

A 1:10 sample dilution was made by pipetting 1.0 ml of brain homogenate into a 10 ml volumetric flask and diluting to 10 ml volume with cold 0.1 M phosphate buffer (pH of 8.0); the sample and buffer solution was then mixed thoroughly. All remaining reagents were brought to room temperature. A 3 ml aliquot of the diluted sample was transferred to a disposable test tube, to which was added 50 μl of 0.01 M 5,5'-Dithio-bis-(2-Nitrobenzoic Acid) at room temperature. The sample was vortexed to mix and 20 μl of 0.075 M acetylthiocholine iodide was added. The sample was again vortexed. The

change in absorbency at 412 nm was recorded by spectrophotometer. In order to verify that the analysis was providing consistent results, three samples of a fresh rabbit brain were analyzed with the adult frog brains, and the ChE activity for each sample was 3.5, 3.6, and 3.8 μmol/min/g, respectively.

In order to verify that tadpole brains could be measured individually, a rabbit brain was obtained from the LAR. Six samples were analyzed ranging in weight from approximately 20 to 120 mg per 1 ml of buffer, and the ChE activities were compared for consistency by size (Table 2-4). The mean ChE activity and standard deviation (SD) for these six samples were 2.65 and 0.42 μmol/min/g, respectively.

2.5.7 Analysis of Dosing Solutions Used in the Tadpole Toxicity Tests

The concentrations of the stock solutions used to formulate the dosing solutions for the tadpole toxicity tests were verified by pipetting 5 μl of each stock solution into a clean, hexane rinsed volumetric flask and bringing to volume with hexane (Table 2-4). All dilution concentrations were in the range of 400 to 600 μg/ml. The solutions were then verified by on two separate columns by gas chromatography, and found to be within 15% of the nominal concentrations (Table 2-4), except for the highest test concentration, which read low on both columns. This sample also had the highest variability between the two gas chomatographs, and may have been at the limits of the linearity of the instrument.

2.6 STATISTICAL ANALYSIS OF DATA

2.6.1. *96-Hour Toxicity Test with Leopard Frogs*

An LC_{50} was estimated by the Spearman-Karber method (Hamilton et al., 1977; Hamilton et al., 1978) for the adult leopard frog toxicity test data and the nominal concentrations in water. The Spearman-Karber method computes the probability of mortality at each concentration tested while ignoring the other test concentrations, and then combines all concentrations to produce an estimate of the LC_{50} and the bounds on the LC_{50} (Salsburg, 1986). This method tends to provide wider confidence bounds than some of the other methods because the method accounts for many shapes of the dose-response curve.

The remaining statistical analyses of the data from the leopard frog study were performed in Statgraphics (Manuguistics, 1997). A χ^2 test was used to evaluate the mortality data, followed by a multiple comparison of proportions. Survival analysis was used to evaluate the time to mortality.

Analysis of variance (ANOVA) was used to compare the leopard frog body and organ weight data to malathion concentrations in water. The number of leopard frogs exhibiting fright behavior was examined with a χ^2 test to evaluate the effects of malathion concentration in water; a multiple comparison of proportions was applied to the data to determine which means were significantly different. Multiple analysis of variance (MANOVA) was used to determine potential effects of dose, time of observation, and day of treatment. The behavior data were evaluated for normality by examining a normal

probability plot. The residuals for behavior against water concentration were examined for homogeneity of variance; in addition, Cochran's C test was used to evaluate the homogeneity of variance of behavior over each of the levels of water concentration. In the event that the assumptions of normality and homogeneity of variance were violated, and standard transformations of the data failed to improve the assumptions, the Kruskal-Wallis (KW) test was applied. The KW test is a nonparametric test that evaluates the median for behavior within each of the different levels, e.g., either water concentration of malathion or test day (Zar, 1984).

ANOVA was applied to the leopard frog cholinesterase data to examine the effects of malathion concentration and time on brain cholinesterase activity. The ANOVA was followed by a Bonferroni comparison to determine which means were significantly different. The leopard frog cholinesterase data were examined for normality with a normal probability plot, both including and excluding the data for the two additional leopard frogs that were dosed with 100 mg/L but were not included in the 96-h toxicity test. Cochran's C test was applied to determine if the standard deviations for cholinesterase activity at each of the levels of malathion concentration in water were significantly different.

2.6.2 *Residue Study with Bullfrogs*

Due to the limited number of adult bullfrogs, and subsequent lack of sufficient numerical data, data from the residue study were not statistically analyzed.

2.6.3 *Survival, Growth and Development in 28-Day Study with Bullfrog Tadpoles*

The body weight, body length, and developmental stage data were analyzed by repeated-measures ANOVA (SAS) to determine the significance of differences among treatments and among days of exposure, as well as their interaction. If significance was indicated ($p<0.05$), a Bonferroni multiple comparison method was used to compare the means and determine which combinations of variables differed significantly by day, treatment, or their interaction. This consists of a series of *t*-tests, adjusted by the Bonferroni correction so that the overall level of significance is less than or equal to the nominal level of significance (Stevens, 1996). The data for body weight, body length, and developmental stage evaluated for normality and homogeneity of variance as described in Section 2.6.1. The dose-response relationship was studied by step-wise regression analysis for each variable for which the ANOVA test was statistically significant ($p<0.05$) for "dose", "day", or the "dose by day" interaction. The significance of each partial regression coefficient was determined with a *t* test (Zar, 1984); partial regression coefficients less than the critical value were deleted from the equation.

A χ^2 test was used to evaluate the survival data. If mortality was found to vary significantly ($p<0.05$) among treatments, a multiple comparison of proportions was conducted by dose. The arcsine-square root transformation of each proportion (p) (Zar, 1984) is:

$$p' = 1/2\left[\arcsin\sqrt{\frac{X}{n+1}} + \arcsin\sqrt{\frac{X+1}{n+1}}\right], \quad (4)$$

where: n = sample size
X = number dead

Two comparisons were made. A procedure analogous to Dunnett's test was used to compare each treatment to the control. The p' for the control was subtracted from the p' for the treatment group and divided by the standard error (SE) to obtain the Dunnett test statistic, q', as follows:

$$q' = \frac{p'_B - p'_A}{\sqrt{\frac{410.35}{n + 0.5}}} \quad (5)$$

The critical value for a one-tailed test is given by $q_{\alpha(1),\infty,k}$ where k is the number of treatments. A procedure analogous to Tukey's test was used to compare the various proportions to each other (Zar, 1984):

$$q = \frac{p'_B - p'_A}{\sqrt{\frac{205.18}{n + 0.5}}} \quad (6)$$

The critical value for a two-tailed test is given by $q_{\alpha,\infty,k}$.

The mortality data were also evaluated by survival analysis, which analyzes the distribution of time to a given event (Afifi and Clark, 1990). This procedure was used to determine if the time to death differed by treatment level.

2.6.4 *6-Day Behavior Study with Bullfrog Tadpoles*

The learning data were expressed as mean response time (sec) or mean latency to respond based on 15 trials/day/tadpole. The distance moved during the avoidance response was recorded. Repeated-measures ANOVA was used to evaluate the data obtained from the learning test, followed by Bonferroni comparisons to determine where significant differences occurred. A dose-response curve was obtained by linear regression as described in 2.6.3.

2.6.5 *Loss of Righting Reflex in All Bullfrog Tadpoles*

The data on the loss of righting reflex were expressed as a dichotomous variable, i.e., the event occurred or it did not on any day. Differences among treatments in the total number of tadpoles that exhibited an impaired righting reflex during the 28-day toxicity test were evaluated by the χ^2 test for homogeneity of proportions, followed by a multiple comparison of proportions as described in Section 2.6.3. The loss of righting reflex data were also evaluated by survival analysis (Afifi and Clark, 1990) to determine if the time to the first loss of righting reflex differed between treatments. The tadpoles in the behavior study were also examined for impaired righting reflex.

2.6.6 *Gross Motor Activity in 6-Day Behavior Study Bullfrog Tadpoles*

The gross motor activity data were expressed as the total seconds of movement during a three minute observation period each day. Repeated-measures ANOVA was used to evaluate the gross motor activity data, followed by a Bonferroni comparison. A

dose- response curve was obtained by multiple linear regression as described above in Section 2.6.3.

2.6.7 *Brain Cholinesterase Activity in 6-Day Behavior Study Bullfrog Tadpoles*

The cholinesterase data were analyzed by ANOVA to look for significant differences among treatments. The data were analyzed for normality and homogeneity of variance as previously described. A dose- response curve was obtained by multiple linear regression as described above in Section 2.6.3.

Table 2-1. Measured Concentrations of Malathion and Malaoxon in Water Samples from the Leopard Frog 96-h Toxicity Test

Treatment	Malathion (mg/L)		Malaoxon (mg/L)		n
	Mean	Standard Deviation	Mean	Standard Deviation	
Control	0.008	0.004	0.03	0.022	6
10	11.68	1.98	9.03	2.00	5
100	135.07	28.1	105.05	14.1	5
1000[a]	602.02	-	715.35	-	1

a - this sample formed a dense emulsion that could have contributed to the low amount of malathion recovered in the analysis

Table 2-2. Experimental Data for the Bullfrog Residue Study.

Frog Number	Sex	Body Weight (g)	Volume of Malathion Administered (ml)	Dose (mg/kg)	Exposure Time (min)
63	F	383.50	0.250	6.50	45.0
64	M	340.20	0.210	6.20	60.0
65	M	321.10	0.200	6.20	0.0[a]
66	F	448.80	0.290	6.50	20.0
67	F	355.20	0.230	6.50	7.0
68	F	374.80	0.240	6.40	120.0
69	M	387.40	0.250	6.40	14.0
70	F	396.60	0.000	0.00	0.0[b]

a - Died during dosing
b - Control frog

Table 2-3. Formulations for the Stock Solutions Used in the Bullfrog Tadpole Toxicity Test.

Stock Solution (mg/L)	Malathion (mg)	Acetone (ml)
Control	0	0
Acetone Control	0	100
500	52.5	100
1000	105.0	100
2000	209.0	100
2500	260.5	100
3000	313.0	100

Table 2-4. Measured Cholinesterase Activity in Small Samples of Rabbit Brain.

Sample Identification	Sample Weight (g)	ChE Activity (μmol/min/g)
A	0.0614	2.3
B	0.1192	2.4
C	0.0590	2.4
D	0.0372	2.9
E	0.0206	3.4
F	0.0335	2.5

Table 2-4. Verification of the Stock Solutions Used in the Tadpole Toxicity Tests.

Stock Solution (mg/L)	Nominal Dilution Concentration[a] (μg/L)	Dilution Concentration GC2 (μg/L)	Dilution Concentration GC4 (μg/L)	Mean Dilution Concentration (μg/L)	Mean Error ± SD (%)
500	500	500	500	500	0.0 ± 0.0
1000	500	585	500	545	8.5 ± 11.4
2000	400	436	488	460	15.4 ± 9.0
2500	500	600	505	550	10.4 ± 13.3
3000	600	300	480	390	35.1 ± 21.8

a - 5 μl of stock solution brought to appropriate volume in hexane

Figure 2-1. Chromatogram of a 1 μg/L Malathion (Retention Time = 4.711) and Malaoxon (Retention Time = 3.939) Standard in Hexane

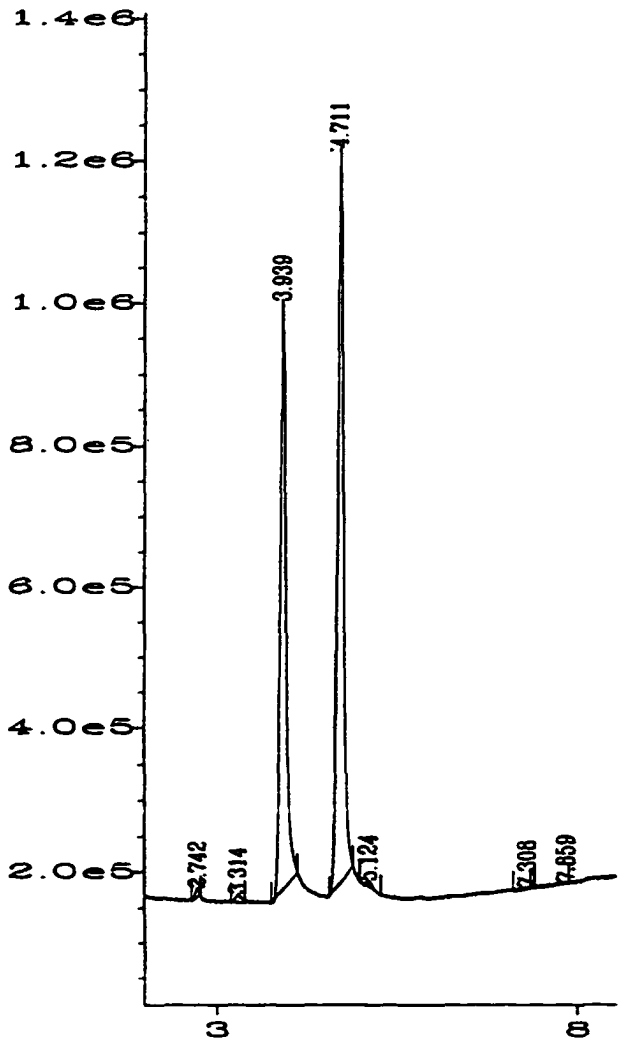
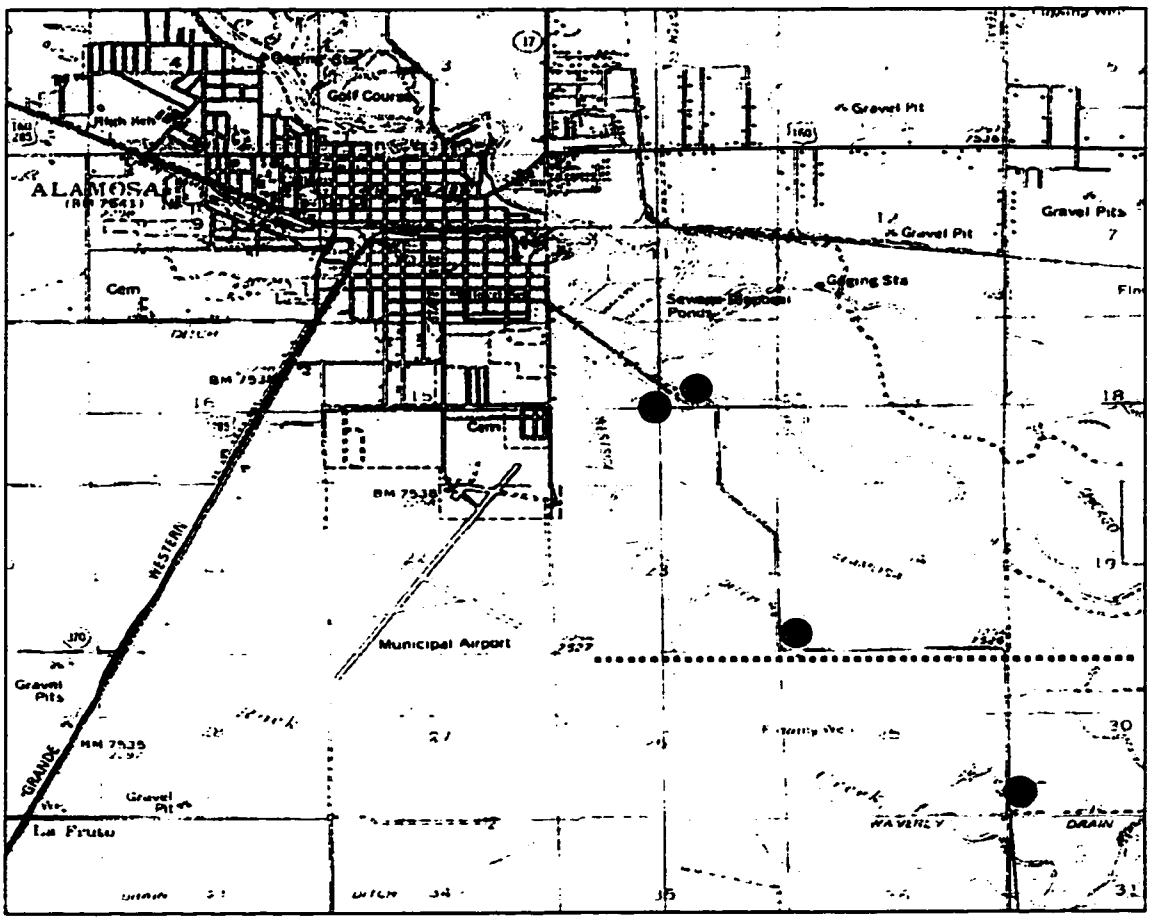


Figure 2-2. Environmental Water Sample Locations Near Alamosa, Colorado.

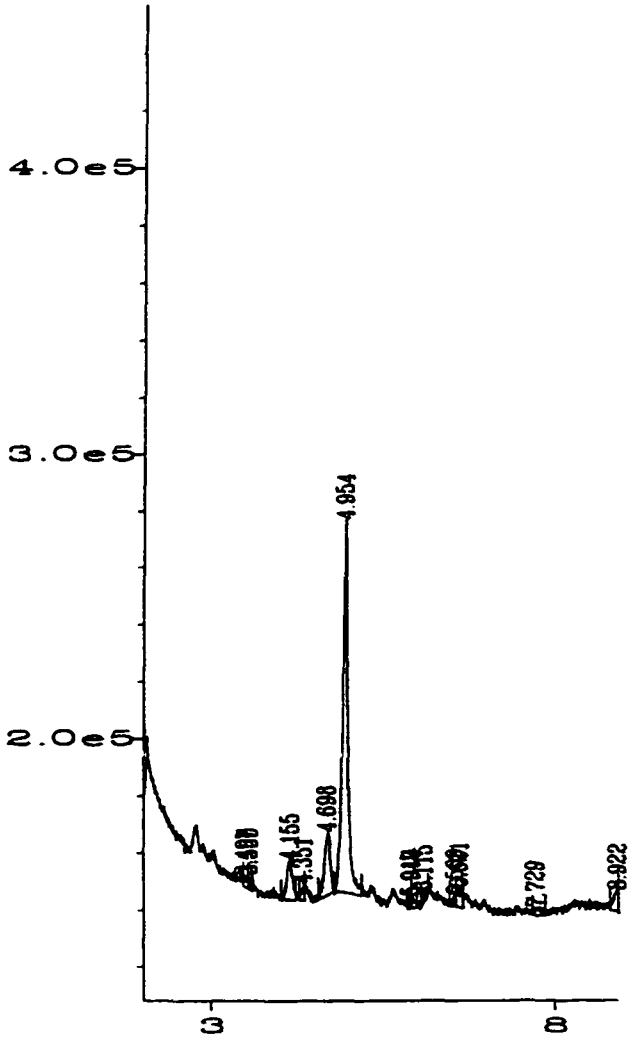


● Sample Locations

● Control Location

•••• Southern boundary of spray zone

Figure 2-3. Chromatogram of Malathion (Retention Time=4.698), Malaoxon (Retention Time = 3.932), and Large Metabolite (Retention Time=4.954) Peaks in Liver Tissue of Leopard Frog No. 9[a,b]



a - Malaoxon was below detection
b - Metabolite tentatively identified by GC-MS as O,O-dimethyl phosphorodithioate

3.0 RESULTS

3.1 96-HOUR TOXICITY TEST WITH LEOPARD FROGS

3.1.1 *Test Conditions*

The test solutions were analyzed for malathion and malaoxon once daily after formulating the solutions. The detection limits for the 250 ml water samples, based on a standard of 100 μg/L for malaoxon and 50 μg/L for malathion, were 0.004 and 0.002 mg/L for malaoxon and malathion, respectively.

Beakers of control reconstituted water apparently picked up trace amounts of pesticide, indicating possible glassware contamination or contamination due to ambient air flow. One sample at both the 10 and 100 mg/L treatments was lost during chemical analysis. The 1000 mg/L treatment was only formulated one day due to rapid mortality of the test frogs, so water concentrations were analyzed only once. There was a dense emulsion formed in the 1000 mg/L sample that could not adequately be broken during analysis, which could explain the low recovery (60%) of malathion from this sample. Large amounts of malaoxon were measured in water, whereas malaoxon is expected to form only 1% of technical grade malathion (Brown et al., 1993). This could be because the technical malathion had been open for several years prior to use in this study.

The room and the water temperature were maintained at 70°F throughout the duration of the test. The pH of the test solutions ranged from 7.1 to 7.2.

3.1.2 *Animal Data*

Leopard frog body and organ weights for each individual animal, as well as the mean and standard deviation (SD) by malathion dose, are presented in Table 3-1. Data represent all frogs used in the 96-h toxicity test. The test concentrations at which each frog was exposed are presented. In addition, data from two frogs (number 17 and 18) that were dosed with malathion by immersion in 100 mg/L for 1.5 h were included in this table.

Body weights were not significantly different ($p>0.05$) between treatment groups at the beginning of the test. There was no significant effect of malathion dose on body weight since body weight data did not differ significantly by treatment ($p>0.05$). The liver weight data also were not significantly different by treatment ($p>0.05$). However, the kidney weight data were significantly different by treatment ($p=0.02$). Comparison of the mean kidney weights indicated that the mean kidney weight for the 1000 mg/L malathion treatment was significantly higher ($p<0.05$) than that for the 10 mg/L malathion treatment. However, mean kidney weight did not follow a dose-response trend, since the control and the 100 mg/L treatment groups exhibited higher kidney weights than did the 10 mg/L treatment group (Table 3-1). There was no significant dose-response in the mean body or organ weights. The body and organ weight data were normal and the variances were evenly distributed.

3.1.3 *Mortality*

The survival times for each individual frog, and percent mortality by malathion treatment level, are reported in Table 3-2. A χ^2 test indicated that mortality was significantly different by treatment group ($p=0.005$). Mortality in the 100 and 1000 mg/L malathion treatments was significantly higher ($p<0.05$) than in the control and 10 mg/L treatments; however, there was no significant difference in mortality between the 100 and 1000 mg/L. Survival analysis indicated that the mean survival time for both the controls and the frogs in the 10 mg/L treatment was 96 h; frogs exposed to 100 mg/L survived 76.8 h, and those exposed to 1000 mg/L malathion in water survived only 1.5 h.

The 96-h LC_{50} estimated from the mortality data and the nominal concentrations in water was 56.2 mg/L (95% confidence interval: 20.75 to 152.41 mg/L). Frog 17 and 18 were not used in estimating the LC_{50} or percent mortality by treatment because these frogs were euthanized after 1.5 h of exposure in order to evaluate the effect of time on uptake into tissues and ChE activity levels.

3.1.4 *Behavior*

Behavior was monitored several times daily by counting the number of fright responses observed in each animal during the first two minutes following entry of the investigator into the animal room. The first day, two observation periods were conducted. Four observation periods were conducted on the second day, six periods on the third day, and

nine periods on the last day. The number of observation periods was increased because the frogs appeared to be more active during the night as opposed to the day.

The behavior data are summarized in Table 3-3. The average number of fright responses for each individual for each observation period was calculated as the total number of fright responses recorded per day divided by the total number of observation periods during that day (Table 3-3). The average responses per observation period per day for each treatment group are also presented. This table demonstrates that there is a general trend towards inactivity for frogs treated with malathion. There was considerable variability in the number of fright responses observed for individual frogs. Frogs 3 and 5, which were in the control and the 10 mg/L treatment, respectively, appeared to be less active than the other individuals in the same treatments. Data were unavailable for the frogs exposed to 1000 mg/L because they died prior to initiation of behavior observations.

Based on a χ^2 of the total number of frogs that exhibited a fright response at some time during the study, there was a significant difference in the number of frogs exhibiting a fright response by malathion treatment ($p=0.0025$). The 100 mg/L malathion treated frogs were significantly less mobile compared to the control or 10 mg/L treated frogs; however, the control group did not differ significantly from the 10 mg/L group.

There were no significant differences in behavior ($p>0.05$) due to malathion treatment or time of observation on Day 1. On Days 2, 3, and 4, frogs in the control group were

significantly more active ($p<0.05$) than frogs in either the 10 or 100 mg/L malathion treatment. Time of day the observation was made was not a significant factor ($p>0.05$) influencing the mean number of fright responses. The interaction of time and day was also not significant ($p>0.05$) on any given day.

There were more observation periods on Day 4 than on the other days, and this is the point in the test at which adverse effects due to exposure could be expected to be maximized. Therefore, these data were examined graphically (Figure 3-1). Although initially it appeared that frogs were more active during evening hours, examination of the data on Day 4 by time of observation and malathion treatment suggest a more complex activity pattern (Figure 3-1). Frogs appeared to be more active in the morning, and again in the evening, than in the mid-afternoon. Most likely due to the small sample sizes and high variability, the differences between observation times were not significant ($p>0.05$).

The results of the behavior observations may be biased because behavior observations were only made twice on Day 1, and both of the observation periods were in the afternoon, as compared to four, six, and nine times on Days 2, 3, and 4 respectively.

3.1.5 *Brain Cholinesterase Activity*

Cholinesterase activity is presented graphically in Figure 3-2 and numerically in Table 3-4. Cholinesterase activity was significantly ($p=0.005$) reduced in frogs treated with malathion (Figure 3-2), as indicated by the results of ANOVA. The frogs exposed to the 10 mg/L

treatment had cholinesterase activity that did not differ significantly from the controls. ChE activity in frogs in the 100 mg/L and 1000 mg/L groups was significantly lower ($p<0.05$) than ChE activity in the controls as indicated by a Bonferroni comparison (Table 3-5).

Survival time also influenced the ChE activity levels in the 16 leopard frogs in the 96 h toxicity test ($p<0.05$) as indicated by ANOVA. However, when all of the treatment levels in the 96-h toxicity test data were evaluated, the means were not different at a 90% or higher confidence level as indicated by a Bonferroni comparison. A larger sample size for ChE activity would likely have provided sufficient data to determine differences among the means. Examination of the all data available for frogs exposed only to 100 mg/L indicated that ChE activities were significantly different by survival time at a 90% confidence level ($p=0.06$) (Table 3-4). Frogs exposed to 100 mg/L for the duration of the 96-hr toxicity test (or until death) had significantly lower cholinesterase activities than the two frogs that were exposed for only 1.5 hr to 100 mg/L. The ChE activity in frogs exposed to 100 mg/L for 1.5 h did not differ significantly ($p=0.21$) from the activity in frogs exposed to 1000 mg/L until death (also 1.5 h). A larger sample size would possibly have changed this result, considering that the mean ChE activity was lower in frogs exposed to 1000 mg/L than in frogs exposed to 100 mg/L for 1.5 hours (Figure 3-2). Mortality and ChE activity were also significantly related ($p<0.05$), as indicated by the results of ANOVA. ChE activity was significantly higher in frogs that survived the 96-h toxicity test as compared to those that died during the test.

ChE activity was linearly related to the log of the water concentration (r^2=54%) when all of the data for the leopard frog toxicity test were evaluated by linear regression (Figure 3-3). The equation for the relationship of ChE activity to malathion concentration in water (MC) was:

$$ChE(\mu mol / min / g) = -1.388 \times Log\ MC\ (mg / L) + 4.925 \quad (7)$$

The linear relationship of ChE activity to malathion concentration in water was similar to equation (7) when only the ChE activities of the survivors were considered (Figure 3-3), although the association was weaker (r^2=-24%) due to the smaller sample size. The equation for the data without the frogs that died during the experiment was:

$$ChE(\mu mol / min / g) = -1.208 \times Log\ MC\ (mg / L) + 5.061 \quad (8)$$

Predictions of ChE activity in relationship to malathion dose are clearly affected by analysis of the data with consideration of mortality; however, the slopes and the intercepts of the two regression lines were not significantly different ($p>0.05$). Because ChE activity was significantly related to both dose and mortality, a larger sample size may have indicated a difference in the dose-response curves with or without data from frogs that died. The water concentration at which ChE activity is one-half that of the controls is 59.73 mg/L based on equation (7), and 142.07 mg/L based on equation (8). This may reflect that some individuals are more tolerant than others.

The cholinesterase data for frogs in the 96-h toxicity test followed a normal distribution. The standard deviations of ChE activity were not significantly different among the treatment groups ($p>0.05$).

3.1.6 *Tissue Residues*

Malathion and malaoxon concentrations were analyzed in liver and kidney tissues of leopard frogs. The livers were analyzed separately, but the kidneys were pooled by treatment because the sample weight was so small. Table 3-5 presents the results of the liver tissue analyses, and Table 3-6 presents the results of the kidney analyses.

Malathion was detected in nearly all (90%) of the livers of leopard frogs exposed to the 100 and 1000 mg/L treatments (Table 3-5). Malaoxon was detected in the livers of only 30% of the frogs. A similar pattern was observed for the kidney samples (Table 3-6). Malathion was detected in kidneys of frogs exposed to the 100 and 1000 mg/L malathion treatments. Malaoxon was not detected in kidney. The kidney data are more limited than the liver data because the kidneys were pooled by treatment in order to have sufficient sample size for chemical analysis.

3.2 RESIDUE STUDY WITH BULLFROGS

Eight bullfrogs were used in an attempt to study uptake and excretion of malathion in adult bullfrogs. Blood, liver, and kidney samples were obtained from each frog and analyzed for malathion and malaoxon. Brain cholinesterase activity was measured in each

frog. The shortest time from dosing to termination was 7 minutes, and the longest time was 120 minutes. Frog 65 died during anesthesia, but this was not noticed until the injection was given and the dose was observed to pool at the injection site.

Table 3-7 presents the data obtained for the bullfrogs used in this experiment. Body and organ weights, dose, and time from dosing to termination were measured for each frog. Bullfrogs were much larger than leopard frogs, which improved their suitability for use in the residue study.

The cholinesterase activity measured in each bullfrog is presented in Table 3-7. ChE activity in brain tissue of untreated bullfrogs (Table 3-7) was similar to that observed in control leopard frogs (Table 3-4). Figure 3-4 presents the ChE activity relative to the time from dose administration to point of termination for each individual frog. ChE activity had not started to recover within the duration of the residue test (Figure 3-4).

Bullfrog blood, liver, and kidney samples were analyzed for malathion and malaoxon. Table 3-8 presents the measured pesticide concentrations and the detection limits for each blood sample. The detection limits were calculated as described above for leopard frogs (Equation 3). The lowest standards that were used were 0.01 μg/ml malaoxon and 0.005 μg/ml malathion. A volume of 5 μl was injected on the GC-FPD. The amount of sample injected on the GC-FPD was calculated as the sample weight (mg) divided by the final volume (μl) (Table 3-8) multiplied by the volume injected (5 μl). Malaoxon peaks

occurred in most blood samples, although only one sample contained malaoxon above the detection limits. Malathion was not detected in blood.

Table 3-9 presents the results of chemical analysis of the liver tissue. Malathion was detected in four of eight liver tissue samples, whereas malaoxon was below detection limits in all eight liver tissue samples. Table 3-10 presents the results of chemical analysis of kidney tissue. Malathion was detected in three of eight kidney tissue samples, whereas malaoxon was not detected in any kidney tissue samples.

A large peak eluted immediately after malathion on the chromatogram (Figure 2-3). A single liver sample was analyzed by GC-MS in order to make a tentative identification of this compound. The metabolite was tentatively identified as O,O-dimethyl phosphorodithioate, a compound that results from hydrolysis of malathion at the S-C linkage in mammals (Muan et al., 1981).

3.3 ENVIRONMENTAL CONCENTRATIONS OF MALATHION IN WATER

The concentrations of malathion detected in the water samples collected from Alamosa, Colorado wetland areas after an aerial spray event are presented in Table 3-11. Pesticide drift appeared to have occurred, since malathion was detected in shallow surface water, yet was not sprayed over the control area (Figure 2-2). There were no residences or other development in the immediate vicinity of the control location. However, other sources of

malathion may have existed. Malaoxon was not detected in any of the environmental water samples (Table 3-11).

3.4 SURVIVAL, GROWTH AND DEVELOPMENT OF BULLFROG TADPOLES IN THE 28-DAY TOXICITY TEST

3.4.1 *Survival of Tadpoles in the 28-Day Toxicity Test*

Survival at different times throughout the study was determined for each treatment group at each day by the number of surviving tadpoles (n); Table 3-12 summarizes survival at weekly intervals throughout the 28 day period. All of the control and acetone control tadpoles, as well as tadpoles in the lower treatments of 500 and 1000 μg/L, survived the 28-day toxicity test (Figure 3-5). The highest two treatment groups exhibited the greatest number of deaths. Based on the χ^2 test for homogeneity of proportions, mortality was significantly related to treatment level ($p<0.001$).

Comparison of the proportions indicated that mortality in the 2000 μg/L treatment did not differ significantly from the control groups; however, both the 2500 and 3000 μg/L treatments had significantly ($p<0.001$) greater mortality than the control groups (Table 3-12). Mortality did not differ significantly between the 2500 and 3000 μg/L treatment groups; however, mortality in the 2500 μg/L malathion treatment was significantly greater ($p<0.05$) than that in the 2000 μg/L test concentration.

The time at which mortality occurred was observed to be related to treatment (Figure 3-5). Mortality occurred first in the 3000 μg/L treatment, followed by the 2500 μg/L treatment, and then by the 2000 μg/L treatment (Figure 3-5). Mean survival time was 28 days for the 0, 500, and 1000 μg/L treatment groups, and, 26, 19.6 and 19.1 days for the 2000, 2500, and 3000 μg/L treatment groups, respectively.

A dose-response curve for mortality (Figure 3-6) was obtained by converting the percent response of tadpoles in the malathion treatment groups to probit units (Zar, 1984; Amdur et al., 1991), and then plotting the probit response against the log of the malathion concentration in water (Figure 3-6). The equation for the dose-response was obtained by linear regression (R^2=64%) of mortality against the log of the malathion concentration in water (MC; μg/L):

$$Mortality\ (probit\ units) = 1.188 \times \left(Ln\ MC + 1\right) - 3.427 \quad (9)$$

3.4.2 *Growth of Tadpoles in the 28-Day Toxicity Test*

Effects on growth were evaluated by measuring body weight weekly throughout the study (Table 3-13). The tadpoles were stratified by body weight at the start of the study, and there was no significant difference ($p>0.05$) in mean body weights between the different treatments on Day 1 as indicated by ANOVA. Based on the results of the repeated-measures ANOVA, body weight throughout the study did not differ significantly between the acetone and control groups ($p=0.50$); therefore, the data for the two control groups were pooled for the following statistical analyses. There was no significant effect

(p=0.41) of malathion treatment on body weight of surviving tadpoles (Figure 3-7) based on the results of repeated-measures ANOVA. The interaction of treatment and day was also not significant (p=0.13). Therefore, the data were not evaluated further by linear regression to obtain a dose-response curve.

Body weight decreased over the duration of the 28-day toxicity test (Figure 3-7); repeated-measures ANOVA indicated that this effect was significant (p=0.002). A Bonferroni comparison of mean body weight by day indicated body weight was significantly lower on Days 8, 15, and 22 than on Day 1.

The cumulative change in body weight (Figure 3-8) expressed as the difference between the initial and final body weight was significantly different by treatment as indicated by ANOVA (p=0.03). A Bonferroni comparison indicated that the change in body weight of surviving tadpoles in the 2000 μg/L treatment was significantly different ($p<0.05$) from that of tadpoles in the 1000 μg/L treatment; however, body weights of surviving tadpoles in all other treatments were not significantly different. The residuals of body weight followed a normal distribution, and the variance was not significantly different ($p>0.05$) between the treatment levels.

The effects of malathion on growth were also evaluated by measuring body length weekly throughout the study (Figure 3-9). Based on the results of the repeated-measures ANOVA, body length did not differ significantly between the acetone and control groups

($p>0.05$); therefore, the data for the two control groups were pooled for the following statistical analyses.

Body length decreased significantly ($p=0.0001$) over the duration of the 28-day toxicity test (Figure 3-9) as indicated by repeated-measures ANOVA. The mean body length was significantly less on all days measurements were made (Days 8, 15, 22, and 28) compared to Day 1. There was a significant effect ($p=0.01$) of malathion on body length as indicated by the results of repeated-measures ANOVA for only one treatment group; the tadpoles in the 2000μg/L treatment group were significantly longer than the controls ($p<0.05$). Although the tadpoles in the 2000 μg/L treatment appeared to be longer than tadpoles in the other treatments (Figure 3-9) throughout the study; this difference was not significant on Day 1 ($p=0.10$) as indicated by ANOVA. Mean body length of tadpoles in the 2000 μg/L treatment was significantly higher ($p<0.05$) compared to the controls only on Day 28 (Table 3-13). Because the body length was not significantly related to treatment level by repeated-measures ANOVA, linear regression was not used to obtain a dose-response curve for these data.

There was a significant effect ($p=0.01$) of malathion treatment on cumulative change in body length of surviving tadpoles (Figure 3-10). Tadpoles in the malathion treatments tended to lose less body length than the controls. The cumulative change in body length of surviving tadpoles in the 2500 μg/L treatment was significantly less ($p<0.05$) than the controls as indicated by a Bonferroni comparison of means. Body length was normally

distributed, and the variance was homogeneous between treatment groups.

Brain weights (Table 3-14) did not differ significantly between the control and acetone control groups. More importantly, there was no significant effect of malathion dose on brain weight (Table 3-14).

3.4.3 *Development of Tadpoles in the 28-Day Toxicity Test*

Effects on development were evaluated by measuring developmental stage weekly throughout the study (Figure 3-11). Based on the results of ANOVA, the developmental stage of the tadpoles in the different treatment groups was not significantly different ($p>0.05$) on Day 1 of the study (Table 3-15). Based on the results of the repeated-measures ANOVA, developmental stage throughout the study did not differ significantly between the acetone and control groups ($p=0.50$); therefore, the data for the two control groups were pooled for the following statistical analyses.

Treatment with malathion delayed development over time as indicated by a significant dose and day interaction ($p=0.003$) when the data were evaluated by repeated-measures ANOVA. On all days on which measurements of developmental stage were made (i.e., Days 8, 15, 22, and 28), the mean developmental stage of the controls and the 500 μg/L was significantly higher than that measured on Day 1. On Days 15, 22 and 28, the mean stage of tadpoles in the 1000 and 2000 μg/L treatments was higher than that on Day 1. The 2500 and 3000 μg/L treatment groups progressed even less, as stage in the 2500

μg/L was significantly higher than on Day 1 only on Days 15 and 22, and stage in the 3000 μg/L did not differ significantly on any day from that measured on Day 1. The effect of malathion on developmental stage was not significant ($p=0.11$) without consideration of time. Developmental stage increased significantly ($p=0.0001$) over time as indicated by repeated-measures ANOVA (Figure 3-11). Tadpole stage was significantly lower ($p<0.05$) on Day 1 compared to each of the other days at which stage was measured.

The cumulative change in developmental stage of the surviving tadpoles was evaluated by comparing the difference in average final stage to average initial stage and by comparing the percent change in stage (Figure 3-12). Tadpoles in the highest two treatments did not progress as far in developmental stage as did the control or lower dose tadpoles (Figure 3-12). The cumulative percent change in stage of surviving tadpoles was significantly different only at a confidence level above 95%; cumulative change in stage was significant at a confidence level of 94% ($p<0.06$) when the data were analyzed by ANOVA, and comparison of the means indicated that tadpoles in the lowest malathion treatment (500 μg/L) had progressed farther in developmental stage than those in the highest treatment ($p<0.1$). A larger sample size could have improved interpretation of the statistical analyses.

3.4.4 *Loss of Righting Reflex in Tadpoles in the 28-Day Toxicity Test*

Tadpoles in this study were considered to have an impaired righting reflex if they rolled over within 15 seconds of the test container being moved, so that the ventral surface was

nearly upward and the animal failed to maintain an equilibrium position. In order to evaluate the righting reflex data statistically, a count of the number of tadpoles in each treatment that lost the righting reflex at any time during the study was made and expressed as a proportion of the initial number of tadpoles in each group (Table 3-16). The number of tadpoles in each treatment that exhibited an impaired righting reflex during the 28-day toxicity test was also expressed as a proportion of the surviving tadpoles on that day (Figure 3-13).

The acetone controls did not differ significantly ($p<0.05$) from the controls (Table 3-16); therefore, the data for both control groups were pooled for the following statistical analyses. Malathion significantly ($p=0.0008$) affected the righting reflex of tadpoles as indicated by a χ^2 test, and the tendency to lose the righting reflex increased with dose (Figure 3-13). The number of tadpoles with an impaired righting reflex in each of the malathion treatment groups was significantly different ($p<0.05$) from the control group as indicated by comparison of the proportions. As seen from Figure 3-14, the proportion of tadpoles exhibiting loss of the righting reflex was linearly associated ($R^2=79\%$) with the log of the malathion concentration in water (MC; μg/L) as follows:

$$Proportion = 0.0599 \times Ln(MC + 1) + 0.4765 \quad (10)$$

In addition, the mean time to loss of the righting reflex was inversely related to dose; the tadpoles in the highest treatment groups exhibited an impaired righting reflex earlier in the

study than did the controls or lower treatment groups ($p<0.01$). The mean time and standard error at which a loss of righting reflex was observed as predicted by a life table analysis are reported in Table 3-16.

3.5 LEARNING BEHAVIOR, GROSS MOTOR ACTIVITY, AND CHOLINESTERASE ACTIVITY IN BULLFROG TADPOLES IN THE 6-DAY BEHAVIOR STUDY

3.5.1 *Survival of 6-Day Behavior Study Tadpoles*

Survival was investigated in the behavior study tadpoles (Table 3-17), which used the same malathion test concentrations that were used in the 28-day growth study. Statistical evaluation of the data is summarized in Table 3-17. Some tadpoles in the higher test concentrations died during the exposure period, and survival was significantly different by treatment, but not by test replicate. An effect on survival relative to the control tadpoles during the 6-day behavior tests was not apparent in the 500 μg/L treatment, but mortality was significantly higher in the 1000 and 3000 μg/L malathion test concentrations than in the control, acetone control or 500 μg/L dose.

3.5.2 *Growth Parameters in 6-Day Behavior Study Tadpoles*

Body weight and length, brain weight, and developmental stage were similar in tadpoles in all malathion test concentrations (Table 3-17), indicating that these variables were unlikely to bias the learning test results. Based on the results of ANOVA, there was no significant difference ($p<0.05$) between the control and the acetone control group with respect to any

of the growth parameters at the end of the 6-day test.

3.5.3 *Loss of Righting Reflex in the 6-Day Behavior Study Tadpoles*

There was no significant difference in the number of tadpoles exhibiting an impaired righting reflex between the control and the acetone control groups. There was no significant difference among the three test replicates in the total number of tadpoles that lost their righting reflex during the course of the study (Table 3-18) as indicated by a χ^2 test. A total of 11, 12, and 16 tadpoles out of the 35 tadpoles per replicate lost their righting reflex at some point during the 6-day behavior study in replicates 1, 2 and 3, respectively.

Since there was no significant difference between the three test replicates, data for all replicates were combined and analyzed by malathion treatment (Figure 3-15). The number of tadpoles in each treatment with an impaired righting reflex during the study increased significantly ($p<0.05$) with malathion dose (Table 3-18). Multiple comparison of the proportions (Zar, 1984) indicated that the number of tadpoles with an impaired righting reflex in the highest treatments (i.e., the 2000, 2500 and 3000 μg/L malathion test concentrations) was significantly higher than in the controls or lower treatments (i.e., the acetone control, 500, or 1000 μg/L treatments). More tadpoles in the 2000 and 3000 μg/L malathion treatment groups lost the righting reflex than in either of the control treatment groups ($p<0.05$). The number of tadpoles with an impaired righting reflex in the 3000 μg/L malathion test concentration was significantly higher than in the 2500μg/L

treatment, as was the number in the 1000 relative to the 500 $\mu g/L$ (Table 3-18).

The effect of malathion exposure on the righting reflex was further investigated with linear regression of the data collected from all three test replicates on Day 1 compared to Day 5 (Figure 3-16). The data indicate that the longer exposure time produced a greater effect as indicated by comparison of the slope of the regression lines. The equation for the proportion of tadpoles exhibiting an impaired righting reflex on Day 1 (R^2=53%) was as follows:

$$Proportion = 0.00004 \times Concentration(\mu g / L) - 0.0107 \quad (11)$$

The equation for Day 5 (R^2=73%) was:

$$Proportion = 0.001 \times Concentration(\mu g / L) - 0.0352 \quad (12)$$

The total number of tadpoles that exhibited an impaired righting reflex at some time during the 6-day behavior study expressed as a proportion of the initial number of tadpoles (Figure 3-16) was linearly related (R^2=79%) to the log of malathion treatment (MC; $\mu g/L$) as follows:

$$Total\ Proportion\ Losing\ Righting\ Reflex = 0.138 \times (Log\ MC + 1) + 0.4765 \quad (13)$$

3.5.4 *Learning Behavior in Tadpoles in the 6-Day Behavior Study*

The time that it took the tadpole to initiate an avoidance or escape response was termed the latency to respond (Figure 3-17) and was measured in seconds following onset of the conditioned stimulus. The data were evaluated by repeated-measures ANOVA, followed

by Bonferroni comparisons to determine where significant differences occurred (Table 3-19). Latency to respond was not significantly different between the control and acetone control tadpoles; therefore, the data for both control treatments were combined and statistically analyzed to evaluate the effects of malathion dose. There was no significant effect of dose ($p=0.11$), or dose by day interaction ($p=0.55$) as indicated by the results of repeated-measures ANOVA (Table 3-19). However, the latency to respond was different by day ($p<0.01$). Both Day 1 and Day 2 latencies were higher than Day 5 latency (Table 3-19). This decrease in latency with time indicates that the tadpoles were exhibiting avoidance learning during the experiment. Evaluation of the data with or without the trials that resulted in a non-response did not indicate different results.

The distance that a tadpole moved during an avoidance or escape response (Table 3-20) was measured by counting the number of 10 cm squares that the animal crossed. The data were evaluated by repeated-measures ANOVA, followed by Bonferroni comparisons to determine where significant differences occurred. Distance moved by the tadpole during an avoidance or escape (Figure 3-17) was not significantly different between the control and acetone control tadpoles; therefore, the data for both control treatments were combined and statistically analyzed by malathion treatment.

There was no significant effect of malathion dose ($p=0.74$) on the distance moved during an escape or avoidance response; however, the distance moved was significantly different ($p<0.001$) by day. In addition, there was a significant dose by day interaction observed

(p=0.05). The distance moved by a tadpole increased significantly from Day 1 to Day 5 (Table 3-20), indicating that the tadpoles were exhibiting avoidance learning. The distance moved on Days 2 and 3 was also significantly lower than on Day 5. The dose by day interactions indicated that the distance moved by tadpoles in the 3000 μg/L malathion test concentration on Days 1 and 2 was significantly less ($p<0.001$) than the distance traveled by tadpoles in the 3000 μg/L malathion test concentration on Day 5. Because malathion is a ChE inhibitor, the tadpoles in the higher treatment could have been overly responsive once an avoidance response was initiated. Evaluation of the data with or without the trials that resulted in a non-response did not indicate different results.

The number of trials that resulted in a non-response (i.e., distance moved was 0 during the trial) was significantly different ($p<0.001$) by dose when the data evaluated with a χ^2 test. The 0, 500 and 1000 μg/L test concentrations had significantly fewer trials that resulted in a non-response than did the 2000, 2500, or 3000 μg/L treatments (Table 3-21).

3.5.5 *Gross Motor Activity in Tadpoles in the 6-Day Behavior Study*

Gross motor activity was measured as the total seconds of movement during a three minute observation period (Figure 3-18). There was no difference between the acetone and control treatments when these data were analyzed by repeated-measures ANOVA (Table 3-22); therefore, the data were combined and analyzed by malathion dose.

There was a significant effect of dose ($p<0.004$), but not day of exposure, on gross motor

activity in tadpoles. Both the 0 and the 500 μg/L test concentrations were significantly

different ($p<0.05$) than the 3000 μg/L test concentration; tadpoles in the controls and the lowest malathion dose were significantly more active than tadpoles in the highest dose.

3.5.6 *Brain Cholinesterase Activity in Tadpoles in the 6-Day Behavior Study*

Brain cholinesterase activity decreased with malathion treatment (Figure 3-19). The acetone and the control groups were not significantly different when analyzed by ANOVA ($p=0.573$); therefore, the data for the two control groups were combined for further statistical analysis.

ANOVA indicated that ChE was significantly different by dose ($p<0.001$). The ChE activities in 0 μg/L tadpoles were significantly higher than in any of the malathion test concentrations. The ChE activity in the 500 μg/L tadpoles was higher than that observed in any of the higher malathion test concentrations. All malathion test concentrations above the 500 μg/L had similar ChE activities. As seen in Figure 3-20, there was a fairly strong linear relationship ($R^2=74\%$) of ChE activity against the log of the malathion concentration in water (MC; μg/L):

$$ChE(\mu mol / min / g) = -0.3037 \times (Log\ MC + 1) + 1.1783 \quad (14)$$

The ChE data were normal, and variances were homogeneous.

Table 3-1. Leopard Frog Body and Organ Weights for Leopard Frogs Used in the 96-h Toxicity Test and the Two frogs Exposed for 1.5 Hours.

Malathion Treatment (mg/L)	Frog Number	Body Weight (g)	Liver Weight (g)	Kidney Weight (g)
0	1	52.70	1.2	0.2
	2	62.30	2.1	0.5
	3	69.70	2.2	0.2
	4	64.40	1.3	0.4
	Mean ±SD	62.3 ± 7.1	1.7 ± 0.5	0.3 ± 0.2
10	5	55.80	1.6	0.2
	6	41.80	1.0	0.2
	7	49.00	1.1	0.2
	8	40.00	0.7	<0.1
	Mean ±SD	46.7 ± 7.2	1.1 ± 0.4	0.2 ± 0.1
100	9	81.70	1.9	0.4
	10	57.10	2.1	0.4
	11	75.30	1.5	0.3
	12	68.20	1.9	0.2
	Mean ±SD	70.6 ± 10.5	1.9 ± 0.3	0.3 ± 0.1
1000	13	85.30	2.5	0.6
	14	50.00	1.5	0.4
	15	51.10	1.4	0.4
	16	58.00	1.6	0.4
	Mean ±SD	61.1 ± 16.5	1.8 ± 0.5	0.5 ± 0.1
100[a]	17	55.80	1.8	0.3
	18	49.00	1.8	0.2

a - Frogs were exposed to 100 mg/L for 1.5 hours to test uptake rates

Table 3-2. Mortality and Survival Time Observed in the Leopard Frog 96-h Toxicity Test.

Malathion Treatment (mg/L)	Frog Number	Survival Time (hr)[a]	Percent Mortality Observed in Each Treatment
0	1	96	0
	2	96	
	3	96	
	4	96	
10	5	96	0
	6	96	
	7	96	
	8	96	
100	9	72	75[b]
	10	47	
	11	92	
	12	96	
1000	13	1.5	100[b]
	14	1.5	
	15	1.5	
	16	1.5	

a - All frogs with survival times of 96-h survived the toxicity test and were terminated at the end of the test period.
b - Mortality significantly different from controls ($p<0.05$)

Table 3-3. Results of Behavior Observations Made During the Leopard Frog 96-h Toxicity Test.

Malathion Treatment (mg/L)	Frog No.	Average Number (± Standard Deviation) of Fright Responses per 2 Minute Observation Period by Day				Total Number of Fright Responses for the 96-h Test
		Day 1	Day 2	Day 3	Day 4	
0	1	1.0 ± 1.4	2.5 ± 2.4	2.7 ± 2.9	2.1 ± 3.0	47
	2	0.5 ± 0.7	4.0 ± 2.2	3.7 ± 1.8	3.7 ± 3.0	72
	3	0.0 ± 0.0	1.0 ± 1.4	0.8 ± 1.0	0.3 ± 0.5	12
	4	0.5 ± 0.7	7.8 ± 6.5	3.3 ± 3.1	2.7 ± 2.4	76
	Mean	0.5 ± 0.8	3.8 ± 4.2	2.6 ± 2.4	2.2 ± 2.7	51.8 ± 29.4
10	5	0.0 ± 0.0	0.5 ± 0.6	0.3 ± 0.8	0.1 ± 0.3	5
	6	1.0 ± 1.4	4.8 ± 3.5	1.2 ± 2.4	2.0 ± 2.2	46
	7	0.0 ± 0.0	3.0 ± 4.1	1.2 ± 1.5	0.7 ± 1.7	25
	8	0.5 ± 0.7	2.0 ± 2.3	2.3 ± 2.7	0.9 ± 1.6	31
	Mean	0.4 ± 0.7	2.6 ± 3.1[a]	1.2 ± 2.0[a]	0.9 ± 1.7[a]	26.8 ± 17.0
100[b]	9	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	NA	0
	10	0.5 ± 0.7	0.0 ± 0.0	NA	NA	1
	11	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0
	12	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0
	Mean	0.1 ± 0.4	0.0 ± 0.0[a]	0.0 ± 0.0[a]	0.0 ± 0.0[a]	0.25 ± 0.5
1000	13	NA	NA	NA	NA	NA
	14	NA	NA	NA	NA	NA
	15	NA	NA	NA	NA	NA
	16	NA	NA	NA	NA	NA

a - significantly fewer avoidance responses than controls when data were evaluated by day ($p<0.05$)
b - proportion of frogs exhibiting a fright response significantly different from controls ($p<0.05$)

Table 3-4. Cholinesterase Activity Measured in Leopard Frog Brain Tissue.

Malathion Treatment Level (mg/L)	Frog Number	Survival Time (h)[a]	Brain Cholinesterase Activity (µmol/min/g)	Mean Cholinesterase Activity (µmol/min/g)	Standard Deviation
0	1	96	8.4	5.0	2.4
	2	96	3.0		
	3	96	4.0		
	4	96	4.4		
10	5	96	3.8	4.1	0.51
	6	96	4.4		
	7	96	4.6		
	8	96	3.5		
100	9	72	0.4	1.0[b,c]	0.91
	10	47	1.2		
	11	92	0.2		
	12	96	2.2		
1000	13	1.5	1.2	1.4[b]	1.3
	14	1.5	0.4		
	15	1.5	3.2		
	16	1.5	0.6		
100[d]	17	1.5	2.5	2.8[e]	0.42
	18	1.5	3.1		

a - Frogs with a survival time <96 h died during the toxicity test; frogs with a survival time of 96 h were terminated at the end of the test.

b - significantly different from control ($p<0.05$)

c - significantly different from 10 mg/L ($p<0.1$)

d - Two frogs used for dermal uptake estimates that were exposed to 100 mg/L for a fixed time period of 1.5 h.

e - significantly different from 100 mg/L frogs in 96-h toxicity test ($p<0.1$)

Table 3-5. Analytical Data for Leopard Frog Liver Tissue.

Malathion Treatment (mg/L)	Frog No.	Survival Time (h)[a]	Sample Weight (g)[b]	Sample Detection Limits Malaoxon (μg/g)	Sample Detection Limits Malathion (μg/g)	Liver Concentrations Malaoxon (μg/g)	Liver Concentrations Malathion (μg/g)
0	1	96	0.85	0.0118	0.0059	BDL	BDL
0	2	96	1.672	0.0060	0.0030	BDL	BDL
0	3	96	1.844	0.0054	0.0027	BDL	BDL
0	4	96	1.036	0.0097	0.0048	BDL	BDL
10	5	96	0.854	0.0117	0.0059	BDL	BDL
10	6	96	1.021	0.0098	0.0049	BDL	BDL
10	7	96	0.968	0.0103	0.0052	BDL	BDL
10	8	96	0.459	0.1218	0.0109	BDL	BDL
100	9	72	1.134	0.0088	0.0044	BDL	0.307
100	10	47	1.105	0.0090	0.0045	0.093	8.480
100	11	92	0.753	0.0133	0.0066	BDL	0.190
100	12	96	1.679	0.0060	0.0030	BDL	0.105
1000	13	1.5	1.795	0.0056	0.0028	BDL	0.155
1000	14	1.5	1.337	0.0075	0.0037	BDL	0.151
1000	15	1.5	0.834	0.0120	0.0060	0.098	0.050
1000	16	1.5	1.384	0.0072	0.0036	BDL	BDL
100[c]	17	NA	1.370	0.0073	0.0036	BDL	0.082
100[c]	18	NA	1.504	0.0066	0.0033	0.057	0.080

a - Frogs with a survival time <96 h died during the toxicity test; frogs with a survival time of 96 h were terminated at the end of the test.
b - Sample weights as measured prior to analytical effort.
c - Two frogs used for dermal uptake estimates that were exposed to 100 mg/L for a fixed time period of 1.5 h.

Table 3-6. Analytical Data for Leopard Frog Kidney Tissue Pooled by Treatment Level.

Treatment (mg/L)	Frog No.	Sample Weight (g)[a]	Sample Detection Limits Malaoxon (µg/g)	Sample Detection Limits Malathion (µg/g)	Kidney Tissue Concentrations Malaoxon (µg/g)	Kidney Tissue Concentrations Malathion (µg/g)
0	1,2,3,4	0.572	0.1748	0.0874	BDL[b]	BDL
10	5,6,7,8	0.144	0.6944	0.3472	BDL	BDL
100	9,10,11,12	0.708	0.1412	0.0706	BDL	1.374
1000	13,14,15,16	1.037	0.0964	0.0482	BDL	8.187
100[c]	17,18[d]	NA	NA	NA	NA	NA

a - Sample weights as measured prior to analytical effort.
b - BDL= Below Detection Limits
c -Two frogs used for dermal uptake estimates that were exposed to 100 mg/L for a fixed time period of 1.5 h.
d - Kidneys were not analyzed due to small sample size

Table 3-7. Bullfrog Body and Organ Weight Measurements, Cholinesterase Activity, Malathion Dose, and Survival Time.

Malathion Dose (mg/kg)	Frog No.	Body Weight (g)	Liver Weight (g)	Kidney Weight (g)	Survival Time (min)	ChE Activity (μmol/min/g)
NA[a]	65	321.08	6.588	0.954	0	3.6
0.0[b]	70	396.60	6.956	0.978	0	4.9
6.2	64	340.17	6.903	1.261	60	1.4
6.4	68	374.84	8.472	1.310	120	1.4
6.5	67	355.20	6.784	1.176	7	0.85
6.5	69	387.40	8.160	0.876	14	1.8
6.5	63	383.50	5.817	0.882	45	1.5
6.5	66	448.80	9.672	1.106	20	2.0

a -This frog died during dosing and the data were not utilized.
b -Control frog

Table 3-8. Analytical Data for Bullfrog Blood Serum.

Malathion Dose (mg/kg)	Frog Number	Sample Weight (g)	Final Volume (ml)	Sample Detection Limits		Serum Concentrations		Percent of Dose Recovered as Malathion
				Malaoxon (μg/g)	Malathion (μg/g)	Malaoxon (μg/g)	Malathion (μg/g)	
NA[a]	65	NA	NA	NA	NA	NA	NA	NA
0.0[b]	70	2.75	5.0	0.018	0.009	BDL[c]	BDL	NA
6.2	64	1.73	5.0	0.029	0.014	BDL	BDL	<0.23
6.4	68	2.24	5.0	0.022	0.011	BDL	BDL	<0.17
6.5	67	2.75	5.0	0.018	0.009	BDL	BDL	<0.14
6.5	69	3.36	5.0	0.015	0.007	BDL	BDL	<0.12
6.5	63	1.33	5.0	0.038	0.019	0.111	BDL	0.08
6.5	66	1.93	5.0	0.026	0.013	BDL	BDL	<0.20

a - This frog died during dosing and the data were not utilized
b - Control frog
c - BDL = below detection limits

Table 3-9. Analytical Data for Bullfrog Liver Tissue.

Malathion Dose (mg/kg)	Frog No.	Sample Weight (g)	Final Volume (ml)	Sample Detection Limits Malaoxon (μg/g)	Sample Detection Limits Malathion (μg/g)	Sample Concentrations Malaoxon (μg/g)	Sample Concentrations Malathion (μg/g)	Percent of Dose Recovered as Malathion
NA[a]	65	6.588	5.0	0.0152	0.0076	BDL[b]	0.008	NA
0.0[c]	70	6.956	5.0	0.0144	0.0072	BDL	BDL	NA
6.2	64	6.903	5.0	0.0145	0.0072	BDL	BDL	<0.12
6.4	68	8.472	5.0	0.0118	0.0590	BDL	BDL	<0.09
6.5	67	6.784	5.0	0.0147	0.0074	BDL	BDL	0.09
6.5	69	8.160	5.0	0.0122	0.0061	BDL	0.279	4.33
6.5	66	9.672	5.0	0.0103	0.0052	BDL	0.048	0.74
6.5	63	5.817	5.0	0.0172	0.0086	BDL	0.014	0.21

a - This frog died during dosing and the data were not utilized.
b - BDL - Below detection limits
c - Control frog

Table 3-10. Analytical Data for Bullfrog Kidney Tissue.

Malathion Dose (mg/kg)	Frog No.	Sample Weight (g)	Final Volume (ml)	Sample Detection Limits Malaoxon (μg/g)	Sample Detection Limits Malathion (μg/g)	Sample Concentration Malaoxon (μg/g)	Sample Concentration Malathion (μg/g)	Percent of Dose Recovered as Malathion
NA[a]	65	0.9542	5.0	0.1048	0.0524	BDL[b]	0.214	NA
0.0[c]	70	0.9784	5.0	0.1022	0.0511	BDL	BDL	NA
6.2	64	1.2608	5.0	0.0793	0.0397	BDL	BDL	<6.42
6.4	68	1.3097	5.0	0.0764	0.0382	BDL	BDL	5.96
6.5	67	1.1757	5.0	0.0851	0.0425	BDL	0.662	10.22
6.5	69	0.8763	5.0	0.1141	0.0570	BDL	BDL	8.84
6.5	66	1.1060	5.0	0.0904	0.0452	BDL	BDL	<7.00
6.5	63	0.8820	5.0	0.1134	0.0567	BDL	0.236	3.62

a -This frog died during dosing and the data were not utilized.
b - BDL = Below detection limits
c - Control frog

Table 3-11. Concentrations of Malathion and Malaoxon in Environmental Water Samples from Alamosa, Colorado.

Sample Identification	Malathion (μg/L)	Malaoxon (μg/L)
1	< 2.00	< 4.00
2	14.98	< 4.00
3	5.04	< 4.00
Control	10.72	< 4.00
Control (replicate)	10.42	< 4.00

Table 3-12. Survival of Bullfrog Tadpoles in the 28-Day Toxicity Test by Treatment and Study Day.

Test Day	Treatment Group[a]	n
1	Control	10
	Acetone	10
	500	10
	1000	10
	2000	10
	2500	10
	3000	10
8	Control	10
	Acetone	10
	500	10
	1000	10
	2000	10
	2500	8
	3000	8
15	Control	10
	Acetone	10
	500	10
	1000	10
	2000	9
	2500	7
	3000	6
22	Control	10
	Acetone	10
	500	10
	1000	10
	2000	9
	2500	5
	3000	6
28	Control	10
	Acetone	10
	500	10
	1000	10
	2000	9
	2500	4[b,c]
	3000	5[b]

a - Each treatment group started the study with 10 tadpoles
b - Total mortality was significantly different than controls ($p<0.001$)
c - Total mortality was significantly different than 2000 $\mu g/L$ ($p<0.05$)

Table 3-13. Growth Data for Surviving Bullfrog Tadpoles (n) in the 28-Day Toxicity Test by Study Day and Treatment.

Test Day	Treatment Group[a]	n	Mean Body Weight[b] (g)	SD	Mean Length[b] (cm)	SD
1	Control	10	7.550	1.610	8.300	0.820
	Acetone	10	8.310	1.800	8.410	0.550
	500	10	7.730	1.580	8.360	0.510
	1000	10	7.440	1.430	8.480	0.800
	2000	10	8.890	2.060	8.920	0.450
	2500	10	7.510	1.390	8.220	0.780
	3000	10	7.310	1.220	8.090	0.610
8	Control	10	7.230	1.690	7.950	0.580
	Acetone	10	7.700	1.550	8.060	0.610
	500	10	7.320	1.530	8.310	0.720
	1000	10	7.070	1.040	7.800[c]	0.700
	2000	10	8.240	1.240	8.650	0.770
	2500	8	7.630	1.460	8.200	0.590
	3000	8	7.130	1.710	8.090	0.850
15	Control	10	6.640	1.770	7.500[d]	0.750
	Acetone	10	7.260	1.320	7.770	0.360
	500	10	7.130	1.340	8.250	0.700
	1000	10	7.130	1.180	7.770	0.600
	2000	9	8.100	1.930	8.520	0.440
	2500	7	7.600	1.310	8.300	0.770
	3000	6	6.600	0.670	7.820	0.720
22	Control	10	7.010	1.420	7.750	0.640
	Acetone	10	7.120	1.230	7.860	0.520
	500	10	7.220	1.390	8.130	0.700
	1000	10	7.230	1.140	8.190	0.540
	2000	9	8.490	1.560	8.730	0.540
	2500	5	6.970	1.330	8.320	0.290
	3000	6	6.580	0.780	7.780	0.660
28	Control	10	7.040	1.760	7.750	0.640
	Acetone	10	7.340	1.270	7.810	0.470
	500	10	7.310	1.420	8.120	0.640
	1000	10	7.510	1.180	8.140	0.390
	2000	9	7.770	1.370	8.620	0.600
	2500	4	6.840	1.160	8.130	0.480
	3000	5	6.380	0.810	7.860	0.610

a - Each treatment group started with 10 tadpoles
b - Significantly different by day ($p<0.05$)
c - Significantly different from 1000 μg/L on Day 1 ($p<0.01$)
d - Significantly different from 0 μg/L on Day 1 ($p<0.01$)

Table 3-14. Brain Weights by Treatment in Bullfrog Tadpoles in the 28-Day Toxicity Test.

Treatment	Mean Brain Weight (mg)	SD	n
Control	42.30	6.67	10
Acetone	43.30	11.61	10
500	39.70	14.37	10
1000	43.40	15.36	10
2000	46.20	13.85	10
2500	54.30	13.43	10
3000	52.90	20.09	10

Table 3-15. Developmental Stage of Surviving Bullfrog Tadpoles (n) in the 28-Day Toxicity Test by Study Day and Treatment.

Test Day	Treatment Group[a]	n	Mean Stage[b,c]	SD
1	Control	10	27.0	1.0
	Acetone	10	27.0	1.0
	500	10	27.0	1.0
	1000	10	27.0	1.0
	2000	10	27.0	1.0
	2500	10	27.0	1.0
	3000	10	27.0	1.0
8	Control	10	32.0[d]	2.0
	Acetone	10	32.0[d]	4.0
	500	10	31.0[d]	3.0
	1000	10	30.0	2.0
	2000	10	30.0	1.0
	2500	8	30.0	2.0
	3000	8	29.0	2.0
15	Control	10	33.0[d]	3.0
	Acetone	10	32.0[d]	4.0
	500	10	33.0[d]	3.0
	1000	10	32.0[d]	3.0
	2000	9	34.0[d]	2.0
	2500	7	32.0[d]	3.0
	3000	6	30.0	2.0
22	Control	10	33.0[d]	3.0
	Acetone	10	32.0[d]	3.0
	500	10	33.0[d]	4.0
	1000	10	32.0[d]	3.0
	2000	9	33.0[d]	3.0
	2500	5	32.0[d]	3.0
	3000	6	30.0	2.0
28	Control	10	33.0[d]	3.0
	Acetone	10	33.0[d]	3.0
	500	10	34.0[d]	3.0
	1000	10	33.0[d]	3.0
	2000	9	33.0[d]	2.0
	2500	4	31.0	3.0
	3000	5	30.0	3.0

a - Each treatment group started with 10 tadpoles
b - Significantly different over time ($p=0.0001$)
c - Significant dose by day interaction ($p=0.0027$)
d - Significantly different from same treatment group on Day 1 ($p<0.05$)

Table 3-16. Proportion of Bullfrog Tadpoles in Each Treatment that Exhibited an Impaired Righting Reflex at Some Point During the 28-Day Toxicity Test.

Treatment	n[a]	Proportion of Tadpoles Losing Righting Reflex	Mean Time to First Loss of Righting Reflex (d)[b]	Standard Error
Control	3	0.30	24.35[c]	1.16[c]
Acetone	6	0.60		
500	9	0.90[d]	19.60	1.70
1000	10	1.00[d]	11.70	2.08
2000	10	1.00[d]	8.50	1.85
2500	8	0.80[d]	11.80	3.02
3000	9	0.90[d]	6.70	2.33

a - all treatment groups began the study with 10 tadpoles in each group
b - life-table analysis
c - data include both control groups
d - significantly different from controls ($p<0.05$)

Table 3-17. Summary of Survival and Growth Parameters Measured in the Behavior Study Bullfrog Tadpoles.

		Survival	Body Weight (g)		Length (cm)		Stage	
Test	Treatment	Total	Mean	SD	Mean	SD	Mean	SD
1	Control	5	8.370	2.000	9.080	0.560	27.0	1.0
	Acetone	5	7.270	1.470	7.960	0.430	27.0	1.0
	500	5	7.580	2.470	8.300	0.840	27.0	1.0
	1000	4	7.830	1.840	8.620	0.740	27.0	1.0
	2000	5	6.850	1.670	7.940	0.850	27.0	1.0
	2500	5	8.520	1.840	8.280	0.600	26.0	1.0
	3000	4	7.370	1.440	8.340	0.630	27.0	1.0
2	Control	5	6.470	1.430	7.480	0.530	33.0	4.0
	Acetone	5	6.750	0.840	8.160	0.680	30.0	2.0
	500	5	7.820	1.680	8.520	0.920	33.0	4.0
	1000	4	7.410	1.850	8.220	1.050	33.0	3.0
	2000	5	7.460	1.270	8.640	0.780	33.0	4.0
	2500	4	7.450	1.500	8.440	0.580	32.0	3.0
	3000	2	7.440	1.580	8.340	0.210	31.0	3.0
3	Control	5	6.270	1.390	7.780	0.860	33.0	5.0
	Acetone	5	6.550	1.380	7.400	0.840	30.0	3.0
	500	5	6.490	1.440	7.860	0.440	32.0	1.0
	1000	5	6.480	1.510	7.520	0.540	30.0	4.0
	2000	4	6.320	1.640	7.500	0.480	31.0	4.0
	2500	5	6.170	1.300	7.480	0.760	32.0	2.0
	3000	4	6.290	1.450	7.400	0.670	31.0	2.0
All	Control	15	7.040	1.800	8.110	0.950	31.0	5.0
	Acetone	15	6.860	1.210	7.840	0.710	29.0	3.0
	500	15	7.300	1.870	8.230	0.760	31.0	4.0
	1000	13[a]	7.240	1.710	8.120	0.880	30.0	4.0
	2000	14	6.880	1.500	8.030	0.830	31.0	4.0
	2500	14	7.380	1.760	8.070	0.740	30.0	3.0
	3000	10[a]	7.030	1.480	8.030	0.680	30.0	3.0

a - significantly different from the control, acetone control, or 500μg/L treatment group

Table 3-18. Proportion of Bullfrog Tadpoles in the 6-Day Behavior Study Exhibiting an Impaired Righting Reflex by Treatment.

Treatment[a] (μg/L)	Proportion[b]
Control[c]	0.17
500	0.00[d]
1000	0.20[e]
2000	0.67[d,e,f]
2500	0.53[d,e,f]
3000	0.87[d,e,f,g]

a - data for all three test replicates combined
b - all treatment groups included 15 tadpoles at the beginning of the study
c - control groups combined for statistical analysis (n=30)
d - significantly different from control ($p<0.05$)
e - significantly different from 500 μg/L ($p<0.05$)
f - significantly different from 1000 μg/L ($p<0.05$)
g - significantly different from 2500 μg/L ($p<0.05$)

Table 3-19. Latency (sec) of Bullfrog Tadpoles to Respond to an Adverse Stimulus by Test Replicate, Day, and Treatment.

		Test Replicate							
		1		2		3		All	
Treatment	Day[a]	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Control	1	13.2	6.1	10.6	2.3	10.9	1.8	11.5	4.0
	2	9.6	2.5	10.4	2.2	10.7	0.7	10.2	2.0
	3	9.4	3.3	10.6	1.3	10.4	1.6	10.1	2.3
	4	10.3	2.7	10.2	2.0	10.1	1.5	10.2	2.2
	5	9.9	2.2	10.4	1.5	9.9	2.3	10.1	2.0
Acetone	1	10.3	3.3	10.0	2.1	10.9	0.9	10.4	2.3
	2	9.6	2.7	10.6	1.7	10.8	0.8	10.3	2.0
	3	10.2	1.9	9.8	2.7	10.5	0.5	10.1	2.0
	4	10.4	1.3	10.1	1.9	10.4	2.0	10.3	1.7
	5	10.3	2.0	10.0	1.7	10.3	1.4	10.2	1.7
500	1	9.6	1.9	11.0	2.1	10.6	2.3	10.4	2.2
	2	10.1	2.7	11.5	3.5	10.0	2.0	10.5	2.9
	3	10.2	2.1	10.5	0.8	10.2	2.2	10.3	1.8
	4	10.0	2.4	10.4	0.9	10.3	1.1	10.2	1.6
	5	10.3	2.9	10.5	1.2	10.0	3.2	10.3	2.5
1000	1	13.0	7.0	11.7	4.4	10.2	1.9	11.6	5.0
	2	9.8	2.7	12.0	4.1	10.5	0.5	10.8	3.0
	3	10.0	2.1	12.0	5.7	10.0	1.9	10.6	3.8
	4	10.6	1.6	10.4	3.3	10.1	1.1	10.4	2.1
	5	10.7	2.5	9.7	2.8	10.0	1.1	10.1	2.2
2000	1	9.4	2.5	13.2	5.9	11.9	2.4	11.5	4.3
	2	13.9	7.2	11.4	1.5	11.2	2.4	12.2	4.7
	3	11.3	4.1	15.0	7.9	10.2	1.9	12.3	5.8
	4	14.4	7.8	12.1	4.4	10.0	2.8	12.3	5.8
	5	10.8	1.3	10.1	1.2	10.2	1.9	10.4	1.5
2500	1	13.2	6.7	17.3	8.6	12.9	5.5	14.5	7.3
	2	13.0	6.5	14.3	6.3	10.8	3.2	12.6	5.7
	3	11.2	1.2	14.2	7.2	9.8	2.9	11.5	4.6
	4	11.6	3.2	12.3	4.4	9.6	2.2	11.1	3.5
	5	10.9	1.8	9.8	2.0	9.3	2.7	10.0	2.3
3000	1	10.0	3.8	15.4	7.3	10.8	2.4	12.0	5.5
	2	15.7	8.0	13.2	5.7	11.4	3.4	13.4	6.2
	3	12.0	3.4	12.3	4.4	8.3	4.1	11.0	4.4
	4	11.7	3.0	14.9	8.1	9.5	4.4	12.0	6.0
	5	10.8	0.6	10.6	0.7	9.4	2.9	10.2	2.0

a - Latency was significantly different ($p<0.05$) on Day 5 compared to Day 1 and 2

Table 3-20. Distance Moved (cm) by Bullfrog Tadpoles During an Avoidance or Escape Response by Test Replicate, Day, and Treatment.

		Test Replicate							
		1		2		3		All	
Treatment	Day[a,b]	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Control	1	26.9	17.3	37.5	27.9	28.9	14.9	31.1	21.2
	2	26.4	16.2	42.4	25.7	30.4	15.6	33.1	20.7
	3	23.3	10.2	40.9	31.5	35.1	22.0	33.1	24.0
	4	28.0	21.8	36.8	32.4	36.9	20.2	33.9	25.6
	5	29.7	22.8	47.9	34.1	38.8	22.0	38.8	27.8
Acetone	1	31.7	22.2	36.8	21.2	24.3	12.4	30.9	19.7
	2	26.0	16.8	41.3	26.4	31.9	18.5	33.1	21.8
	3	31.7	23.8	29.1	14.3	26.4	11.8	29.1	17.5
	4	31.9	17.9	37.7	20.8	24.9	12.7	31.5	18.2
	5	30.1	16.4	38.4	18.1	26.8	14.8	31.8	17.1
500	1	28.8	12.6	28.1	17.2	28.9	17.9	28.6	16.0
	2	25.1	18.6	30.7	23.2	28.5	16.4	28.1	19.7
	3	29.1	17.2	27.5	20.7	30.8	19.5	29.1	19.1
	4	26.8	11.4	26.4	15.3	32.8	15.4	28.7	14.4
	5	22.8	9.7	37.1	23.1	36.7	29.0	32.2	23.0
1000	1	27.1	18.7	31.3	20.6	21.1	12.1	26.5	17.9
	2	23.7	11.7	31.2	24.4	30.5	24.7	28.5	21.4
	3	29.6	18.0	25.7	14.8	38.3	31.7	31.2	23.2
	4	28.3	13.1	23.5	9.7	30.8	15.3	27.8	13.4
	5	25.3	11.6	40.3	31.6	39.2	24.3	35.3	24.8
2000	1	25.2	12.5	28.9	22.0	30.5	18.8	28.2	18.3
	2	24.9	14.8	36.0	19.5	34.7	21.2	31.7	19.1
	3	28.8	14.9	24.7	18.8	30.7	18.7	27.9	17.6
	4	22.5	14.4	27.7	15.5	32.2	15.2	27.1	15.5
	5	28.1	9.3	39.2	18.8	33.0	18.1	33.5	16.4
2500	1	24.5	22.7	26.9	23.2	27.7	13.5	26.4	20.2
	2	24.8	14.9	29.2	17.5	33.1	20.0	29.0	17.9
	3	29.6	12.4	21.0	13.9	25.2	11.9	25.6	13.1
	4	23.3	12.6	26.7	13.5	29.1	16.2	26.3	14.4
	5	29.3	17.1	42.0	23.1	29.9	13.7	33.1	18.8
3000	1	23.7	17.2	35.5	29.5	30.9	14.4	30.0	21.8
	2	21.7	16.6	25.5	16.1	29.7	16.1	25.6	16.6
	3	31.7	13.3	25.5	12.0	26.5	13.0	27.7	12.9
	4	28.2	10.0	18.8	14.6	24.7	16.5	23.9	14.4
	5	28.5	12.1	42.3	32.1	31.2	16.6	32.3[c,d]	19.9

a - Distance on Day 5 was significantly higher from that on Day 1, 2, or 3 ($p<0.05$)
b - Distance on Day 4 was significantly higher from that on Day 1 ($p<0.05$)
c - Significantly higher than on Day 1 ($p<0.05$)
d - Significantly higher than on Day 2 ($p<0.05$)

Table 3-21. Number of Behavior Trials with a Non-Response Compared to the Total Number of Behavior Trials by Malathion Treatment.

Treatment (μg/L)	Total Number of Trials with a Non-response	Total Number of Trials
0	6	2250
500	3	1125
1000	19	1080
2000	57[a]	1065
2500	65[a]	1065
3000	58[a]	975

a - Significantly different from control, 500, or 1000 μg/L treatment groups ($p<0.05$)

Table 3-22. Gross Motor Activity in Bullfrog Tadpoles Averaged by Test Replicate and Time and Presented by Malathion Treatment.

Treatment	Mean Gross Motor Activity (sec)	SD
Control	14.33	17.33
Acetone	12.52	12.57
500	14.47	15.32
1000	7.84	9.86
2000	7.25[a,b]	12.17
2500	6.49[a,b]	10.23
3000	4.28[a,b]	7.23

a - Significantly different from combined control group ($p<0.05$)
b - Significantly different from 500 μg/L treatment group ($p<0.05$)

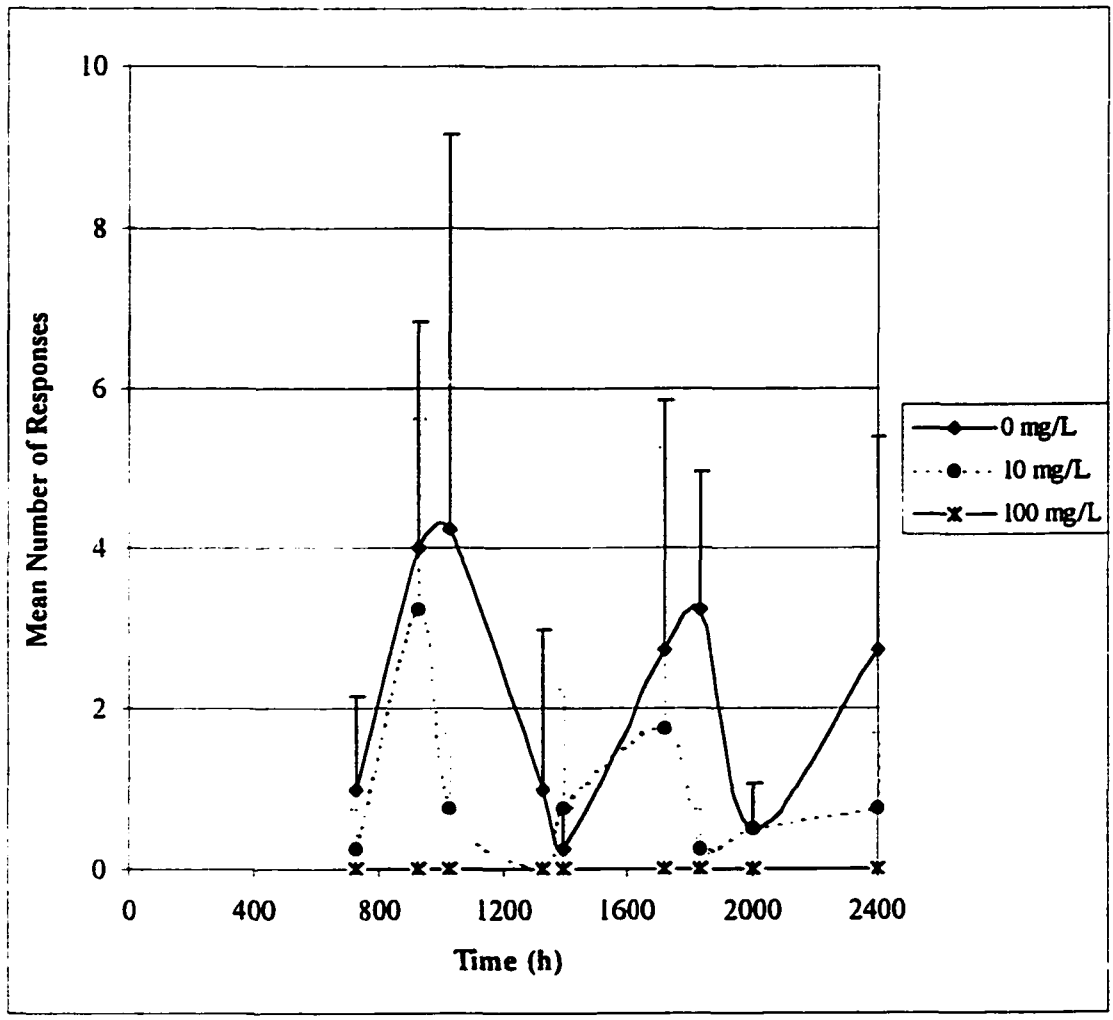


Figure 3-1. Mean (+SD) Number of Leopard Frog Fright Responses on Day 4 by Observation Time (h) and Malathion Treatment.

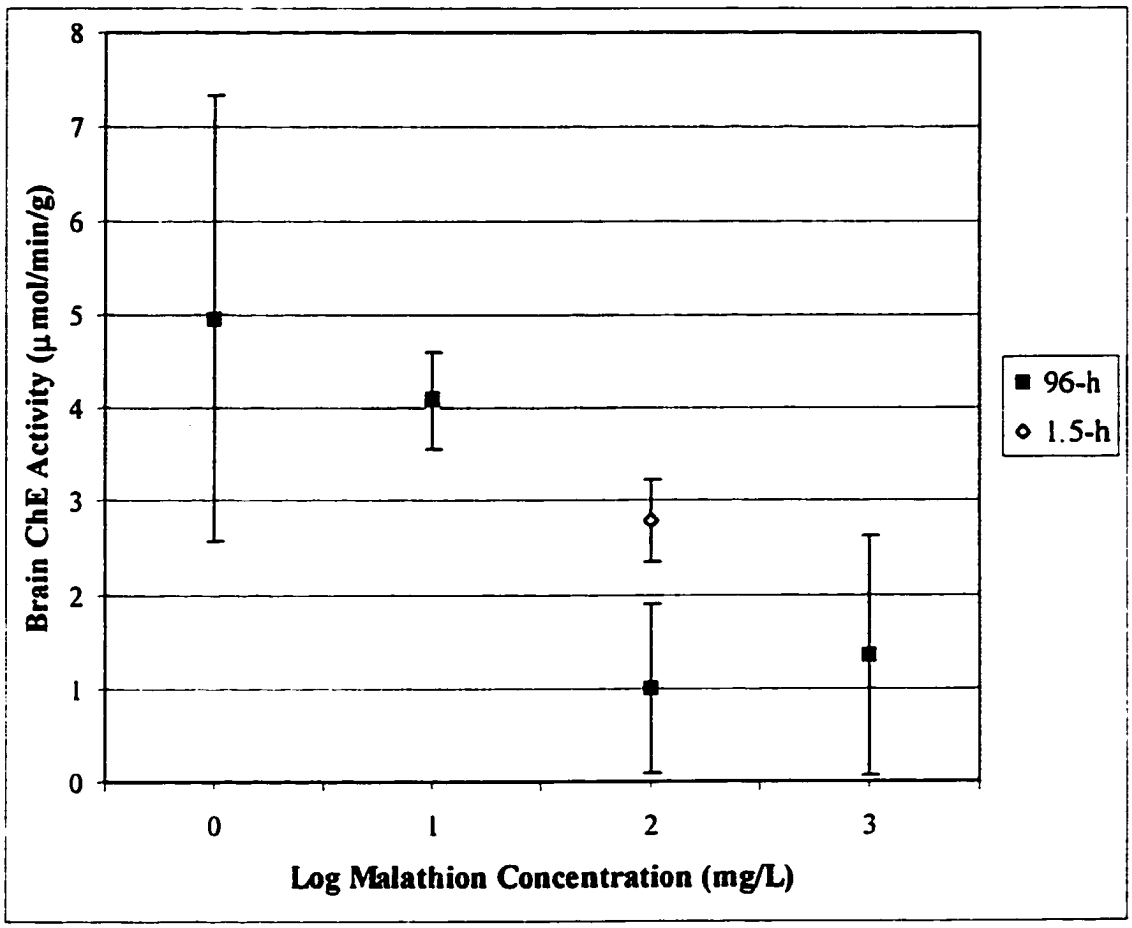


Figure 3-2. Mean (±SD) Measured Cholinesterase Activity in Leopard Frog Brain Tissue Versus the Log of the Nominal Malathion Concentration in Water, Where a 1 was Added to Each Concentration so that the Data Could be Presented on a Log Scale

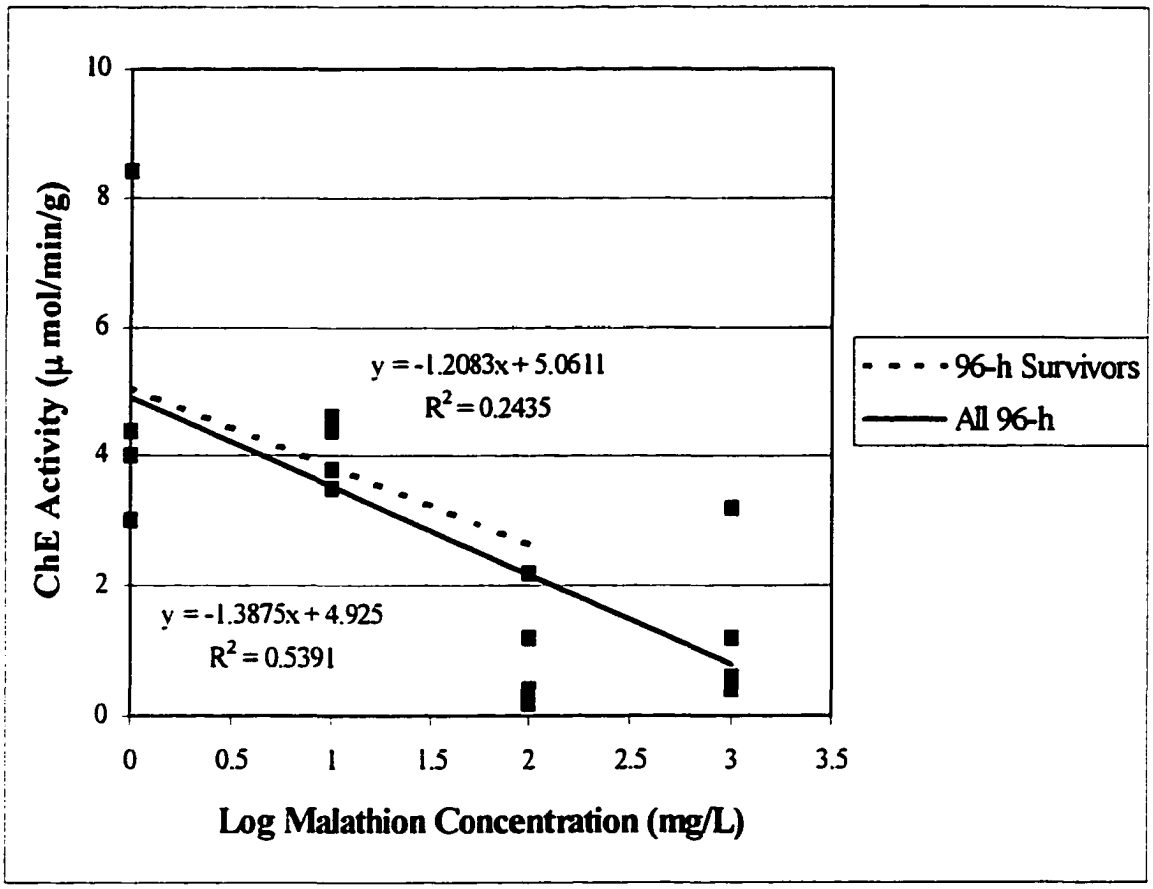


Figure 3-3. Linear Regression of Leopard Frog ChE Activity Compared by Mortality and the Log of the Nominal Concentration of Malathion in Water, Where a 1 was Added to Each Concentration so that the Data Could be Presented on a Log Scale

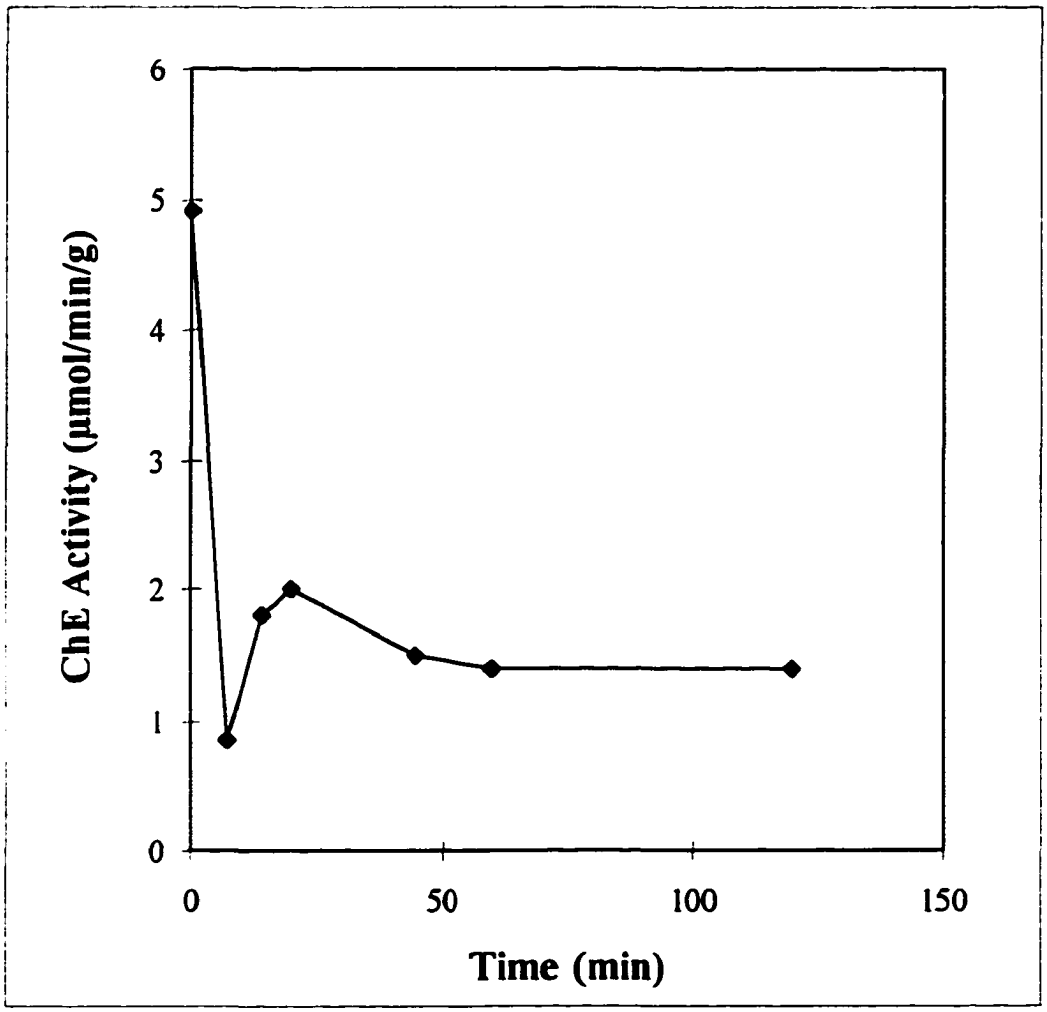


Figure 3-4. ChE Activity Measured Over Time in Brain Tissue of Adult Bullfrogs Dosed Intravenously with Malathion at 0 mg/kg (Time 0) or 6.43 ± 0.12 mg/kg (Time>0).

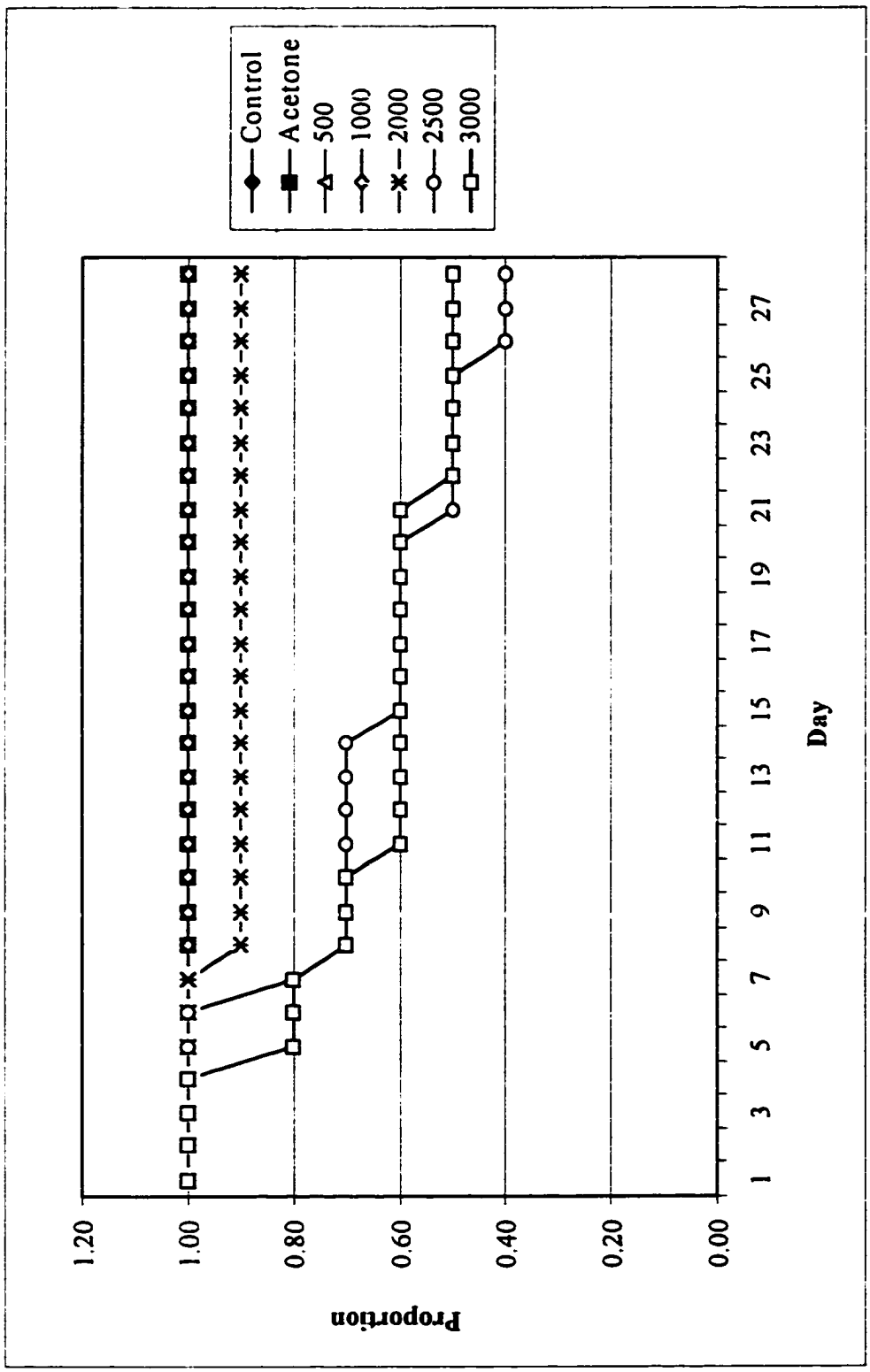


Figure 3-5. Survival of Bullfrog Tadpoles in the 28-Day Toxicity Test by Day and Malathion Treatment.

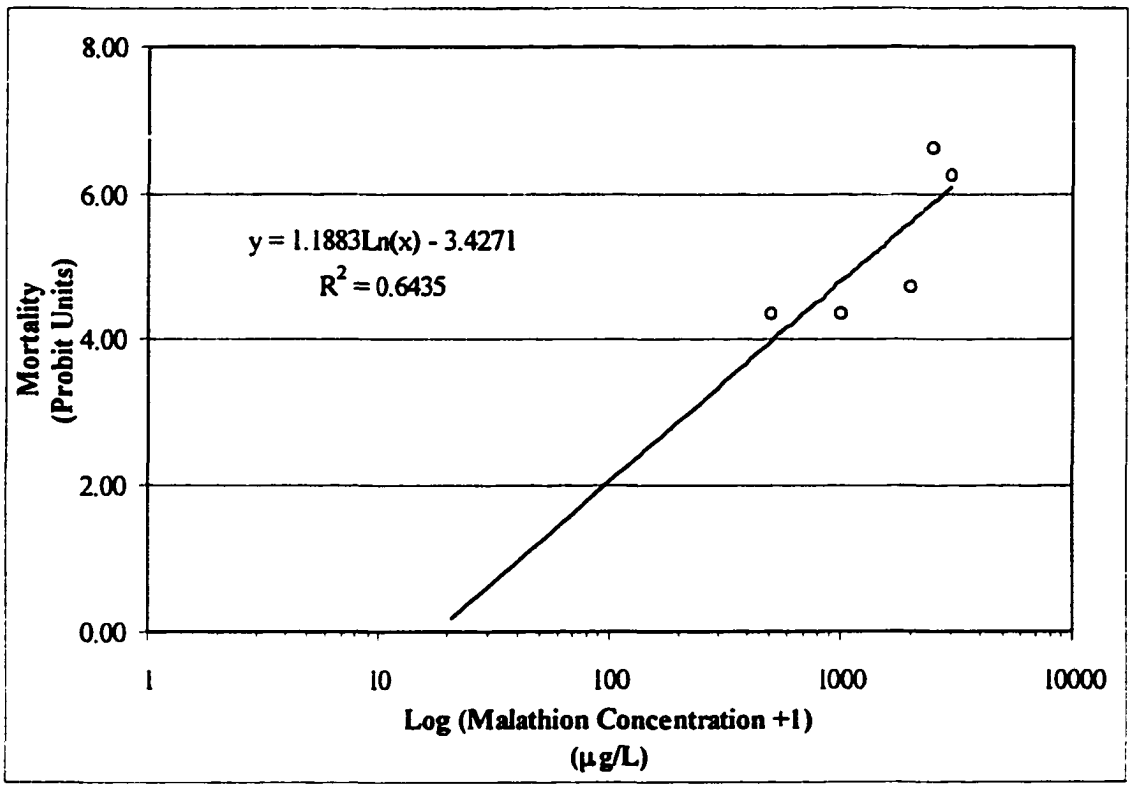


Figure 3-6. Dose Response Curve for Bullfrog Tadpole Mortality in the 28-Day Toxicity Test Plotted Against the Log of the Malathion Concentration in Water

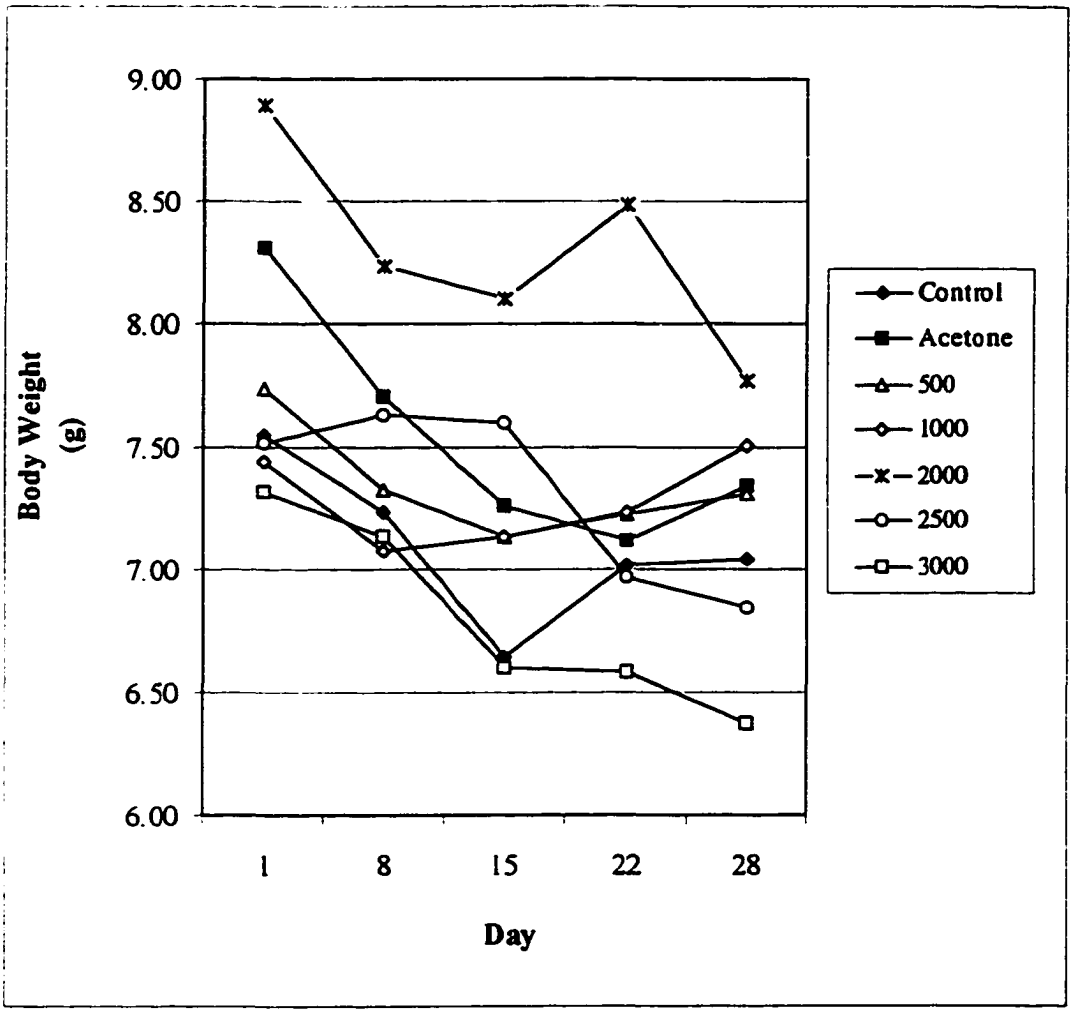


Figure 3-7. Mean Body Weight of Surviving Bullfrog Tadpoles by Day and Malathion Treatment.

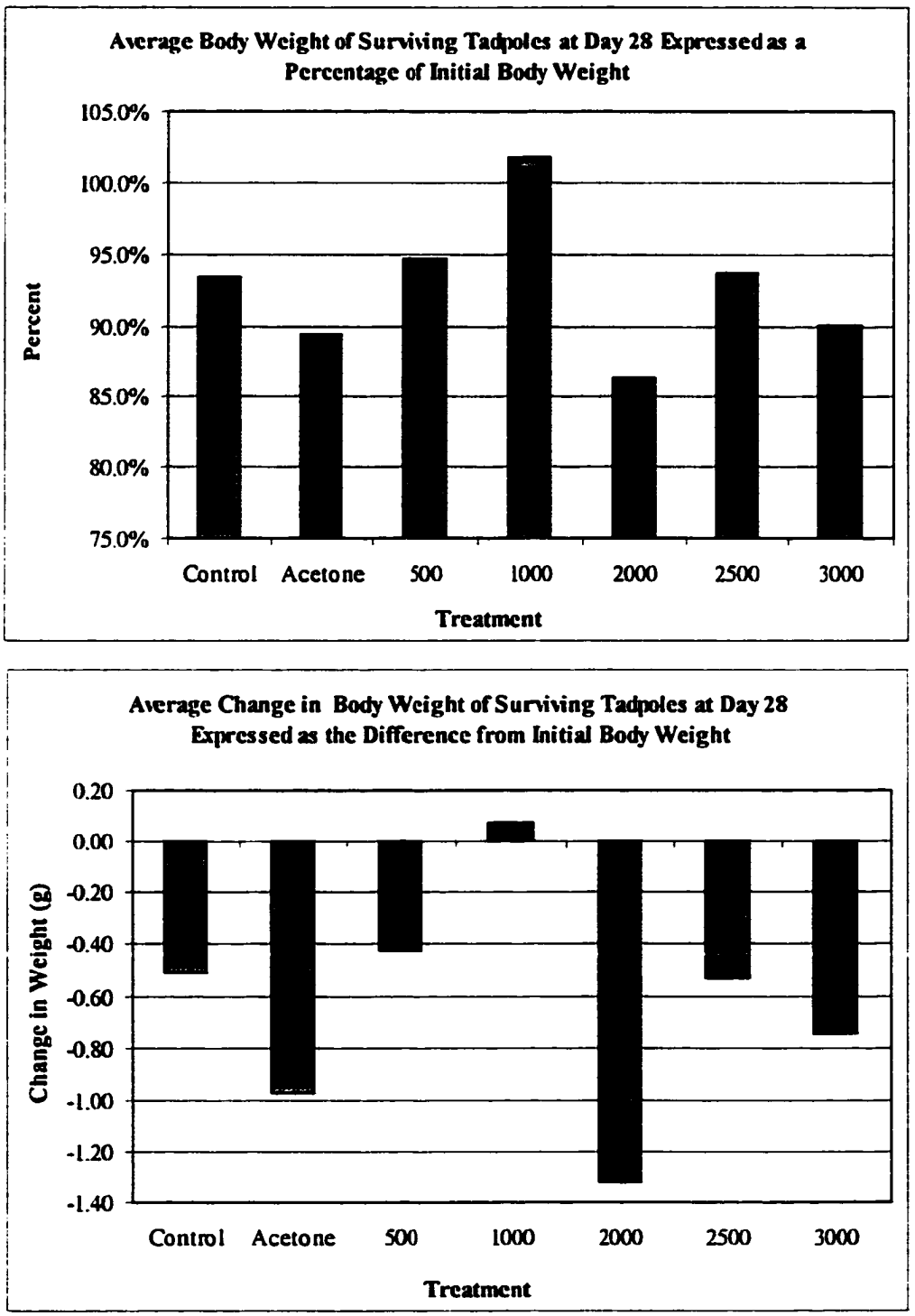


Figure 3-8. Cumulative Change in Body Weight of Surviving Bullfrog Tadpoles by Malathion Treatment

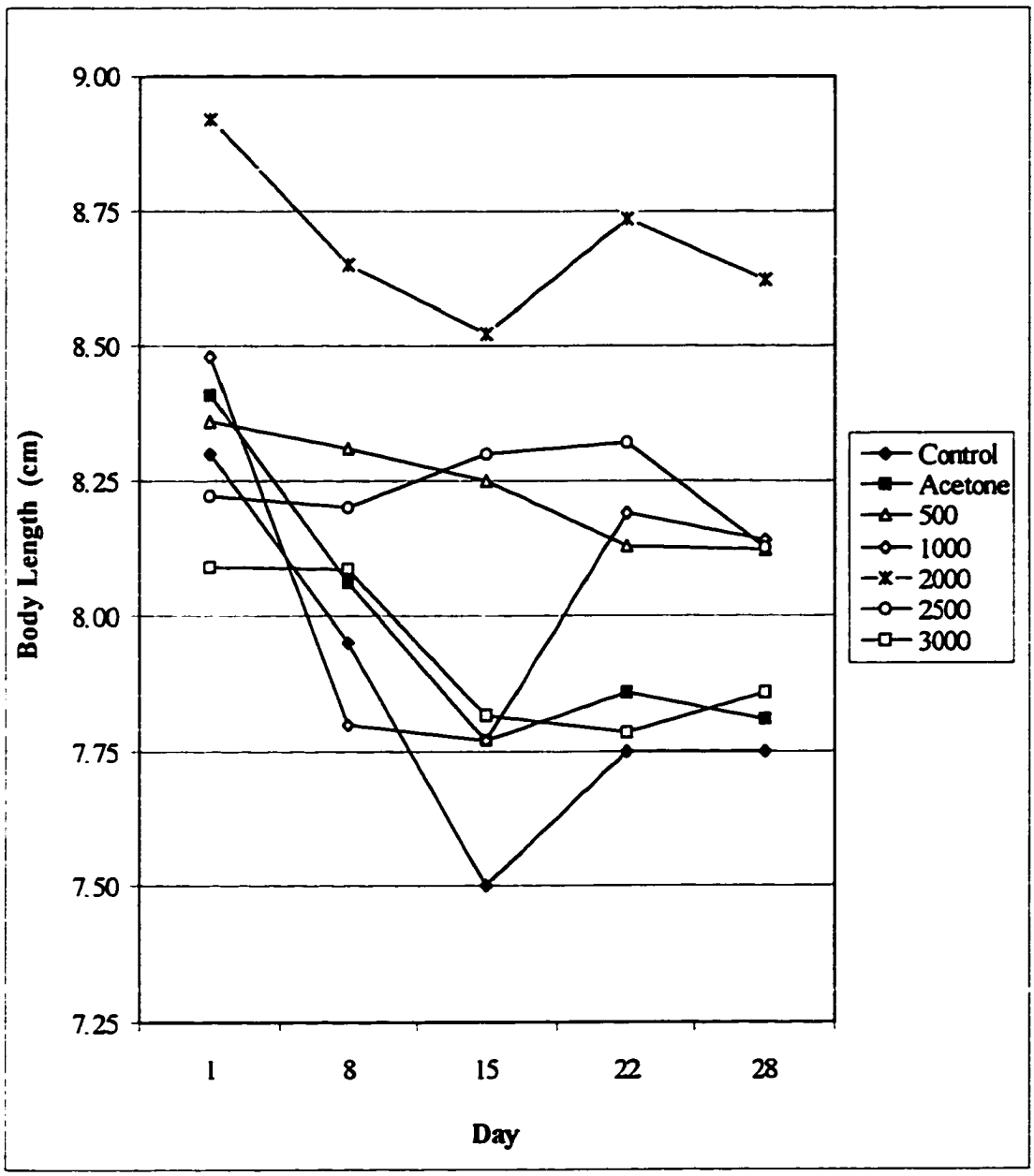
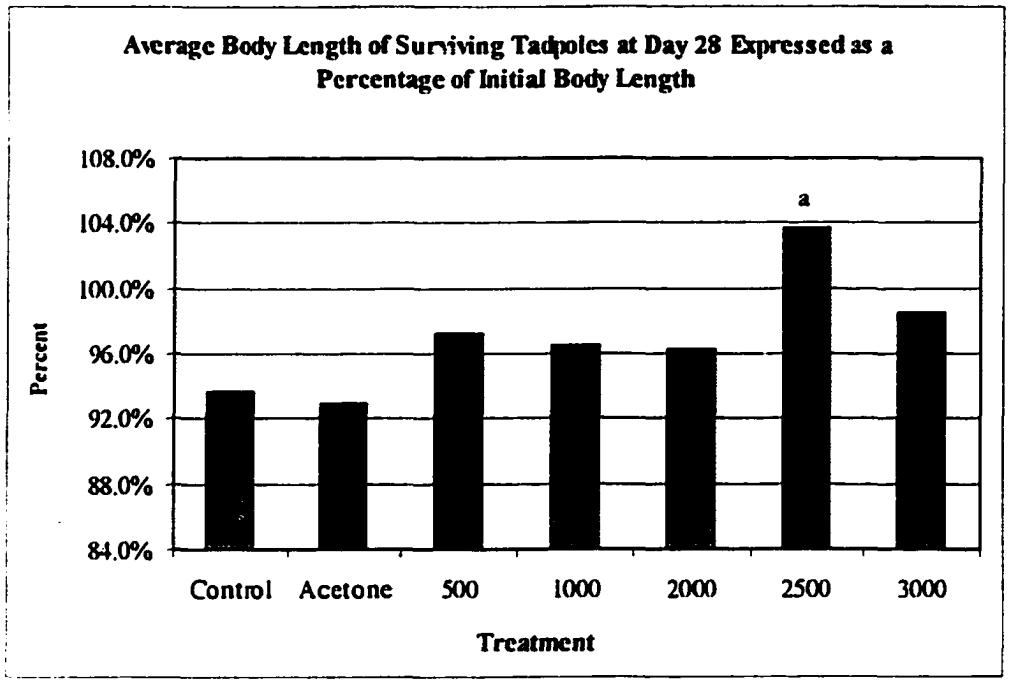
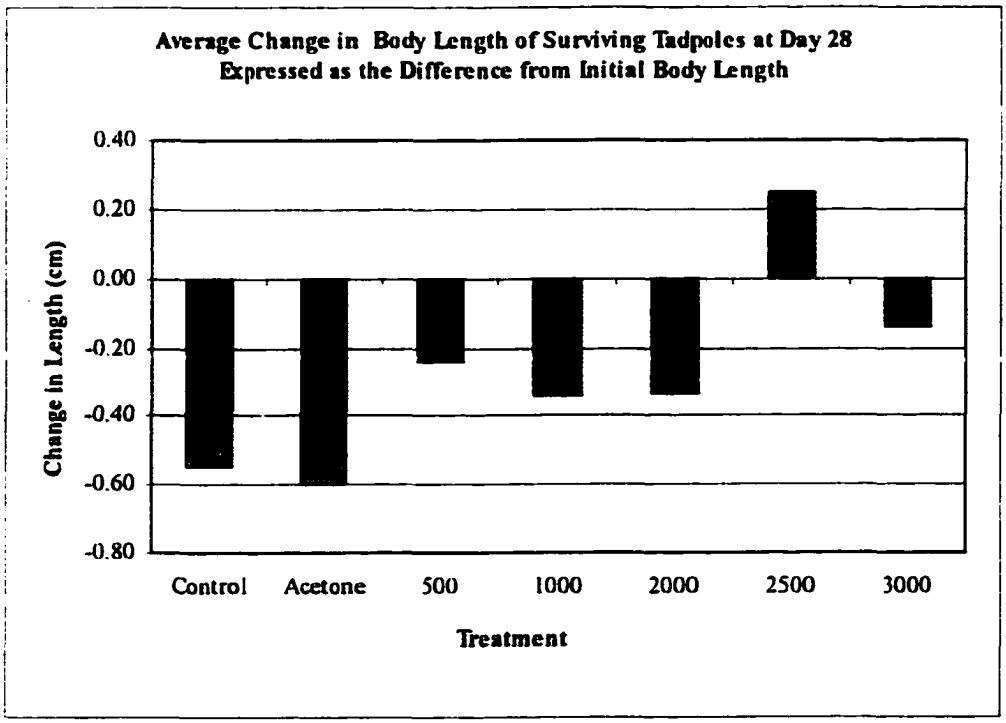


Figure 3-9. Mean Body Length of Surviving Bullfrog Tadpoles by Time and Malathion Treatment





a-Treatment was significantly different ($p<0.05$) from controls

Figure 3-10. Cumulative Change in Body Length of Surviving Bullfrog Tadpoles by Malathion Treatment

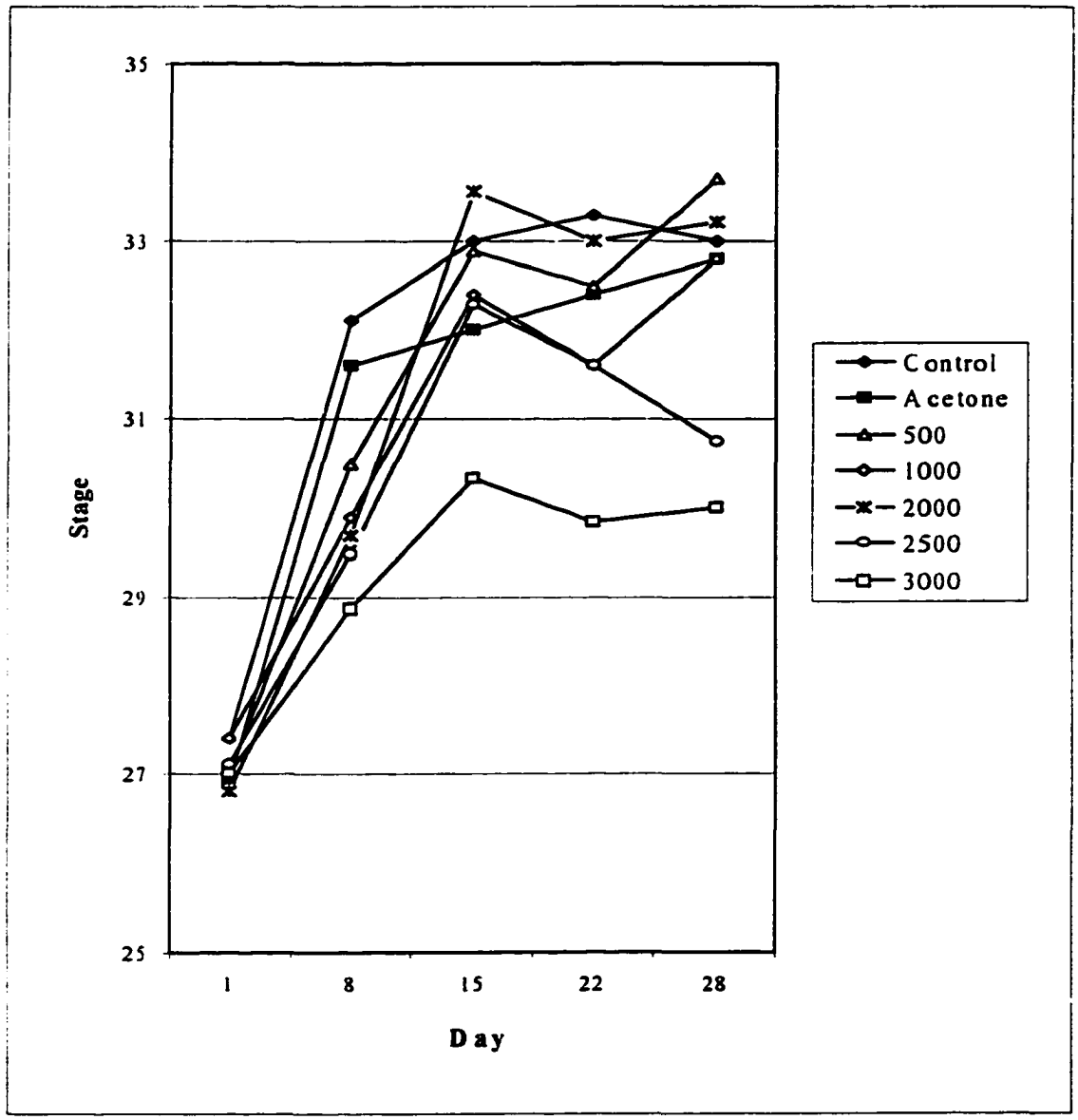
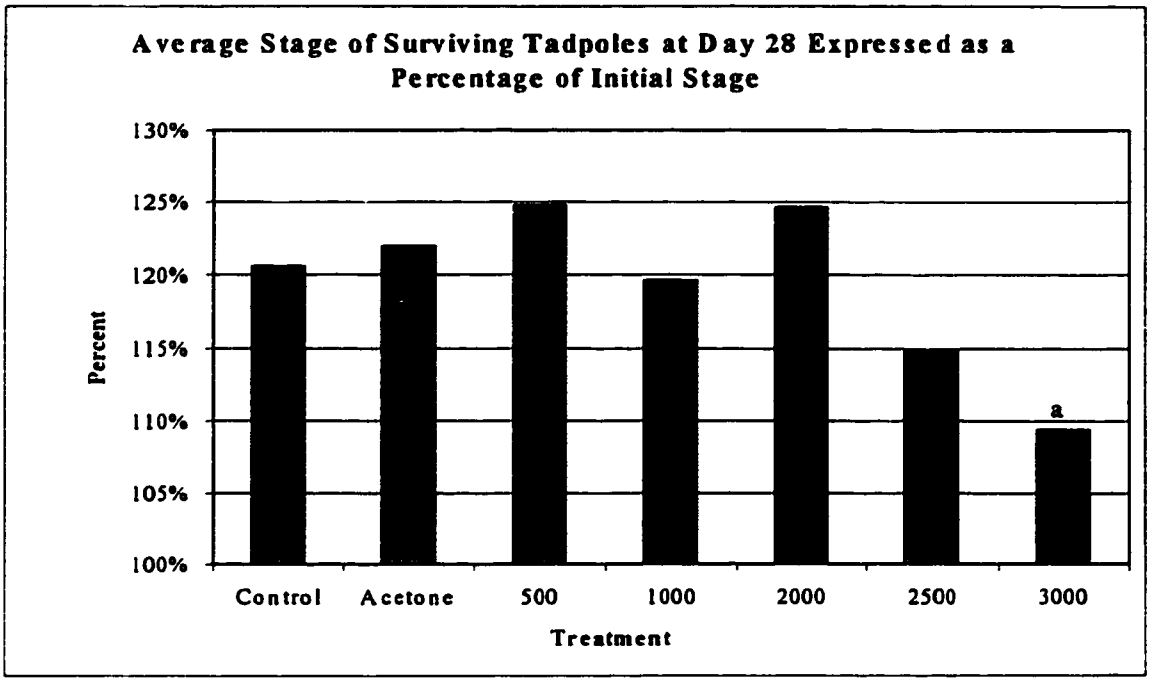
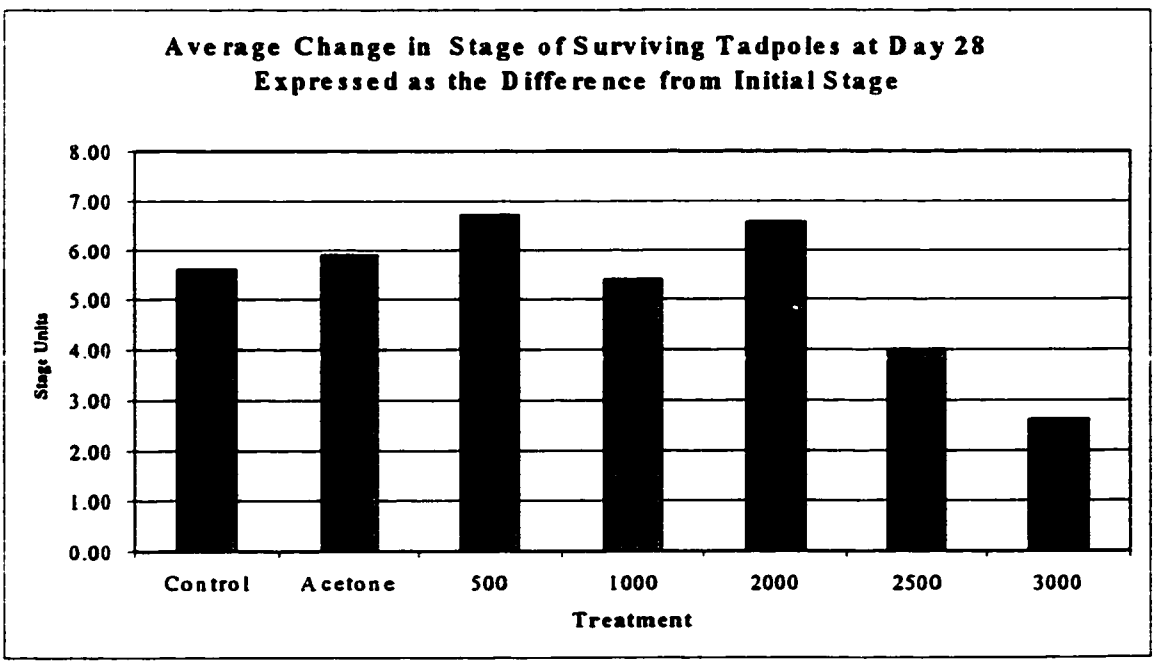


Figure 3-11. Mean Developmental Stage of Surviving Bullfrog Tadpoles by Malathion Treatment and Time





a - Significantly different from control ($p<0.1$)

Figure 3-12. Cumulative Change in Bullfrog Tadpole Developmental Stage by Malathion Treatment

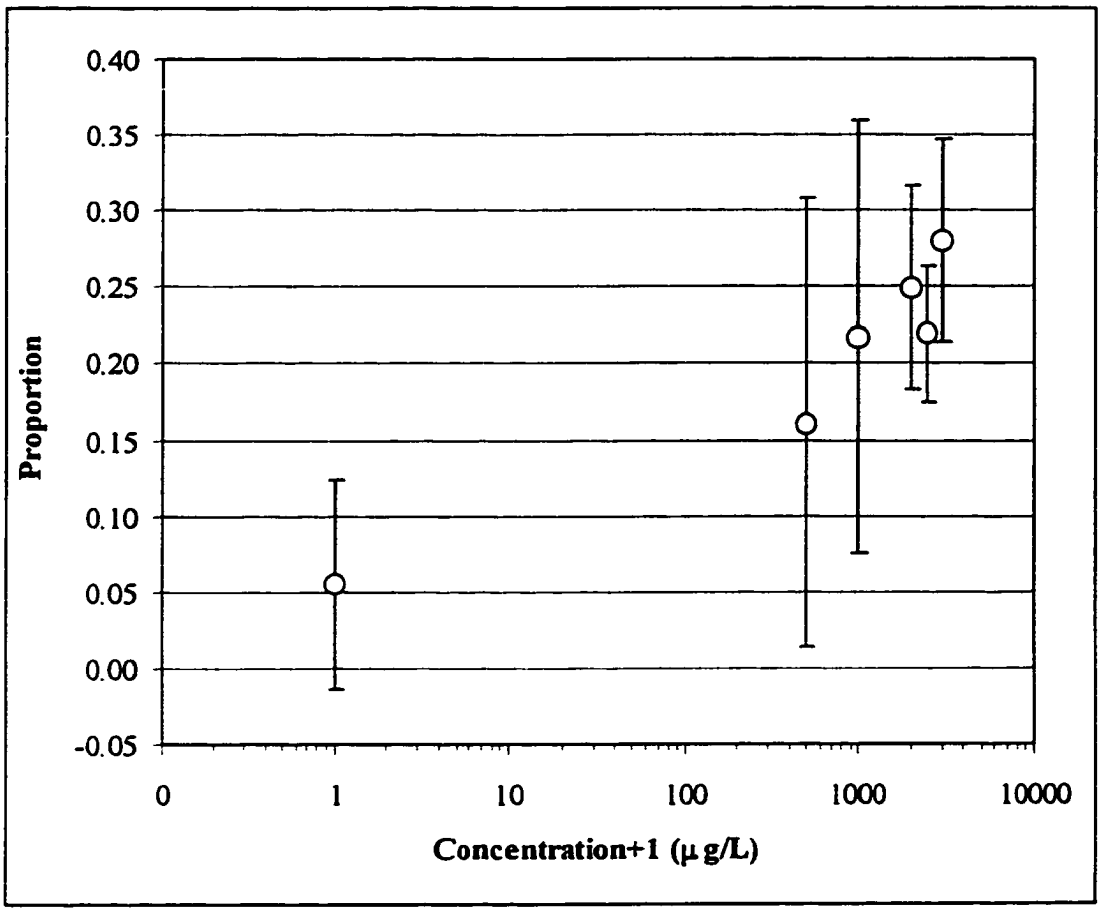


Figure 3-13. Mean Proportion (and Standard Deviation) of Surviving Bullfrog Tadpoles Exhibiting Loss of Righting Reflex During the 28-Day Toxicity Test Averaged by Time Plotted Against Malathion Treatment on a Log Scale

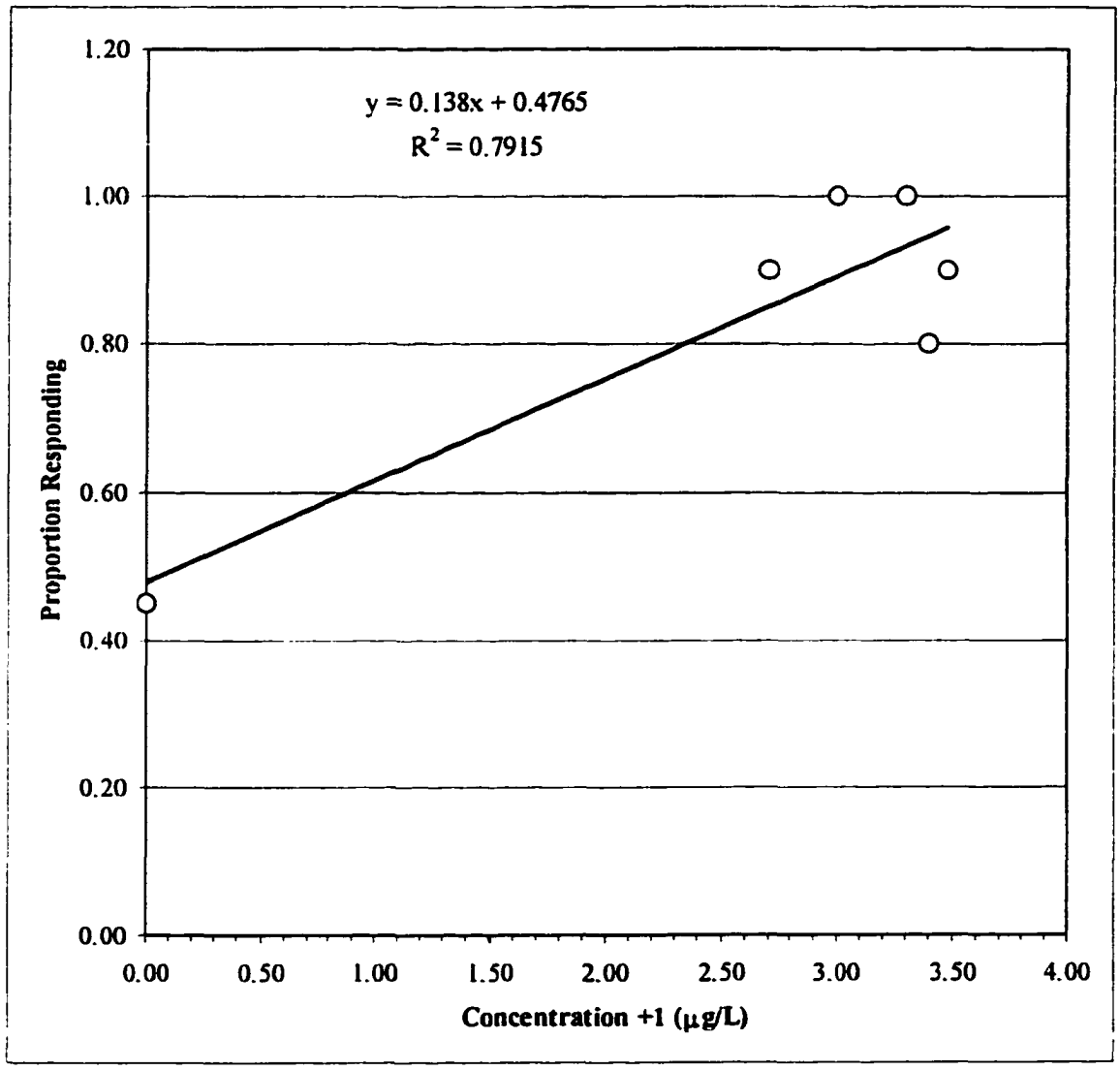


Figure 3-14. Linear Regression of the Total Number of Bullfrog Tadpoles in the 28-Day Toxicity Test Exhibiting an Impaired Righting Reflex Expressed as a Proportion Plotted Against Malathion Treatment on a Log Scale

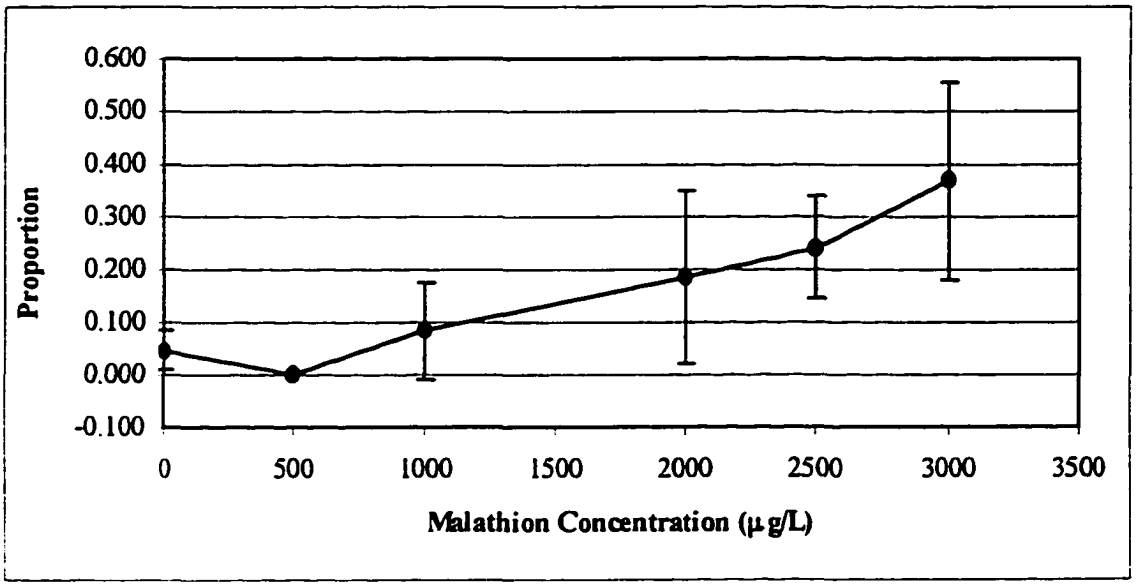


Figure 3-15. Mean Proportion (and Standard Deviation) of Surviving Bullfrog Tadpoles in the 6-Day Behavior Study Exhibiting Loss of the Righting Reflex Averaged by Day and Replicate Against Malathion Treatment

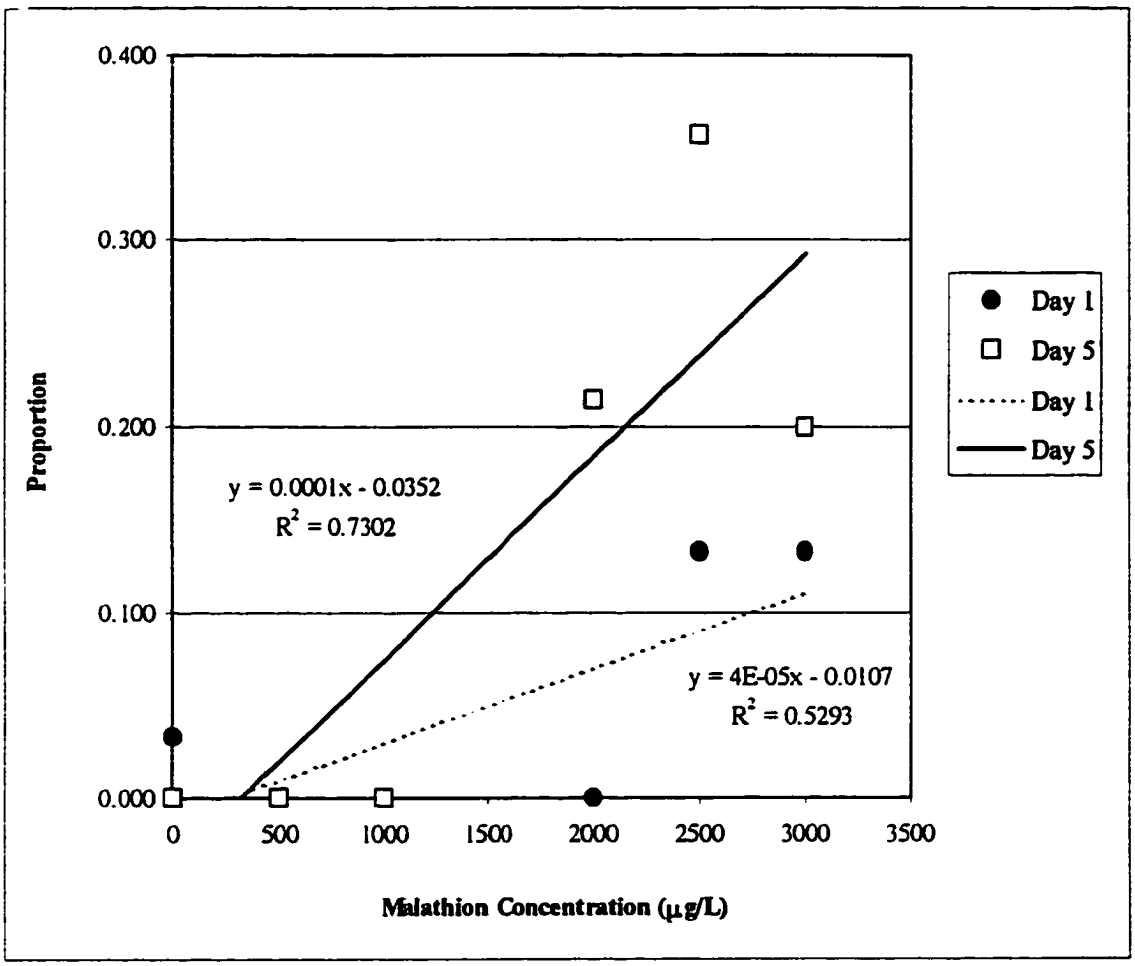


Figure 3-16. Linear Regression of the Proportion of Bullfrog Tadpoles Exhibiting an Impaired Righting Reflex on Day 1 Compared to the Regression on Day 5 for Data from all Three Test Replicates Combined, Where all Treatment Groups Began the Study with 15 Tadpoles

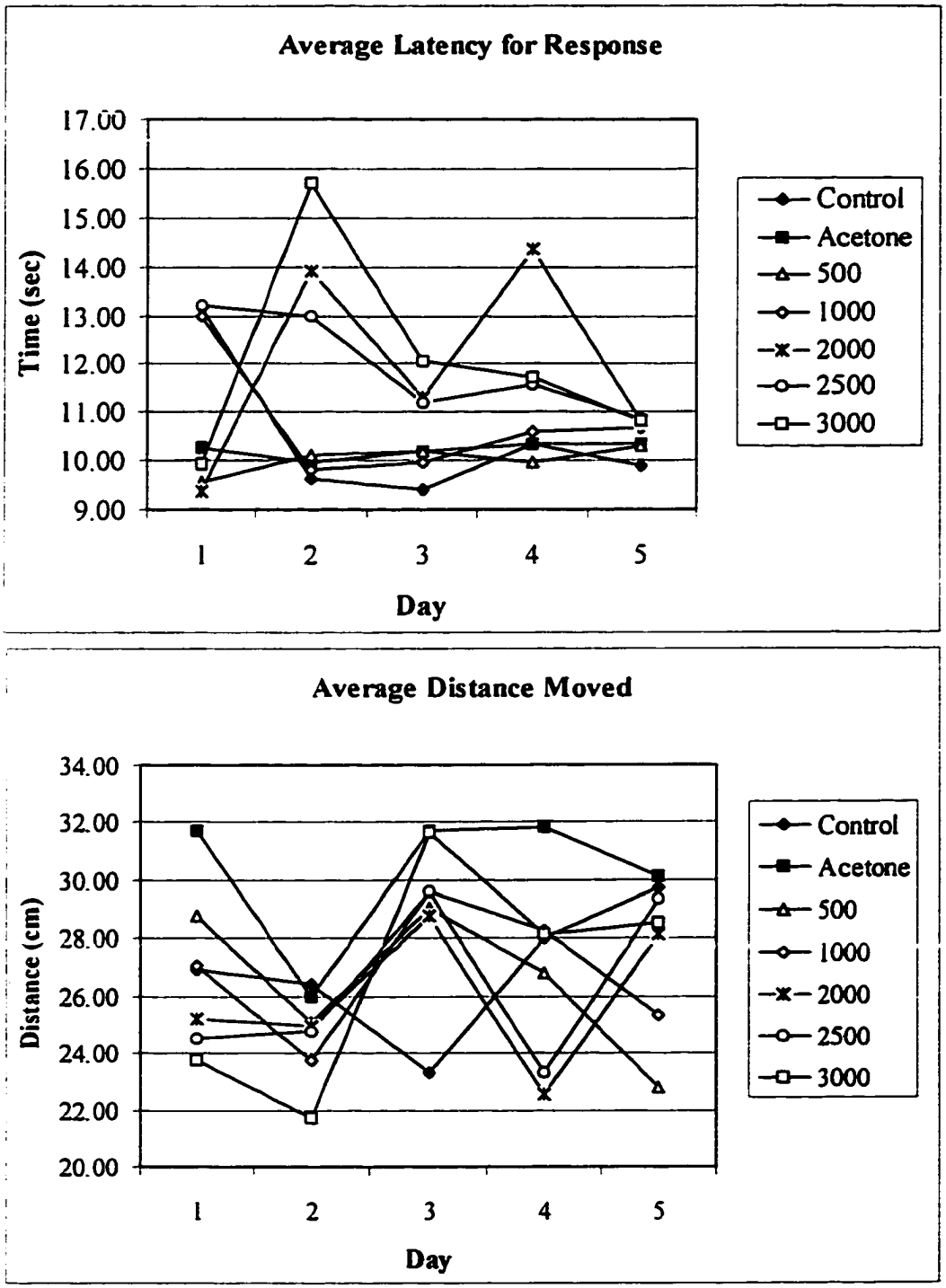


Figure 3-17. Average Latency to Respond and Distance Moved by Bullfrog Tadpoles During an Avoidance or Escape Response

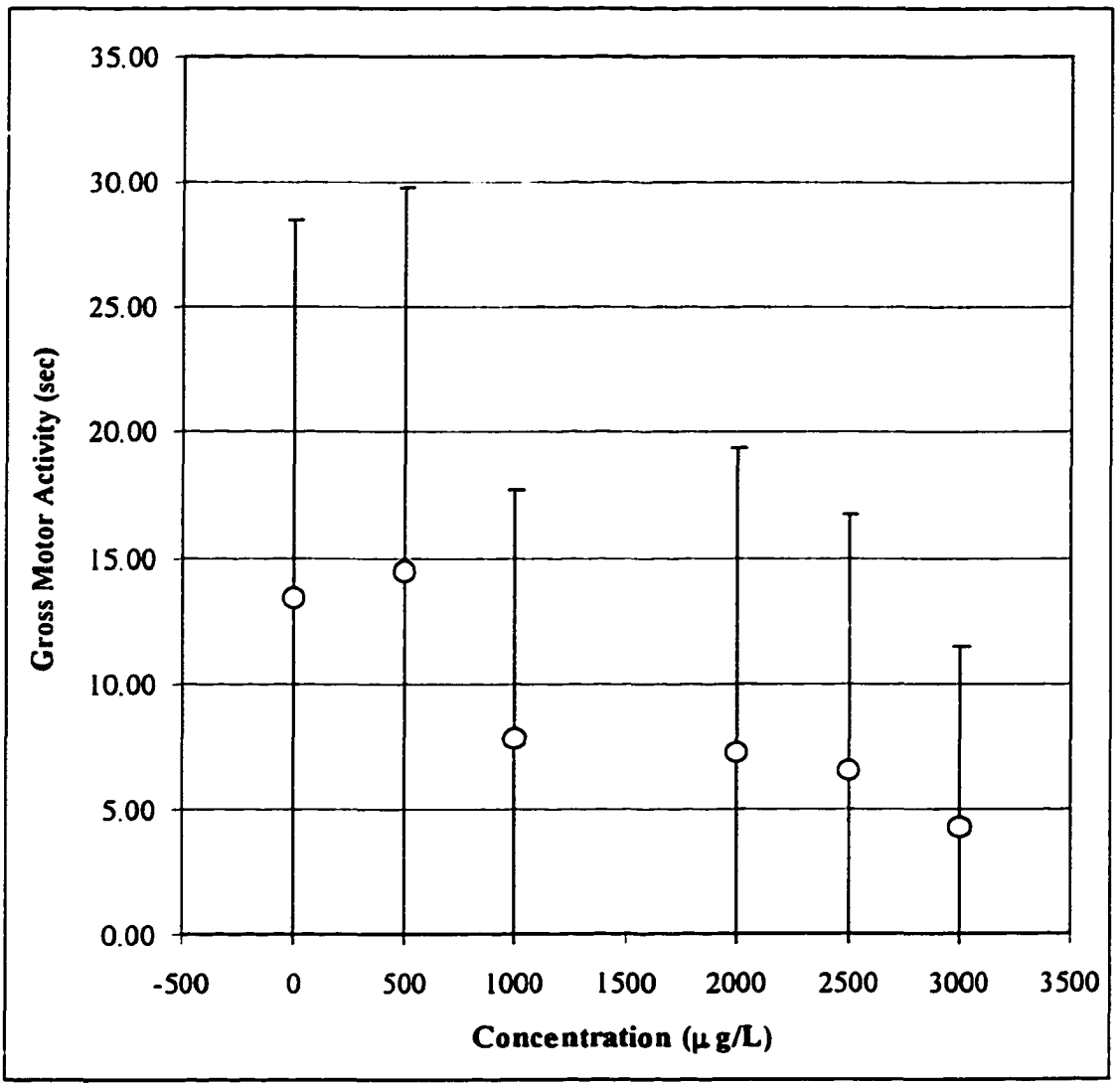
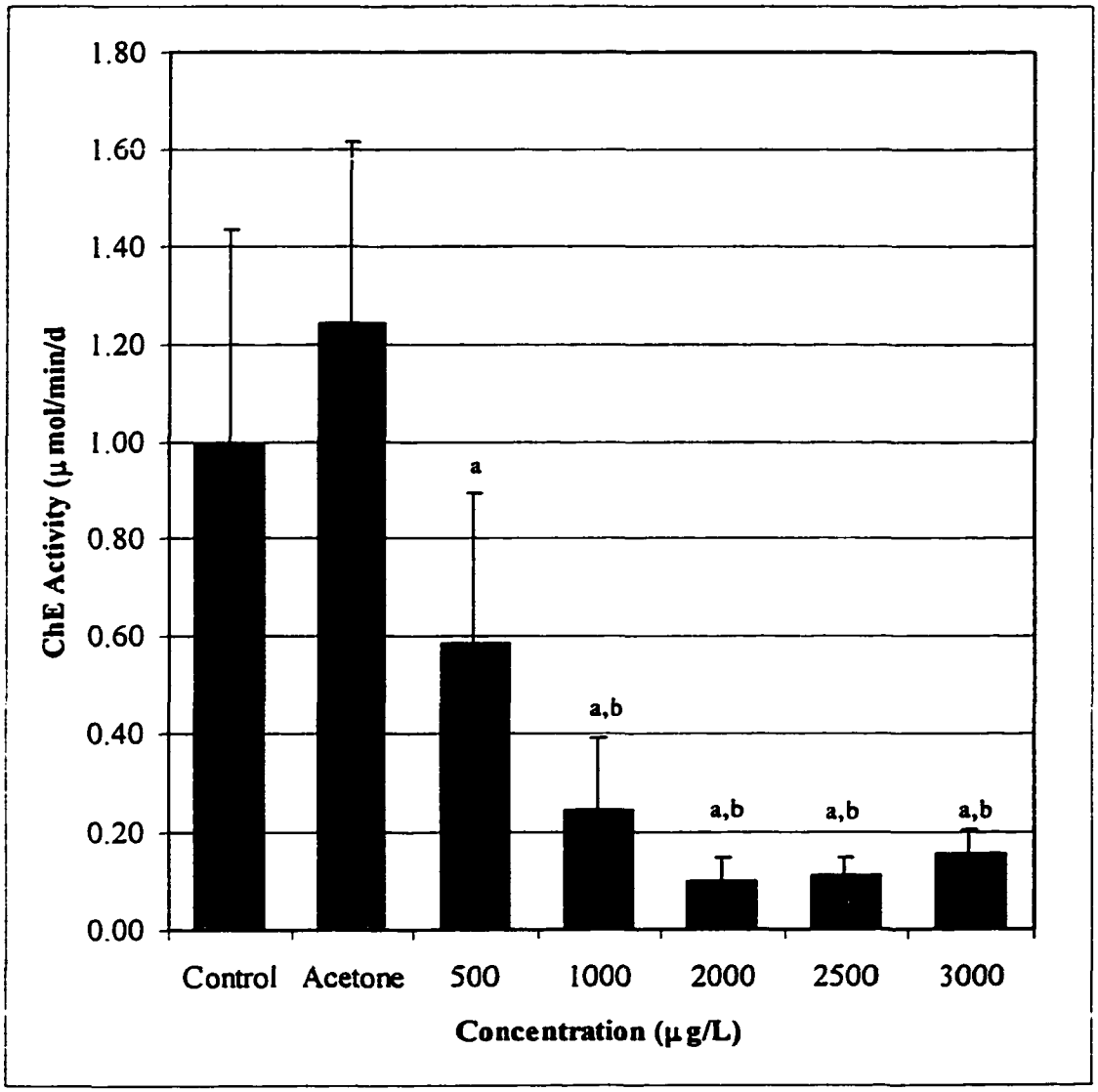


Figure 3-18. Mean and Standard Deviation of Gross Motor Activity for Bullfrog Tadpoles in the 6-Day Behavior Study Averaged by Test Day and Test Replicate



a - Significantly different from control group ($p<0.05$)
b - Significantly different from 500 μg/L treatment group ($p<0.05$)

Figure 3-19. Average ChE Activity in Bullfrog Tadpole Brains by Malathion Treatment

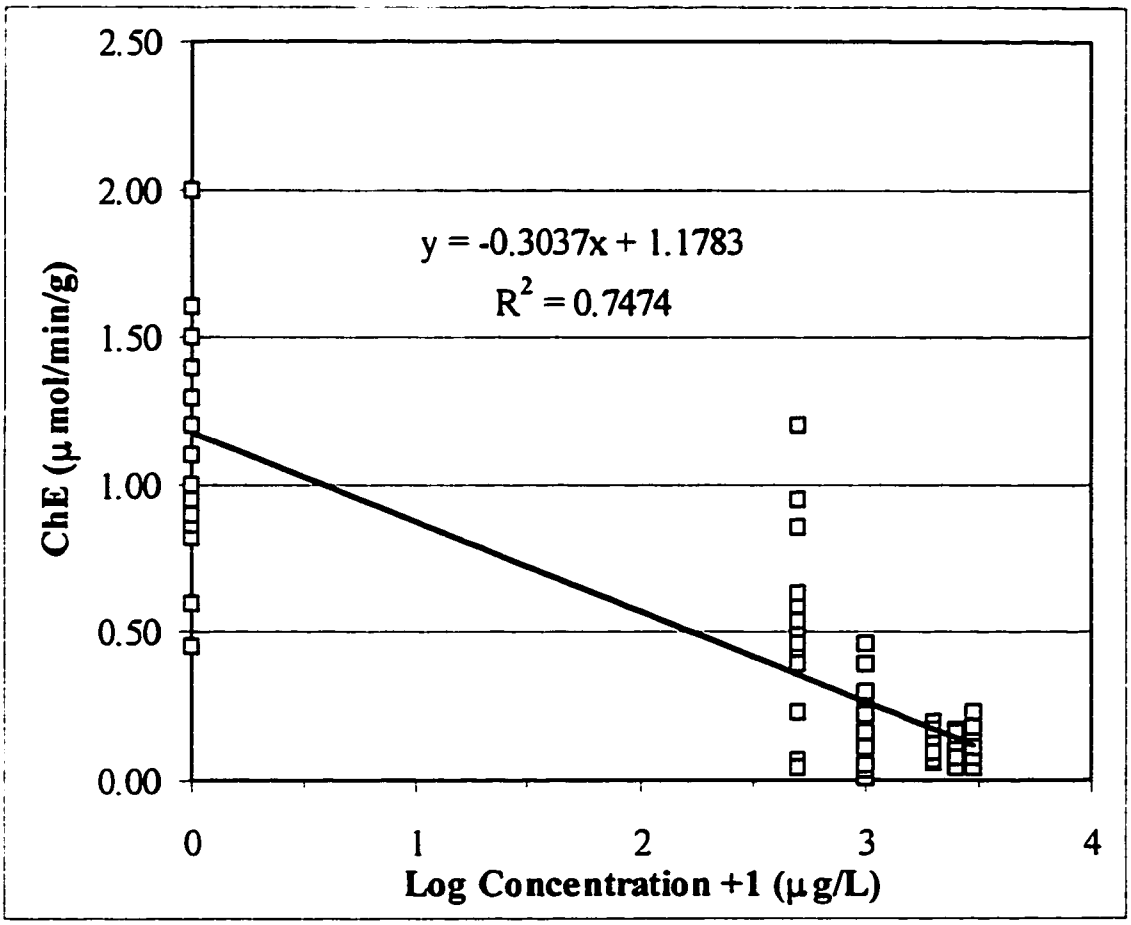


Figure 3-20. Linear Regression of ChE Activity in Bullfrog Tadpole Brains by Malathion Treatment

4.0 DISCUSSION AND CONCLUSIONS

This current investigation was conducted to predict acute lethal and sublethal adverse effects of malathion on leopard frogs. Malathion is a commonly used organophosphorus insecticide with reported half-lives in surface water ranging from 15 hours to nearly five days (Montgomery, 1997), which gives sufficient time for acute or subacute toxicity in aquatic life to occur. Amphibian populations are in general declining (Carey, 1993; Pechmann et al., 1991), and research into lethal and sublethal effects of pesticides may help explain amphibian losses occurring in wetlands.

This study investigated toxicity of malathion by measuring adult leopard frog behavior, mortality, tissue residues, and ChE inhibition following a 96-h exposure to malathion in water. In addition, the uptake and excretion of malathion in adult bullfrogs were investigated by measuring the concentrations of malathion and malaoxon in tissue over a two hour time period. The response of ChE in bullfrog brain to malathion exposure was examined in intravenously injected bullfrogs. The potential effects of malathion on growth, survival, and behavioral responses were investigated in bullfrog tadpoles.

4.1 EXPECTED ENVIRONMENTAL WATER CONCENTRATIONS

Water samples were collected during the current investigation following a spraying of malathion a mosquito control in Alamosa, Colorado to determine potential levels of exposure to wild populations of amphibians. Maximum malathion concentrations detected in surface water samples collected between 6 to 7 hours after spraying were 14.98 μg/L (0.015 mg/L). Sampling was done 6 to 7 hours after spraying in order to maximize aerial drift into wetland areas but minimize environmental degradation of pesticide. The 96-h LC_{50} for leopard frogs is much higher than ambient concentrations measured from water samples collected after the ultra-low volume aerial spraying of malathion in Alamosa, Colorado.

Overt toxicity to adult leopard frogs is unlikely based on both current pesticide application practices in Colorado and measured concentrations in environmental samples. The measured environmental concentrations were also lower than various reported toxic effects levels for other species of amphibians ranging from a low of 0.20 mg/L for a 96-h LC_{50} to 47.3 mg/L for 100% mortality in 5 days (Mayer and Ellersieck, 1986; Mohanty-Hejmadi and Dutta, 1981; De Llamas et al., 1985). Malaoxon was not detected in water samples from wetland areas near Alamosa, Colorado.

4.2 LETHALITY OF MALATHION IN ADULT FROGS

A 96-h LC_{50} of 56.2 mg/L malathion for adult leopard frogs was obtained from the current study. Comparison of toxicity data for fish to the current study results is more relevant

than comparing studies utilizing oral administration or injection in mammals, since the route of exposure for the studies with fish is typically immersion, often under static test conditions, as was the method of exposure utilized in the current study. However, since fish are also exposed to chemicals in water by absorption across gill membranes, they may be more sensitive than adult frogs. Based on these results, adult leopard frogs are much less sensitive to the acute effects of malathion than are fish. Beyers et al. (1994) reported a 96-h LC_{50} for Colorado squawfish (*Ptychocheilus lucius*) and bonytail (*Gila elegans*) of 9.14 mg/L and 15.3 mg/L, respectively. Alam and Maughan (1992) obtained a malathion 96-h LC_{50} for common carp (*Cyprinus carpio*) and *Barilius vagra* of 12.8 to 13.8 mg/L, and 7.39 to 7.66 mg/L, respectively.

Given that the acutely lethal concentrations of malathion are so high, and the half-life relatively short, there is little possibility that adult anurans would be killed due to applications for pest control purposes. Racke (1992) reports that malathion concentrations in water following direct application to control aquatic pests are in the range of 1 to 10 μg/L, a concentration much lower than the LC_{50} from the current study. The current study indicated that a malathion concentration of 10 mg/L was not lethal for a 96-h exposure, whereas measured environmental water concentrations following an aerial spray event would range between 10 and 20 μg/L, or less than 1% of a no observed effect level for mortality.

Mortality in leopard frogs was related to a ChE inhibition level of about 50% relative to that of the controls, which is consistent with observations made with other species (Munroe et al., 1991; Ludke et al., 1975). ChE activity measured in leopard frogs exposed to malathion was predicted to be half that of the controls at a malathion concentration of 58.77 mg/L, or very close to the LC_{50} of 56.2 mg/L obtained from this study. Wallace (1992) also points out that numerous correlations have been made between the concentration required to inhibit 50% ChE activity *in vitro* and the acute LD_{50} measured *in vivo* for various organophosphorus pesticides. Thus, although adult leopard frogs may be fairly resistant to the acute effects of malathion, as indicated by the high LC_{50} observed in this study, mortality still occurs at inhibition rates similar to that of other species.

4.3 MALATHION EFFECTS ON ADULT LEOPARD FROG BEHAVIOR

Avoidance behavior was significantly affected by water concentrations of malathion of 100 mg/L, and a trend toward immobility was apparent in frogs in the 10 mg/L treatment group as well. There was high mortality in the 100 mg/L treatment group, although activity was altered well before mortality in this group occurred. The observations made in the current study are supported by results from other investigations with various species. Behavioral responses in toads dosed with organophosphate compounds (OP) by injection indicated general paralysis, loss of righting reflexes, and coma (Edery and Schatzberg-Porath, 1960). Behavioral symptoms in deer mice (*Peromyscus maniculatus*), poisoned with the organophosphorus pesticide terbufos, included tremors in the

extremities, rapid breathing, motionless crouching, lack of avoidance response, and loss of righting reflex (Block et al., 1993).

Buerger et al. (1994) reported the acute effects of organophosphate toxicity in northern bobwhites (*Colinus virginianus*). Birds exposed to the phosphorothioate methyl parathion exhibited lethargy, ataxia, diarrhea, salivation, muscle tremors, and opisthotonos (Buerger et al., 1994). Starlings (*Sturnus vulgaris*) exposed to chlorfenvinphos had altered posture at ChE activities less than 88% of normal, decreased flying, singing, and resting when ChE levels were less than 61% normal, but increased flying when ChE levels were 90% of normal (Hart, 1993). The author stated that, while inhibition of ChE activity at more than 50% of normal correlates with altered behavior, different behaviors have different sensitivities to enzyme inhibition.

In leopard frogs, data from the current study indicate that reduction of ChE activity by approximately 20% correlates with a measurable change in avoidance behavior. The ChE activity in the controls was 5 μmol/min/g, and in the 10 mg/L malathion treatment group the ChE activity was 4.1 μmol/min/g; the number of avoidance responses by frogs in the 10 mg/L malathion treatment was approximately half that of the controls.

It is difficult to predict whether the decrease in behavioral responses would affect population success in wild populations. The effects on behavior could make wild frogs more susceptible to predation by a failure to avoid predators. Conversely, the lack of

movement could also protect poisoned individuals from predator detection. A tendency toward lack of movement could make individuals less likely to engage in feeding behavior. A study with the toad *Bufo woodhousii* (Walton, 1988) and the frog *Dendrobates auratus* (Pough and Taigen, 1990) indicate that successful prey capture is related to the distance moved, suggesting that malathion exposed frogs might not be successful foragers.

4.4 TISSUE RESIDUES IN ADULT LEOPARD FROGS AND BULLFROGS

Malathion and malaoxon were rarely detected in tissues of adult leopard frogs or bullfrogs, despite the fact that test concentrations for both species were high. This is consistent with the results from other studies. Edery and Schatzberg-Porath (1960) determined that organophosphorus parent compound was absent due to metabolic processes occurring in liver, kidney, and blood from toad *(B. viridis)* blood within four minutes following injection. However, pesticides were not chemically analyzed in blood in the study conducted by Edery and Schatzberg-Porath (1960); blood was withdrawn from dosed amphibians and injected into mice, followed by analysis of blood ChE activity in the mice. The method is likely to lack precision and accuracy by current analytical standards. However, these results do support the findings of the current study, which failed to detect malathion in blood serum seven minutes after injection of bullfrogs.

Malathion is apparently rapidly cleared by bullfrogs, as indicated by the data collected for serum, liver, and kidney over a time course of two hours. Of the tissues studied, the highest percent of the dose recovered as malathion was measured in kidney. Most of the

initial intravenous dose (>87%) was not recovered as parent compound, and only minimal amounts of malaoxon were found. However, other metabolites were present. One metabolite tentatively identified in bullfrog liver tissue was O,O-dimethyl phosphorodithioate, which is also a metabolite found in mammals (Muan et al., 1989).

The two adult species of anuran studied appeared similar in the disposition of malathion and malaoxon. Malathion was detected more frequently than malaoxon in the liver and kidney of both species. It is not possible to say that both species had the same rate of accumulation since the route of administration was different.

Hall and Henry (1992) report that some organophosphates can accumulate to high levels in amphibians. Bioconcentration of organophosphates in zebra mussel *(Dreissena polymorpha* P.) were up to 10 times higher than in ambient water, regardless of whether mussels were alive or dead (Dauberschmidt et al., 1996; Keller and Ruessler, 1997). The maximum observed bioconcentration factor (concentration in tissue divided by concentration in water) for leopard frogs in the current study was 0.08, indicating that malathion residues were not accumulating to any great extent in frog liver or kidney over the duration of the exposure. Even though frogs were exposed to high concentrations of pesticide relative to concentrations that would be expected in ambient water, only concentrations above the LC_{50} resulted in measurable levels of malathion in liver or kidney.

4.5 CHOLINESTERASE ACTIVITY

ChE activity was determined in individual brains of adult leopard frogs and bullfrogs, and bullfrog tadpoles. The mean cholinesterase activity levels measured in adult leopard frogs (5 μmol/min/g) were similar to those found in adult bullfrogs (4.9 μmol/min/g). The mean ChE activity in bullfrog tadpoles was 1.0 μmol/min/g, or approximately two times higher than that measured in adult frogs. However, the analyses were not performed at the same time, although they were performed with the same methodology by the same laboratory. The rabbit brain "standards" that were run with the amphibian brain tissue differed significantly between the analysis of the adult versus tadpole brains, indicating that perhaps the data are not comparable. Conversely, the rabbit brain ChE activities do not represent intra-specific variability, as only one animal was used to provide the brain tissue; therefore, at least part of the difference may be due to intra-specific variability and not variability due to the analytical method.

Although the absolute activities may not be comparable between the leopard frogs and the bullfrog tadpoles, the concentrations of malathion at which the activity was reduced to one half the control activity are comparable. Bullfrog tadpoles were 345 times more sensitive to malathion-induced effects on ChE activity than were adult leopard frogs under the current test conditions; a malathion concentration in water that produced one- half maximal activity was 0.17 mg/L for bullfrog tadpoles and 58.77 mg/L for adult leopard frogs. Different test conditions could yield different results since ChE activity is influenced by exposure time and mortality.

The concentration expected to depress leopard frog ChE to the point that toxicity due to organophosphates would be diagnostic of poisoning is only slightly higher than the 96-h LC_{50} of 56.2 mg/L. These results are similar to those reported in studies with other classes of animals, for example, the percent of ChE activity relative to controls at the malathion LD_{50} dose was 44.4% for lizards (Hall and Clark (1982). This seems to indicate that other biological endpoints to diagnose pesticide effects in the field would be more useful, since lethality is so high at a concentration that inhibits ChE activity to one half that of the controls.

Bullfrog ChE activity did not begin to recover within the two hour duration of the residue study. Block et al. (1993) reported that deer mice *(Peromyscus maniculatus)* dosed with a formulation containing terbufos as the active ingredient did not exhibit AChE recovery within 96 hours.

4.6 SURVIVAL, GROWTH, AND DEVELOPMENT OF BULLFROG TADPOLES

Malathion significantly affects tadpole survival. Survival was significantly related to malathion dose, although acetone had no effect on survival of tadpoles. A test concentration of 2000 μg/L was identified as the no observed effect level (NOEL), since survival in this test concentration was not significantly different than that in the controls. However, more tadpoles died in the 2000 μg/L test concentration than in the 0, 500, or 1000 μg/L test concentrations. This suggests that if sample size were higher, or exposure

longer, a significant difference might be observed between the 2000 and the 0 μg/L test concentrations.

The 2500 μg/L malathion test concentration was identified as the lowest observed effect level (LOEL), since this was the lowest concentration at which a statistically significant difference was observed in survival relative to the controls. However, at this concentration, 60% of the test population died. This amount of toxicity in a wild population could have adverse effects on survival of the population. For example, one guideline for an environmental pesticide concentration that indicates a level of concern for birds, mammals, or aquatic organisms is a concentration of one-half the LC_{50} (Kendall, 1993). The actual impact on the potential population growth rate or the probability of local extinction depends on numerous factors including species, population size, reproductive strategy, physical environment, and temporal and spatial distribution of pesticide contamination (Slade, 1993).

Malathion appears to affect tadpole growth and development, although the data are difficult to interpret. There was no significant effect of malathion or acetone exposure on body weight. Body weight did decrease significantly with time ($p=0.002$). Some physiological effect that was not measured as part of this study may have influenced the body weight of malathion exposed tadpoles, since some tadpoles in the higher test concentrations appeared bloated. However, there is no way to test this hypothesis without measurements of percent moisture content of the whole body. If the study duration had

been longer, a significant difference might have been observed, since on Day 28 tadpoles in the three highest malathion treatments were decreasing in body weight more than those in the lower treatments or the controls. Great individual variation in growth rate, even under laboratory conditions where temperature is maintained at a constant level and food is provided *ad libitum*, is a known intrinsic characteristic of amphibian growth (Jørgensen, 1992). Brain weight was not influenced by malathion or acetone treatment. A NOEL of 3000 μg/L was observed for body weight and brain weight changes, and no LOEL was obtained.

Other investigations have also reported that tadpoles lose body mass as developmental stage increases. Studies with the anurans *Phyllobates subpunctatus* and *Bufo boreas i* indicated body mass for both species began to decrease at about the Gosner (1960) developmental stage 20 (Funkhouser and Mills, 1969; Sivula et al., 1972; Feder, 1982). Body mass of *B. boreasi* decreased until stages 30 to 33, and then began to increase until Stage 40. Data *for P. subpunctatus* were reported only until stage 25, but mass decreased steadily to this point. These normal fluctuations of body mass with stage could explain some of the results of the current investigation on the potential effects of malathion on growth. Since not all tadpoles develop at the same rate, development could have been expected to influence mean measured body mass in the different treatment groups. If malathion was delaying development, as the data indicate, tadpoles in the malathion treatments would not lose as much mass due to developmental changes; however, if the tadpoles were not feeding due to toxicity, this could have resulted in loss of mass without

a change in developmental stage. The mean values for body weight on Day 28 suggests that this hypothesis is plausible, since tadpoles in the control group were lighter than tadpoles in the low malathion treatments, but not in those the higher treatments.

Body length was measured as one variable that was associated with the status of tadpole development, since as the developmental stage progresses, tadpoles reabsorb the tail as they become gradually become froglets (Burggren and Just, 1992). There was no effect of acetone exposure on body length, however, length was significantly affected by both time and malathion treatment. Tadpoles lost length over time ($p<0.001$), and a significant dose by day interaction was observed ($p<0.05$). Only the 0 and 1000 μg/L treatments were significantly shorter ($p<0.05$) at some point during the study than they were initially, although this does not indicate a clear dose-response effect. The 2500 μg/L treatment group did not lose as much cumulative length as did the controls. A higher sample size or a longer exposure period could have improved the interpretation of the statistical analyses. A NOEL of 2000 μg/L, and a LOEL of 2500 μg/L, for effects on body length was obtained.

Developmental stage increased significantly with time, although tadpoles in the two highest treatments did not progress as far in developmental stage as did those in the control treatment group. There was no effect of acetone treatment on developmental stage. While the developmental stage of tadpoles in the 0 and 500 μg/L malathion test concentrations was significantly higher on all days later in the study than initially,

development was delayed in the higher treatment groups. Tadpoles in the 1000 μg/L and higher treatment groups lagged behind the controls by Day 8, although by Day 15 the 1000 , 2000, and 2500 μg/L treatment groups were similar to the controls. At the end of the 28 day study, the stage of tadpoles in the 2500 and 3000 μg/L treatment groups was significantly lower than the control group. The 2000 μg/L malathion test concentration is the highest concentration at which development appeared the same as the controls, and thus would be considered the NOEL. The 2500 μg/L treatment would be considered the LOEL

Tadpole development has long been known to be controlled by the endocrine system, particularly the thyroid and pituitary glands and their hormones, and for normal metamorphosis, thyroid hormones must increase continuously in the blood (Burggren and Just, 1994). Thyroid hormones in bullfrog tadpoles begin to increase dramatically around Gosner (1960) Stage 41, and approach climax at Gosner (1960) Stage 44 (Burggren and Just, 1994). Malathion disrupts the function of the thyroid gland in rats (Akhtar et al., 1996), resulting in a significant decrease of the circulating hormones T3 and T4 in blood and a rise in thyroid stimulating hormone (TSH) levels. In the study with rats, serum T4 was depressed by a factor of 2 in rats exposed for up to 21 days. There were no other data available to indicate how quickly an effect on serum T3 or T4 might occur. Tadpoles in the current study were in the early stages where thyroid hormone levels begin to increase. In the current study, exposure to malathion delayed progression of developmental stage relative to the controls. It is possible that the rather minimal effects

observed on developmental stage in the current study would have been drastically emphasized had the study been continued until the tadpoles approached climax; it is even possible that tadpoles may have failed to complete metamorphosis. Adrenocorticotropic hormone (ACTH) and prolactin are also influential in tadpole metamorphosis (Burggren and Just, 1994); however, no information relating to the effects of malathion on these hormones was available.

Developmental delay could decrease survival potential, since failure to metamorphosis at the appropriate time could lead to failure to breed at appropriate times. Delay in development from an aquatic to an air-breathing stage could decrease survival, if ephemeral ponds dry up and the frogs cannot migrate. The gills begin to lose function at Gosner (1960) Stages 26 to 30, and the tadpole becomes an air-breathing animal at Gosner (1960) Stage 40 (Burggren and Just); thus, for amphibians in arid environments that depend on rapid development for survival, a developmental delay could be detrimental.

Loss or impairment of the righting reflex was significantly affected in tadpoles in the lowest test concentration of 500 μg/L, as well as at all higher treatments, compared to the controls. An animal's ability to orient itself properly in the environment could influence its survival potential. Loss of equilibrium posture even temporarily could potentially decrease swimming speed during an escape movement. Feeding behavior could be impaired if an animal is oriented with the ventral surface up; predation could be affected as well since a

tadpole with a light ventral surface could be more visible. A NOEL was not obtained, but a LOEL of 500 μg/L indicates that this is one of the most sensitive parameters measured in the 28-day toxicity test.

4.7 BEHAVIOR EFFECTS IN TADPOLES

Learning behavior was inhibited in fish following exposure to the organophosphate pesticide parathion. Since data on whether or not tadpoles also exhibit inhibition of learning behavior due to exposure to OPs were lacking, a behavioral test was conducted with malathion. The ability to avoid an adverse stimulus in the environment could influence an individual's survival. In adult leopard frogs, behavior was sensitive to the time at which it was observed. The same may be true for tadpoles. Activity can be an indication of morbidity due to exposure, and inactivity could influence a tadpole's susceptibility to predation and also influence its ability to find food.

Effects on survival were observed in the tadpoles exposed to malathion in water in the behavioral study for periods up to six days. Overall mortality was not significantly different between the 28-day growth and development test and the 6-day behavior test; however, mortality was observed at a lower test concentration in the 6-day experiment as compared to the 28-day experiment. A NOEL and LOEL of 500 and 1000 μg/L were observed, respectively, for survival in the behavior study tadpoles. This is lower than the NOEL of 2000 μg/L observed for the growth study tadpoles.

Growth parameters did not change significantly during the behavior experiments parameters; therefore,.these variables are unlikely to bias the results of the behavior measurements. The highest test concentration of 3000 μg/L was the NOEL for the 6-day exposures for the growth. In addition, the three replicates were not significantly different.

The righting reflex was significantly affected at the 1000 μg/L treatment compared to the controls. This resulted in an NOEL of 500 and a LOEL of 1000 μg/L for the 6-day exposure period. This differs from the apparent effect on righting reflex in the tadpoles in the 28-day toxicity test, where 500 μg/L was the LOEL. One exception for this finding is that righting reflex appeared to become more impaired over time so that the shorter exposure duration was not as toxic. The environmental half-life for malathion in water ranges from 15 hours to 5 days (Montgomery, 1996); therefore the results from the 6-day toxicity test may be more applicable to field situations than those from the 28-day toxicity study.

Effects on learning behavior were measured by measuring latency and distance moved during an avoidance response. Tadpoles did learn during the conditioning test as evidenced by a significant decrease in the latency to respond over time. The response time increased significantly between Days 1 and 2, and 5. The latency was not affected significantly by dose, however. The data suggest that the three highest doses responded more slowly than the lower test concentrations until day 5, when all malathion test concentrations were observed to exhibit a similar latency to respond as the controls.

These findings differ from those of Strickler-Shaw and Taylor (1991) who studied the effects of lead exposure on tadpoles; lead exposure reduced the latency to respond. Malathion would be expected to have more complicated effects on behavior than lead, since as a ChE inhibitor, at low doses the effects would mimic ACh effects and lead to nervous system hyper-stimulation. At higher doses, the effects of extreme overstimulation would be expected to dominate the behavioral response.

Distance moved was found to significantly increase over time ($p<0.05$), and although the overall test for dose was not significant, the dose by day interaction was statistically significant. Only the tadpoles in the highest treatment group showed a significant difference in the distance moved during an avoidance response over time; the tadpoles in the 3000 μg/L test concentration moved farther on Day 5 than on Day 1 or 2. A plausible explanation for these results is that tadpoles in the highest test concentration experienced some level of morbidity, and were less active; however, learning did occur, and the avoidance response was stronger than the tendency toward inactivity. This hypothesis is supported by the fact that the total number of behavior trials at which no avoidance response was made increased by treatment level ($p<0.001$), and indicated that treatment concentrations in excess of 1000 μ/L were different than the controls.

Gross motor activity was also inhibited by malathion treatment. The 3000 μg/L treatment group was less active than the either the control group or the 500 μg/L treatment group. The NOEL for gross motor activity was 2500, and the LOEL was 3000 μg/L. These

findings further support the hypothesis that there was a stronger tendency toward inactivity in malathion-treated tadpoles..

The ChE activity was significantly lower in tadpoles in the 500 μg/L test concentration relative to controls. The ChE activity was reasonably well explained by the linear regression of ChE against the log of the malathion concentration in water (R^2=74.7%). The malathion concentration producing a 50% level of inhibition in bullfrog tadpoles was 170 μg/L; however, mortality in tadpoles was not evident at ChE activities 50% that of controls. Mortality in tadpoles occurred at 1000 μg/L, at which point ChE activity predicted from the regression equation was 0.27 μg/min/g, or about 25% of the control activity. These findings differ from those for the adult leopard frogs, where a predicted inhibition of 50% relative to the controls occurred at a test concentration approximately that of the LC_{50}.

This is consistent with the somewhat conflicting physiological effects that could be expected from malathion exposure. Decreased ChE activity in tadpoles at the ≥500 μg/L treatments could lead to overstimulation of the central nervous system, which could increase the distance moved. But general morbidity and lethargy could decrease the tendency to move. Since the gross motor activity data indicated a tendency toward immobility, it is not surprising that latency was higher in malathion exposed tadpoles.

4.8 CONCLUSIONS

The study resulted in a 96-h LC_{50} of 56.2 mg/L for adult leopard frogs, indicating that this species of frog is less sensitive to malathion than are many other aquatic animals. Frog avoidance response is a more sensitive, sublethal indicator of toxicity than is ChE activity, given that frogs exposed to the lowest test concentration (10 mg/L) exhibited significantly decreased numbers of fright responses than did control frogs. ChE activity was not significantly depressed in frogs at the 10 mg/L malathion treatment. The highest bioconcentration factor for malathion in liver or kidney was 0.08, indicating that malathion did not bioconcentrate in liver or kidney of leopard frogs.

Malathion was rapidly removed from bullfrog tissues in a time period of less than 2 hours following injection; only malaoxon was detected in blood. In contrast, malathion was detected in liver and kidney, whereas malaoxon was not detected in these tissues. Only samples collected within the first 45 minutes following dosing contained pesticide concentrations above detection in organ tissues, indicating that malathion does not bioaccumulate in adult frogs. Ingestion of adult frogs by predators is unlikely to be a significant exposure pathway for malathion poisoning in wildlife or birds.

Survival, growth, and development of bullfrog tadpoles was affected by malathion exposure. In addition, righting reflex was impaired. General activity levels were decreased, as were ChE activity levels in individual tadpole brains. Learning behavior was not significantly affected by malathion, although this could be confounded by the various

and often opposing physiological effects expected due to malathion poisoning. The lowest observed adverse effect level (LOAEL) for bullfrog tadpoles exposed to malathion was 500 μg/L based on impairment of the righting reflex. A no observed adverse effect level (NOAEL) was not identified.

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