DISSERTATION

TOXICITY OF CARBARYL AND MALATHION TO COLORADO SQUAWFISH AND BONYTAIL

Submitted by

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WE HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER OUR SUPERVISION BY DANIEL W. BEYERS ENTITLED TOXICITY OF CARBARYL AND MALATHION TO COLORADO SQUAWFISH AND BONYTAIL BE ACCEPTED AS FULFILLING IN PART REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY.

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ABSTRACT OF DISSERTATION

TOXICITY OF CARBARYL AND MALATHION TO

COLORADO SQUAWFISH AND BONYTAIL

Toxicity of technical carbaryl, Sevin-4-Oil, and technical malathion to federally endangered Colorado squawfish (*Ptychocheilus lucius*) and bonytail (*Gila elegans*) was estimated by 4-d renewal-acute, 32-d early life-stage (ELS), and 1-d *in vivo* brain acetylcholinesterase-inhibition tests. To measure toxicant concentrations, an analytical method was developed using C_8 solid-phase extraction columns to extract carbaryl and malathion from well and pond water. Mean percent recovery over a concentration range of 1 to 10,000 µg/L was consistently between 80 and 100. Well-water samples fortified with carbaryl and malathion were solid-phase extracted, and analytes were stored on solid-phase extraction columns at -4 °C for 30 d without significant decomposition.

Optimal assay conditions for analysis of brain acetylcholinesterase activity in Colorado roundtail chub (*Gila robusta robusta*) were determined for the pH-stat method. Optimal conditions were: 10 mg brain tissue per reaction vessel; temperature, 30 °C; substrate concentration, 11 mM; and pH, 7.5. Enzyme-inhibition studies confirmed that hydrolysis of acetylcholine was primarily by acetylcholinesterase.

Effect concentrations for 32-d ELS and 1-d acetylcholinesterase-inhibition tests were estimated by analysis-of-variance hypothesis

testing and linear-plateau regression. Four-d median lethal concentrations were estimated by probit analysis. Median lethal concentrations for technical carbaryl, Sevin-4-Oil, and technical malathion were 1.31, 3.18, and 9.14 mg/L for Colorado squawfish and 2.02, 3.31, and 15.3 mg/L for bonytail, respectively. No-observed-effect concentrations (NOEC) in 32-d ELS tests of technical carbaryl and technical malathion were 445 and 1680 µg/L for Colorado squawfish and 650 and 990 µg/L for bonytail, respectively. Threshold concentrations for Colorado squawfish in ELS tests were 364 µg/L carbaryl and 455 µg/L malathion. Threshold concentrations for bonytail were 217 µg/L carbaryl and 521 µg/L malathion. The NOECs for Colorado squawfish in acetylcholinesterase-inhibition studies were 29.3 µg/L carbaryl and 371 µg/L malathion. Threshold concentrations estimated for Colorado squawfish in acetylcholinesterase-inhibition studies were 7.40 µg/L carbaryl and 150 µg/L malathion. Linear-plateau regression consistently gave lower estimates of effect concentrations than those estimated by hypothesis testing. Linear-plateau regression models accounted for a significant amount of sample variance and provided an appropriate description of the observed concentration-response relation.

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DEDICATION

For my wife Julie.

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PREFACE

Grasshopper populations in many western states annually attain economically damaging levels. To control these infestations, the United States Department of Agriculture - Animal and Plant Health Inspection Service applies insecticides to thousands of hectares of rangeland and cropland every year. Occasionally, treated areas abut or encompass aquatic habitats, and although no-spray buffer zones are established around these ecologically sensitive areas, pesticide may be deposited by accidental drift or mobilized from upland areas by runoff. Potential effects of accidental pesticide deposition on federally threatened or endangered fishes in the Colorado River Basin are a recent concern.

Carbaryl (1-naphthyl methylcarbamate), Sevin-4-oil (a formulation containing 49 % carbaryl and petroleum distallates), and malathion (diethyl mercaptosuccinate, S-ester with O, O-dimethyl phosphorodithioate) are three synthetic organic insecticides commonly used in grasshopper control operations. The toxicity of these chemicals to federally endangered Colorado squawfish (*Ptychocheilus lucius*) and bonytail (*Gila elegans*) was investigated as part of an ongoing program to study effects of large-scale insecticide applications on nontarget organisms.

This dissertation has been organized into three chapters, each of which is in manuscript form for submission to the peer-reviewed journal "Environmental Toxicology and Chemistry". Chapter 1, entitled "Solid-

Phase Extraction of Carbaryl and Malathion From Pond and Well Water", was published, with slight modification, in Environmental Toxicology and Chemistry (volume 10:1425-1429) prior to completion of this dissertation. The chapter describes a new quantitative method for measuring the concentration of carbaryl and malathion in water samples.

Chapter 2 describes optimal assay conditions for measurement of brain acetylcholinesterase activity in Colorado roundtail chub (*Gila robusta robusta*). Unlike Colorado squawfish and bonytail, Colorado roundtail chub remain abundant in the upper Colorado River Basin and were studied as a surrogate for the protected fishes.

Chapter 3, entitled "Toxicity of Carbaryl and Malathion to Two Federally Endangered Fishes as Estimated by Regression and Analysis of Variance", reports the toxicity of technical carbaryl, Sevin-4-Oil, and technical malathion to Colorado squawfish and bonytail as estimated by 4-d acute, 32-d early life-stage, and 1-d *in vivo* acetylcholinesterase-inhibition tests. Results of these tests were analyzed by regression and analysis of variance, and effect-concentration estimates were compared.

Lastly, a conclusion discusses the importance of this research in relation to grasshopper control operations. Suggestions for future research are presented.

CHAPTER 1

SOLID-PHASE EXTRACTION OF CARBARYL AND MALATHION FROM POND AND WELL WATER

ABSTRACT

An analytical method was developed using C_8 solid-phase extraction columns to extract carbaryl and malathion from well and pond water. Mean percent recovery over a concentration range of 1 to 10,000 μg/L was consistently between 80 and 100. Well-water samples fortified with carbaryl and malathion were solid-phase extracted, and analytes were stored on solid-phase extraction columns at -4 °C for 30 d without significant decomposition. The method was field tested by collecting pond water before and after an application of malathion. It provided significantly higher recovery of malathion than a liquid-liquid extraction procedure.

INTRODUCTION

The insecticides carbaryl and malathion are used to control grasshopper infestations on rangeland and cropland in many western states. Effects of these pesticide applications on nontarget aquatic organisms are a recent concern. Use of carbaryl and malathion is preferred because of their high efficacy and low environmental persistence. However, the latter property makes preservation of these chemicals in aquatic-environmental samples difficult. Effective procedures for preservation of these pesticides in water samples are available, but they require equipment and methods that are bulky and difficult to employ in remote locations (i.e., glass bottles and preservation by acidification and freezing).

Solid-phase extraction (SPE) is an alternative procedure that has been used extensively to remove nonpolar organic chemicals from water [1-7]. In this procedure, a water sample containing the chemical compound of interest is passed through a column containing a sorbent that has an affinity for nonpolar compounds (Figure 1). The target compound is bound by the column sorbent, and the extracted water is discarded. The target compound is then eluted from the column and analyzed by appropriate methods. Use of SPE as a method of sample collection (i.e., conducting the extraction step in the field) has many potential benefits. For example, sample volume and weight are greatly

reduced, and adjustments of pH or addition of salting-out agents to preserve samples are not required.

We developed and verified an analytical method using SPE to extract technical carbaryl (1-naphthyl methylcarbamate, 99 %), and technical malathion (diethyl mercaptosuccinate, S-ester with O, O-dimethyl phosphorodithioate, 93 %) from water. The objectives of this research were to (1) show that the method was accurate over a wide range of concentrations, (2) demonstrate that carbaryl and malathion could be extracted from water and stored on SPE columns at -4°C for 30 d without significant decline in residue levels due to decomposition, and (3) implement the method in the field.

METHODS AND MATERIALS

Solid-phase extraction procedure

To keep the methodology as simple as possible, no adjustments of sample pH were made prior to extraction. Preliminary trials in which carbaryl and malathion were extracted from well-water samples having pHs of 6 and 8 (a range which encompasses the pH of most natural surface waters) showed that recovery was not significantly different ($p = 0.53$, and $p = 0.12$, respectively).

The C_8 columns used in this study contained 10 g of sorbent and had a column volume of 60 ml (Analytichem International). Large SPE columns were selected for purposes of convenience in the field. The large bore (26 mm) permitted water samples to be dispensed onto columns without special equipment. Columns were conditioned with 10 ml of n-hexane, 10 ml of methanol, and 10 ml of deionized water. Sample extraction was initiated immediately after the conditioning step. Extraction time for 500-ml samples ranged from 6 to 17 min (83 to 29 ml/min) for all samples. Extraction was facilitated by use of a vacuum (380 mm Hg). Columns were dried by drawing air through them for 5 min using vacuum. Columns were eluted with 10 ml of n-hexane (as a wash step) followed by 2X10 ml of a methylene chloride:acetonitrile:n-hexane (50:3:47, v/v) elution mixture. In preparation for gas chromatographic analysis, eluates were solvent-exchanged to n-hexane and adjusted to final volume. Gas chromatographic

analyses were conducted using a Hewlett Packard model 5890A gas chromatograph equipped with a 30 m x 0.53 mm i.d. x 1.5 micron film thickness DB5 surface-bonded, cross-linked fused-silica column connected to a nitrogen-phosphorus detector (NPD) and a 30 m x 0.53 mm i.d. x 1.5 micron film thickness DB1 surface-bonded, cross-linked fused-silica column connected to a flame photometric detector (FPD) with a phosphorus filter. Gas chromatographic operating conditions for the NPD were: helium carrier gas flow, 32 ml/min; air flow, 104 ml/min; hydrogen flow, 5 ml/min; injector temperature, 270°C; isothermal oven temperature, 200°C; and detector temperature, 220°C. Operating conditions for the FPD were: helium carrier gas flow, 20 ml/min; air flow, 115 ml/min; hydrogen flow, 79 ml/min; injector temperature, 270°C; isothermal oven temperature, 200°C; and detector temperature, 200°C. Carbaryl and malathion were analyzed using the NPD and FPD, respectively.

Concentration trials

To demonstrate that the SPE method was accurate over a range of concentrations, carbaryl and malathion were extracted from well-water samples containing 1, 10, 100, 1,000, and 10,000 µg/L of each pesticide. Analytical reference standards of carbaryl and malathion were obtained from the U.S. Environmental Protection Agency, Pesticides and Industrial Chemicals Repository. Water-soluble fortification standards were prepared by dissolving and/or diluting neat standards in pesticide-grade acetone. Fortification of 500-ml well-water samples was accomplished by addition of 1.0 ml of a fortification standard. Fortified water samples were vigorously agitated for 1 min and were not extracted for at least

30 min. Five samples were fortified at each concentration level. Each water sample was extracted and analyzed as described above. Linear-regression analysis was used to determine the relation between percent recovery and pesticide concentration.

Storage trials

To demonstrate that carbaryl and malathion could be extracted from water and then stored on SPE columns at -4 °C without significant decomposition, 15 well-water samples fortified to 10 µg/L carbaryl and malathion were extracted. After extraction, each SPE column was randomly assigned to one of three storage treatments: 1) immediate elution and analysis on day of extraction, 2) elution and analysis 15 d after extraction, or 3) elution and analysis 30 d after extraction. Solid-phase extraction columns assigned to the latter two treatment groups were wrapped in n-hexane-rinsed aluminum foil and sealed in glass jars before being stored in darkness at -4 °C. All columns were eluted and analyzed as described above, but columns that had been held at -4 °C were allowed to come to room temperature for at least 1 h before being eluted. Linear regression analysis was used to determine the relation between percent recovery and time in storage.

Field trials

To test the SPE method under field conditions, arrangements were made with a local health department to collect water from a pond before and after an application of malathion. For comparison, samples were

extracted using a modified EPA liquid-liquid extraction (LLE) method [8] and the SPE method described above.

Before pesticide application, an 8-L water sample was collected from the pond and prefiltered through a Whatman #4 filter. Four 1-L subsamples were preserved for LLE by transfer to 1-L amber glass bottles and addition of 10 g Na_2SO_4 and H_2SO_4 to a pH $\leq$ 3, as recommended by Plumb [9]. Prior to preservation, one of the bottled samples was fortified by addition of 1 ml of a malathion standard to a concentration of 10 μg/L. The remaining 4 L of prefiltered water were extracted by SPE. Prior to SPE, one of the water samples was fortified by addition of 1 ml of a malathion standard to a concentration of 10 μg/L. Solid-phase extraction was completed in the field. After extraction, SPE columns were individually wrapped in n-hexane-rinsed aluminum foil and placed in resealable plastic bags.

Approximately 40 min after pesticide application, a 6-L sample of pond water was prefiltered. Three 1-L subsamples were preserved for LLE, and three 1-L subsamples were immediately extracted by SPE. Bottled water samples and SPE columns were stored overnight in darkness at 4 °C. All samples were analyzed within 12 h of collection. Malathion recoveries from SPE and LLE were compared by Student's t-test.

RESULTS

Concentration trials

Mean percent recoveries by SPE for carbaryl and malathion at all concentrations ranged from 84 to 106 and 81 to 96, respectively (Table 1). There was no significant linear relation ($p = 0.1$) between percent recovery and carbaryl concentration (Figure 2). For malathion, there was a significant linear relation ($p = 0.002$, $r^2 = 0.34$). The least-squares regression line of best fit for malathion had the form: percent recovery = 93.8 + -2.6 log concentration.

Storage trials

Mean percent recoveries for carbaryl and malathion at three storage times ranged from 86 to 89 and 81 to 92, respectively (Table 2). There was no significant linear relation between percent recovery and storage time for carbaryl or malathion ($p = 0.2$ and $p = 0.9$, respectively).

Field trials

SPE and LLE provided acceptable recoveries (107 % and 76 %, respectively) of malathion from field-fortified samples. Mean concentrations of malathion in field samples collected after pesticide application were 1.35 µg/L (SD = 0.18) for SPE and 0.53 µg/L (SD = 0.12) for LLE. Malathion concentrations in samples extracted by

SPE were significantly higher than those extracted by LLE ($p = 0.003$). Concentrations of malathion in field samples collected before pesticide application were less than the quantitation limit of 0.08 μg/L.

DISCUSSION

The SPE method described in this paper worked well during all phases of the research. Recovery of carbaryl and malathion from well water was consistently between 80 and 100 %. For carbaryl, percent recovery was not affected by pesticide concentration. However, recovery of malathion was significantly correlated with concentration; specifically, percent recovery decreased with increasing pesticide concentration. Mean recovery for the highest malathion concentration studied (10,000 µg/L) was 84 %. It is unlikely that concentrations as high as those used in this study will be encountered in the field under normal conditions, but such levels are encountered in laboratory toxicity tests.

Because field-collected samples are typically stored for several days or weeks before being analyzed, the amount of sample decomposition while in storage should be known. In this study, well-water samples fortified to 10 µg/L carbaryl and malathion were solid-phase extracted and stored on SPE columns for up to 30 d without significant loss. This concentration was selected as a level which may result from current pesticide application practices. The method should work equally well with higher concentrations of carbaryl and malathion, but preliminary trials should be conducted before extensive use.

Compared to a LLE procedure, SPE gave higher recovery of malathion from fortified pond-water samples and higher concentration estimates

from field samples collected after pesticide application. In fortified samples, the estimated malathion concentration, as given by LLE, was approximately 29 % lower than the estimate by SPE. However, in pond-water samples collected after pesticide application, the estimated malathion concentration, as given by LLE, was approximately 61 % lower than that given by SPE. The cause of this difference is uncertain. One explanation is that the addition of 1 ml of acetone to the fortified samples (via fortification standard) slowed malathion degradation or prevented sorption of the pesticide to sample-container surfaces. A second explanation is that the concentration of malathion on the SPE column may have been enriched by trace amounts of malathion in the air which passed through the column during the 5-min drying step. In retrospect, this factor could have been controlled by reducing or eliminating the drying step, or by including a reagent blank when extracting samples in the field. We omitted the reagent blank because extensive laboratory work had shown it to be unnecessary.

Solid-phase extraction can be successfully conducted in the field and offers several advantages over the traditional method of collecting and preserving water samples in glass bottles for laboratory extraction by LLE. Field-extracted samples require less space, and are easily cooled and packaged for shipment to analytical laboratories. Solid-phase extraction columns are lightweight and unbreakable. When properly preserved, the compound(s) of interest may be stored on SPE columns without significant decomposition. In addition, analysis in the laboratory is simplified, requiring only elution of the material of

interest from the column and analysis of the eluate by appropriate methods.

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Table 1. Mean percent recovery (± SD) of carbaryl and malathion extracted from well water with C_8 solid-phase extraction, n = 5.

	Concentration (ug/L)				
	1	10	100	1000	10000
Carbary	106±10.	86±4.0	85±2.5	89±4.3	94±3.7
Malathion	96±3.0	92±2.8	81±6.4	90±3.0	84±2.5

Table 2. Mean percent recovery (± SD) of carbaryl and malathion extracted from well water with C_8 solid-phase extraction at three storage times. All samples fortified to 10 µg/L, n = 5.

	Time in storage (d)		
	0	15	30
Carbaryl	86±4.0	86±0.8	89±4.5
Malathion	92±2.8	81±7.2	92±2.3

Figure 1. Diagram of solid-phase extraction column, approximately 1/2 actual size.

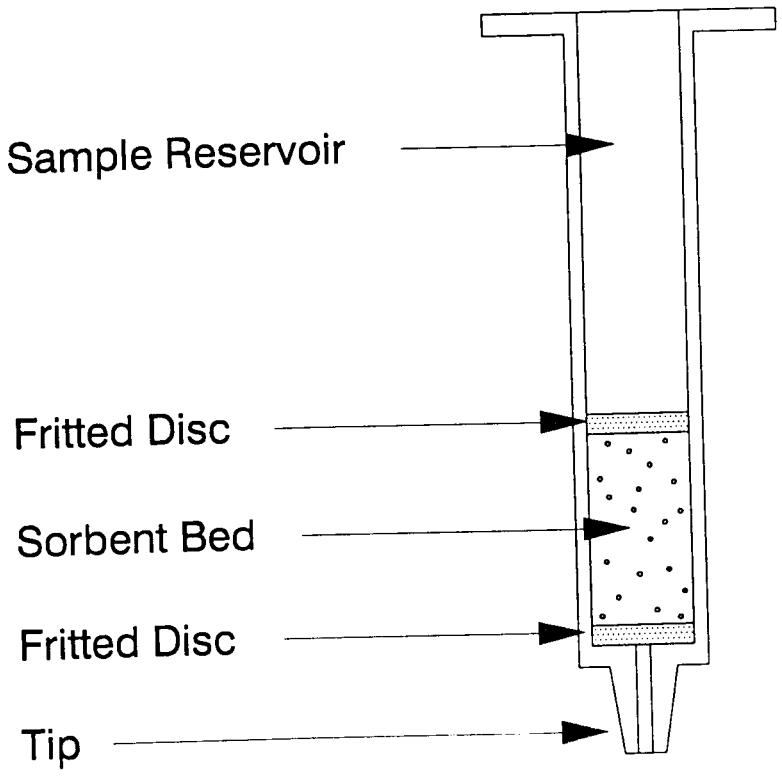
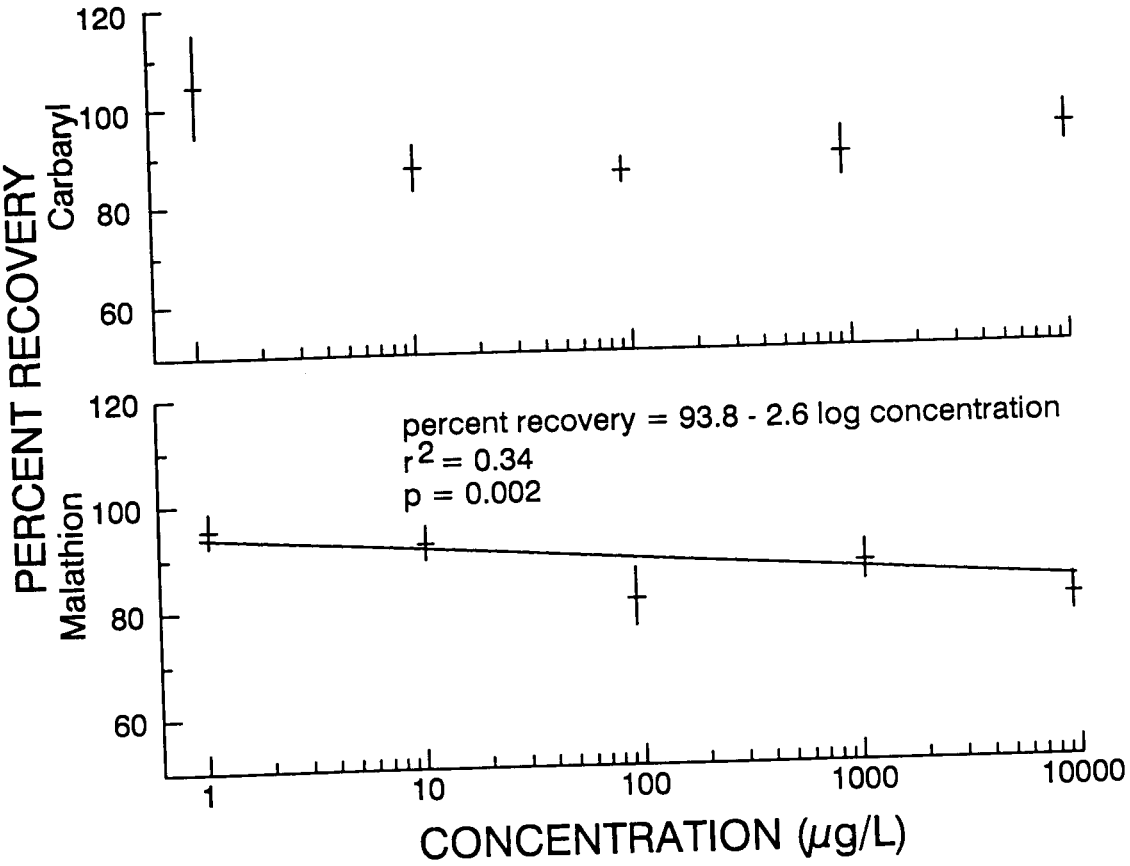

Sample Reservoir
Fritted Disc
Sorbent Bed
Fritted Disc
Tip

Figure 2. Relation between percent recovery and concentration for carbaryl and malathion. The least-squares regression line of best fit is illustrated for malathion. Mean percent recovery ($\pm$ SD) is shown for each concentration, n = 5.


PERCENT RECOVERY
Carbaryl
Malathion
120
100
80
60
percent recovery = 93.8 - 2.6 log concentration
r2 = 0.34
p = 0.002
1
10
100
1000
10000
CONCENTRATION (µg/L)

CHAPTER 2

OPTIMAL ASSAY CONDITIONS FOR ANALYSIS OF BRAIN ACETYLCHOLINESTERASE IN COLORADO ROUNDTAIL CHUB

ABSTRACT

Optimal assay conditions for analysis of brain acetylcholinesterase in Colorado roundtail chub (*Gila robusta robusta*) were determined for the pH-stat method. Optimal assay conditions were: 10 mg brain tissue per reaction vessel; temperature, 30 °C; substrate concentration, 11 mM; and pH, 7.5. Brain-enzyme activity was reduced 99 % by *in vitro* exposure to 100 mM eserine. Enzyme-inhibition studies confirmed that hydrolysis of acetylcholine was primarily by acetylcholinesterase.

INTRODUCTION

Acetylcholinesterase (AChE)-inhibiting insecticides are applied to rangeland and cropland throughout the Colorado River Basin. In response to concerns about potential effects of these insecticides on three federally endangered minnows, humpback chub (*Gila cypha*), bonytail (*Gila elegans*), and Colorado squawfish (*Ptychocheilus lucius*), we proposed to use a closely related species, the Colorado roundtail chub (*Gila robusta robusta*) as a surrogate for the rare fishes in biomonitoring programs. One aspect of biomonitoring involved measurement of brain AChE activity in 1-year-old Colorado roundtail chub collected from control and treatment sites before and after insecticide application.

Use of fish brain AChE inhibition as a measure of exposure to carbamate and organophosphate pesticides was intensively studied during the late 1960's and early 1970's [1-5]. Both classes of pesticides exert their toxic effects by inhibiting the neurotransmitter AChE, thereby causing disruption of the central nervous system. Because estimates of AChE activity can be affected by factors other than pesticide exposure, several potential sources of error have been described [6-10]. Experimental design and proper storage procedures can control most sources of experimental error, but one source of error that cannot be eliminated by these methods is use of improper enzyme-assay conditions for the animal of interest.

Like most physiological and biochemical tests used in aquatic toxicology, techniques used to measure brain AChE activity in fish were originally developed for study of enzymes in mammals [11]. Consequently, assay conditions (e.g., temperature, pH, and substrate concentration) may be inappropriate for study of enzymes from poikilothermic organisms. Several studies of optimal assay conditions for fishes have been conducted [2-3, 12-14]. However, none of the previously studied fishes are closely related to the native fishes of the Colorado River Basin.

To ensure accurate and sensitive analysis of brain AChE activity, we determined optimal assay conditions and confirmed that AChE was the enzyme responsible for observed substrate hydrolysis. The objectives of this study were to determine the (a) relation between rate of substrate hydrolysis and enzyme concentration; (b) temperature, substrate concentration, and pH at which rate of enzymatic hydrolysis was maximized; and (c) contribution of AChE to total substrate hydrolysis.

METHODS AND MATERIALS

Experimental animals

Fertilized eggs of Colorado roundtail chub were obtained by stripping eggs and sperm from wild adults captured in the Yampa River, Colorado, during June 1989. After fertilization, eggs were allowed to water-harden for 4 h before being packaged and transported by automobile to laboratory facilities at Colorado State University. Upon arrival, fertilized eggs were acclimated to culture-facility water and transferred to a Heath incubator. Hatching occurred approximately 5 d after fertilization. After hatching, larvae were transferred to flow-through troughs. At onset of first feeding, larvae were fed a mixture of live $\leq$ 24-h-old brine shrimp nauplii (Aquarium Products, Glen Burnie, MD) and commercially prepared flake diet (Tetramin, TetraWerke, West Germany) two or three times a day.

Physical and chemical conditions

Culture water was supplied by a well and had the following characteristics: dissolved oxygen, 7.1 mg/L; pH, 7.6; temperature, 19.0 °C; alkalinity, 259 mg/L as $CaCO_3$; hardness, 344 mg/L as $CaCO_3$; and specific conductance, 754 μS/cm. Cool-white fluorescent lamps were the only source of illumination, and a 16:8-h light:dark photoperiod was maintained.

Brain acetylcholinesterase assay

Colorado roundtail chub were sacrificed by immersion in ice water, weighed, measured, and prepared for brain AChE-activity assay. Study fish had an average wet weight of 6.3 g and total length of 88 mm. Whole brains were excised by removing the top of the cranium, severing the spinal cord at the base of the medulla, and cutting all cranial nerves at their origins on the brain. A brain-homogenate stock was prepared. Individual brains were pooled, weighed (± 1 mg), homogenized, diluted with distilled water, and held on ice until they were assayed (< 30 min).

Assays were conducted using a Sargent-Welch recording pH stat (Sargent-Welch Scientific Company, Skokie, IL) and the pH-stat method [3, 15]. The pH-stat is an automatic titrator and it was used to maintain pH in a reaction vessel where acetylcholine was being hydrolyzed by brain AChE into acetic acid and choline. The rate of addition of base (NaOH) to maintain pH was recorded and used to calculate rate of substrate hydrolysis. Enzyme substrate was acetylcholine iodide (Sigma Chemical Company, St. Louis, MO). Enzyme-activity units (AU) were defined as the activity of AChE which hydrolyzed 1 μM of substrate/mg brain tissue/min. Human blood-serum reference standards (Fisher Scientific, Orangeburg, NY) were assayed periodically to provide quality assurance. Estimates of activity of reference standards were within 5 % of reported values.

To determine the relation between rate of substrate hydrolysis and assay variables (i.e., brain-homogenate concentration, temperature, substrate concentration, and pH), three 0.15-ml aliquots of brain

homogenate were analyzed at each assay condition. Assay variables were studied in a stepwise fashion, with newly determined optimal assay conditions incorporated into subsequent analyses. The first assay variable studied (and optimized) was substrate concentration, followed by brain-homogenate concentration, pH, and temperature. Initial analytical conditions for determination of optimal substrate concentration were based on results reported by Coppage [3] and were: 10 mg brain tissue per reaction vessel; temperature, 20 °C; and pH, 7.0. Each assay took approximately 6 min, including time required to warm reaction vessels to assay temperature. Nonenzymatic hydrolysis of acetylcholine was estimated by adding all reagents except brain tissue to a reaction vessel and measuring the rate of hydrolysis for 10 min.

Determination of the contribution of AChE to total substrate hydrolysis was accomplished by *in vitro* exposure of brain homogenate to eserine (99 % active; Aldrich Chemical Company Inc., Milwaukee, WI), a selective AChE inhibitor. Eserine concentrations of 0 (control), 1, 10, and 100 μM were incubated with brain homogenate for approximately 30 min at 23 °C before addition of acetylcholine and enzyme-activity determination. Average AChE activity in each treatment (n = 3) was calculated. Assay conditions for inhibition studies were: 10 mg brain tissue per reaction vessel; temperature, 30 °C; substrate concentration, 11 mM; and pH, 7.5.

Statistical analysis

Linear regression analysis was used to evaluate the relation between rate of substrate hydrolysis and brain-homogenate concentration.

Raw data and residual plots were examined to confirm that the regression model was appropriate and statistical assumptions were not violated. Statistical analyses were conducted using SAS [16] statistical software. Optimal conditions for other assay variables were determined empirically.

RESULTS AND DISCUSSION

Rate of hydrolysis of acetylcholine was linearly related to brain-homogenate concentration (Figure 1a). Brain-homogenate concentration in subsequent analyses was 10 mg brain tissue/reaction vessel (2.0 mg/ml). This concentration was selected because it was low enough to permit analysis of individual fish brains and high enough to provide a reliable and measurable rate of enzymatic hydrolysis.

The observed optimum temperature for AChE activity was 30 °C (Figure 1b). No nonenzymatic hydrolysis of acetylcholine was observed at this temperature. Enzyme activity declined rapidly at higher temperatures. The observed temperature optimum was consistent with the optima for sheepshead minnow (*Cyprinodon variegatus*) and cascudo (*Hypostomus punctatus*) [3, 14].

Optimum substrate concentration for AChE was 11.0 mM (Figure 1c). Enzyme activity increased with acetylcholine concentration to the optimum and then declined. This response to substrate concentration is characteristic of AChE which is inhibited by excess substrate [17]. Optimal substrate concentration for brain AChE of other fishes was similar. A substrate concentration of 10 mM was optimal for bluegill (*Lepomis macrochirus*), channel catfish (*Ictalurus punctatus*), cutthroat trout (*Oncorhynchus clarki*), and sheepshead minnow [2-3, 13]. For cascudo, the optimal substrate concentration was 7 mM [14]. The optimal substrate concentration determined in other studies was selected because

it was one of the test concentrations. True optimal substrate concentrations for all fishes studied may be identical, but because they were not studied under identical experimental conditions, slightly different optima were observed.

Acetylcholinesterase activity also varied with pH (Figure 1d). The highest rate of substrate hydrolysis occurred at a pH of 7.5. Similarly, activity of AChE in sheepshead minnow and cascudo was maximized at pH ranges of 7.0 to 7.5, and 7.3 to 7.6, respectively [3, 14]. Nonenzymatic hydrolysis of acetylcholine was not detected at pH of 7.5 in our studies.

Enzyme activity of brain homogenate was inhibited by *in vitro* exposure of brain homogenate to eserine. Acetylcholinesterase activity in 1, 10, and 100 mM eserine treatments was reduced to 14, 6, and 1 % of that observed in the control treatment. This degree of inhibition was consistent with that reported by Coppage [3]. Eserine is a selective AChE inhibitor, and the observed reduction of enzyme activity showed that hydrolysis of acetylcholine was primarily by AChE. That AChE is the primary enzyme hydrolyzing acetylcholine under these test conditions has been confirmed in other studies [3, 13-14].

Observed optimal brain AChE-assay conditions for Colorado roundtail chub were: 10 mg brain tissue per reaction vessel; temperature, 30 °C; substrate concentration, 11 mM; and pH, 7.5. These conditions were surprisingly similar to those for other freshwater and estuarine fishes given variability of water quality and temperature that is observed in aquatic habitats and diverse evolutionary histories of the fishes studied.

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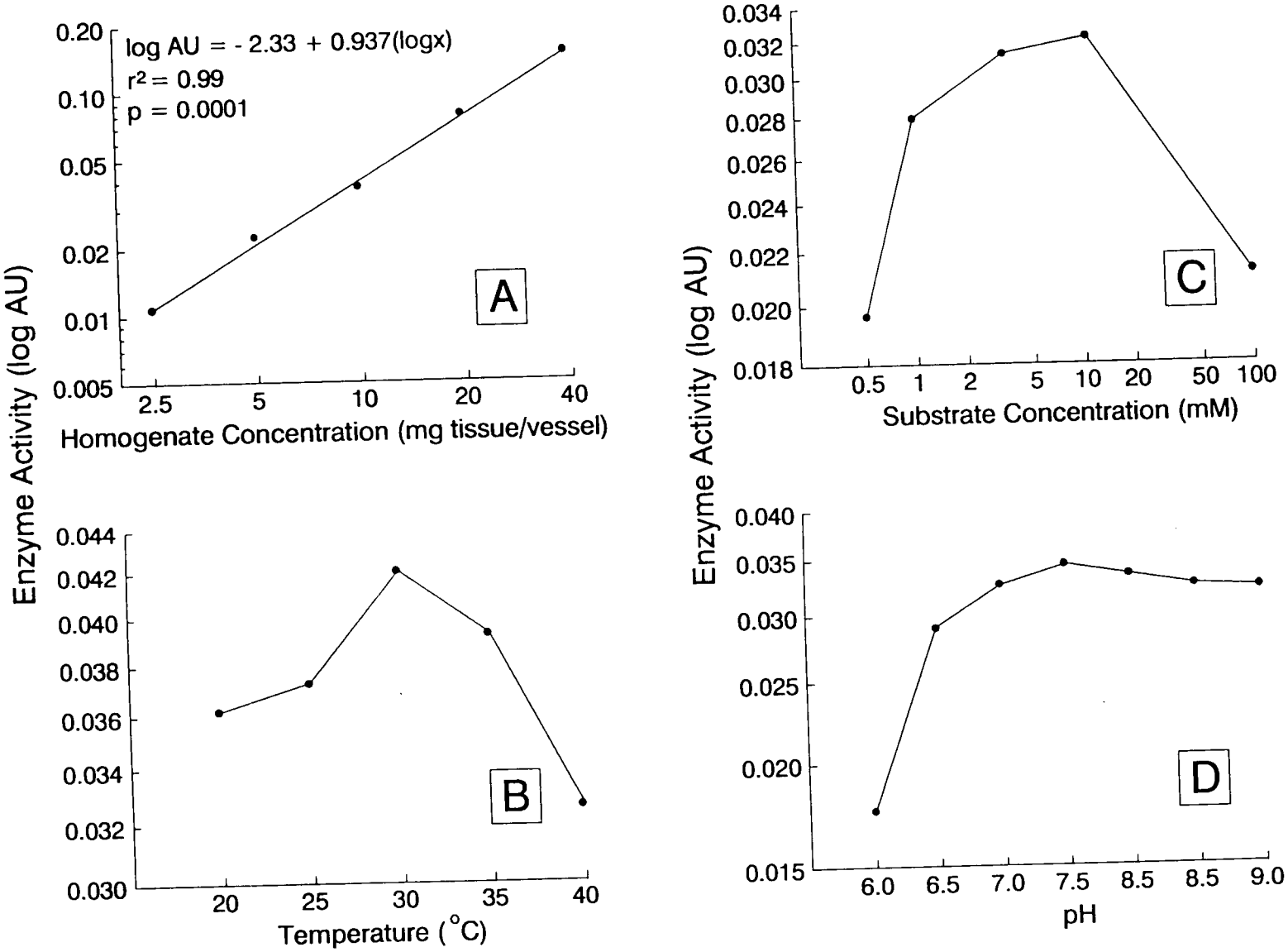
Figure 1. Relation between rate of hydrolysis of acetylcholine and enzyme-assay variables. Data points represent means of three replicate determinations. AU = μM substrate hydrolyzed/mg brain tissue/min.


log AU = - 2.33 + 0.937(logx)
r2 = 0.99
p = 0.0001
A
Enzyme Activity (log AU)
Homogenate Concentration (mg tissue/vessel)
B
Temperature (°C)
C
Substrate Concentration (mM)
D
pH

CHAPTER 3

TOXICITY OF CARBARYL AND MALATHION TO TWO FEDERALLY ENDANGERED FISHES AS ESTIMATED BY REGRESSION AND ANALYSIS OF VARIANCE

ABSTRACT

Toxicity of technical carbaryl, Sevin-4-Oil, and technical malathion to federally endangered Colorado squawfish (*Ptychocheilus lucius*) and bonytail (*Gila elegans*) was estimated by 4-d renewal-acute, 32-d early life-stage (ELS), and 1-d *in vivo* brain acetylcholinesterase-inhibition tests. Median lethal concentrations were estimated by probit analysis. Effect concentrations for ELS and acetylcholinesterase-inhibition tests were estimated by analysis of variance and an alternative procedure utilizing a linear-plateau regression model. Linear-plateau regression estimated a threshold concentration above which toxic effects began to occur. Median lethal concentrations and 95 % confidence limits (in parentheses) for carbaryl, Sevin-4-Oil, and malathion were 1.31 (1.23, 1.40), 3.18 (2.87, 3.52), and 9.14 mg/L (8.36, 10.0) for Colorado squawfish and 2.02 (1.78, 2.25), 3.31 (3.06, 3.55), and 15.3 mg/L (14.4, 16.4) for bonytail. No-observed-effect concentrations (NOEC) for Colorado squawfish were 445 µg/L carbaryl and 1680 µg/L malathion. The NOECs for bonytail were 650 µg/L carbaryl and 990 µg/L malathion. Threshold concentrations for Colorado squawfish in ELS tests were 364 µg/L carbaryl and 455 µg/L malathion. Threshold concentrations for bonytail were 217 µg/L carbaryl and 521 µg/L malathion. The NOECs for Colorado squawfish in acetylcholinesterase-inhibition studies were 29.3 µg/L carbaryl and 371 µg/L malathion. Threshold concentrations estimated for Colorado squawfish in acetylcholinesterase-inhibition studies were 7.40 µg/L

carbaryl and 150 μg/L malathion. Estimates of effect concentrations from linear-plateau regression were consistently lower than those estimated by hypothesis testing. Linear-plateau regression models adequately described the observed concentration-response relation.

INTRODUCTION

The insecticides carbaryl and malathion are used to control grasshopper infestations on rangeland and cropland throughout the western United States. Both chemicals exert their toxic effects by inhibiting the neurotransmitter acetylcholinesterase (AChE), thereby causing disruption of the central nervous system. Insecticides used to control grasshopper infestations may pose a particular threat to fishes because although no-spray buffer zones are observed around aquatic habitats, pesticide may be deposited by accidental drift or mobilized from upland areas by runoff [1].

The Colorado squawfish (*Ptychocheilus lucius*) and bonytail (*Gila elegans*) are large riverine-adapted minnows that historically occurred throughout the Colorado River Basin [2]. Populations of both species have declined as a result of construction of reservoirs and other management practices in the basin [3]. In response to the rapid decline and threat of extinction of these fishes, the United States Fish and Wildlife Service authorized listing of Colorado squawfish and bonytail as federally endangered species in 1967 and 1980, respectively. Although the scarcity and federally endangered status of these fishes has made study of their habits in the wild difficult, some information on life history and behavior has been collected [4, 5]. During the first several months of life (June through October), larvae of Colorado squawfish and bonytail occupy shallow, low-velocity, near-shore

habitats. Because these habitats have low rates of water exchange, pesticides deposited in them may be present long enough for toxic effects to occur. Grasshopper control programs and life-history patterns of Colorado squawfish and bonytail overlap such that potentially sensitive life stages (less than 30 d old) are at risk of exposure.

Toxicity of carbaryl and malathion to fish and aquatic invertebrates has been thoroughly studied [6], but none of the previously tested fishes is closely related to the native fishes of the Colorado River Basin. Because of uncertainty of predicting the sensitivity of Colorado squawfish and bonytail to carbaryl and malathion, we estimated toxicity of these chemicals using prescribed methods for 4-d acute [7] and 32-d early life-stage (ELS) toxicity tests [8]. We also conducted studies of brain AChE inhibition in Colorado squawfish after *in vivo* exposure to carbaryl or malathion for 24 h. In contrast to standard toxicity tests, AChE-inhibition studies provided a measure of toxic effects at a scale consistent with the duration of exposure and concentration range that may result from aerial pesticide applications. Data from toxicity tests were analyzed using analysis-of-variance (ANOVA) hypothesis testing and an alternative procedure utilizing a linear-plateau regression model. Stephan and Rogers [9] described the computational and conceptual advantages of using regression analysis for concentration-response data. We present comparisons of the effect-concentration estimates obtained by the two alternative statistical methods. The purpose of this study was to estimate the toxicity of technical carbaryl, Sevin-4-Oil (a formulation

of carbaryl), and technical malathion to Colorado squawfish and bonytail. The objectives were to (a) estimate 4-d median lethal concentrations; (b) estimate and compare effect concentrations for survival, growth, and *in vivo* brain AChE inhibition; and (c) compare estimates of effect concentrations obtained by hypothesis testing and regression analysis.

METHODS AND MATERIALS

Experimental animals

Fertilized eggs of Colorado squawfish and bonytail were obtained from Dexter National Fish Hatchery, Dexter, New Mexico. The hatchery maintains reproducing populations of Colorado squawfish and bonytail to prevent their extinction should some event result in extirpation of existing wild populations. Hatchery researchers artificially induced spawning of Colorado squawfish [10] and bonytail [11], and young fish from excess reproduction were available for experimental purposes.

Fertilized eggs were allowed to water-harden for 48 h before being packaged and transported via same-day commercial air-freight to Denver, Colorado, and then by automobile to laboratory culture facilities at Colorado State University. Upon arrival, fertilized eggs were acclimated to culture facility water temperature (19 °C) and transferred to a Heath incubator. Hatching occurred approximately 4 and 5 d after fertilization for Colorado squawfish and bonytail, respectively. After hatching, larvae were transferred to flow-through troughs, where they were maintained until selected for a toxicity test. After onset of first feeding (approximately 11 d after fertilization), larvae were fed live $\leq$ 24-h-old brine shrimp nauplii (Aquarium Products, Glen Burnie, MD) two or three times a day.

Toxicity testing overview

Because we had no previous knowledge of the sensitivity of Colorado squawfish or bonytail to the toxicants, 24-h range-finding tests were conducted. The lowest concentration lethal to all test organisms in 24 h was the highest test concentration in 4-d renewal-acute tests. Results of 4-d renewal-acute tests were used to select a toxicant concentration range for 32-d ELS tests. The lowest toxicant concentration in renewal-acute tests that caused abnormal behavior (i.e., erratic swimming, lethargy, or loss of equilibrium) was the highest toxicant concentration in ELS tests. The lowest-observed-effect concentration (LOEC) estimated by ELS tests was the highest test concentration in 1-d AChE-inhibition studies.

Toxicity tests were conducted with Colorado squawfish and bonytail during 1989 and 1990, respectively. Because young Colorado squawfish and bonytail were only available on an annual basis, toxicity tests were conducted sequentially. Unfortunately, by the time ELS tests were initiated, considerable ontogenetic development had occurred, and embryos and protolarvae [12] were not present during the exposure period. Mesolarval, metalarval, and juvenile life stages were present during the exposure period.

Exposure systems

Range-finding and 4-d renewal-acute tests were conducted using 1-L glass beakers containing 0.75 L of toxicant solutions. Contents of each beaker were renewed every 24 h. The dilution factor was 0.75.

Early life-stage and AChE-inhibition tests were conducted using a continuous-flow mini-diluter exposure system [13]. The diluter maintained a 0.5 dilution factor and provided a volume of 0.055 L/min to replicate aquaria. Aquaria were 10 x 20 x 15 cm high, and depth of test solutions was 12 cm.

In both exposure systems, treatments were assigned to two replicate exposure chambers using a randomized block design. Test animals were randomized to one of seven treatment groups: five toxicant concentrations, a solvent control, and a dilution-water control. Cool-white fluorescent lamps were the only source of illumination, and a 16:8-h light:dark photoperiod was maintained.

Physical and chemical conditions

Dilution water for all toxicity tests was supplied by a well on the Colorado State University campus and was vigorously aerated for approximately 24 h while being heated to a test temperature of 22±1 °C. In renewal-acute and AChE-inhibition tests, alkalinity, hardness, and specific conductance were measured at the beginning and end of the exposure period. Dissolved oxygen and pH were monitored daily, and water temperature was measured continuously. For 32-d ELS tests, alkalinity, hardness, pH, and specific conductance were measured weekly. Dissolved oxygen was measured daily, and water temperature was measured continuously. Dilution water characteristics for all tests, except Colorado squawfish renewal-acute tests, had the following ranges: dissolved oxygen, 6.1-7.0 mg/L; pH, 7.9-8.2; temperature, 21.2-22.7 °C; alkalinity, 237-259 mg/L as $CaCO_3$; hardness, 344-378 mg/L as $CaCO_3$; and

specific conductance, 720-780 μS/cm. Dilution water used in Colorado squawfish renewal-acute tests underwent a different aging process; its characteristics were: dissolved oxygen, 7.1-7.2 mg/L; pH, 8.5-8.6; temperature, 22.0-22.8 °C; alkalinity, 104-110 mg/L as $CaCO_3$; hardness, 212-216 mg/L as $CaCO_3$; and specific conductance, 600 μS/cm.

Toxicant solutions

Technical carbaryl (1-naphthyl methylcarbamate, 99 %) and Sevin-4-Oil (a formulation containing 49 % carbaryl and petroleum distillates) were obtained from Rhône-Poulenc (Research Triangle Park, NC). Technical malathion (diethyl mercaptosuccinate, S-ester with O, O-dimethyl phosphorodithioate, 93 %) was obtained from American Cyanamid Company (Princeton, NJ). Technical carbaryl and technical malathion will henceforth be referred to as carbaryl and malathion. Stock solutions were prepared by dissolving each toxicant in pesticide-grade acetone or acetone-dilution-water mixtures. Renewal-acute exposure concentrations were prepared by pipetting the desired amount of toxicant stock into beakers containing 0.75 L dilution water. Test solutions were stirred and transferred to exposure chambers within 30 min of preparation. In flow-through tests, toxicant stock solutions were delivered to the diluter via peristaltic pump. The amount of acetone in any exposure concentration never exceeded 0.5 ml/L.

Analytical procedures

Toxicant concentrations in renewal-acute and flow-through AChE-inhibition tests were measured twice during the exposure period.

Toxicant concentrations in ELS tests were measured weekly (four occasions). Samples for analysis were taken from alternate replicate exposure chambers. In renewal-acute tests, toxicant concentrations nearest the median lethal concentration were also measured 24 h after renewal to estimate the amount of toxicant breakdown during the 24-h period between renewals. Toxicants were extracted with solid-phase extraction and analyzed with gas chromatography [14]. Extracted samples were stored at -4 °C until they could be analyzed.

Exposure conditions

The 4-d renewal-acute tests with Colorado squawfish and bonytail were initiated with 26- and 6-d-old (post-fertilization) larvae, respectively. Mean wet weight and total length at start of renewal-acute tests were 4 mg and 9.4 mm for Colorado squawfish and 2 mg and 6.8 mm for bonytail. Initial weights and lengths were determined by measuring 20 fish sacrificed and preserved at the start of the exposure period. Renewal-acute tests with the three toxicants were conducted simultaneously. Ten larvae were placed in replicate exposure chambers for a total of 20 per test concentration. Obviously deformed or abnormal larvae were not selected. Larvae were not fed within 24 h of the start of a renewal-acute test or during the 4-d exposure period. Survival and behavior were monitored at least daily. At conclusions of renewal-acute tests, surviving fish were sacrificed by administering an overdose of MS-222 (Argent Chemical Laboratories, Redmond, WA) and preserved in 10 % formalin.

Early life-stage tests with Colorado squawfish and bonytail were initiated with 41- and 48-d-old larvae, respectively. Mean wet weight and total length at the start of tests were 9 mg and 12 mm for Colorado squawfish and 4 mg and 8.6 mm for bonytail. Thirty Colorado squawfish and 40 bonytail larvae were placed in replicate exposure chambers. Larvae were acclimated to conditions within exposure chambers for 48 h before the toxicant-metering system was activated. Larvae were fed live $\leq$ 24-h-old brine shrimp nauplii two or three times a day. Approximately 100 nauplii/fish/feeding were introduced into exposure chambers, and the number of nauplii was adjusted to account for mortality of test animals. Larvae were not fed within 24 h of conclusion of a test. Exposure chambers were siphoned as required to remove debris. Survival and behavior were observed daily; however, small size and rapid deterioration of dead larvae made accurate counting difficult. Therefore, counts of fish surviving at conclusion of an exposure period were used to estimate survival. Upon conclusion of a test, fish were sacrificed by administering an overdose of MS-222 and preserved in 10 % formalin. Preserved fish were blotted, counted, and weighed ($\pm$ 1 mg).

Flow-through AChE-inhibition studies were only conducted with Colorado squawfish. Mean wet weight and total length of Colorado squawfish were 8.0 g and 74 mm. Because of their large size, only two fish were placed in each replicate exposure chamber. Fish were acclimated to conditions within exposure chambers for 48 h and were not fed within 24 h of, or during, the 1-d exposure period. At conclusion of the exposure period, fish were sacrificed in ice water, weighed, measured, and prepared for brain AChE assay.

Brain acetylcholinesterase assay

Brain AChE activity was measured immediately after conclusion of the toxicant exposure period. Three fish were selected from each exposure concentration. Whole brains were excised by removing the top of the cranium, severing the spinal cord at the base of the medulla, and cutting all cranial nerves at their origin on the brain. Individual brains were weighed (± 1 mg), homogenized, diluted with distilled water, and held on ice until they were assayed (< 30 min). Assays were conducted using a Sargent-Welch recording pH stat (Sargent-Welch Scientific Company, Skokie, IL) and the pH-stat method [15, 16]. Enzyme assay conditions for Colorado squawfish were those determined for the closely related Colorado roundtail chub (*Gila robusta robusta*) (Chapter 2), and were: pH, 7.5; temperature, 30 °C; 10 mg brain tissue/reaction vessel (2.0 mg/ml); and substrate concentration, 0.011 M (acetylcholine iodide, Sigma Chemical Company, St. Louis, MO). Enzyme activity units (AU) were defined as the activity of AChE which hydrolyzed 1 μM of substrate/mg brain tissue/min. Human blood-serum reference standards (Fisher Scientific, Orangeburg, NY) were assayed periodically to provide quality assurance. Estimates of activity of reference standards were within 5 % of the reported value.

Statistical analysis

Median lethal concentrations for mortality in renewal-acute tests were estimated by probit analysis [17]. Toxicity of carbaryl was compared to that of Sevin-4-Oil by calculating a ratio of the median lethal concentrations estimated for each toxicant. A ratio greater than 1.0 suggested that Sevin-4-Oil was more toxic than carbaryl; a ratio less than 1.0, that Sevin-4-Oil was less toxic [6]. Ratios were based on active ingredient.

Two methods of analysis, hypothesis testing and regression analysis, were used to analyze survival, growth (as weight), and AChE activity in 32-d ELS and 1-d AChE-inhibition tests. For hypothesis testing, survival data were analyzed twice so that effects of two alternative statistical transformations could be assessed. First, the angular transformation:

not true, I really used:

$$p' = \frac{\text{arcsine}\sqrt{p}}{\sqrt{(n+0.5)}}$$ $\arcsin\sqrt{(p+0.375)/(n+0.75)}$

but this may not have been best choice. See me if you have questions.

where p = proportion surviving was applied to stabilize the variance [18]. Alternatively, the logistic transformation:

$$\text{logit} = ln\ \{(np + 0.5) / (nq + 0.5)\}$$

where n = the initial number of animals in a replicate, p = the proportion surviving, and $q = 1 - p$, was applied to survival data [19].

Statistical weights (w) for logistic transformed survival values were calculated using the formula:

$$w = \{1/(np + 0.5) + 1/(nq + 0.5)\}^{-1}$$

Survival, growth, and AChE-activity of fish in solvent controls and dilution-water controls were compared by calculating a t-statistic and comparing it to a two-tailed Student's critical value. If effects of the solvent and dilution-water controls were not significantly different ($p = 0.05$ for all statistical comparisons), data from these two treatments were pooled for subsequent analyses. After pooling control treatments, all data were subjected to Shapiro-Wilk's Test for normality and Bartlett's Test for homogeneity of variance [18]. No additional transformations were required to meet assumption of normality or homogeneity of variance. Following formal testing of statistical assumptions, angular-transformed survival and AChE-activity data were subjected to one-way ANOVA. Logistic-transformed survival and growth data were subjected to weighted one-way ANOVA. Weighting factors for growth data were equal to the number of fish comprising the sample from each replicate. Treatments that had significantly different effects compared to controls were identified by calculating a t-statistic for comparison to a one-tailed Dunnett's critical value. No-observed-effect concentrations (NOEC) and lowest-observed-effect concentrations (LOEC) were estimated for survival, growth, and AChE activity.

For regression analysis, a linear-plateau regression model, also called hockey stick [20] or threshold model [21], was fit to survival,

growth, and AChE activity as a function of toxicant concentration. An assumption for use of this model was that there be a toxicant concentration (threshold) below which toxic effects were not exhibited and above which a concentration-response was observed. Of particular interest in this analysis was estimation of the threshold and its confidence interval, since it represented the toxicant concentration at which effects began to be manifested. The linear-plateau regression model had the form:

$$y = \begin{cases} \beta_0 + \beta_1 x_0 & \text{for } x \leq x_0 \\ \beta_0 + \beta_1 x & \text{for } x \geq x_0 \end{cases}$$

where x_0 represented the threshold concentration.

Prior to regression analysis, survival data were subjected to the logistic transformation. Measured toxicant concentrations were $\log_2$ transformed. No other transformations of data were made. As in hypothesis testing, survival and growth data were analyzed using weighted analyses. The multivariate-secant, nonlinear regression method was used to simultaneously (1) fit a line through data that comprised the plateau (i.e., zero slope), (2) fit a second line through data that showed a concentration-response (i.e., non-zero slope), and (3) estimate the threshold concentration. Data and residual plots were examined to confirm that regression models were appropriate and statistical

assumptions were not violated. All statistical analyses were conducted using SAS statistical software [22].

RESULTS

4-d renewal-acute tests

Median lethal concentrations and 95 % confidence limits (in parentheses) for Colorado squawfish and bonytail exposed to carbaryl were 1.31 (1.23, 1.40) and 2.02 mg/L (1.78, 2.25), respectively (see Appendix A for summaries of measured toxicant concentrations and mortality). Median lethal concentrations for Colorado squawfish and bonytail exposed to Sevin-4-Oil were 3.18 (2.87, 3.52) and 3.31 mg/L (3.06, 3.55), respectively. Toxicity of Sevin-4-Oil (49 % carbaryl) was approximately one-half that of carbaryl. The ratio of median lethal concentrations of carbaryl to Sevin-4-Oil was 0.840 and 1.24 for Colorado squawfish and bonytail, respectively. Ratios between 0.5 and 1.5 are considered to be within the range of normal experimental variation [6]; therefore, no synergistic or antagonistic toxic effects due to formulation of carbaryl as Sevin-4-Oil were observed. Median lethal concentrations for Colorado squawfish and bonytail exposed to malathion were 9.14 (8.36, 10.0) and 15.3 mg/l (14.4, 16.4), respectively. Malathion was approximately 7 times less toxic to Colorado squawfish and bonytail than carbaryl.

Carbaryl and malathion concentrations declined during the 24-h period between renewals. Initial concentrations of carbaryl and

malathion in test solutions nearest the median lethal concentrations were 1.95 and 14.5 mg/L; final concentrations were 0.817 and 9.20 mg/L, respectively. Dilution water had a pH of 8.2, and temperature was 22 °C.

Early life-stage and acetylcholinesterase-inhibition tests

As in 4-d renewal-acute studies, carbaryl was consistently more toxic than malathion (Table 1). For hypothesis tests involving survival and growth, growth was the most sensitive measure of toxic effects; therefore, only NOECs for growth are presented below. Estimates of effect concentrations for survival were identical regardless of whether the angular or logistic transformation was used. No-observed-effect concentrations in 32-d ELS tests were: 445 µg/L carbaryl and 1680 µg/L malathion for Colorado squawfish; and 650 µg/L carbaryl and 990 µg/L malathion for bonytail. No-observed-effect concentrations for Colorado squawfish in 1-d AChE-inhibition studies were 29.3 µg/L carbaryl and 371 µg/L malathion. Threshold concentrations in 32-d ELS tests as estimated by linear-plateau regression were: 364 µg/L carbaryl and 455 µg/L malathion for Colorado squawfish; and 217 µg/L carbaryl and 521 µg/L malathion for bonytail. Threshold concentrations estimated for Colorado squawfish in 1-d AChE-inhibition studies were 7.40 µg/L carbaryl and 150 µg/L malathion (see Appendix A for summaries of measured toxicant concentrations, survival, weight, and AChE activity).

For each toxicant and endpoint, the distribution of data as a function of concentration was such that a linear-plateau regression model was appropriate. In all cases, the regression accounted for a

significant amount of the total variation. Regression analyses permitted compilation of a family of linear-plateau functions for each test species and toxicant (Figure 1). Typically, the least sensitive endpoint was survival and the most sensitive was AChE inhibition.

DISCUSSION

4-d renewal-acute tests

A concern responsible for initiation of our toxicological studies was that Colorado squawfish or bonytail might be super-sensitive to carbaryl or malathion. To evaluate the relative sensitivity of Colorado squawfish and bonytail, we compared median lethal concentrations estimated in our study to those summarized by Mayer and Ellersieck [6]. Compared to other commonly studied fishes, Colorado squawfish and bonytail were approximately as sensitive to carbaryl as cutthroat trout (*Oncorhynchus clarki*), rainbow trout (*Oncorhynchus mykiss*), Atlantic salmon (*Salmo salar*), and brook trout (*Salvelinus fontinalis*). They were two to ten times more sensitive than fathead minnow (*Pimephales promelas*), channel catfish (*Ictalurus punctatus*), and bluegill (*Lepomis macrochirus*). In contrast, Colorado squawfish and bonytail were one to two orders of magnitude less sensitive to malathion than cutthroat trout, rainbow trout, and bluegill; and approximately as sensitive to malathion as fathead minnow and channel catfish. These comparisons provide some basis for discussing relative toxicity of carbaryl and malathion to Colorado squawfish and bonytail, but two factors that strongly influence toxicity of carbaryl and malathion should be considered. The first is related to size and life stage of test organisms. The relation of toxicant sensitivity to body size is inconsistent [23]; however, early life stages are generally more

sensitive to toxicants [24]. The acute toxicity tests summarized by Mayer and Ellersieck [6] involved "fingerling fish" weighing from 200 to 1500 mg [25]. Therefore, we expected Colorado squawfish and bonytail to be relatively sensitive to carbaryl and malathion as a result of a life-stage effect. This prediction was supported for toxicity of carbaryl, but not for malathion. However, the effect of a second factor that modifies toxicity, that of pH, should be considered.

Carbaryl and malathion hydrolyze rapidly in waters having pH greater than 7 [26, 27]. The pH of test solutions in our renewal-acute studies ranged from 7.9 to 8.6 and concentrations of carbaryl and malathion declined by 58 and 37 %, respectively, in 24 h. Under these conditions, our estimates of median lethal concentrations may have been underestimated by relatively short exposure to target concentrations. However, breakdown products of carbaryl and malathion have been shown to be more toxic than their respective parent compound [28, 29]. Thus, the net toxic effect of rapid hydrolysis of carbaryl and malathion is uncertain.

Use of renewal-acute test results to compare interspecific sensitivity is confounded by the effects of pH, life-stage differences, and body-size differences. These factors also complicate reliable prediction of the effects of carbaryl or malathion on Colorado squawfish and bonytail in natural conditions. Fortunately, estimation of median lethal concentrations was only a preliminary step in our assessment of toxicity of carbaryl and malathion to Colorado squawfish and bonytail. Flow-through ELS tests and AChE-inhibition studies provided more

sensitive measures of effects and permitted more reliable comparisons of interspecific sensitivity.

Early life-stage tests

Interspecific comparisons of toxicant sensitivity based on ELS tests are of greater value than those based on renewal-acute tests because the design of ELS tests minimizes potential confounding effects. For example, accumulation of toxicant breakdown products was precluded by the flow-through nature of ELS tests. In addition, ELS tests measured sensitive endpoints (i.e., reduced growth), thus, minimizing effects due to life-stage and size differences that may have confounded comparisons based on renewal-acute tests.

Colorado squawfish and bonytail were approximately as sensitive to carbaryl as the fathead minnow [30]. The NOEC and LOEC for fathead minnow were 210 and 680 µg/L, a range which overlaps that estimated for both endangered fishes. For fathead minnow exposed to malathion, the NOEC and LOEC were 200 and 580 µg/L [31]. These effect concentrations were approximately five to eight times lower than those estimated for Colorado squawfish or bonytail. However, toxicity to Colorado squawfish and bonytail may have been relatively underestimated for carbaryl and malathion because the exposure period was short (32 d compared to 9 and 10 months for fathead minnow) and reproductive effects were not studied. Long-term toxicity of malathion to two other species, flagfish (*Jordanella floridae*) and bluegill, has been studied [32, 33]. In a 30-d exposure, flagfish were more sensitive to malathion than Colorado squawfish or bonytail and had a LOEC of 24.7 and 10.9 µg/L for survival

and growth, respectively. Bluegill were also relatively sensitive to malathion: Eaton [33] estimated a LOEC of 7.4 μg/L based on the development of spinal deformities in adults. Considering the range of species sensitivity encompassed by existing long-term exposure studies, Colorado squawfish and bonytail fall near the tolerant end of the spectrum and may be roughly equivalent to fathead minnow in sensitivity to AChE-inhibiting pesticides.

Regression analysis versus hypothesis testing

Although threshold concentrations estimated by linear-plateau regression were consistently lower than NOECs from hypothesis testing, the two statistical methods did not lead to vastly different conclusions. Six of ten calculated threshold concentrations were within a factor of two of the NOECs; the remaining estimates were within a factor of four; and, in two cases, the upper limit of the confidence interval for a threshold concentration contained the NOEC.

The tendency of linear-plateau regression to produce lower estimates of effect concentrations may suggest that the procedure underestimated the concentration at which toxic effects began to accrue; however, inspection of Figure 1 shows that fitted regression models and estimated thresholds were appropriate. A more likely explanation for the discrepancy between regression and hypothesis-testing estimates is related to experimental design. A weakness of our study was that we used only two replicates per test concentration in ELS tests. Statistical power would have been increased had there been more replication, but other disadvantages of the hypothesis-testing approach

(i.e., sensitivity to the selected level of significance, sensitivity to variability of data, and dependence on concentration interval) would not have been corrected. The influence of these factors has been thoroughly summarized by Stephan and Rogers [9]. Unlike regression analysis, hypothesis testing does not utilize concentration-response relations in toxicological data. Hypothesis testing uses a pair-wise comparison procedure to determine if the difference between the mean response at a given concentration and the mean for the control is greater than the minimum statistically significant difference. In contrast, linear-plateau regression uses all of the data to simultaneously estimate a threshold concentration. Regression analysis also has advantages of being designed to evaluate the relation between a dependent variable and a quantitative independent variable, and of providing parameter estimates for an equation that best describes the relation. The resulting equation can be used to interpolate effects to untested concentrations. Interpolation allows estimation of a concentration that corresponds to a specified magnitude of effect (e.g., concentration that produces a 5 % reduction in growth) or the magnitude of effect corresponding to a given concentration. The linear-plateau regression model is especially useful because it describes a concentration response and reflects that, for certain toxicants, there may be a threshold below which toxic effects are not observed.

Justification for the linear-plateau regression model is based on the threshold concept [34, 35]. A basic tenet of this concept is that toxic effects appear only when toxicant-induced changes in an organism exceed the organism's ability to compensate by homeostatic mechanisms.

A variety of protective mechanisms have been identified in fish: metallothionein [36-37], non-metallothionein metal binding proteins [38], induction of mixed-function oxidase system [39], and mucus barriers [40]. These mechanisms decrease toxic effects by sequestering, eliminating, or reducing absorption of toxicants; but they may be overwhelmed by high concentrations or long-term exposure.

In reality, a threshold model may not correctly represent the toxicology of carbaryl and malathion. A curvilinear model may be more appropriate, but aquatic toxicology data are often inadequate to describe higher-order relationships. Given a mechanistic basis, a more complicated model may be justified; lacking this, a parsimonious model is preferred. Even if the concentration-response relation is higher-order, a linear-plateau model may provide a relatively close approximation of the level of effect at any given concentration while providing an estimate of the concentration at which toxic effects are first manifested.

Nonlinear regression models are typically more difficult to specify and estimate than linear models. Recent improvements in statistical packages have increased the efficiency of nonlinear regression. We used the NLIN procedure in SAS [22]. A derivative-free method, called multivariate-secant or false-position method, was especially useful because it did not require specification of partial derivatives of the model with respect to each parameter. Other factors that probably contributed to success of our analyses were (a) use of a relatively simple regression model and (b) analysis of data that were

distributed such that there were at least two treatment responses with which to characterize plateau and concentration-response lines.

Utility of acetylcholinesterase-inhibition studies

Measurement of AChE inhibition allowed study of toxic effects on a scale consistent with duration of exposure and concentration range that may occur in the field. Another aspect of environmental realism of 1-d AChE-inhibition studies was that the duration of exposure was too short to permit test organisms to physiologically adapt to toxicants. In 4-d renewal-acute and 32-d ELS tests, we observed that incidence of sublethal effects (e.g., partial loss of equilibrium or failure to feed) was initially high but decreased over time. Mortality accounted for a portion of the observed decrease, but many fish were able to regain equilibrium or begin feeding even though toxicant concentrations were unchanged. Development of tolerance to organophosphorus and carbamate insecticides has been studied, and mechanisms responsible for this phenomenon may be structural modification of the active site of AChE [41] or reduced sensitivity of postsynaptic cholinergic receptors [42].

There has been some controversy over use of brain AChE inhibition as evidence of exposure to AChE inhibitors. Gibson et al. [43] reported that fish that became moribund after a 30-min exposure to 750 µg/L parathion showed only 25 % AChE inhibition, while those exposed to 20 µg/L for 13 h showed 57 % inhibition. A key factor in these studies was that duration of exposure was much longer at the lower concentration. The degree of brain AChE inhibition in fish is dependent on duration of exposure during the first 72 h [44-45]. Protective

mechanisms like the blood-brain barrier may retard transfer of AChE inhibitors to the brain, making it one of the last organs to be affected. Toxic effects resulting from relatively short-duration exposure (i.e., minutes or hours) to AChE inhibitors may be manifestations of inhibition of neural transmitters in other organs (e.g., liver or hepatopancreas) or in respiratory tissues.

Long-duration toxicity tests do not always emphasize the most sensitive endpoints [46]. However, many researchers consider results of these tests to be conservative estimates of potential effects [47]. Our results showed a discrepancy between effect concentrations estimated from survival and growth and those estimated from measurement of brain AChE inhibition. The LOEC for Colorado squawfish in AChE-inhibition studies was approximately 18 times lower for carbaryl and five times lower for malathion than the LOEC estimated from growth or survival. In most laboratory toxicity tests, food is generally super-abundant, easily obtained, and effects due to predator avoidance or competition are not incorporated. Constant environmental conditions alleviate other sources of physiological stress, allowing test organisms to devote nearly all their resources to compensating for toxic effects. These testing shortcomings may contribute to underestimating toxicity because of lack of ecological realism in standardized laboratory toxicity tests.

The mode of action of most toxicants in fish is unknown; therefore, physiological effects must be inferred from reduced survival, growth, or reproduction. Chemicals that inhibit AChE provide a unique opportunity to evaluate current aquatic toxicology methods because their mode of action is known. Although the biological significance of a

given reduction (e.g., 5 %) of AChE activity may be controversial, AChE activity can be used as a direct measure of physiological effects and provides a conservative estimate of effect concentrations. Accuracy of predictions based on current methods could be tested by incorporating study of AChE inhibitors and AChE inhibition into future method-development and method-evaluation programs.

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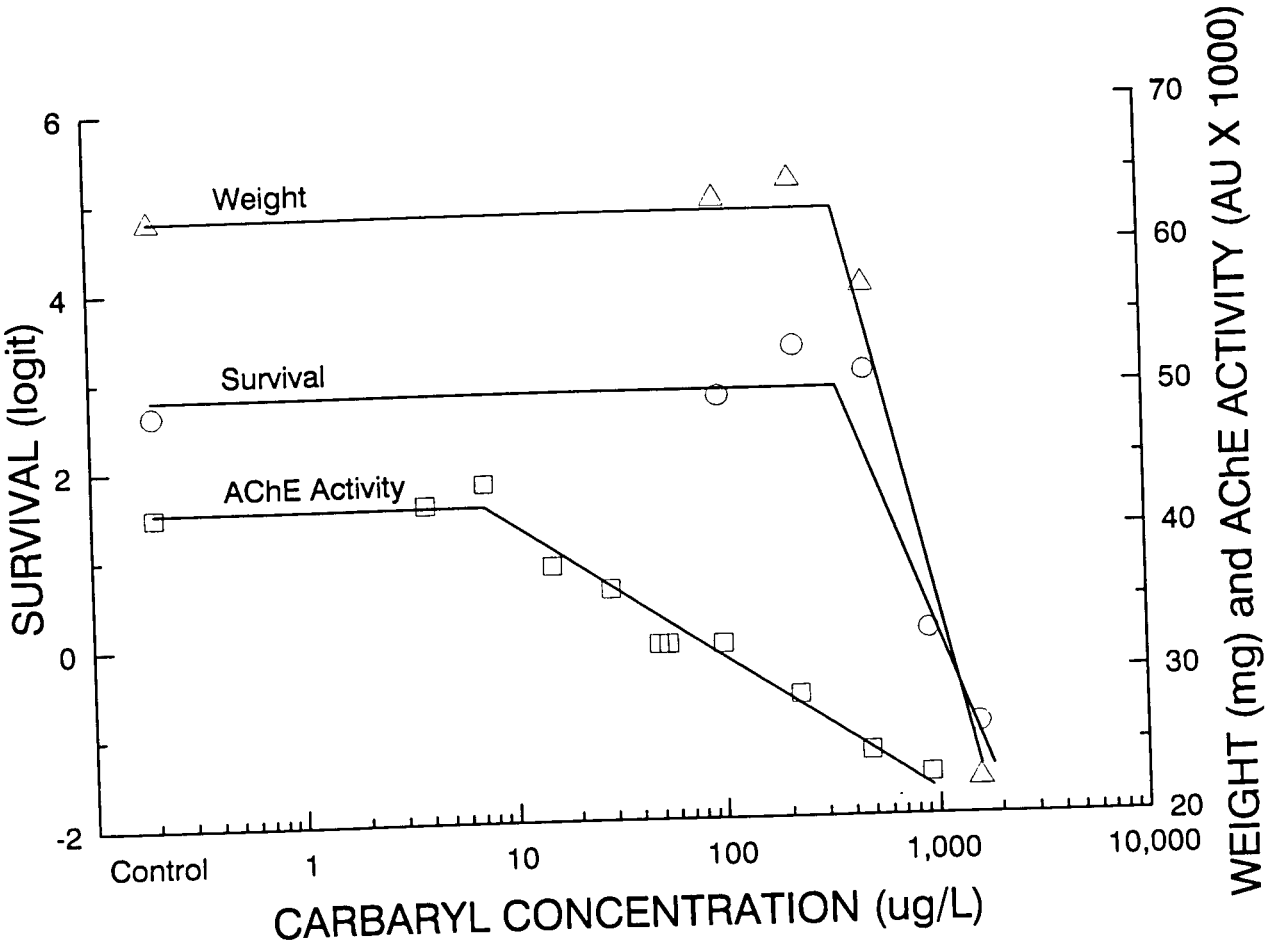
Table 1. Threshold and effect concentrations estimated from exposure of Colorado squawfish (CS) and bonytail (BT) to technical carbaryl and technical malathion. Plateau, slope, and intercept estimates for the linear-plateau regression equation: $y = \text{intercept} + \text{slope}[3.322(\log_{10}\text{concentration})]$. AChE = acetylcholinesterase.

Measure	Plateau	Slope	Intercept	Threshold[a, b]	NOEC[a]	LOEC[a]
Survival[c, d]						
Carbaryl						
CS	2.76	- 1.88	18.8	364 (203, 653)	445	866
BT	3.57	- 6.73	65.6	593 (566, 621)	650	1240
Malathion						
CS	3.18	- 0.833	10.5	455 (236, 786)	1680	3510
BT	3.39	- 3.20	36.9	1420 (936, 2160)	2000	4060
Weight[c, e]						
Carbaryl						
CS	62.6	-18.9	224	364 (301, 440)	445	866
BT	66.3	- 6.95	120	217 (180, 262)	650	1240
Malathion						
CS	46.0	-21.7	275	1470 (1410, 1520)	1680	3510
BT	64.0	-18.9	234	521 (487, 557)	990	2000
Brain AChE[f, g]						
Carbaryl						
CS	0.0419	- 0.00281	0.0500	7.40 (4.93, 11.1)	29.3	49.1
Malathion						
CS	0.0393	- 0.00720	0.0914	150 (83.8, 270)	371	707

[a] μg/L.
[b] Threshold values and their 95 % confidence limits (in parentheses) were derived from regression and back-transformed for comparison to no observed effect concentrations (NOEC) and lowest-observed-effect concentrations (LOEC).
[c] 32-d exposure period.
[d] Units of dependent variable = logits.
[e] Units of dependent variable = mg.
[f] 1-d exposure period.
[g] Units of dependent variable = μM substrate hydrolyzed/mg brain tissue/min.

Figure 1. Estimated linear-plateau regression lines for Colorado squawfish exposed to technical carbaryl. Length of exposure was 32 d for survival and weight, and 1 d for acetylcholinesterase (AChE) activity. Each point represents a mean: n = 2 for survival and weight; n = 3 for AChE activity. AU = μM substrate hydrolyzed/mg brain tissue/min.


SURVIVAL (logit)
6
4
2
0
-2
Weight
Survival
AChE Activity
Control
1
10
100
1,000
10,000
CARBARYL CONCENTRATION (ug/L)
WEIGHT (mg) and AChE ACTIVITY (AU X 1000)
70
60
50
40
30
20

CONCLUSION

In recent years, potential for exposure of Colorado squawfish and bonytail to insecticides as a result of grasshopper control operations has been reduced. A combination of relatively low grasshopper densities due to natural cycling of grasshopper populations, modification of pesticide application practices (e.g., the use of bran baits), and use of integrated pest management have decreased risk of exposure to synthetic insecticides. Whether these practices will be sufficient to control grasshopper densities during years of peak grasshopper abundance is unknown. If less invasive measures fail, aerial applications of synthetic insecticides will probably be used, and their effects will once again be of concern.

This study emphasized estimation of the toxicity of carbaryl and malathion to Colorado squawfish and bonytail using laboratory toxicity tests. Toxicant concentrations that significantly reduced survival, growth, and acetylcholinesterase activity were determined using standard procedures. Results of laboratory tests showed that concentrations of carbaryl and malathion, equivalent to those that may result from large-scale aerial applications, had toxic effects on young Colorado squawfish. This finding has some relevance when considering potential effects on wild Colorado squawfish and bonytail because in addition to being the most sensitive life stage, young Colorado squawfish and bonytail occupy shallow near-shore habitats that have low rates of water

exchange. Pesticides deposited in these habitats may be present for sufficient time for toxic effects to occur. However, extrapolation of laboratory results to the field is beyond the scope of this research and can be more accurately accomplished by ecological risk-assessment procedures.

Ecological risk assessment is a natural extension of laboratory toxicity testing. It integrates information from field and laboratory and provides an appraisal of the actual or potential effects of a contaminant. Because carbaryl and malathion have been so thoroughly studied, an ecological risk assessment for Colorado squawfish and bonytail would be relatively easy to complete. The only additional information required is an estimate of the potential distribution of life stages and species of concern. Sufficient information probably exists to complete this process for many of the threatened or endangered fishes of the Colorado River Basin.

Given the rare status of many fishes in the Colorado River Basin, some advocates may argue that it would be prudent to establish a moratorium on the use of synthetic pesticides near its rivers and streams. However, this alternative would be unacceptable to ranchers and farmers in the basin, and is probably not justified. Ecological risk assessment can provide justified guidelines for use of carbaryl and malathion that would allow effective control of insect outbreaks without significantly affecting populations of threatened or endangered fishes.

APPENDIX A

SUMMARIES OF EXPOSURE CONDITIONS AND MEASURED RESPONSES FOR COLORADO SQUAWFISH AND BONYTAIL EXPOSED TO TECHNICAL CARBARYL, SEVIN-4-OIL, AND TECHNICAL MALATHION

Table A1. Water quality characteristics of dilution water used in acute and chronic exposure studies of the toxicity of three insecticides to Colorado squawfish. Values are: mean (standard error).

	4-Day Exposure			32-Day Exposure	
Toxicant:	Technical carbaryl	Sevin-4-oil	Technical malathion	Technical carbaryl	Technical malathion
Dissolved oxygen (mg/L)	7.2(0.081)	7.1(0.081)	7.2(0.23)	6.1(0.13)	6.3(0.10)
pH	8.5(0.055)	8.6(0.050)	8.6(0.055)	8.1(0.071)	8.2(0.089)
Temperature (°C)	22.0(0.00)	22.8(0.12)	22.0(0.00)	21.4(0.037)	21.7(0.059)
Alkalinity (mg/L)	104(1.49)	110(1.32)	104(1.49)	249(3.42)	237(6.48)
Hardness (mg/L)	212(2.16)	216(3.38)	212(2.16)	362(3.42)	353(4.24)
Conductivity (μS/cm)	600(2.9)	600(0.0)	600(2.9)	780(7.6)	770(9.4)

Table A2. Water quality characteristics of dilution water used in acute and chronic exposure studies of the toxicity of three insecticides to bonytail. Values are mean (standard error).

	4-Day Exposure			32-Day Exposure	
Toxicant:	Technical carbaryl	Sevin-4-oil	Technical malathion	Technical carbaryl	Technical malathion
Dissolved oxygen (mg/L)	6.9(0.13)	6.8(0.17)	6.7(0.13)	7.0(0.050)	7.0(0.050)
pH	7.9(0.016)	7.9(0.016)	7.9(0.016)	8.0(0.038)	8.0(0.038)
Temperature (°C)	21.2(0.48)	21.2(0.48)	22.2(0.12)	21.8(0.10)	22.7(0.061)
Alkalinity (mg/L)	256(2.70)	256(2.70)	256(2.70)	259(3.04)	259(3.04)
Hardness (mg/L)	378(1.60)	378(1.60)	378(1.60)	344(1.62)	344(1.62)
Conductivity (μS/cm)	720(6.0)	720(6.0)	720(6.0)	754(20)	754(20)

Table A3. Summary of mortality of Colorado squawfish exposed to three insecticides for 4-d exposure period. Ten animals per replicate, two replicates per toxicant concentration. Measured toxicant concentrations are mean (standard error), n = 2.

Measured toxicant concentration (µg/L)	Cumulative dead per replicate			
	1 day	2 day	3 day	4 day
Technical carbaryl				
4210 (74.8)	10, 10			
3050 (79.6)	10, 6	10, 10		
2190 (105)	1, 0	10, 10		
1560 (52.9)	0, 0	4, 2	8, 6	10, 9
1180 (6.60)	0, 0	0, 0	2, 0	2, 1
solvent control	0, 0	0, 0	0, 0	0, 0
control	0, 0	0, 0	0, 0	0, 0
Sevin-4-oil				
4880 (86.5)	0, 0	7, 7	10, 10	
3980 (124)	0, 0	0, 1	4, 5	7, 7
3010 (137)	0, 0	0, 0	1, 1	3, 3
2100 (78.1)	0, 0	0, 0	1, 0	2, 2
1730 (156)	0, 0	0, 0	0, 0	0, 0
solvent control	0, 0	0, 0	0, 0	0, 0
control	0, 0	0, 0	0, 0	0, 0
Technical malathion				
14000 (201)	1, 0	2, 0	4, 4	10, 10
10000 (201)	0, 0	0, 0	0, 0	7, 8
8200 (355)	0, 0	0, 0	1, 1	2, 2
6200 (81.6)	0, 1	0, 1	0, 1	0, 1
5050 (170)	0, 0	0, 0	0, 0	3, 0
solvent control	0, 0	0, 0	0, 0	0, 0
control	0, 0	0, 0	0, 0	0, 0

Table A4. Summary of mortality of bonytail exposed to three insecticides for 4-d exposure period. Ten animals per replicate, two replicates per toxicant concentration. Measured toxicant concentrations are mean (standard error), n = 2.

Measured toxicant concentration (µg/L)	Cumulative dead per replicate			
	1 day	2 day	3 day	4 day
Technical carbaryl				
4780 (254)	0, 0	1, 3	8, 10	10, 10
3640 (262)	1, 0	4, 0	8, 4	10, 10
2760 (25.5)	0, 1	0, 2	0, 4	6, 8
1950 (85.9)	0, 0	1, 0	2, 1	7, 4
1650 (39.9)	0, 0	0, 0	1, 0	2, 3
solvent control	0, 0	0, 0	0, 0	0, 0
control	0, 0	0, 0	0, 0	1, 0
Sevin-4-oil				
8690 (274)	0, 0	9, 4	10, 10	
7310 (284)	1, 1	4, 3	9, 10	10, 10
5040 (192)	0, 0	3, 1	8, 6	10, 10
3980 (319)	0, 0	0, 1	4, 8	9, 9
3010 (22.5)	0, 0	0, 0	0, 0	2, 3
solvent control	0, 0	0, 0	0, 0	0, 0
control	0, 0	0, 0	1, 0	1, 1
Technical malathion				
34400 (986)	3, 2	7, 7	10, 9	10, 10
25800 (1540)	2, 0	3, 0	10, 8	10, 10
19000 (357)	0, 1	0, 1	8, 8	9, 10
14500 (234)	0, 0	0, 0	0, 0	2, 5
11600 (358)	0, 0	0, 0	0, 0	0, 0
solvent control	0, 0	0, 0	0, 0	0, 0
control	0, 0	0, 0	0, 0	0, 0

Table A5. Summary of survival and mean wet weight of Colorado squawfish exposed to technical carbaryl for 32 days. The initial number of animals in each replicate was 30. Measured toxicant concentrations are mean (standard error), n = 4.

Measured toxicant concentration (µg/L)	Replicate	Number surviving	Weight (mg)
1580 (126)	a	11	20.8
1580 (126)	b	2	24.0
866 (65.5)[a]	a	17	59.4
866 (65.5)[a]	b	14	68.2
445 (33.4)	a	29	56.5
445 (33.4)	b	29	57.6
210 (25.6)	a	29	66.4
210 (25.6)	b	30	62.6
116 (22.8)	a	28	60.3
116 (22.8)	b	30	66.2
solvent control[b]	a	27	62.6
solvent control[b]	b	30	57.0
control[b]	a	29	62.0
control[b]	b	29	68.3

[a]Data from this concentration excluded from regression analysis because of failure to adjust food ration for test-animal mortality.
[b]Toxicant concentration not verified by chemical analysis.

Table A6. Summary of survival and mean wet weight of Colorado squawfish exposed to technical malathion for 32 days. The initial number of animals in each replicate was 40. Measured toxicant concentrations are mean (standard error), n = 4.

Measured toxicant concentration (μg/L)	Replicate	Number surviving	Weight (mg)
3510 (165)	a	24	18.7
3510 (165)	b	28	18.8
1680 (37.0)	a	34	42.8
1680 (37.0)	b	36	40.8
881 (43.2)	a	32	46.0
881 (43.2)	b	39	42.6
394 (9.30)	a	39	46.0
394 (9.30)	b	37	38.0
212 (14.7)	a	40	45.7
212 (14.7)	b	38	49.5
solvent control[a]	a	38	47.7
solvent control[a]	b	40	44.2
control[a]	a	39	45.8
control[a]	b	39	45.1

[a]Toxicant concentration not verified by chemical analysis.

Table A7. Summary of survival and mean wet weight of bonytail exposed to technical carbaryl for 32 days. The initial number of animals in each replicate was 40. Measured concentrations are mean (standard error), n = 4.

Measured toxicant concentration (µg/L)	Replicate	Number surviving	Weight (mg)
1240 (53.8)	a	0	0.0
1240 (53.8)	b	2	47.4
650 (37.5)	a	36	56.7
650 (37.5)	b	39	56.8
348 (7.37)	a	40	61.9
348 (7.37)	b	40	59.7
158 (6.42)	a	40	68.2
158 (6.42)	b	40	68.3
80.6 (4.04)	a	40	60.7
80.6 (4.04)	b	40	64.8
1.42 (1.12)[a, b]	a	38	69.8
1.42 (1.12)[a, b]	b	39	64.2
0.350 (0.350)[a]	a	40	65.7
0.350 (0.350)[a]	b	39	68.7

[a]Trace contamination of dilution-water and solvent controls from air inside vented enclosure.
[b]Solvent control.

Table A8. Summary of survival and mean wet weight of bonytail exposed to technical malathion for 32 days. The initial number of animals in each replicate was 40. Measured toxicant concentrations are mean (standard error), n = 4.

Measured toxicant concentration (µg/L)	Replicate	Number surviving	Weight (mg)
7950 (1080)	a	0	0.0
7950 (1080)	b	0	0.0
4060 (463)	a	5	8.14
4060 (463)	b	2	12.3
2000 (184)	a	35	25.2
2000 (184)	b	39	29.2
990 (74.0)	a	40	42.0
990 (74.0)	b	39	44.5
522 (52.0)	a	36	66.3
522 (52.0)	b	39	68.0
0.950 (0.323)[a, b]	a	40	62.0
0.950 (0.323)[a, b]	b	40	60.0
1.20 (1.00)[a]	a	39	68.3
1.20 (1.00)[a]	b	39	66.0

[a]Trace contamination of dilution-water and solvent controls from air inside vented enclosure.
[b]Solvent control.

Table A9. Summary of brain acetylcholinesterase (AChE) measurements from Colorado squawfish exposed to technical carbaryl for 1 d. Measured toxicant concentrations are mean (standard error), n = 2. AU = μM substrate hydrolyzed/mg brain tissue/min.

Measured toxicant concentration (μg/L)	Replicate	AChE activity (AU)
924 (80.7)	a	0.0228
	b	0.0252
	c	0.0207
481 (28.0)	a	0.0294
	b	0.0220
	c	0.0222
225 (0.706)	a	0.0298
	b	0.0242
	c	0.0315
99.0 (13.1)	a	0.0317
	b	0.0344
	c	0.0304
54.6 (11.0)[a]	a	0.0352
49.1 (2.85)	a	0.0344
	b	0.0300
	c	0.0322
29.3 (2.70)	a	0.0337
	b	0.0378
	c	0.0368
15.4 (0.658)	a	0.0335
	b	0.0369
	c	0.0431
7.43 (1.03)	a	0.0399
	b	0.0426
	c	0.0486
3.93 (0.249)	a	0.0409
	b	0.0462
	c	0.0398
0.834 (0.746)[b]	a	0.0416
	b	0.0470
	c	0.0513
0.395 (0.0940)[b]	a	0.0389
	b	0.0402
	c	0.0457
0.200 (0.0380)	a	0.0367
	b	0.0415
	c	0.0426
0.200 (0.0951)	a	0.0390
	b	0.0335
	c	0.0437

[a]Replicates b and c lost.
[b]Solvent control.

Table A10. Summary of brain acetylcholinesterase (AChE) measurements from Colorado squawfish exposed to technical malathion for 1 d. Measured toxicant concentrations are mean (standard error), n = 2. AU = μM substrate hydrolyzed/mg brain tissue/min.

Measured toxicant concentration (μg/L)	Replicate	AChE activity (AU)
1330 (25.5)	a	0.0195
	b	0.0197
	c	0.0161
708 (29.1)	a	0.0297
	b	0.0145
	c	0.0142
371 (4.80)	a	0.0288
	b	0.0349
	c	0.0329
175 (2.83)	a	0.0374
	b	0.0374
	c	0.0376
84.6 (6.76)	a	0.0423
	b	0.0430
	c	0.0346
0.624[a, b]	a	0.0383
	b	0.0401
	c	0.0401
0.200 (0.001)	a	0.0411
	b	0.0367
	c	0.0377

[a]Solvent control.
[b]Measured concentration of one replicate.