Proposal to:

CA DEPT. OF WATER RESOURCES / IEP
http://www.water.ca.gov/iep/

Submitting Organization:

The Regents of the University of California
Office of Research, Sponsored Programs
1850 Research Park Drive, Suite 300
University of California
Davis, California 95618

Title of Proposed Research:

Adverse Outcome Pathway Characterization for Three Neurotoxic Pesticides and their Mixt

Total Amount Requested:	Proposed Duration:	Desired Starting Date:
$217,052	1 year	01/01/13
Principal Investigator:	**Department:**	**Phone Number:**
Swee J. Teh	VM: Anatomy, Physiology &	530-754-8183

Checks Made Payable to:

The Regents of the University of California

Send Checks to:

Cashier's Office
University of California Davis
PO BOX 989062
West Sacramento, California 95798-9062

Send Award Notice to:

Office of Research
Sponsored Programs
1850 Research Park Drive
University of California
Davis, California 95618
(530) 754-7700 / FAX (530) 754-8229
vcresearch@ucdavis.edu

Approvals:

Swee Teh 9/4/12
Principal Investigator Date

Fern Tabe 9/4/12
Department Chair Date

KC Kent Lloyd 9/4/12
Dean, College/School Date
(if required)

Other Endorsement Date
(optional)

9-4-12
Official Signing for Organization Date
Erlita Oliver
Contracts and Grants Analyst, Sponsored Programs

I. Name of the Principal Investigator with affiliation:
Dr. Swee Teh - School of Veterinary Medicine, University of California, Davis.

II. Name of Co-Investigator(s), with affiliation(s) and role in proposed project:
There is no Co-investigator in this project. However, this study is a collaboration across multiple labs. Roles will be divided as such: Toxicity tests, enzyme analyses and histopathology will occur in Dr. Teh's Lab (above), molecular analysis in Dr. Connon's Lab (School of Veterinary Medicine, UC Davis), and chemical analyses of water samples in Dr. Young's Lab (Department of Civil Engineering, UC Davis).

III. Project title: Adverse Outcome Pathway Characterization for three Neurotoxic Pesticides and their Mixtures in *Menidia beryllina*

IV. Total budget: The total budget for this project is $217,052 (54% indirect costs)

V. Study duration: Funding is requested for a one-year period

VI. Priority research topic and questions addressed and overall relevance:
Our research addresses topic II: *Physical and chemical habitat effects on fish populations,* and will focus on lethal and sublethal effects of pesticides individually and in mixtures on Inland silverside, *Menidia beryllina* (question D).

Relevance: Contaminants are prolific in the aquatic environment and regulations capable of minimizing species and environmental impacts must be developed. One major challenge is to gain understanding of biological effects and interactions of contaminants and other stressors, especially for those that commonly occur and have been linked to toxicity (Lydy *et al.* 2004). This study will generate novel information about the mechanisms of effects (adverse outcome pathways) of three neurotoxic pesticides of concern in the Sacramento San Joaquin Delta, bifenthrin, chlorpyrifos and fipronil, both applied singly and in binary mixtures. Adverse outcome pathways will be investigated via multiple endpoints over the course of 4-day exposures and a recovery period, including effects on molecular and cellular levels (gene and enzyme activity), sublethal endpoints (growth, histopathology, and swimming behavior), and organism responses (deformity and mortality) (Figure 1). Our results will build the basis for understanding the mechanisms by which neurotoxic pesticides and their mixtures cause impairment in fish. These findings will help to understand the effects of environmental drivers, such as pesticides, to conservation and management actions within in the Delta Plan.

VII. Overall project purpose:

By using the standard indicator species *M. beryllina*, the proposed study provides a comprehensive analysis of the lethal, sublethal and mixture toxicity of three neurotoxic pesticides of concern; bifenthrin, chlorpyrifos and fipronil.

In detail, the following research questions will be investigated:

1. What are the adverse outcome pathways of bifenthrin, chlorpyrifos and fipronil over acute exposures?
   We hypothesize that genes and enzymes associated with neurological function will respond across all three neurotoxic pesticides according to their mechanisms of action, and that physical effects (reduction in mobility and growth) will follow such molecular responses, and ultimately result in mortality.
2. How do three neurotoxic pesticides interact in mixtures, and how do these interactions manifest on multiple endpoints including mortality, swimming behavior, growth and gene and protein activity?
   It is our hypothesis that the additive responses seen on lethal and sublethal endpoints in other standardized toxicity test species will be apparent in *M. beryllina*, as well as on the selected neurological gene and enzyme targets.
3. What are the long-term effects of low-level acute pesticide exposures after 14 and 28 days of recovery?
   Our hypothesis is that low-level exposures to pesticides will continue to cause long-term health effects, even after the toxicants are withdrawn and fish are placed in clean water.

This study will inform water quality regulations that support the protection of the Sacramento-San Joaquin Delta ecosystem. We will publish at least one peer-reviewed publication in a journal of significant impact; e.g. Environmental Toxicology and Chemistry or other scientific journals with similar impact factors.

VIII. Project background and conceptual model:

Background: Resident fish species of the Sacramento-San Joaquin River Delta are exposed to a mixing pot of contaminants and stressors in the freshwater regions of the estuary, often during their most sensitive life stages. Recent declines in many of these species may be due to the cumulative effects of multiple sublethal or low-level stressors. As such, the proposed study aims to investigate the adverse outcome pathways (AOP) of three neurotoxic pesticides, bifenthrin, chlorpyrifos and fipronil, and their binary mixtures, on the inland silverside (*M. beryllina*). *M. beryllina* is an EPA approved indicator species for estimating toxicity of effluents and receiving waters to estuarine organisms (U.S.EPA 2002). It is one of three species in the atherinid family that are amenable to laboratory culture; and one of four atherinid species used for chronic toxicity testing. *M. beryllina* is an important species within the estuarine food web and populates a variety of habitats throughout coastal North America including the SSJ Delta (Clark *et al.* 1985;

Middaugh and Hemmer 1992, Matern et al. 2002). Their habitat usage and life history characteristics are comparable to the delta smelt.

Bifenthrin, chlorpyrifos and fipronil were selected for this study because they are all neurotoxins with different neurological target sites of action, and are abundantly used in agriculture, are highly toxic to fish, and are pervasive in the environment (Sprague and Nowell 2008; Trimble *et al.* 2009; CEPA 2009). While bifenthrin and chlorpyrifos are well documented contaminants of concern in the SSJ Delta (Budd *et al.* 2007; Weston and Lydy 2010), fipronil is of rising ecological scrutiny. Fipronil is widely used in structural pest control and other urban and agricultural applications (Oros and Werner 2005) and is present in runoff from metropolitan areas throughout the United States (Sprague and Nowell 2008). A study conducted by the Department of Pesticide Regulation in 2008 implicated fipronil as the most detected pesticide (among a suite of 41 measured) in urban drains across California (Cowley et al. 2008). Sprague and Nowell also found fipronil to be the most abundantly detected pesticides in urban runoff at selected sites across the U.S., and particularly one of the most potent to fish (2008).

Previous work in our lab has demonstrated additive toxicity between chlorpyrifos and bifenthrin on both lethal and sublethal endpoints in the aquatic amphipod, *Hyalella azteca* (Callinan *et al.*, in prep), despite their slight differences in target sites of action. The proposed study will provide a cross-phyla comparison between *H. azteca* and *M. beryllina* with regards to the toxic effects of these pesticides on mortality, swimming and growth for both individual chemicals, and their binary mixtures. Fipronil will be included in this study to both characterize the adverse outcome pathway in fish for this pesticide of rising concern, and also to determine whether a similar pattern of additive toxicity occurs when mixed with other neurotoxic pesticides, such as chlorpyrifos or bifenthrin.

Enzymes including acetylcholinesterase, sodium (Na+)/ potassium (K+) ATPase, and the ethoxyresorufin-O-deethylase (EROD) pathway have previously been shown to be reliable biomarkers for neurotoxic effects of pesticides (Dutta and Arends 2003; Kakko et al. 2003; Binelli et al. 2006). Based on these findings, we will investigate enzyme responses as well as associated gene activity following pesticide exposures.

Conceptual Model: Our conceptual model is graphically illustrated in Figure 1. An AOP is a conceptual construct that portrays existing knowledge concerning the associations between a toxicant's activity at one or more biological target sites and the resulting effects on the organism, community and ecosystem. The pathway theoretically starts with a direct molecular initiating event (e.g., a molecular interaction between pyrethroids and sodium channels) and an adverse outcome at a biological level of organization relevant to risk assessment such as mortality and development (Figure 2) (Ankley *et al.* 2010). Such associations provide the critical foundation for greater use of predictive approaches in ecotoxicology and ecological risk assessment. With the help of this conceptual model we will be able to establish the relevance of molecular initiating

events and intermediate endpoints to outcomes that ultimately affect organism fitness (e.g., survival and development). We will obtain mechanistic and behavioral data at different levels of biological organization; gene transcription (qPCR), enzyme response, cellular damage, growth and swimming behavior; that will be useful for predicting and modeling responses on an organism, and ultimately, community and population level.

IX. Estimated number of all FESA and ESA-listed fishes that would be captured by the field component of your study: Fish used in this study will be purchased commercially and are not listed under the FESA or ESA.

X. Project description:
The objectives of our proposal will be achieved in a single task which is designed to answer our specific research questions.

Task: Test the individual and mixture effects of bifenthrin, chlorpyrifos and fipronil on *M. beryllina* survival, growth, swimming behavior, molecular activity, enzyme activity and cellular stress:

Investigator(s) responsible for carrying out the task: Dr. Swee Teh and Graduate Students, Krista Callinan and Simone Hasenbein; with assistance from Dr. Connon. A lab manager (Linda Deanovic), junior specialist, and student assistants will support the study completion.

Specific questions the task seeks to address:
What are the adverse outcome pathways for bifenthrin, chlorpyrifos and fipronil on *M. beryllina* over acute exposures? How do the three neurotoxic pesticides interact in mixtures, and how do these interactions manifest through multiple endpoints including gene and protein activity, growth abnormality, histopathology, swimming behavior, and mortality? What chronic effects do we see in low concentrations of these chemicals, and their mixture, after a 14 and 28 day recovery periods?

Approach and methods:
Overview
Adverse outcome pathways for chlorpyrifos, bifenthrin and fipronil and their mixture will be tested using 10-14 day-old *M. beryllina* larvae.

Our mixture design, illustrated in Figure 3, is based on the toxic unit (TU) approach to assess the joint toxicity of the binary mixtures of the assessed pesticides. In this model, the 96-hour LC_{50} of each chemical equals 1 TU. The LC_{50} for each pesticide (chemical A and B) will be obtained from both in-house single-chemical tests (subtask A) and U.S. EPA Ecotox database. According to the toxic unit approach (theory), the sum of the TUs from each component of a mixture describes the toxicity of a mixture by:

$$1TU = 1/2\ LC_{50}(A) + 1/2\ LC_{50}(B).$$

Pesticides will be applied in a factorial design of mixture concentrations containing both equitoxic and non-equitoxic treatments. A series of individual pesticide concentrations will also be tested within each experiment (Figure 3).

A detailed description of our methods is provided below. In brief, five treatment concentrations of each individual chemical and a four-by-four factorial of mixture treatments, as well as a vehicle and non-vehicle control, will be included in each experiment. One replicate beaker per treatment will be sampled after six hours of exposure for gene analysis, to capture changes in expression at the onset of exposure but prior to acclimation or lethality, leaving four replicate beakers for the remainder of the test. After 96 hours, the remaining four replicate beakers will be sampled to test for swimming behavior, weight, and gene and protein activity. Fish from each replicate beaker will be split between swimming and weight measures, and gene and enzyme assays. Gene and enzyme assays will be conducted on the same split samples for the most congruent results. Extra replicates beakers for a few selected low-level treatments will be added at the initiation of each experiment to be tested for recovery, as well as chronic and developmental effects. Fish from these replicates will be transferred to clean water and tested for an additional 28 days.

The time table of subtasks proposed for this study is outlined in Table 1, which can be found in the Appendix.

Subtask A: *Optimize experimental conditions for M. beryllina and test LC_{50}s*
Experimental conditions for *M. beryllina* will be tested and optimized through preliminary exposures and analyses, based on standard EPA protocols (EPA WET method 1006.0, U.S. EPA 2002). Lethal concentrations will then be tested over a series of 96-hour exposures. Each 1 Liter treatment beaker will contain 750 ml of treatment water, with four replicates of 15 fish per beaker (density based on EPA WET method recommendations). Seven or more concentrations will be tested per chemical. Treatments will be renewed (75% of the water replaced with fresh treatment water), fed and scored daily. Results will be analyzed for LC_{50}, LOEC and NOEC data using CETIS (version 1.7) statistical software.

Subtask B. *Acute Mixture Toxicity Tests – Lethality (dependant on subtask A)*
Toxicity tests with *M. beryllina* will be conducted at the Aquatic Health Program Laboratory, UC Davis. Control (matrix) water will be prepared based on U.S. EPA guidelines (U.S. EPA 2002) and used for all exposures. Stock solutions will be made in methanol (vehicle control) and all treatments will be spiked with a combination of stock and methanol as needed to attain 0.01% vehicle control. Fish embryos will be purchased from Aquatic Biosystems (Fort Collins, CO) and hatched and reared in test control water using protocols outlined in the U.S. EPA guidelines.

Tests will be initiated using 10-14 day old larvae. Treatment concentrations will include five levels of each individual chemical (to test for individual chemical effects) and a four by four factorial of mixture concentrations (to analyze for mixture interactions). Fifteen fish will be tested per beaker containing 750 mL of treatment water, with four replicate beakers per treatment. Test chambers will be lightly aerated (<100 bubbles/min) and incubated in a temperature-controlled environmental chamber or water bath maintained at 20°C ±1°C under a 16-hour light: 8-hour dark photoperiod. Fish will be fed *Artemia* nauplii once per day at least one hour prior to 75% water renewal, at which time mortality will be scored and beakers randomized.

Water quality parameters of temperature, electrical conductivity, pH, dissolved oxygen will be measured on all samples at test initiation, prior to and after water change, and at test termination. Samples for pesticide analysis will be preserved by the addition of 10 ml/L dichloromethylene immediately after treatment waters are prepared (day 0) and stored at 4°C. Analyses will be performed by Simone Hasenbein in Dr. Thomas Young´s laboratory (Department of Civil & Environmental Engineering, UC Davis). Samples will be extracted, and analyzed using gas chromatography negative chemical ionization mass spectrometry (GC-NCI-MS).

Dose response curves, LC_{50}, LOEC (low effect concentration) and NOEC (no effect concentration) data will be generated. Lethal and sublethal mixture toxicity data will also be analyzed for interactions using generalized linear models (GLM) and will be statistically tested for fit against two theoretical models of additivity, the model of Concentration Addition and the model of Independent Action (Loewe and Muischnek 1926, Bliss 1939). Additive models will be tested for fit via a modified multiple logistic regression analysis. All model analyses will occur using the program R.

Subtask C: *Swimming and growth abnormality (dependant on subtasks A and B)*
Swimming behavior and condition factor (weight and length) will be measured on a subsample of fish at the onset of each experiment, after the 96-hour exposures and during the recovery study. Individual fish will be collected, placed in a well (on a custom built five-welled plate) filled with their treatment water and recorded by video camera for two minutes. Image analysis will be performed using Ethovision Behavior Software XT 6 (Noldus Information Technology Inc. Leesburg, VA). Organisms will be tracked based on the method of dynamic subtraction at a speed of 29 data-points per second. Quantitative behavioral performance parameters such as distance moved, velocity and levels of mobility will be analyzed. Other more complex analyses including rotation, angular velocity, meander and turn angle may also be measured.

Following swimming analysis, fish will be rinsed using a fine-meshed sieve to retain the larvae and allow any residue of *Artemia* and/or salts to be removed. Larvae will be examined grossly for deformity, immediately sacrificed with MS-222, then weighed and measured for analysis of condition factor.

Effect concentrations, including EC_{50}s, LOECs and NOECs will be calculated for both sublethal endpoints. Generalized linear models will be used to test for significant pesticide effects on either endpoint as well as for significant mixture interactions. Mixture responses will be further characterized by fitting data to either the model of CA or IA, as described under subtask B.

Subtask D: *Molecular analysis (dependant on subtasks A and B)*
Fish will be sampled immediately prior to exposure tests (Foe *et al.*), as well as six and 96 hours after test initiation. Fish will be euthanized with a lethal dose of MS-222 and frozen at -80°C until RNA extraction for quantitative PCR. Exposed samples will be analyzed against the corresponding controls. RNA expression of genes related to neuromuscular processes (e.g. Creatine Kinase, aspartoacylase, Na+/K+ ATPase, calcium channels and others associated with the enzymes mentioned) will be quantified using qPCR. Samples will be prepared in Dr. Connon's lab, and assessments conducted at the Lucy Whittier facility at UC Davis. Samples will be placed in 384-well plates and amplified in an automated fluorometer (ABI PRISM 7900 Sequence Detection System, Applied Biosystems).

SDS 2.2.1 software (Applied Biosystems) will be used to quantify gene amplification in real-time. Gene expression levels will be calculated using the relative quantification 2(-Delta Delta CT) method (Livak and Schmittgen 2001). Beta-actin, a commonly used house-keeping gene, will be used as reference to normalize expression of the entire suite of genes. We will perform cluster analysis and gene expression profiling to aid mechanistic interpretation of responses. Principal component analysis (PCA) will be conducted in conjunction to correlation heatmaps, to ascertain factors (such as the toxicants in a mixture) responsible for response variability.

Subtask E: *Enzyme analysis (dependant on subtasks A and B)*
Preserved fish will be homogenized and split between molecular and enzyme analysis. Sample and preservation methods are described under Subtask D. Acetylcholinesterase, Na^+/K^+ ATPase and EROD will be tested using methods based on standard protocols from the literature. Acetylcholinesterase analyses will follow methods described in McCormick and Bern (1989); Na^+/K^+ ATPase analyses will be based on the Ellman method (Ellman et. al. 1961); and EROD will be based on Nilsen et. al. (1998). All analyses will be optimized for *M. beryllina* prior to the initiation of this study. Negative (buffer only) and positive control samples will be run in concert within each test, and where applicable internal and external standards will be implemented. Samples will be analyzed using a Decan Infinite 200 UV Spectrophotometer. Protein content will be measured (Lowry et. al. 1951) and activity will be normalized to total protein. Generalized linear models statistics will be employed to test for pesticide effects on enzyme activity using the program R. ANOVA statistics, followed by a Dunnetts post hoc analysis, will be used to test for differences between treatments.

Subtask F: *Recovery (dependant on subtasks A and B)*

Recovery and chronic toxicological effects will be tested in a 28-day follow-up study using fish from extra replicates amended to selected low-level treatments (¼ TU) and their mixture in each experiment (Figure 3). In total, the recovery study will include four treatments: One binary mixture, their two respective individual concentrations, and the control. Following the 96-hour pesticide exposure, fish will be transferred to clean tanks containing control water only. Eighty percent of water will be renewed and water chemistry measured daily to ensure optimal conditions. On day 14 and 28 of recovery, swimming behavior, growth, enzymatic activity and histopathology (analysis of tissue and cellular stress) will be analyzed as detailed above by collecting 50% of surviving fish per tank. Abnormal development and mortality will be scored daily. Growth and histopathological analysis will follow standard protocol of the Aquatic Health Program as described by Teh et al. (2004).

Interaction with existing monitoring surveys or other studies:

This study is a collaboration between graduate students and researchers in Dr.Teh's lab, and assistance from Dr. Connon's lab at UC Davis, and builds upon previous contaminant and mixture investigations conducted over the past two years, including: "The Toxicity and Interactions among Common Aquatic Contaminants in Binary Mixtures," (Callinan *et. al.* 2012) and "Effect assessment of tertiary pesticide mixtures on the amphipod *Hyalella azteca* and the midge *Chironomus tentans*" (Hasenbein *et. al.* 2012). This project provides in depth analyses of pesticide effects on a potentially useful model species, *M. Beryllina,* and will complement investigations concurrently proposed to IEP by Connon et. al. "What is sensitivity? A multi-species comparison."

Feasibility:

Personnel

The feasibility of this project is very high. Dr. Swee Teh and Dr. Richard Connon are highly qualified and suitably experienced to carry out all aspects of this investigation. The proposed molecular, physiological and behavioral and histopathological methodologies are routinely used by both laboratories and the majority of equipment needed to conduct this study is readily available.

Dr. Teh is a comparative aquatic toxicologist/pathologist with extensive field and laboratory research experience in carcinogenesis, ecotoxicology, endocrine disruption, enzymatic and histopathologic biomarker studies. He has extensively studied the development and application of acute and chronic toxicity test protocols and the interpretation of toxicity data, using protocols involving both vertebrate and invertebrate species.

Dr. Connon is currently researching sublethal molecular and behavioral endpoints in fish at the University of California Davis, and is highly experienced at both ecological and molecular aspects of the proposed study. He will be responsible for training and overseeing molecular analyses in his laboratory.

Krista Callinan is a PhD student under the Graduate Group of Pharmacology and Toxicology. She has worked at the Aquatic Health Program Laboratory over the past three years conducting aquatic toxicity tests using multiple standardized methods. Krista's PhD research focuses on investigating and modeling the interactions between toxic chemicals in mixtures and their physiological effects on aquatic species. Using *Hyalella azteca* as a model test species, Krista has successfully characterized and quantified interactions between the most commonly used pyrethroid pesticides, as well as pyrethroids in combination with chlorpyrifos, copper and ammonia (Callinan et. all. 2012, publications in prep). Krista is also interested in molecular measures of contaminant stress, and is currently working with Sai Krithika, out of the Teh lab, to optimize methods for AChE, Na^+/K^+ ATPase and EROD analyses for *Pimephales promelas* and *M. beryllina.* Sai has extensive experience with such analyses through her Master's research and current investigations in the Teh lab, and has and will serve as an excellent mentor for these techniques.

Simone Hasenbein is a PhD student that is fully funded (stipend and student fees) by the Bavarian Research Foundation, Germany. She has conducted several toxicity tests within her ongoing PhD research. Chemical analyses will be conducted in Dr. Thomas Young's laboratory (Department of Civil Engineering at UC Davis) where the proposed methods are of common practice. Simone Hasenbein has been trained by Dr. Young's staff to conduct the analyses of water samples, and has conducted these assessments during her previous study "Effect assessment of tertiary pesticide mixtures on the amphipod *Hyalella azteca* and the midge *Chironomus tentans*" granted by the California State Water Resources Control Board to Dr. Connon and Simone Hasenbein (Agreement No. 06-447-300).

Facilities and equipment

The UCD Aquatic Health Program (AHP) Laboratory of the School of Veterinary Medicine (University of California Davis, www.ucdavis.edu/apc/atl) is a State-certified lab with over 20 years of experience in investigating surface water quality and aquatic ecosystem health in watersheds throughout California. AHP activities focus on conducting aquatic organism toxicity tests, as defined by the United States Environmental Protection Agency (US EPA), using standard as well as resident aquatic species (Werner *et al.* 2004; Werner *et al.* 2010; Connon *et al.* 2011). Facilities include a 3200 ft^2 main laboratory building fully equipped to conduct various toxicant exposure and surface water monitoring studies, supplied with non-chlorinated well water, deionized water, distilled water and Milli-Q purified water. Five temperature-controlled water baths totaling 153 ft^2 provide exposure space for a number of fish species. Additional equipment housed in the lab include dissecting scopes, three scales, three pH meters, three electrical conductivity meters, three YSI dissolved oxygen meters, hardness and alkalinity titration stations and an autoclave. In addition, there is a 280 ft^2 negative pressure room equipped with a 6 ft^2 fume hood and a vented, temperature controlled exposure chamber.

The biochemical and molecular laboratory, managed by Dr. Connon is located in Haring Hall, Rooms 2160 and 1341, at the University of California, School of Veterinary Medicine, Dept. of Anatomy, Physiology and Cell Biology, and occupies approx. 1000 ft^2. It is fully equipped for biochemical and molecular biomarker analyses. Routine RNA extractions, cDNA synthesis and quantitative polymerase chain reaction (qPCR), poly acrylamide gel electrophoresis (PAGE), and a number spectrophotometric protein assays, are performed by staff and students. Available equipment includes, but is not limited to an Eppendorf thermocycler, vacuum centrifuge DNA concentrator, refrigerated centrifuge, nano-drop for nucleotide and protein concentration, molecular and biochemical electrophoresis systems, gel densitometer, UV imaging system, various cell incubators, 96-well spectrophotometer, 384 and 86-well fluorometer, a sterilizing biosafety and an externally vented flow hood. Furthermore this laboratory is set up with all necessary equipment to perform manual microarray hybridizations. Microarray scanning is carried out at facilities within the University.

Deliverables

We will provide brief quarterly reports, which will describe the activities undertaken, accomplishment of the milestones, and any problems encountered in the performance of the work. A final report will be prepared to include a description of methods, a summary of raw data and associated statistical analyses, results of all work to ensure QA/QC, and a discussion of the results including conclusions of the study. The final report will be submitted to the IEP within a year of the start of the project.

This study will result in at least one peer-reviewed publication in a journal of significant impact; e.g. Environmental Science and Technology or other scientific journals with similar impact factors. Findings will be presented at scientific conferences. Targeted annual conferences include Delta Science Program, Society of Environmental Toxicology and Chemistry (SETAC), and related conferences and workshops. Further this study will be a chapter of both graduate students' PhD Theses.

Detailed Budget					
CATEGORIES	**Role/Title**		**% Effort**	**01/01/13 - 12/31/13**	**TOTAL**
A. PERSONNEL (Allows 3% COLA)				2013-14	
Swee J. Te h	PI/Researcher	101,190	3%	3,036	**3,036**
Linda Deanovic	SRA IV	71,990	17%	12,238	**12,238**
Krista Callinan	Grad Stud. Researcher	51,972	47%	24,427	**24,427**
To Be Named	Junior Specialist	34,680	50%	17,340	**17,340**
Student Assistants (4 @ 8.50/hr)			25%	17,680	**17,680**
SUBTOTAL PERSONNEL COSTS				74,721	**74,721**
B. FRINGE BENEFITS (Based on UCD Proposed Blended Rates)				**2013-14**	**TOTAL**
Swee J. Te h	PI/Researcher			965	**965**
Linda Deanovic	SRA IV			5,642	**5,642**
Krista Callinan	Grad Stud. Researcher			318	**318**
To Be Named	Junior Specialist			7,994	**7,994**
				230	**230**
TOTAL FRINGE BENEFITS				15,148	**15,148**
TOTAL PERSONNEL COSTS				**89,869**	**89,869**
C. TRAVEL					
PIs and staff to attend Scientific Meeting				1,000	**1,000**
TOTAL TRAVEL COSTS				1,000	**1,000**
D. EQUIPMENT: None Requested					
TOTAL EQUIPMENT COSTS				0	**0**
E. SUPPLIES					
Mixture Toxicity Testing				20,000	**20,000**
Enzymatic Biomarkers				5,000	**5,000**
Molecular analysis				10,082	**10,082**
Biohazard disposal				4,000	**4,000**
Publication costs				1,000	**1,000**
TOTAL SUPPLY COSTS				40,082	**40,082**
F. CONTRACT/SERVICE AGREEMENT					
TOTAL CONTRACTUAL COSTS (not exempt from F&A)				**0**	**0**
G. OTHER EXPENSES					
Graduate Student Fee Remission (Projected 7% annual increase)				15,387	**15,387**
TOTAL OTHER COSTS (exempt from F&A)				15,387	**15,387**
H. TOTAL DIRECT COSTS (sum of a through g)				146,338	**146,338**
Modified Total Direct (less fees/tuition)				130,951	**130,951**
I. Indirect Costs/Charges (54%, MTD)				70,714	**70,714**
J. TOTAL PROJECT COSTS (sum of h & I)				217,052	**217,052**

Budget Justification

A. Personnel

Swee J. Teh, PhD (3%) is the Principal Investigator (PI) and will be responsible for details of contract management and execution, as well as ensuring coordination among tasks. Staff Research Associate, Linda Deanovic (17%) and graduate student, Krista Callinan (47%) will be the onsite supervisor for the majority of laboratory. Ms. Callinan, with the help of a junior specialist (50%) and 4 student assistants (25%) will execute a number of toxicity tests and exposures. Together, they will also be responsible for maintaining organism cultures, entering and analyzing test data, and organizing electronic files. Dr. Teh, along with graduate student Callinan and a junior specialist, will be responsible for all biomarker analyses. Student Assistant will also assist with day-to-day activities including washing glassware, labeling glassware and measuring water quality parameters. Quarterly and annual reports will be generated as contract deliverables which require the participation of the PI, SRA, and graduate student.

B. Fringe Benefits

Fringe benefits rates and annual increases are defined by the University's composite fringe benefit rates for fiscal years 2013-2014.

C. Travel

Travel will cover costs associated with meetings where key findings will be presented. Generally, these will occur in the greater Sacramento area.

D. Equipment

None requested

E. Supplies

Mixture toxicity recharges services and test organisms ($20,000). Laboratory supplies include 1) biochemical and histopathological biomarker reagents, enzyme, antibodies, histological slides and stains ($5,000); 2) molecular and chemical analysis ($10,082), 3) Biohazard disposal ($4,000) and 4) publication costs ($1,000).

F. Other Expenses

Graduate student fees will support the single graduate student's tuition costs ($15,387).

G. Indirect Costs

F&A is calculated using the UCD Rate Agreement dated 06/27/2011.

Appendix

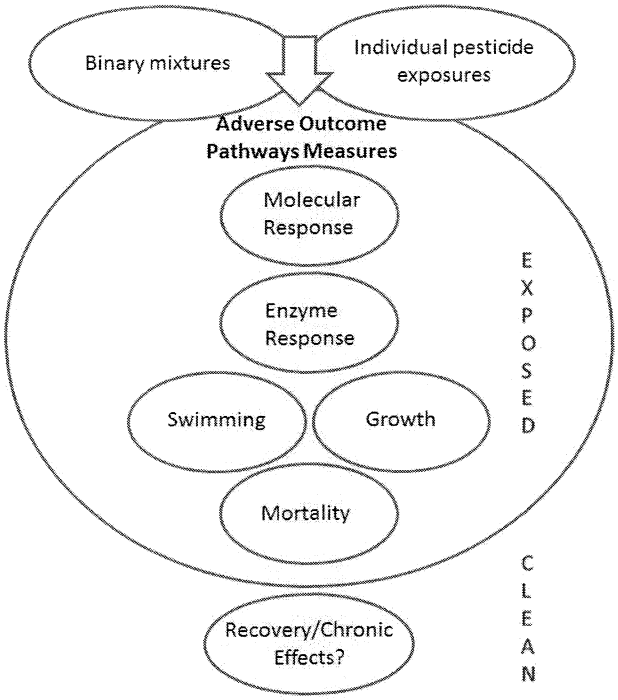

Figure 1. Conceptual Model of endpoints measured following pesticide exposures to binary mixtures and individual pesticides. Fish will be exposed to pesticides for 96 hours and then transferred to clean water where recovery and potential chronic effects will be measured.

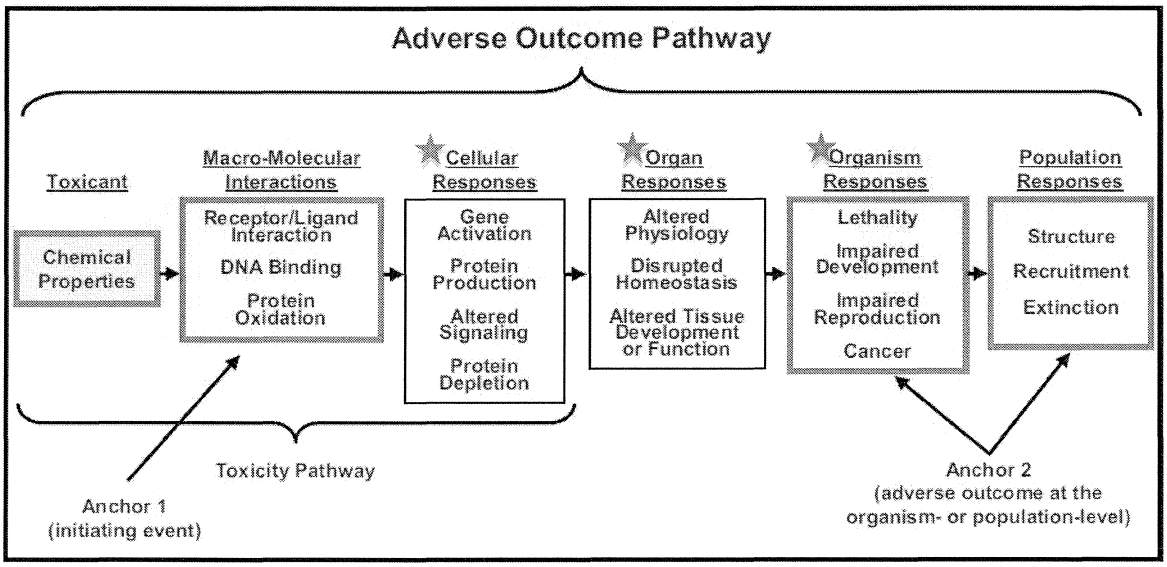

Figure 2. Diagram of key features of an adverse outcome pathway (AOP). Each AOP begins with a molecular initiating event in which a chemical interacts with a biological target (anchor 1) leading to a sequential series of higher order effects to produce an adverse outcome with direct relevance to a given risk assessment context (e.g., survival, development etc.; anchor 2) (Ankley *et al.* 2010). Endpoints measured within the proposed study are starred.

Chemical B	Chemical A				
	0	1/4	1/2	1	1.5
	1/4	1/2	3/4	1.25	
	1/2	3/4	1	1.5	
	1	1.25	1.5	2	
	1.5				

Figure 3. Experimental design. Blue shading represents the levels of individual chemical treatments and grey shows mixture treatments. Numbers indicate toxic units (TUs) based on the 96-hour LC50 for each chemical. Mixture TUs are theoretical assuming dose additivity (TU approach). Treatments with a darker shade are those that will be analyzed for recovery (subtask f).

Table 1. Time schedule for testing adverse outcome pathways.

Exposure period	Score Mortality	Swimming and Growth	Molecular Analysis	Enzyme Analysis	Histopathology
0 hours	x	x	x	x	
6 hours			x		
96 hours	x[1]	x	x	x	x[2]
18 day (14 days recovery)	x[1]	x	x	x	x
32 days (28 days recovery)	x[1]	x	x	x	x

[1]to be scored daily
[2]only recovery treatments

Literature cited

Ankley, G.T., Bennett, R.S., Erickson, R.J., Hoff, D.J., Hornung, M.W., Johnson, R.D., Mount, D.R., Nichols, J.W., Russom, C.L., Schmieder, P.K., Serrrano, J.A., Tietge, J.E. and Villeneuve, D.L. (2010). "ADVERSE OUTCOME PATHWAYS: A CONCEPTUAL FRAMEWORK TO SUPPORT ECOTOXICOLOGY RESEARCH AND RISK ASSESSMENT." Environmental Toxicology and Chemistry **29**(3): 730-741.

Binelli, A., Ricciardi, F., Riva, C. and Provini, A. (2006). "New evidences for old biomarkers: Effects of several xenoblotics on EROD and AChE activities in Zebra mussel (Dreissena polymorpha)." Chemosphere **62**(4): 510-519.

Budd, R., Bondarenko, S., Haver, D., Kabashima, J. and Gan, J. (2007). "Occurrence and Bioavailability of Pyrethroids in a Mixed Land Use Watershed." J. Environ. Qual. 36(4): 1006-1012.

CEPA, 2009. Relative Risk Evaluation for Pesticides Used in the Central Valley Pesticide Basin Plan Amendment Project Area. California Environmental Protection Agency, RegionalWater Quality Control Board, Central Valley Region, Sacramento, CA

Clark, J.R., Patrick Jr, J.M., Middaugh, D.P. and Moore, J.C. (1985). "Relative sensitivity of six estuarine fishes to carbophenothion, chlorpyrifos, and fenvalerate." Ecotoxicology and Environmental Safety **10**(3): 382-390.

Connon, R.E., Deanovic, L.A., Fritsch, E.B., D'Abronzo, L.S. and Werner, I. (2011). "Sublethal responses to ammonia exposure in the endangered delta smelt; Hypomesus transpacificus (Fam. Osmeridae)." Aquatic Toxicology **105**(3–4): 369-377.

Cowley, J., He, L.M., Ensiminger, M., and Kelley, K. Monitoring Pesticides in Surface Waters in Urban Areas during Baseflow and Storm Events in California. Poster Presentation, NorCal SETAC. May 3, 2012.

Dutta, H.M. and Arends, D.A. (2003). "Effects of endosulfan on brain acetylcholinesterase activity in juvenile bluegill sunfish." Environmental Research **91**(3): 157-162.

Foe, C.G., Deanovic, L., Hinton, D. and California. Central Valley Regional Water Quality Control Board (1998). Toxicity identification evaluations of orchard dormant spray storm runoff. New series no. 7. [Sacramento], California Regional Water Quality Control Board, Central Valley Region: x, 45, 13 p.

Kakko, I., Toimela, T. and Tahti, H. (2003). "The synaptosomal membrane bound ATPase as a target for the neurotoxic effects of pyrethroids, permethrin and cypermethrin." Chemosphere **51**(6): 475-480.

Livak, K.J. and Schmittgen, T.D. (2001). "Analysis of relative gene expression data using real-time quantitative PCR and the 2-DELTADELTACT method." Methods (Orlando) **25**(4): 402-408.

Lydy, M.J., Belden, J.B., Wheelock, C.E., Hammock, B.D. and Denton, D.L. (2004). "Challenges in regulating pesticide mixtures." Ecology and Society **9**(6).

Matern, S.A., P.B. Moyle, and L.C. Pierce. 2002. Native and alien fishes in a California estuarine marsh: twenty-one years of changing assemblages. Transactions of the American Fisheries Society. 131: 797-816.

Middaugh, D.P. and Hemmer, M.J. (1992). "Reproductive Ecology of the inland silverside, *Menidia beryllina*, (Pisces, Atherinidae) from Blackwater Bay, Florida." Copeia(1): 53-61.

Oros, D.R. and Werner, I. (2005). Pyrethroid insecticides an analysis of use patterns, distributions, potential toxicity and fate in the Sacramento-San Joaquin Delta and Central Valley. SFEI contribution 415. Oakland, Calif., San Francisco Estuary Institute: 112 p.

Sprague, L.A. and Nowell, L.H. (2008). "Comparison of pesticide concentrations in streams at low flow in six metropolitan areas of the United States." Environmental Toxicology and Chemistry **27**(2): 288-298.

Trimble, A.J., Weston, D.P., B., B.J. and Lydy, M.J. (2009). "Identification and evaluation of pyrethroid insecticide mixtures in urban sediments." Environmental Toxicology and Chemistry **28**(8): 1687-1695.

U. S. Environmental Protection Agency (2002). "Short-term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters to Marine and Estuarine Organisms". EPA-821-R-02-014. Washington, DC.

Werner, I., Deanovic, L.A., Miller, J., Denton, D.L., Crane, D., Mekebri, A., Moore, M.T. and Wrysinski, J. (2010). "Use of vegetated agricultural drainage ditches to decrease toxicity of irrigation runoff from tomato and alfalfa fields in California, USA." Environmental Toxicology and Chemistry: n/a-n/a.

Werner, I., Zalom, F.G., Oliver, M.N., Deanovic, L.A., Kimball, T.S., Henderson, J.D., Wilson, B.W., Krueger, W. and Wallender, W.W. (2004). "Toxicity of storm-water runoff after dormant spray application in a french prune orchard, Glenn county, California, USA: Temporal patterns and the effect of ground covers." Environmental Toxicology and Chemistry **23**(11): 2719-2726.

Weston, D.P. and Lydy, M.J. (2010). "Urban and Agricultural Sources of Pyrethroid Insecticides to the Sacramento-San Joaquin Delta of California." Environmental Science & Technology **44**(5): 1833-1840.