ERP Proposal Application Form

For DFG use only	
Proposal No.	Region

Section 1: Summary Information

1. Project title:	A Systems Biology Assessment of EDCs in the Delta
2. Applicant name:	Dr. Richard E. Connon
3. Contact person:	David Miller
4. Address:	The Regents of the University of California, Office of Research, Sponsored Programs 1850 Research Park Drive, Suite 300 University of California
5. City, State, Zip:	Davis, California 95618-6153
6. Telephone #:	(530) 754-7700
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8. Email address:	vcresearch@ucdavis.edu
9. Agency Type:	Federal Agency ☐ State Agency ☐ Local Agency ☐ Nonprofit Organization ☐ University (CSU/UC) ■ Native American Indian Tribe ☐
10. Certified nonprofit organization:	Yes ■ No ☐ *(Organizational DUNS: 047120084)*
11. New grantee:	Yes ■ No ☐
12. Amount requested:	$486,411
13. Total project cost:	$486,411
14. Topic Area(s):	Ecosystem Water and Sediment Quality
15. ERP Project type:	Research (1), Monitoring (2)
16. Ecosystem Element:	Contaminants
17. Water Quality Constituent:	Toxicity of Unknown Origin and Contaminants (1), Pesticides (2)
18. At-Risk species benefited:	Delta smelt
19. Project objectives:	Our approach will generate data on the molecular, behavioral and population-level response of *Menidia beryllina* to the endocrine disruptors bifenthrin & ibuprofen, and treated wastewater from three Delta outfalls. We expect a measurable response at all three tiers
20. Time frame:	3 years (July 2011 to June 2014)

Section 2: Location Information

Note: *The location details provided below are applicable to sampling sites or sites at which research work will be conducted.*

1. Township, Range, Section: and the 7.5 USGS Quad map name.	Fairfield-Suisun Treated Wastewater Outfall Fairfield South (USGS 7.5 quad map) Solano County Central Contra Costa County Treated Wastewater Outfall Vine Hill (USGS 7.5 quad map) Contra Costa County City of Benicia Treated Wastewater Outfall Benicia (USGS 7.5 quad map) Napa County
2. Latitude, Longitude (in decimal degrees, Geographic, NAD83):	Boynton Slough (Fairfield-Suisun POTW) 38.212947,-122.054014 Suisun Bay (Central Contra Costa County POTW) 38.042652, -122.091808 Carquinez Strait (Benicia POTW) 38.043344,-122.150831
3. Location description:	Aquatic Toxicology Laboratory, University of California, Davis Dr. Connon's Laboratory, Aquatic Toxicology Program, University of California, Davis Center for Marine Science, University of North Carolina Wilmington
4. County(ies):	Solano County, CA (field & lab work) ; New Hanover, NC (lab work only)
5. Directions:	We will reach our field sites (Benicia outfall, Central Contra Costa outfall, Suisun-Fairfield outfall) by boat: Martinez Marina, 7 North Court Street, Martinez, CA (http://maps.google.com/maps?f=d&source=s_d&saddr=830+S+Street+Sacramento,+CA+95811&daddr=7+North+Court+Street,+Martinez,+CA+94553-1109+(Martinez+Marina)&hl=en&geocode=FTuKTAIdExHC-CmtTnlcJNGagDHp5OEIEFt5sA%3BFdo6RAId0VS4-CFhihGDPEITnw&mra=ls&sll=38.397644,-121. 997681&sspn=1.618696,3.422241&ie=UTF8&ll=38.270532,-121.819153&spn=0.864672,1.711121&z=9) Suisun City Public Launch, 800 Kellogg Street, Suisun City, CA (http://maps.google.com/maps?f=d&source=s_d&saddr=830+S+Street+Sacramento,+CA+95811&daddr=800+Kellogg+Street,+Suisun+City,+CA&hl=en&geocode=FTuKTAIdExHC-CmtTnlcJNGagDHp5OEIEFt5sA%3BFS11RwIdBtW5-CkbSWYlUxSFgDEpwSBMOjO20w&mra=ls&sll=38.270532,-121.819153&sspn=0.864672,1.711121&ie=UTF8&z=10)
6. Ecological Management Region:	Bay Region: Suisun Marsh / San Francisco Bay
7. Ecological Management Zone(s):	Bay Region: Suisun Marsh / San Francisco Bay
8. Ecological Management	Bay Region: Suisun Marshlands & Bay

Unit(s):	
9. Watershed Plan(s):	Not applicable
10. Project area:	Sampling will be performed within a 30 meter x 30 meter area at each outfall
11. Land use statement:	Not applicable
12. Project area ownership:	% Private_____ % State_____ % Federal_____ Not applicable *Enter ownership percentages by type of ownership.*
13. Project area with landowners support of proposal:	**Not applicable as publicly accessible**

Section 3: Landowners, Access and Permits

1. Landowners Granting Access for Project: (Please attach provisional access agreement[s]) **Not applicable**	
2. Owner Interest:	
3. Permits:	
4. Lead CEQA agency:	
5. Required mitigation:	Yes ☐ No ☐

Section 4: Project Objectives Outline

1. List task information:

Goal 6. Water & Sediment Quality (Objective 1): The data generated from our study will provide further evidence to support the enforcement and strengthening of the California Water Code and the Clean Water Act, particularly for the control of pyrethroid pesticides and pharmaceuticals. Through our findings we also hope to encourage municipalities to adopt advancing technology that provides more effective removal of organic contaminants from treated wastewater prior to discharge.

2. Additional objectives:

Goal 1. Endangered and Other At-Risk Species and Native Biotic Communities (Objective 1): An additional objective is to further establish *Menidia* spp. (*M. beryllina, M. audens*) as bioindicators of endocrine disruption for the Bay-Delta. *Menidia* possess a number of attributes (see Background) that make it a good surrogate for threatened species such as the Delta smelt. By determining the impact of endocrine disrupting compounds on *Menidia* spp. we can gain further insight into the issues that could be contributing to population decline in important native fishes.

3. Source(s) of above information:

Appendix D: Ecosystem Restoration Strategic Goals and Objectives

Section 5: Conflict of Interest

To assist ERP staff in managing potential conflicts of interest as part of the review and selection process, we are requesting applicants to provide information on who will directly benefit if your proposal is funded. Please provide the names of individuals who fall in the following categories:

- Persons listed in the proposal, who wrote the proposal, will be performing the tasks listed in the proposal, or who will benefit financially if the proposal is funded; and/or
- Subcontractors listed in the proposal, who will perform tasks listed in the proposal, or will benefit financially if the proposal is funded.

Primary Contact for Proposal:	Dr. Richard E. Connon
Primary Investigator:	Dr. Richard E. Connon
Co-Primary Investigator:	
Supporting Staff:	Ms. Susanne M. Brander
Supporting Staff:	Project Scientist I (not named)
Supporting Staff:	SRA IV (not named)
Supporting Staff:	SRA II (not named)
Supporting Staff:	Student Assistant(s) III (not named)
Subcontractor:	Dr. John Colbourne, Center for Genomics & Bioinformatics (CGB), Indiana University

Provide the list of names and organizations of all individuals not listed in the proposal who helped with proposal development along with any comments.

Last Name	First Name	Organization	Role
White	Will	UNC Wilmington	Modeling

Section 6: Project Tasks and Results Outline

1. Detailed Project Description

Project purpose: This proposal directly addresses requirements specified by the Delta Regional Ecosystem Restoration Implementation Plan (DRERIP): Priority 2, "*Research that Tests Hypotheses Identified in the DRERIP Evaluation of the Bay Delta Conservation Plan (BDCP) Conservation Measures and National Research Council Operations Criteria and Plan (OCAP) Biological Opinion Review and Address Uncertainties*", and aims to "*Address Potential Factors Affecting Productivity*"; namely the contaminants of emerging concern, and toxicity of unknown origin, that are known to affect reproductive and development systems in a ubiquitous fish species; the inland silverside; *Menidia beryllina*, as a comparable surrogate for the endangered delta smelt; *Hypomesus transpacificus*.

Endocrine disrupting chemicals (EDCs) can mimic, enhance or block the effects of endogenous hormones and are known to cause a number of physiological and behavioral abnormalities in fishes (Rempel & Schlenk, 2008). EDCs originate from a variety of sources and are widespread in the aquatic environment, but are particularly concentrated downstream of treated wastewater outfalls (Kolpin et al., 2002, Pait & Nelson, 2002, Anderson et al., 2006). Examples of hormonal disruptions produced by EDCs in fishes include altered secondary sexual characteristics, males producing egg proteins (vitellogenin, choriogenin), and reduced sperm quality (Bogers et al., 2006, Metcalfe et al., 2001, Singh & Singh, 2008). One of the most sensitive – but least commonly measured – indicators of reproductive endocrine disruption is altered mating behavior (Bell, 2001, Clotfelter et al., 2004). These types of changes may be predictive, as changes in reproductive behavior can potentially translate to changes at the population level (Rankin & Kokko, 2007, Cotton & Wedekind, 2009).

Although theory and empirical data confirm that EDCs can cause declines in fish populations and impact mating behavior (Gurney, 2006, Kidd et al., 2007, Clotfelter et al., 2004), predicting such effects at sites where EDCs are detected is difficult. This is because many EDC studies test single chemicals, such as nonylphenol and bisphenol-A (Bonefield-Jorgensen et al., 2007, Soares et al., 2008) on a few standard species (e.g., zebrafish) at unrealistically high concentrations and use a single molecular biomarker as an endpoint. Therefore a need exists to study compounds that may act as endocrine disruptors through less direct pathways, as they may have impacts that are currently being overlooked. Alternatively, field-based studies typically examine realistic mixtures of EDCs using native fishes, but fail to establish a mechanistic link between the molecular action of EDCs and higher level effects. Additionally, most behavioral EDC studies are performed on the same species of pair-spawners (Schoenfuss et al., 2008) whose altered behavior is difficult to extrapolate to group-spawners, a type of mating behavior exhibited by many fishes (Helfman et al., 2009). Consequently, there is a vital need to strengthen our ability to predict population and behavioral level consequences using molecular biomarkers of EDC effects on more environmentally relevant species, while evaluating a larger range of compounds (Anderson et al., 2006, Cotton & Wedekind, 2009, Jobling & Tyler, 2006, Clotfelter et al., 2004).

To this end, we plan to investigate EDC effects on individual gene and protein expression and link these to changes in reproductive behavior in the silverside (*M. audens, M. beryllina*), a small estuarine fish, exposed to two endocrine disrupting contaminants commonly present in treated wastewater and surface waters downstream of outfalls: bifenthrin, a pyrethroid pesticide, and ibuprofen, a non-steroidal anti-inflammatory drug (NSAID) (Weston & Lydy, 2010, Buser et al., 1999). Both of these compounds, particularly bifenthrin which has been found in > 95% of sediment samples in California often at high mg/kg concentrations, also tend to adsorb to sediments (Bondarenko et al., 2004, Tixier et al., 2003). Therefore these compounds are also of interest because they are among those that could be mobilized during upcoming restoration events that are planned for a number of locations in the Bay-Delta region (NRC 2010). Concurrently, we will also include more environmentally relevant exposures by sampling at several Delta wastewater treatment outfalls. The Benicia, Fairfield-Suisun, and Central Contra-Costa facilities were chosen because they each discharge into the brackish or estuarine aquatic habitats that silversides abound in and the three facilities together represent a wide breadth of discharge volumes. Finally, we will evaluate the potential for endocrine activity in water samples and compounds alone, using *in vitro* techniques: the yeast estrogen screen (YES assay), the yeast androgen screen (YAS assay) and the CALUX assay (chemical activated luciferase gene expression assay).

Bifenthrin has recently been shown to have both estrogenic and anti-estrogenic properties (Jin et al. 2008, Brander et al., unpublished data), and ibuprofen influences reproduction by mediating levels of prostaglandin, a lipid mediator that among other roles, acts as an important reproductive pheromone in fishes (Sorenson & Goetz, 1993). While studies evaluating single biomarkers have been conducted for both compounds, by developing a whole genome microarray for the silverside we can evaluate the entire suite of genes that may be impacted. Although focusing solely on genes

responding to EDC exposure will further our understanding of the impacts of these chemicals, other parameters, such as behavioral performance that could affect spawning would largely be ignored. Genome-wide assessments therefore can be used to develop a specific "fingerprint" (Benninghoff & Williams, 2008, Tyler et al., 2008) of gene expression, encompassing the many functional cellular pathways that may be affected. The resulting profiles will, to some extent, permit cross-species comparisons, and enable equivalencies to be determined to that of other fishes exposed to bifenthrin and ibuprofen, e.g. the delta smelt. The assessment of genomic responses to single compounds can identify specific effects on the endocrine system, as well as highlight each compound's overall mode of action. By comparing this fingerprint to that of fishes exposed to treated wastewater from Delta outfalls, we can informatively measure the effects of a multitude of mixed stressors, and begin to understand endocrine toxicity effects that are attributable to different contaminant classes.

The inland silverside has already been established as an EDC bioindicator species by Brander et al. (in prep), resulting in the development of a high-throughput protein assay and identification of seven endocrine-related genes. These already optimized assays, together with the three cell lines (CALUX, YES and YAS) mentioned above, and modeling techniques in development by White & Brander (in prep, described below) will allow predictions of impacts on population dynamics to be made from evaluations of changes at three different levels of biological organization (molecular, cellular and behavioral). By employing a systems biology approach (Heckmann et al., 2008; Garcia-Reyero & Perkins, 2010) to link molecular level responses to changes in higher level functions (behavior), we are addressing the complexity of the problem at hand.

Research questions, goals and objectives: The proposed study will address the following questions: What genomic pathways are affected in small euryhaline fish when exposed to ubiquitous, yet less frequently studied, EDCs such as bifenthrin and ibuprofen. Is exposure to bifenthrin or ibuprofen represented by a specific "fingerprint" of gene expression? If so, what endocrine mechanism(s) appear to be altered? How do changes in reproductive behavior, protein expression and cellular activity following exposure to bifenthrin and ibuprofen mechanistically translate to alterations in gene expression? What are the population level implications of this for *M. beryllina* and other Bay-Delta fish species?

Our goals are to develop, validate and utilize a systems biology approach to assess the impacts of EDCs in the Delta. We will first develop and apply a microarray to conduct a detailed assessment of the impact of bifenthrin, ibuprofen (as emerging contaminants of concern), as well as on contaminant mixtures present in wastewater effluent, on *M. beryllina*. Second, we will measure specific proteomic responses using readily available protein assays for choriogenin and chorion. Third, we will assess the effects of exposure upon the reproductive fitness. We will also integrate validated human and yeast cell assays for the EDC assessment, into this approach, and in doing so, will develop monitoring tools that can be applied to assess site-specific reproductive fitness of wild populations in the Bay-Delta System.

Our objectives are:

1. To construct a microarray for *M. beryllina*, thus enhancing the limited availability of molecular tools for this species, which is currently confined to a few select sequences developed in previous studies.
2. To conduct *M. beryllina* exposures to bifenthrin and Ibuprofen to elicit and identify responses at genomic and proteomic levels, with a focus on endocrine system disruption, and include assessments of reproductive behavior.
3. To conduct *M. beryllina* exposures to treated water effluents from three sites in the Bay-Delta system, and assess genomic and proteomic responses.
4. To apply the use of validated cell assays (CALUX, YES and YAS) to determine EDC effects of bifenthrin, ibuprofen and three selected treated water effluents.
5. To utilize modeling efforts from a concurrent, but separately funded project, to further assess the effects of bifenthrin and ibuprofen exposures by integrating reproductive success and population dynamics.

We hypothesize that environmentally relevant concentrations of bifenthrin and ibuprofen, and treated wastewater will alter the transcription of key endocrine-related genes and protein expression in the *M. beryllina*, and that reproductive behavior, and ecological fitness will be subsequently altered. We also hypothesize that bifenthrin and ibuprofen will have either or both estrogenic and anti-estrogenic activity in in-vitro human cell lines (as determined by the CALUX assay), but that only bifenthrin will have estrogenic activity in the yeast estrogen screen (YES assay). Treated wastewater may have estrogenic, anti-estrogenic, androgenic and anti-androgenic activity. Finally, we anticipate that modeling results will establish significant links between alterations in transcription level of a select group of genes, changes in mating behavior and population dynamics.

2. Background and Conceptual Models

Study species: The inland silverside are a species particularly suited to address our research questions for a variety of reasons:

- Ease of study: They are available commercially, are euryhaline and ubiquitous in our study area, and have been successfully been maintained in field caging experiments (Bennett, 2005).

- Habitat and range: Their habitat and diet engender contact with both sediment-bound and soluble contaminants, their home range is relatively small and thus can provide site specific information,

- Life-cycle: Their reproductive biology is well known (Middaugh & Hemmer, 1992). They have a short life-span (1 year), and their habitat usage and life history characteristics are comparable to the delta smelt, which allows for toxicological responses to EDC exposure to be assessed in an analogous manner.

- Robustness: They are less susceptible to handling and display a lower level of sensitivity to contaminant exposure than the delta smelt. As such it is possible to investigate response mechanisms to exposure, at concentrations several magnitudes higher than that found in the environment, yet utilize this information to interpret environmentally relevant equivalent toxic effect (equitoxic) concentrations on the delta smelt.

- Existing methodology and data: *Menidia beryllina* is an EPA approved indicator species for acute marine aquatic toxicology testing and short-tem chronic toxicity estimating of marine estuarine organisms. A suite of *Menidia-specific* assays for EDC activity investigations are available from previous research conducted by our research team, (Brander et al, in preparation). Recent studies have found that *Menidia spp*. sex ratios are influenced by exogenous estrogen exposures (Duffy et al., 2009), and results from work conducted with silversides in 2009 – 2010 suggest that they are being impacted by endocrine disrupting contaminants in Suisun Marsh, and that both genomic and proteomic biomarkers can be linked to population level trends (Figure 1).

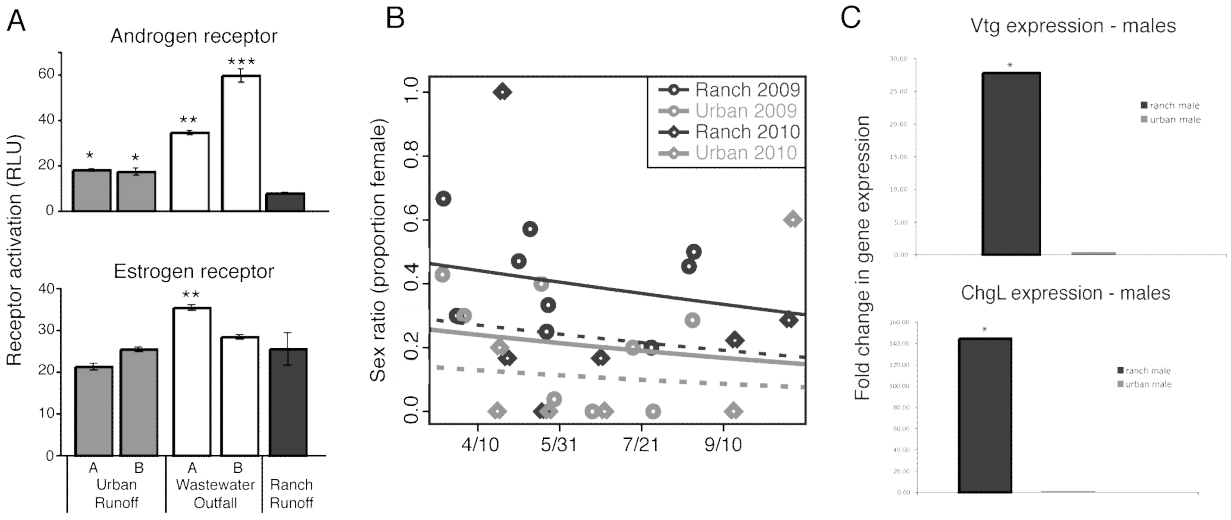

Fig. 1. Endocrine disruption in Menidia beryllina in Suisun Marsh, CA. A) androgenic (top) and estrogenic (bottom) activity at sites exposed to different potential EDC sources. Data collected using CALUX cell-line assay. B) Sex ratio of populations exposed to cattle ranch or urban runoff over the course of two spawning seasons. C) Estrogen-induced gene expression in males caught from the urban and ranch sites in 2009. The urban site had greater androgenic activity and thus had fewer females available for spawning. The ranch site had only estrogenic activity and therefore males had higher expression of estrogen-induced egg proteins.

Contaminants of emerging concern: New classes of chemicals; current use pharmaceuticals, pesticides, and industrial chemicals, that are potentially present in recycled water, are referred to contaminants of emerging concern (CECs), and methods to assess toxicological effects of many of these compounds are either limited or currently unavailable (Anderson et al., 2010). Of particular interest within these classes of chemicals, are the potential effects on fish reproduction. The U.S. Environment Protection Agency has recently recommended that endocrine disruptor screening be conducted on CECs (USEPA, 2009), and that wastewater be monitored, to assess compounds, such as

pharmaceuticals personal care products, and other contaminants present in municipal wastewater, with potential endocrine disruption activity, as specified by the California Department of Public Health, concerning recycled water (Anderson et al., 2010).

Contaminants of emerging ecotoxicological concern (CEECs) include current synthetic pyrethroids, such as bifenthrin due to its current use and broad aquatic toxicity range (Nillos et al., 2008), and the pharmaceutical; ibufrofen, as it is not fully metabolized by humans and can enter the sewage system as the parent compound or metabolites (Buser et al., 1999):

Bifenthrin, is one of the most frequently detected pyrethroid pesticides in aquatic ecosystems in California (Amweg et al., 2006). It is used in agriculture, but also is increasingly used for landscaping and structural pest control under the trade name Talstar (Weston et al., 2005, 2009, Werner & Moran 2008, Domagalski et al., 2010). Bifenthrin's primary mechanism of action is nervous system disruption, the consequences of which are convulsions, paralysis and eventual mortality (Beggel et al., 2010, Brander et al., 2009, Werner & Moran 2008). In addition to the intended mechanism, a number of recent studies demonstrate that some pyrethroids, including bifenthrin, have endocrine activity. Results from *in vitro* assays reveal that pyrethroids can act as estrogens, anti-estrogens and/or anti-androgens (Chen et al., 2002, Kim et al., 2004, Sun et al., 2007, Zhao et al., 2008, 2010). Furthermore, pyrethroid metabolites, generated via photolysis, hydrolysis, or metabolic processing are reported to have greater endocrine activity than parent compounds (Tyler et al., 2000, McCarthy et al. 2006). Pyrethroids also have considerable endocrine activity *in vivo*, particularly bifenthrin, permethrin and permethrin metabolites. These can induce vitellogenin or choriogenin, egg proteins normally found only in mature females, in male or juvenile medaka, zebrafish, and silversides (Wang et al., 2007, Jin et al., 2008, Nillos et al., 2010, Brander unpublished data). A recent study conducted in Northern California found concentrations of bifenthrin in stormwater run-off as high as 73 ng/L (Weston et al., 2009), well above the concentration shown to induce choriogenin production in juveniles (Brander unpublished data).

Ibuprofen is a non-steroidal anti-inflammatory drug (NSAID) that is present in many over-the-counter (OTC) medications. It acts as a temporary inhibitor of cyclooxygenase enzymes (COX-1 & COX-2), which mediate the metabolism of arachidonic acid into a variety of products, such as prostaglandins (Grosser et al. 2006). Prostaglandins play an integral role in inflammation and reproduction (Simmons et al., 2004). In fishes, prostaglandins are important for oocyte maturation (Sorbera et al., 2001) and are expelled as urinary pheromones by female fish during periods of spawning to stimulate male sexual behavior (Appelt & Sorenson, 2007). Exposure to low levels of ibuprofen, which is not completely removed during wastewater treatment (Buser et al., 1999), has been show to impact several aspects of reproduction in both vertebrates and invertebrates (Heckmann et al. 2006, 2008; Han et al. 2010). For example, medaka exposed to ibuprofen over a period of 4 weeks had fewer days with eggs (Flippen et al., 2007). Another study with medaka found that fish exposed for 120 days from egg stage to concentrations as low as 100 ng/L had delayed hatching of eggs when placed in parental pairs. At higher concentrations ibuprofen caused induction of vitellogenin in males, fewer broods per pair, more eggs per brood, and altered levels of estrogen and testosterone (Han et al., 2010). Ibuprofen has been detected at treated wastewater outfalls in the San Francisco Bay area at ng/L concentrations (Harold et al., 2009).

Wastewater effluent sampling sites: The wastewater treatment plant outfalls to which the response to environmentally relevant concentrations of bifenthrin and ibuprofen will be compared are located in the Western Delta (Figure 2). These three outfalls represent facilities that serve small (Benecia), medium (Fairfield-Suisun), and large (Contra Costa) populations.

The Location; including geographical coordinates, and volume of effluent processed daily by the Publicly Owned Treatment Works (POTW) are summarized below:

- Boynton Slough (Fairfield-Suisun POTW)
  38.212947,-122.054014 (17.5 million gal / day)
- Suisun Bay (Central Contra Costa County POTW)
  38.042652, -122.091808 (45 million gal / day)
- Carquinez Strait (Benicia POTW)
  38.043344,-122.150831 (4.5 million gallons / day).

Figure 2. Location of wastewater treatment plant outfalls for the City of Benicia, Fairfield and Suisun Cities, and Central Contra Costa County in the Western Delta.

Genomic techniques: The proliferation of different genomic approaches over the past decade, through a combination of advances in biological, instrumental and bioinformatic techniques, has generated a means of characterizing biological processes in response to stressors, yielding unparalleled information on the molecular and biochemical status of organisms (Ankley et al., 2008). Genomic approaches include studies of genotypes (DNA), transcriptomics (mRNA), proteomics (protein), and more recently metabolomics (metabolic signatures from resulting activity). Here we use the term genomics to signify transcriptomics. Messenger RNA transcription is the first step in gene regulation. Transcriptomics, otherwise known as "global analysis of gene expression", examines the genes and biochemical pathways involved in various biological processes. Whilst these techniques are highly sensitive indicators of an organisms' interaction with their environment because they capture the physiological responses initiated at the molecular level, it should be noted that RNA is not the final product of a gene, and gene function is carried out by the resulting protein. Protein expression, however, is influenced and regulated by many processes downstream of mRNA synthesis. Thus a most effective use of transcriptomics is candidate gene discovery complemented by detailed functional analyses of resulting candidates in an ecologically relevant context. Transcriptional expression levels can be utilized to understand the functional network of the biochemical pathways they are involved in, and thus are useful in the characterization of biological processes of interest, such as in the assessment of EDC impacts on reproductive pathways.

Genome-wide assessments can provide species-specific and sensitive, mechanistic information on the overall health of an organism, as homeostatic responses are often preceded by alterations in gene expression (Connon et al., 2008; Geist et al., 2007; Heckmann et al., 2008). In particular microarray gene profiling is a powerful tool for defining genome-wide effects of stressors on biological function. This tool can help further our understanding of ecological and physiological processes underlying organismal responses to environmental perturbations (Connon et al., 2009; Denslow et al., 2007; Heckmann et al., 2008). The analytical value of microarrays as screening tools and their application in the fields of environmental biology and ecotoxicology is rapidly growing. Genomic applications are a fruitful research avenue in the pursuit of ecological insight into critical questions of ecosystem resilience in the face of anthropogenic activities and global climate change. Notably, the research community at large is recognizing the synergism between genomics, organismal biology and global change biology with many fish profile science conferences highlighting the role of genomics in contaminants and as an overall health condition indicator.

Gene expression studies carried out over short-term exposures have been successful in the assessment and understanding of the effects of contaminants on reduced fecundity and embryonic arrest, somatic growth, and population dynamics of a variety of terrestrial and aquatic organisms (Ahluwalia et al., 2004; Connon et al., 2008; Heckmann et al., 2008; Soetaert et al., 2007). A good example linking mechanistic effects with higher level responses is presented in Heckmann et al (2008) who were successful in linking molecular and phenotypic responses with population growth rates in *Daphnia magna* (Heckmann et al., 2008). In this study, the effects of ibuprofen on reproduction were seen at the molecular, organismal and population level; with measured responses indicating interruption of crustacean eicosanoid metabolism, prostaglandin synthesis, and disruption of signal transduction affecting juvenile hormone metabolism and oogenesis.

Few molecular biomarkers are currently understood well enough to be used on their own to provide conclusive evidence of environmental stressor impacts on aquatic species in field monitoring. Vitellogenin and choriogenin, are two primary exceptions, as both gene transcription and protein activity have received significant attention in fish reproduction studies. This project aims to provide evidence for stronger linkages between EDC exposures and genome-wide responses, with respect to reliable alterations in physiology, and/or performance.

Microarrays and 454 Sequencing: DNA sequencing technology has formed the foundation for molecular biology, genetics and genomics. Historically, the method of choice has been Sanger sequencing (Maxam and Gilbert, 1977). The rise of the genomic era in the 1990's was characterized by technological advances that allowed for feasible deciphering of the complete genetic codes of living organisms. Whole-genome sequencing is a huge undertaking, requires tremendous resources and is generally confined to model organisms. However, "Next Generation" sequencing technologies offer a major step forward for ultra-high throughput sequencing, particularly important for non-model organisms lacking in genomic resources. These systems use nanotechnologies to generate many small sequence reads that are considerably cheaper than traditional Sanger technology. Recently developed oligonucleotide microarrays for model fish species contain in excess of 15,000 genes [e.g. zebrafish (*Danio rerio*) 37,157 target genes, medaka (*Oryzias latipes*) 26,689 target genes, and the standard test species, fathead minnow (*Pimephales promelas*) representing 17,588 genes]. Based on the successes of our previous genomic work (Connon et al, 2009, 2011, Brander et al, in preparation), we propose to develop a *M. beryllina* microarray, using ultra-high throughput sequencing (namely 454 sequencing) coupled with specific in situ oligonucleotide synthesis, and generate a comprehensive and fully annotated microarray for conducting genome-wide studies.

Enzyme-linked Immunosorbant Assay (ELISA) for Chorion / Choriogenin: ELISAs are high throughput assays performed in a 96-well plate that are designed to measure the amount of a particular antigen in a sample. This is done by using an antibody specific to the antigen of interest for detection. Most measurements of protein biomarkers used to evaluate whether endocrine disruption is occurring in a particular organism are done via ELISA (Palumbo et al., 2008). The objective for this particular ELISA is to utilize a biomarker of estrogenic endocrine disruption more sensitive than vitellogenin, the estrogen-induced egg yolk protein abnormally expressed in males or juveniles following exposure to suspected EDCs that is most commonly measured (Rempel & Schlenk, 2008). To this end, we will measure expression of chorion (Chg, precursor = choriogenin), an egg coat protein demonstrated to be more sensitive than vitellogenin (Celius & Walther, 1998, Arukwe et al., 2000), using an antibody derived from *M. audens* oocytes (Brander et al., in prep). Like vitellogenin, this protein is normally only induced by endogenous estrogen in mature females (Oppen-Berntsen et al., 1990, 1992). In comparison to Vtg, Chg expression has been shown to be a more sensitive marker of response the xenoestrogens DDT and bisphenol-A (BPA) (Celius & Walther, 1998, Arukwe et al., 2000). Although the mechanism behind choriogenin's higher sensitivity is not yet known, presumably it is because the coat of the oocyte is laid down prior to deposition of the yolk in a normal mature female responding to endogenous estrogen (Wourms 1976). This assay is highly sensitive and can detect exposures equivalent to 0.01 mg/ml chorion. This is several orders of magnitude more sensitive than an ELISA run with a commercially available salmonid antibody (Figure 3). The results of this assay will be linked to those of the genomic analysis.

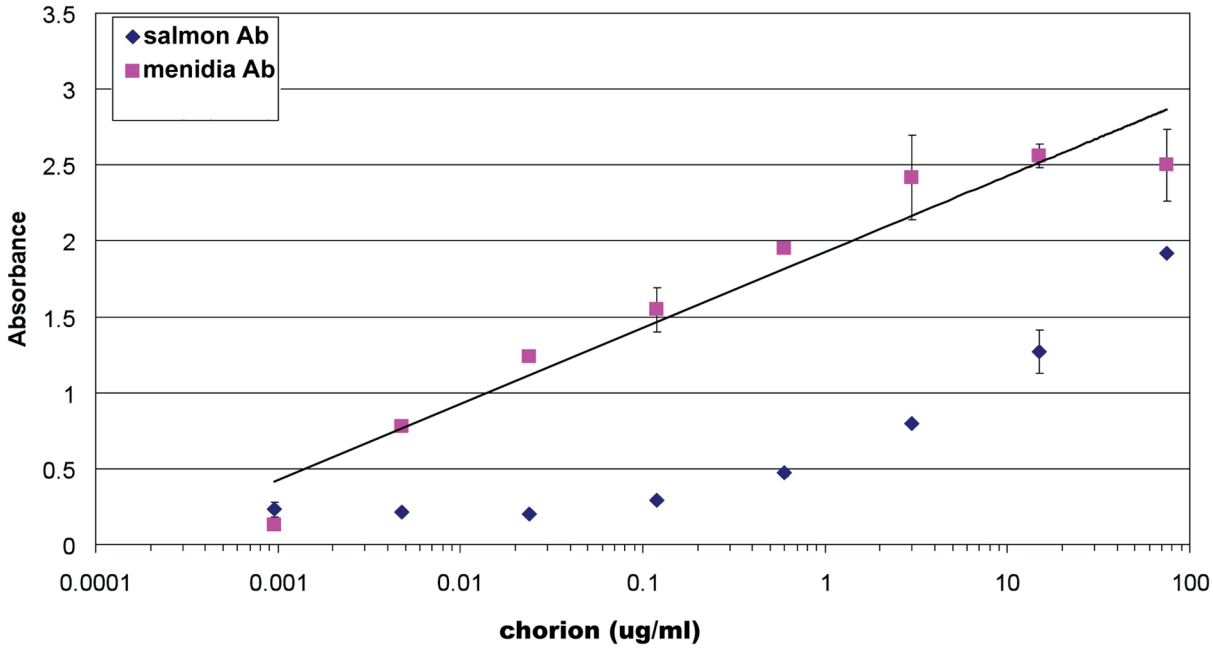

Figure 3. Comparison of ELISAs run with a commercially available salmonid antibody and an antibody created from Menidia chorion. Samples run were whole body homogenate spiked with chorion (egg coat).

Estrogen and Androgen Sensitive Cell Lines: *In vitro* systems such as the recombinant yeast strains developed by Routledge and Sumpter (1996) and Sohoni and Sumpter (1998) have been utilized in a number of studies to identify compounds that can modify the transcriptional activity of the human estrogen receptor (hER), or androgen receptor (hAR) and thus have become useful tools in screening for chemicals that may interfere with normal hormonal signaling *in vivo*. Yeast cells do not contain nuclear receptors, so the stable integration of exogenous nuclear receptor coding DNA sequences (i.e. here, the estrogen or androgen receptor), and an expression plasmid carrying a reporter gene, provides an estrogen- or androgen-inducible expression system, capable of detecting chemicals that can affect the transcriptional activity of these receptors, and thus infer their potential for interfering with the normal regulation of their target genes *in vivo*.

In the hER recombinant yeast screen, the DNA sequence encoding the hER is stably integrated into the genome of *Saccharomyces cerevisiae* (Routledge and Sumpter, 1996). Upon activation of the hER by an active ligand such as 17-β-

estradiol or estrogenic-mimicking chemicals, the hER becomes transcriptionally active, allowing it to bind estrogen receptor response elements (EREs) on expression plamids upstream of a *lacZ* reporter gene. This reporter gene encodes the bacterial enzyme β-galactosidase, which is secreted into the medium, and is capable of cleaving a chromogenic substrate provided in the medium, clorophenol red β-D-galactopyranoside (CPRG). The hAR recombinant yeast screen (Sohoni and Sumpter (1998) utilizes a similar technique but contains the human androgen receptor instead of estrogen receptor, and contains androgen receptor responsive elements upstream of the reporter gene instead of the ERE, to allow detection of androgenic chemicals capable of affecting the transcriptional activity of the androgen receptor.

The CALUX assay differs from the more frequently utilized YES or E-Screen in that cells natively express either the estrogen receptor (ER) or androgen receptor (AR) and can detect compounds that exert effects either by binding directly to a steroid receptor or indirectly (Rogers & Denison, 2000), by altering transcription factors or via crosstalk with other receptors (Tilghman et al., 2010, Palumbo et al., 2009), for example. Additionally with the CALUX assay it is possible to examine the potential for additivity, antagonism, or synergism of a contaminant with endogenous hormones. By using both the YES, YAS, and CALUX assays concurrently, we will better be able to discern whether bifenthrin and ibuprofen are actually binding to the estrogen or androgen receptor, or if instead activity is induced by activation of an alternative cellular pathway.

Our **conceptual model** is summarized in Figure 4, below.

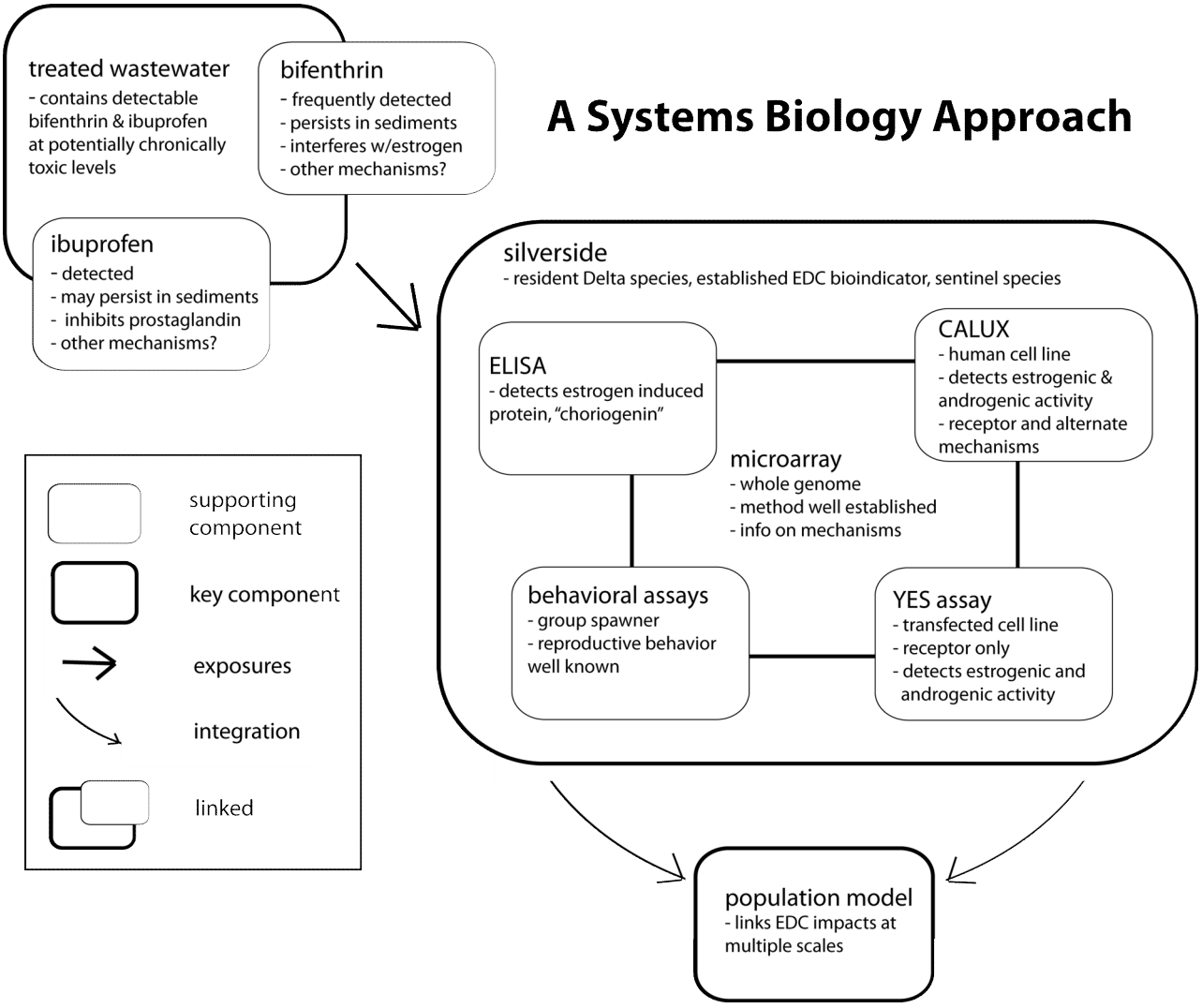

Figure 4. Conceptual model of a systems biology approach detailing the integration of key components (genomic assessments of treated wastewater), supporting components (reference toxicants, and validated assays), their combined use towards understanding mechanisms and pathways affected by endocrine disrupting chemicals (EDCs) that have negative impacts on population dynamics.

The key component of our investigation, and primary focus, is the assessment of the potential detrimental effects of contaminants present in treated wastewater upon endocrine activity, from a genome-wide perspective. We will assess the impact of bifenthrin, ibuprofen (as emerging contaminants of environmental concern) upon the reproductive fitness of the inland silverside (*M. beryllina*), to further understand mechanisms and adverse outcome pathways resulting from EDC exposure. By further obtaining mechanistic and behavioral data at different levels of biological organization; gene transcription (microarray, qPCR), protein expression (ELISA) and behavioral assays, we can begin to predict and model responses that will contribute towards understanding resulting effects leading to changes in population dynamics.

3. Approach and Scope of Work

Our overall approach and scope of work interlinks several tasks and methodologies into specific objectives, in order to address our research questions. Firstly, we will use 454-sequencing technology to generate genome-wide fully annotated sequence data required to construct an oligonucleotide microarray for the delta smelt (Objective 1). Sequencing and annotation will be sub-contracted to Dr. Colbourn at the Center for Genomics and Bioinformatics, Indiana University, and microarrays will be designed and constructed by Agilent Technologies Inc., Santa Clara, California (details below). Second, we will expose *M. beryllina* to environmentally relevant concentrations of bifenthrin and ibuprofen, and determine gene expression profiles via microarray, and protein assays, as well as investigate effects on reproductive behavior (Objective 2). Necessary quantitative PCR of select genes will be performed to validate microarray responses. Third, we will expose *M. beryllina* to wastewater effluents from three pre-selected sites in the Bay-delta and assess genomic and proteomic responses (Objective 3). Chemical analyses, targeted specifically, but not solely, at bifenthrin and ibuprofen will be conducted on these samples. Fourth, we will assess endocrine activity on both compounds as well as on effluent waters using validated cell assays to determine specific effects (Objective 4). Finally, we will utilize models developed in a parallel study, in collaboration with Dr. White at the Department of Biology and Marine Biology, University of North Carolina, Wilmington (UNCW).

Microarray Development and qPCR: One of the goals of this proposal is to enhance upon the limited genomic tools available for *M. beryllina* to allow for a more thorough assessment of EDC effects on this species. We will thus sequence the transcriptome, or the suite of expressed mRNA transcripts, of *M. beryllina* exposed to a number of stressors and environmental conditions. The gene sequences generated will then be annotated (i.e. protein name and function assigned) using computer software and available sequence for other species (Center for Genomics and Bioinformatics, Indiana University). From this well-annotated transcriptome sequence, a custom-designed oligonucleotide microarray will be built as a tool for research investigating the genomic pathways affected by exposure to EDCs, and thus the underlying physiological mechanisms that may affect reproduction. It will immediately result in a publication in a genomics or bioinformatics journal but perhaps more notably will provide a state of the art platform for environmental genomics-based research in the inland silverside. In order to capture a diversity of mRNA transcripts, we will utilize archived RNA from previous *M. beryllina* studies conducted in our laboratories (Brander et al. in preparation) as well as from preliminary exposures to bifenthrin and ibuprofen conducted immediately upon starting the proposed research. This approach will increase our ability to capture sequence information for the expression of rare transcripts, specific to our intent. Total RNA will be pooled into a single sample and shipped on dry ice to the Center for Genomics and Bioinformatics (CGB) facility at Indiana University, where it will undergo quality assessment, cDNA library construction, sequencing using 454 technology and sequence assembly and annotation (see budget justification, for sub-contract information on 454 project costs provided by the CGB facility). To develop the microarray platform, annotated gene sequence data will be uploaded into a java-based oligonucleotide probe design software program called YODA (Yet-another Oligonucleotide Design Application) (Nordberg, 2005). This program will generate three unique, 60 base pair probes for each gene in regions of high binding efficiency. The probe set from all the genes will then be uploaded into eArray, a web-based microarray design program offered by Agilent Technologies, for creation of a microarray platform with the appropriate internal controls and triplicate probe replication. This process will result in a well-designed microarray that will be commercially manufactured by Agilent Technologies and made openly available to the research community interested in conducting genomics studies on *M. beryllina*. Agilent Technologies do not charge for the development and construction of the microarrays, instead, researchers purchase the slides dependent on experimental requirements (see budget justification, for per-slide cost details and requirements). We will then utilize this technology on selected samples from bioassays described below.

Bioassays: A 14 day bioassay with *M. beryllina* (45-60 days old at test initiation), static with daily renewal, will be conducted with ibuprofen, bifenthrin and site water from the Benicia, Fairfield-Suisun and Contra Costa outfalls. Fish will be obtained from Aquatic Biosystems, Fort Collins, Colorado. Bifenthrin and ibuprofen will be obtained from Sigma Aldrich. A bifenthrin stock solution will be made in methanol, and an ibuprofen stock dissolved in water, as ibuprofen chloride is soluble in water at pH>7. Three concentrations each of bifenthrin and ibuprofen will be spiked into lab control water consisting of distilled water and filtered sea water at a salinity of 5 + 1 ppt. We will assess 5 replicates per treatment, with 10 fish per replicate. Fish will be maintained in 2L glass beakers with 1L test water in each at a 14 hr /10

hr light/ dark cycle with temperature at 21 + 2°C. Each beaker will be individually aerated and dissolved oxygen, ammonia and pH will be measured daily prior to water changes. Fish will be fed live artemia each day at least one hour prior to water change. At test termination survivors will be euthanized with a lethal dose of MS-222, rinsed in distilled water, immediately snap frozen in liquid nitrogen and samples stored at -80°C, for subsequent analyses. We will homogenize whole fish from each replicate together in approximately 5 mL liquid nitrogen with a ceramic mortar and pestle and once ground to a powder, samples will be split equally for RNA and Protein extractions. (See specific methodology, below)

Effluent water collection and exposure: Enough water to conduct the 14 day bioassays with a daily water change will be obtained within a 50 meter radius from each of the following wastewater treatment outfalls in the West Delta / East SF Bay region. Water will be analyzed for a suite of contaminants, including bifenthrin and ibuprofen. All collected water will be stored at 4°C until use, and usage will begin within 96 hours as per EPA protocols. Exposures will be conducted as detailed above, with three serial dilutions of each site sample. Dilutions will be made with control water consisting of distilled water and filtered sea water at a salinity of 5 + 1 ppt. The rationale for conducting serial dilutions is that past data have indicated that correlations between level of gene transcription and contaminant concentrations are more informative than single data points, and can indicate functional response thresholds (Connon et al, in preparation).

Behavioral response assays: Twenty *M. beryllina*, large enough to be sexually mature, will be housed in an aquarium (~100 gal) and allowed to group-spawn on artificial cordgrass substrate. Spawning behavior will be observed and the eggs reared to hatching to quantify the per capita fecundity and proportional viability from that spawning event (Middaugh et al., 1993). This basic procedure will be repeated for three separate experiments. Work will be conducted in aquaria in PI White's lab on the main UNCW campus, or preferably in aquaria with flow-through seawater at CMS. Pesticide exposures will be performed in dedicated, non-flow-through aquaria.

° First, commercially reared, environmentally naïve *M. beryllina* will be allowed to spawn in a series of ≥ 10 trials with varying sex ratios. This will establish the relationship between sex ratio and reproductive output in a non-EDC exposed population and provide baseline observations of spawning behavior. Menidia cannot be sexed visually, so typical male-female size differences will be used to guess at the sex ratio in each trial, then following the trial, fish will be sacrificed to inspect their gonads. Trials will be repeated until a suitable range of sex ratios (approx. 0.1 to 0.9) is obtained. Typical spawning functional response curves (Rankin & Kokko, 2007) will be fit to the data using maximum likelihood.

° Second, commercially reared *M. beryllina* will be exposed to a range of concentrations of bifenthrin and ibuprofen for 14 days. Spawning trials will then be conducted with fish from each treatment, allowing an estimate of the alteration in spawning behavior and reproductive output for each concentration. As before, trials will be repeated until a suitable range of sex ratios is obtained (sex ratio based on gonad morphology, although EDC-exposed fish may be infertile). Sub-sampling of individuals will be conducted for choriogenin and vitellogenin, gene and protein assessments.

Specific Methodology:

Genomic Assessments: We will conduct both microarray and confirmatory quantitative polymerase chain reaction (qPCR) assessments on fish exposed to bifenthrin and ibuprofen, and water sampled at the three selected sites. For this we require a total of 10 microarray slides, printed on an Agilent 4 plex, thus four samples can be hybridized per slide (the budget justification indicates a requirement of 16 slides, which includes slides for training and quality assurance). Quantitative PCR assessments will be conducted on select genes that respond significantly in the microarray assessments. Following exposures, fish will be euthanized with a lethal dose of MS-222, livers dissected and frozen at -80ºC until RNA extraction for quantitative PCR and microarray analyses of gene expression. We will investigate responses in a subset of exposed fish from each of the described bioassays. For microarray assessments, aliquots of RNA from livers of ten fish from non-lethal exposures, from each of four replicates will be pooled, while qPCR assessments will be conducted on RNA from livers from each individual fish. Quantitative PCR will focus particularly on genes identified by the microarray studies as associated with reproduction, thought not solely, and will incorporate the assessment of genes identified in previous studies (Brander et al. in preparation); e.g. choriogenin, vitellogenin, estrogen and androgen receptors. We will also assess a select subset of these genes to investigate responses in fish from reproductive studies described below.

Microarray Assessments: Total RNA will be isolated from whole fish homogenates (aliquoted between genomic and protein assessments; above) in TRIZOL reagent (Invitrogen) according to manufacturer's instructions. RNA samples will undergo an additional column clean-up step to remove tRNA and any small-size degraded mRNA. Ten microagrams of total RNA from each sample will be reversed transcribed (RT) using anchored oligo-dT, random primers and amino-allyl dUTP. Following RT, the RNA template will be removed from the reaction by incubation at 65°C for 15 min in the presence of 0.2M NaOH and 0.1M EDTA. Single stranded cDNA is then labeled by indirect coupling with 1µL of Alexa

fluor 555 (reference) or 647 (experimental) succinimidyl ester dyes (Invitrogen) for 1h at room temperature. Unincorporated fluorophors will be removed using a RNA clean-up kit (Qiagen, Valencia, CA, USA). Samples will be quantified spectrophotometrically to ensure high quality cDNA synthesis and dye incorporation before continuing onto the slide hybridization. cDNA will be fragmented prior to being applied to the slide following Agilent's Gene Expression Hybrization Kit (Protocol G4140-90050). Samples will be loaded separately onto each of the four arrays on the slide and hybridized for 17h at 65°C. After hybridization, slides will be washed by immersion in Agilent's Gene Expression Wash Buffers. Slides will be scanned on an Axon GenePix 4000B scanner (Axon Instruments, Molecular Devices). Note: we have not found it necessary to perform dye swaps using the Agilent microarray, which saves a considerable amount in terms of extra microarrays (Todgham and Hofmann, 2010; O'Donnell et al., 2009). Data from the oligonucleotide microarrays used in this project will be gathered using GenePix Pro 4.0 software (Axon Instruments) to measure the ratio of Alexa fluor 555 to 647 fluorescence quantified for each "spot" on the arrays. We will use normalization by design methods to statistically analyze our microarray data. It involves using the experimental design to adjust for array-to-array differences and labeling bias. Here, a gene-by-gene normalization of dye bias is performed. After this analysis, we carry out the empirical Bayes method on the results of each gene-by-gene analysis to moderate variances and, therefore, the test statistics. Additionally, we will calculate adjusted p-values. Thus, most of the methodology, in the end, is very similar to other microarray analysis, and only the normalization differs (Jarrett and Ruggiero, 2008).

Quantitative PCR: qPCR will be used to verify microarray responses. Total RNA will be extracted as described for microarray applications (above). Complementary cDNA will be synthesized from 1µg total RNA using 100 units of SuperScript III, 600 ng random hexamer primers, 10 U RnaseOut (Rnase inhibitor), and 1 mM dNTPs (all Invitrogen, Carlsbad, CA, USA) in a final volume of 20 µL. The reverse transcription reaction will be carried out at 50°C for 50 min and terminated by heating for 5 min to 95°C with immediate cooling on ice. Samples will be diluted with nuclease-free water to a total volume of 120µL and stored at -20 °C for subsequent qPCR assessments. Quantitative PCR measurements will be performed using TaqMan Universal PCR Mastermix (Applied Biosystems) 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). We will use SDS 2.2.1 software (Applied Biosystems) to quantify gene amplification in real-time. Gene expression levels will be calculated using the relative quantification 2(-Delta Delta CT) method as described in (Livak and Schmittgen, 2001). Beta-actin, a commonly used house-keeping gene, will be used as a primary reference upon which to normalize expression of the entire suite of genes, though the inclusion of three or more reference genes will be considered favorable, but their selection is stressor-dependent and as such will be determined during preliminary assessments. We will perform cluster analysis and gene expression profiling on both microarray and subsequent qPCR verification data to aid mechanistic interpretation of responses. Quantitative PCR data for each individual fish from all tests will be combined into one annotated dataset and subjected to agglomerative hierarchical clustering using Genesis software version 1.7.5 (Sturn et al., 2002). Profiles of gene expression will be generated using cosine correlation, with complete linkage clustering, based on data trends and mean distances, to assess response differences between species to respective treatments. Principal component analysis (PCA) will be conducted in conjunction to genomic profiles to ascertain resulting effects of stressors and respective concentrations. We will then compare genomic responses to protein assessments of choriogenin and vitellogenin towards establishing a systems biology investigative approach.

Protein extractions: Total protein will be extracted from whole fish homogenates (aliquoted between genomic and protein assessments; above). A 50 mM tris-HCl homogenization buffer with protease inhibitor (Roche COmplete Mini) will be added at 2X weight : volume (g : mL). We then will thoroughly homogenize each sample with a BRAND tissue-miser for 60 seconds. Homogenate will be centrifuged for one hour at 14,000 rpm (need to convert) and the resulting supernatant removed and centrifuged again at 10,000 rpm for 15 minutes to ensure removal of all solids. The final supernatant will be stored immediately at -80°C. A BCA protein assay (brand) will then be used to quantify the protein content of each sample.

Enzyme-Linked Immunosorbent Assays (ELISA): we will apply a specifically developed ELISA for choriogenin protein quantification in *Menidia beryllina* (Brander et al in preparation). A Menidia polyclonal antibody, produced and optimized from Menidia chorion according to the protocol described in Brander et al. in prep), will be used to measure the relative amount of choriogenin expressed in whole body homogenate (WBH). This is an indirect ELISA, developed based on methodology from Palumbo et al. 2009, which will quantify the response in each sample relative to Menidia chorion and to the positive control, ethinylestradiol. To perform the ELISA, we will first dilute WBH in 1:10 in coating buffer (need recipe here, Roche COmplete Mini) and load 50 µL sample per well. Standards of known concentrations of chorion will be used as an internal control. After coating for 3 hours at 4°C, wells will be washed twice with 200 µL each phosphate buffered saline (PBS) and 200 µL PBS + 5% powdered milk will be added to each well. Sample wells are blocked for 1.5 hours, followed by a second 2X PBS wash. Menidia chorion primary polyclonal antibody diluted at 1:1000 in PBS with protease inhibitor (Roche COmplete Mini) is then added at 50 µL per well and will be incubated overnight at 4°C. Following incubation with primary, wells will be washed four times with 200 µL PBS and 50 µL per well TMB (3,3',5,5' –

tetramethybezidine). Plates will then incubated in the dark for 20-30 minutes until wells cease increasing in color, and 50 µL sulfuric acid (2N) is added to each well to stop the reaction. Wells are read in a (plate reader brand name) at an absorbance of 590 nM

Recombinant Yeast Estrogen and Androgen Screen (YES and YAS): The yeast *Saccharomyces cerevisiae* cell lines containing the human estrogen (hER) or androgen receptor (hAR) are a gift from Professor J. P. Sumpter, Brunel University, UK. Yeast estrogen and androgen assays will be performed as described previously by Routledge and Sumpter (1996) and Sohoni and Sumpter (1998). We will test for estrogenicity and androgenicity using the same concentrations of test chemicals as were used in fish bioassays, with the addition of a series of multiple lower and higher concentrations in 3-fold dilutions. Minimal medium (pH 7.1) will be prepared by adding 13.61 g KH_2PO_4, 1.98 g $(NH_4)_2SO_4$, 4.2 g KOH, 0.2 g $MgSO_4$, 1 ml $Fe_2(SO_4)_3$ stock solution (40 mg/50 ml H_2O), 50 $mg.L^{-1}$ L-leucine, 50 mg L-histidine, 50 mg adenine, 20 mg L-arginine-HCl, 20 mg L-methionine, 30 mg L-tyrosine, 30 mg L-isoleucine, 30 mg L-lysine-HCl, 25 mg L-phenylalanine, 100 mg L-glutamic acid, 150 mg, L-valine, and 375 mg L-serine to 1 L of purite double-distilled water. Aliquots of 45 ml will be dispensed into 250-ml flasks and sterilized by autoclaving at 121°C for 10 min, and stored at room temperature. A vitamin solution will be prepared by adding 8 mg thiamine, 8 mg pyridoxine, 8 mg pantothenic acid, 40 mg inositol, and 20 ml of biotin solution (2 mg/100 ml H_2O) to 180 ml double-distilled water, and filter sterilized through 0.2-µm pore size Whatman PURADISC filters. A 10-mg/ml stock solution of clorophenol red β-D-galactopyranoside (CPRG), and of 20 mM copper (II) sulfate solution, will be prepared in double-distilled water and both also filter sterilized. Solutions of 20% w/v of D-(+)-glucose, 4 mg/ml L-aspartic acid will, and 24 mg/ml L-threonine will be prepared and sterilized by autoclave at 121°C. All stock solutions will be stored at 4°C. Growth medium will be prepared freshly on the day of use from a mixture of the above stock solutions. 5 ml of glucose solution, 1.25 ml L-aspartic acid solution, 0.5 ml vitamin solution, 0.4 ml L-threonine solution, and 125 ml copper (II) sulfate solution will be added to 45 ml minimal medium in a sterile cell culture flask. The growth medium will be inoculated with 5µl of the concentrated stock yeast (hER or hAR yeast) and incubated and 0.5 ml of the chromogenic substrate CPRG to 50 ml fresh growth medium and mixed gently. Estradiol (E_2) or dihydrotestosterone (DHT) standards will be prepared in methanol and used with hER and hAR yeast lines respectively. Working E_2 standards will range from 0.006 to 100nM and working DHT standards will range from 0.1nM to 10µM. Stock standards and test chemicals will be prepared at 10x higher concentrations than working concentrations to allow for the dilution upon addition of growth medium when a test plate is set up. 20µl of standards and test chemicals will be aliquoted to a sterile 96-well tissue culture plate in duplicates, with the addition of vehicle and empty well controls. The methanol will be evaporated in a sterile hood before addition of 200µl growth medium, inoculated with hER or hAR yeast cells, to all wells and mixed on a microtiter plate shaker. The plates will be incubated at 32ºC in a humidified incubator for 48-72 hours. During the color development, the medium will be checked periodically at an absorbance of 540nm to obtain data with the best contrast. After incubation, control wells will appear light orange in color, due to some background expression of β-galactosidase, and turbid, due to growth of the yeast. Positive wells are indicated by a deep red color accompanied by turbid yeast growth. Clear wells (containing no growth) indicate lysis of the cells. Light absorption data at 540nm will allow for extrapolation of estrogenic or androgenic activity by the test chemicals when compared to the standard curve data.

Chemical-Activated LUciferase eXpression (CALUX) assay: CALUX assays will be run using the same concentrations as were used in fish bioassays, with the addition of two higher concentrations each for bifenthrin and ibuprofen. We will test for estrogenic, androgenic, anti-estrogenic and anti-androgenic activity. Recombinant human ovarian cancer cells (BG1Luc4E2, ER-"–positive) and human AR-responsive, recombinant human cells [T47D androgen responsive element (ARE) will be grown and maintained as previously described (Rogers and Denison 2000). ER-responsive cells contain a stably integrated, ER-responsive firefly luciferase reporter plasmid, pGudLuc7ERE. T47D-ARE cells contain a stably integrated AR-responsive firefly luciferase reporter gene plasmid, pGudLuc7ARE. BG1Luc4E2 cells wil be maintained in estrogen-stripped media for 5 days before being plated. Both cell lines will then be plated into white, clear bottomed 96-well tissue culture dishes at 75,000 cells/well and allowed to attach for 24 hr. Plated cells will be incubated with carrier solvent (EtOH; 1% final solvent concentration), either E_2 (1 nM) or testosterone (T) (10 µM), the indicated compound (for measurement of agonist activity), or the indicated compound plus 1 nM E_2 or 10 µM T (for measurement of antagonist activity) for 24 hr at 37°C. For luciferase measurement, sample wells will washed twice with PBS, followed by addition of cell lysis buffer (Promega, Madison, WI); the plates will then be shaken for 20 min at room temperature to allow cell lysis. We will measure luciferase activity in each well using a Lucy2 microplate luminometer (Anthos, Durham, NC) with automatic injection of Promega stabilized luciferase reagent.

Mathematical modeling of responses to EDC exposure (Separately Funded Project): As part of a separately funded project, and collaboration with Dr. White (UNCW), a model of the population impacts of EDCs will be developed. This will consist of a nonspatial, discrete-time model of fish population dynamics that includes key demographic processes (e.g., natural mortality, size-fecundity relationship, space-limited spawning of benthic eggs, and reproduction). The primary state variable will be population density of both males and females (Cotton & Wedekind, 2009, Rankin & Kokko, 2007), and the focus of the modeling effort will be determining whether mean lifetime reproductive success is sufficient to ensure

persistence (Hilborn & Walter, 1992, White et al., 2010a,b). The effects of EDCs on operational sex ratio and per-capita fecundity will be incorporated using functional forms derived from the cell-line assay, field data, and behavioral experiments (25, 26, 27). The results of this model will, where appropriate be applied to the assist the interpretation of results from the experiments described above.

4. Deliverables

- A detailed Study Plan will be provided within the first 30 days of starting the project.
- A *Menidia beryllina* microarray will be developed and will be commercially available to the research community at large within the first year of the project.
- Genomic data (Sequences), where possible, will be made available to the research community at large, through the National Center for Biotechnology Information (NCBI) database.
- Microarray data will be made publically available through the Gene Expression Omnibus (GEO) repository.
- A biomarker suite will be developed as a tool to monitor EDC impacts on *Menidia beryllina* reproduction.
- Progress reports will be produced as stipulated by contract with the Department of Fish and Game.
- Staff will participate in Contaminants Workshops of the Interagency Ecological Program
- Research will be presented at State and National Congresses of the Society for Environmental Toxicology and Chemistry, The American Fisheries Society, and Delta Stewardship Program meetings (obtaining necessary authorization where applicable).
- Three or more peer-reviewed journal publication(s) are anticipated to result from this research.
- Final Report

5. Feasibility

Feasibility of this project is very high. Dr. Connon, Ms. Brander and other personnel involved, are highly qualified and suitably experienced to carry out all aspects of this investigation (see qualifications, below). The proposed molecular, biochemical, physiological and behavioral methodologies are of common use in our laboratories and all the equipment needed to conduct this project is readily available. Furthermore, an Assistant Project Scientist currently conducting research as a volunteer in Dr. Connon's laboratory brings further endocrinology skills in the form of modified *Saccharomyces cerevisiae* designed to specifically report endocrine activity via estrogen or androgen receptors. The project scientist has further experience with fish bioassays and both the biochemical and molecular assessment proposed, and as such will bring together the various components of the project. Ms. Brander is currently completing her Ph.D. dissertation and is scheduled to finish by summer 2011. She will be a Post-Doctoral Scholar at UC Davis, whilst also taking up a position as Adjunct Professor (without salary) at the University of North Carolina at Wilmington (UNCW) from September 2011, where our collaborator Dr. White, a theoretical ecologist in the Department of Biology and Marine Biology, is developing modeling systems to investigate the impact of EDCs on population dynamics. Ms. Brander will conduct all aspects of reproductive studies in conjunction with Dr. White, thus creating collaborative, multi-disciplinary study approach.

Menidia beryllina genome sequencing and annotation will be conducted by staff at the Center for Genomics and Bioinformatics (CBG), Indiana University, who perform these processed routinely. The microarray design and printing will be carried out by Agilent Technologies, Inc. Santa Clara, Ca. who specialize in the creation of platforms and methodologies for genome-wide research. Thus the CGB will be sequencing and annotating the delta smelt genome towards the construction of commercially available microarrays available to the research community at large, thus the developed tool not be limited to this research, nor researchers herewith.

The proposed work will build on, and benefit considerably from results of previous and ongoing studies (Brander et al, in preparation), carried out at various laboratories throughout UC Davis, assessing specific effects of EDCs utilizing *M. beryllina* as a surrogate for the delta smelt. Furthermore, the proposed research will strengthen and be strengthened by collaborations with Dr. Cole at Bodega Bay Marine Laboratory (UC Davis), who has recently received a Delta Science post-doctoral fellowship to study the impacts of EDCs on wild *M. beryllina* throughout the Sacramento San-Joaquin Delta, and plans to integrate studies along with this research proposal.

Facilities and equipment:

The Aquatic Toxicology Laboratory (ATL) 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. ATL 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. ATL follows protocols and quality assurance criteria established by the US EPA and the State of California's Surface Water Ambient Monitoring Program (SWAMP). The UCD-ATL is a 3200 ft² facility consisting of four individual laboratories and is fully equipped to conduct toxicant exposure and surface water monitoring studies. Available equipment includes multiple temperature and light controlled environmental chambers (32 ft³ each) for incubation and experiments, orbital shaker tables, vacuum pumps for sample filtration, a Coulter Counter for the enumeration of cells, a fluorometer for chlorophyll A analysis and two compound microscopes. The 1500 ft² wet lab is equipped with multiple temperature controlled 32 ft³ environmental chambers for culture, maintenance and experimentation of small fish and invertebrate experiments. A 375 ft² section is reserved for conducting the EPA *P. promelas* and *O. mykiss* tests and contains two temperature controlled 29 ft² water baths and two 13.5 ft³ environmental chambers. There are also 320 ft³ of refrigerated storage with an additional 8 ft³ of explosion proof refrigerated storage. Additional equipment housed in the wet lab includes pH meters, electrical conductivity meters, dissolved YSI oxygen meters, hardness and alkalinity titration stations and an autoclave. A 280 ft² negative pressure room is used primarily for exposure experiments with hazardous chemicals, and equipped with a 6 ft² fume hood and a vented, temperature controlled exposure chamber containing 14 individual tanks. A 180 ft² photo period and temperature controlled room is used for the EPA *C. dubia* bioassays. Bench space and light boards provide space for daily water renewals and shelves provide incubation areas for the organisms.

Dr. Connon's Molecular and Biochemical laboratory, at the University of California Davis, School of Veterinary Medicine (http://www.vetmed.ucdavis.edu/apc/wernerlab/subpage/connon.html) is fully equipped for biochemical and molecular biomarker analyses. Routine RNA extractions, cDNA synthesis and quantitative polymerase chain reaction (qPCR), microarray assessments, 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 UCD, detailed below.

Facilities at the University of North Carolina at Wilmington (UNCW) Center for Marine science include a shared research space with an equipment room housing freezers (-20 and -80 ^{o}C), freeze-driers, autoclaves, an ice machine, and other laboratory equipment. Several large (120 sq ft) walk-in temperature-controlled chambers are available. The same building houses wet laboratory space which includes twenty five 15 gallon partitioned assay aquaria flushed with flow-through filtered seawater, 3 fully programmable light/temperature incubators, and several mesocosm tanks appropriately sized for behavioral observations. Ample space for static aquaria used to run contaminant exposures is also available.

Computers: Computer systems with statistical, graphics and text processing software packages (SigmaPlot, SigmaStat, Systat, Statview, ARC GIS, Excel, Word, Powerpoint, Adobe Photoshop) are available for data analysis, data storage, publications and presentations. Computer systems with specialized bioinformatics software (Primer express, R statistics with LIMMA GUI for microarray analyses, Genesis, GenePixPro, SDS, Chromas, BioEdit,) along with image software analyses (EthoVision XT V6.0) are available for data acquisition and analysis.

Office: Approx. 2400 ft² of office space is available for PIs, research staff, and graduate students. All offices are equipped with computers, printers, copiers and other necessary office equipment, and all computers have internet access. Office, publishing and accounting support are provided by the department.

Other: Dr. Connon has access on a fee per service basis to the Lucy Whittier Molecular & Diagnostic Core Facility [http://www.vetmed.ucdavis.edu/vme/taqmanservice/] at UCD which offers services for qPCR analyses and support regularly used by this team.

6. Relevance to the CALFED ERP

Relevance to this PSP: The integrated research study detailed in this proposal is particularly relevant to the CALFED ERP towards better understanding the Delta system and linkages between restoration management, species, and water quality. This proposal focuses on previously identified uncertainties regarding BDCP Conservation Measures and the OCAP Biological Opinion by holistically evaluating adverse outcome pathways through integrating a number

of levels of biological organization; gene transcription and protein expression, survival, reproductive behavior which can be used in life-cycle assessments and population studies. Furthermore, it integrates existing and validated cell assays to specifically measure direct effects of water samples upon endocrine activity. Our proposal addresses and incorporates questions regarding potential factors affecting productivity (e.g. contaminants) and assessing the response of fauna to ecological characteristics of the Bay-Delta. Additionally, our approach is interdisciplinary, collaborative, and integrative, and will focus results towards application for restoration and performance monitoring.

Relevance to CALFED Issues Outside this PSP: One of the main goals of this study is to develop whole-genome assessment tool (a microarray) for *Menidia* spp. with a focus on contaminants; particularly on those of emerging concern (CECs). Bifentrhin and Ibuprofen are contaminants that are known to be present in the Bay-Delta System, and are amongst classes of contaminants that require further investigation, and require tools that can interpret responses in the context of ecologically relevant adverse outcomes. The overall relevance overall Bay-Delta issues, is that these tools will be applied on a surrogate, that shares many commonalities to the delta smelt, and as such, it is expected that data obtained in this proposal will be informative towards potential EDC impacts on the this and other endangered and at-risk species in the Bay-delta System. An important aspect of this study is the validation of the tools from a systems biology perspective, including measurements at different levels of biological organization. Furthermore, this research is highly positively executable and will also provide valuable monitoring tools to assess potential EDCimpacts on the Sacramento-San Joaquin Bay-Delta system.

7. Expected quantitative results (project summary):

- We will elicit and identify the response of *M. beryllina* to bifenthrin, ibuprofen and treated wastewater at the genomic (microarray) and proteomic (choriogenin) levels, with a focus on endocrine system disruption.
- We will assess reproductive behavior and the impact of sex ratio on reproductive success.
- We will determine EDC effects of bifenthrin, ibuprofen and three selected treated wastewater effluents using validated cell assays (CALUX and YES).
- We will employ modeling efforts from a concurrent, but separately funded project to further assess the effects of bifenthrin and ibuprofen exposures. We will do this by integrating the results of the CALUX, YES, ELISA, and microarray to predict reproductive success and population dynamics.

8. Other products and results:

We will construct a microarray for *M. beryllina*, thus enhancing the limited availability of molecular tools for this species, which is currently confined to a few select sequences developed in previous studies. Development of a microarray will further establish *Menidia* spp. as a bioindicator for brackish and estuarine habitats. *Menidia* spp. are an environmentally relevant surrogate for threatened Bay-Delta fishes.

9. Qualifications

The researchers included in this proposal are highly qualified and suitably experienced to carry out all aspects of this investigation, and the proposed methodologies are of common use at the respective UC Davis laboratories. Our individual research expertise has been applied to contaminant, physiological, genetic, disease and ecological issues with respect to the Pelagic Organism Decline (POD) and other projects of concern. Dr Connon's research on the Bay-Delta System, since his involvement in the POD program since 2007, has so far resulted in six publications concerning the effect of contaminants and disease on various fish species (published and submitted) and a further three manuscripts in preparation. Ms. Brander has spent the past 4 years researching endocrine disruption in the Bay-Delta, and her dissertation "Developing *Menidia* spp. as a bioindicator of estrogenic and androgenic endocrine disruption" includes three publications currently in preparation.

Dr. Richard Connon is a project scientist working at the Aquatic Toxicology Laboratory at UC Davis, currently researching sublethal molecular and behavioral endpoints in fish at the University of California Davis. He will act as Project Director and will be responsible for performing and overseeing experiments and analyses at UC Davis, and is expected to become a Research Scientist as from July 1, 2011. Trained as an Environmental Scientist and Ecotoxicologist, he is experienced in conducting toxicological tests on microorganisms, and both invertebrates and vertebrates. His past research involves ecological, toxicological, biochemical, molecular and a large number of environmentally relevant parameters, including reproduction and population growth rate investigations in *D. magna* carried

out at the University of Reading, UK. Published studies on exposure of *D. magna* to a number of stressors (agrochemicals, pharmaceuticals and urban contaminants) have demonstrated the ability to link transcriptional responses with those observed at higher levels of organization; reproductive value, development and population growth rates. Dr Connon has developed biochemical biomarkers in *D. magna* that were successfully applied to field studies on jet fuel spillages at Heathrow Airport, London, UK and linked detrimental effects of exposure between the biomarker activity and reproduction. Studies investigating molecular responses linked to population growth rates on a heavy metal (cadmium) and a pharmaceutical (ibuprofen) were published in 2008. Further work carried out in characterizing the ecological niche of *D. magna* using population growth rate was published in 2007. Recent work by Dr Connon, developing microarrays for a Californian endangered endemic fish species; delta smelt, successfully linked gene expression with swimming behavior anomalies and was published in 2009 and 2010. He has also identified key genes relating to ammonia toxicity (submitted) and urban wastewater contaminants, and has successfully identified disease-specific genomic profiles in outmigrant juvenile steelhead in the Columbia River (submitted). Dr Connon is highly experienced at molecular, microbiological and ecological aspects of the proposed study. Current and upcoming contracts awarded to Dr. Connon, as PI, include: the Refinement and Application of Novel Molecular and Biochemical Biomarkers to Determine Sublethal Contaminant "Exposure and Effects in Archived Delta Smelt Samples" (IEP contract number: R10AC20097, 09/2010-12/2011, and "Physiological Mechanisms of Environmental Tolerance in Delta Smelt (Hypomesus Transpacificus): From Molecules To Adverse Outcomes" (Bay Delta Stewardship contract in review).

Ms. Susanne Brander is a doctoral candidate at the University of California, Davis, Bodega Marine Laboratory and is now Adjunct Faculty at the University of North Carolina, Wilmington. She is currently completing her dissertation work: "Developing *Menidia* spp. as a bioindicator for estrogenic and androgenic endocrine disrupting contaminants," and is expected to receive her Ph.D. in Pharmacology and Toxicology in 2011. She is also trained and has worked as an Environmental Scientist, receiving an M.S. in Environmental Science & Policy from Johns Hopkins University in 2005 and working as an Environmental Analyst and Project Scientist for Weston Solutions, Inc. from 2004 – 2006. She is experienced in conducting biochemical assays on fishes and invertebrates in the laboratory and also has extensive experience in the field collecting organisms and designing ecotoxilogical experiments. Her most recent work involves developing *Menidia* spp. (*M. audens, M. beryllina*) as a bioindicator for endocrine disruption in the Sacramento / San-Joaquin Delta. A publication from her dissertation research that is currently in preparation indicates that endocrine responses at the cellular and molecular levels are linked to population level responses in *Menidia audens* from Suisun Marsh. She plans to expand this work to new applications in North Carolina, where Menidia spp. are native to rivers, estuaries and bays. Brander's knowledge of toxicology and endocrinology complements Dr. Connon's background in molecular biology and enhances the scope of the project.

Dr. John Colbourne (sub-contractor) is an Associate Scientist, Associate Director, Center for Genomics & Bioinformatics (CGB), at the University of Indiana. Dr. Connon has collaborated with Dr. Colbourn on past research concerning the sequencing of the *Daphnia* genome, this high-profile collaboration of researchers throughout the world (https://daphnia.cgb.indiana.edu/Home%3bjsessionid=CED9D4D32B7C3A075D7998E03E5E5910), has recently resulted in *Daphnia spp*. becoming an NIH model species, and a publication to be published in Science, spearheaded by Dr. Colbourne. The CGB offers a wide range of options to researchers. The genome sequencing and microarray services are available through a chargeback service. CGB also contracts out scientist time for consultation in both bioinformatics (for programming or analysis) and genomics (for running an experiment on high performance lab equipment). Project and consultation costs are negotiated on a case-by-case basis with the objective of minimizing costs of start-up projects that will lead to joint grant applications. In cases where joint funding is not appropriate, as in the case of this proposal, charges are structured to recover personnel, consumables, and overhead/administrative costs. Current CGB staff includes 30 full-time scientists and programmers and an administrative staff of 3. In addition, the CGB supports collaborative projects that currently involve 13 other scientists from the departments of Biology and Mathematics, and the School of Informatics. Although genomic facilities exist at the University of California, the microarray printing facility has closed down as a result of budget cuts. The service and expertise provided by the CGB is world renown.

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Section 7: Project Budget

1. Detailed Project Budget

BUDGET	Total 3 years	Year 1			Year 2			Year 3		
Staff Level		*Hourly Rate*	*Total Hours*	*Total Year 1*	*Hourly Rate*	*Total Hours*	*Total Year 2*	*Hourly Rate*	*Total Hours*	*Total Year 3*
Assistant Researcher (R. Connon, PI)	$53,843	$33.17	520	$17,248	$34.50	520	$17,938	$35.88	520	$18,656
Post-doctoral Scholar (S. Brander)	$58,891	$18.14	1040	$18,866	$18.87	1040	$19,620	$19.62	1040	$20,405
SRA IV	$18,037	$33.40	173	$5,778	$34.74	173	$6,009	$36.13	173	$6,250
Asst. Project Scientist I	$91,979	$23.61	1248	$29,465	$24.55	1248	$30,644	$25.54	1248	$31,870
SRA II	$10,720	$19.85	173	$3,434	$20.64	173	$3,571	$21.47	173	$3,714
Student Assistant III	$13,532	$8.50	510	$4,335	$8.84	510	$4,508	$9.19	510	$4,689
Personnel Subtotal	$247,002			$79,127			$82,292			$85,583
Fringe Benefits										
Assistant Researcher (R. Connon, PI)	$15,802		27.00%	$4,657		30.20%	$5,417		30.70%	$5,727
Post-doctoral Scholar (S. Brander)	$11,865		19.60%	$3,698		20.10%	$3,944		20.70%	$4,224
SRA IV	$7,810		40.20%	$2,323		44.20%	$2,656		45.30%	$2,831
Asst. Project Scientist I	$26,994		27.00%	$7,956		30.20%	$9,254		30.70%	$9,784
SRA II	$4,641		40.20%	$1,380		44.20%	$1,578		45.30%	$1,682
Student Assistant III	$176		1.30%	$56		1.30%	$59		1.30%	$61
Benefits Subtotal	$67,288			$20,070			$22,908			$24,310
Personnel Total (salary + benefits)	$314,290			$99,197			$105,200			$109,893
Operating Costs										
Microarray development - Sub-Contractor	$22,763			$22,763						
Validation and training	$5,000			$5,000						
Genomic assessments	$22,376						$18,376			$4,000
Bioassays & Reproduction	$9,900			$4,976			$3,274			$1,650
Cell Assays & ELISA	$4,200			$2,100			$1,050			$1,050
Travel and per diem	$8,000			$2,000			$3,500			$2,500
Office Supplies & Publications	$2,600			$300			$500			$1,800
Operating Costs Total	$74,839			$37,139			$26,700			$11,000
Total overhead @ 25%	$97,282			$34,084			$32,975			$30,223
Total Costs for Project	$486,411			$170,420			$164,875			$151,116

2. Budget justification:

Total budget costs: $486,411 over three years.

1. Personnel Services Cost: $314,290 plus overheads applied to all personnel at 25% ($78,573) as required by the University of California at Davis (Total: $392,863**)**.

Personnel: Included in the funds requested are salary and benefits [520h/year, total $69,645] representing 25% effort, for Dr. Connon (PI), for project management activities including, supervision of the project and training/assistance with molecular work, data handling, report preparation and project oversight and outreach. Salary and benefits are requested

for Susanne Brander (as Post-Doc) [1040h/year, total $70,756] representing 50% effort, to conduct reproductive and behavioral studies and oversee integration of research components, such as cell assays and genomic assessments, and produce manuscripts for publication. Personnel requests also include an Assistant Project Scientist I, [1248h/year, total $118,973] representing 60% effort, to conduct all genomic assessments and endocrine system specific cell based assays, and produce manuscripts for publication. We also request salary and benefits for UC Davis Aquatic Toxicology Laboratory staff; a Staff Research Associate (SRA) IV [173h/year, total $25,847] and SRA II [173h/year, total $15,361], both representing 8% effort, along with support from Student Assistant(s) III [510h/year, total $13,708], for assistance with conducting genomic research, fish handling and exposures, sample processing and general laboratory assistance. Although the salary lines on this proposal may appear relatively high, a genomics focused project combined with physiological outcomes, and confirmatory cell assays requires this level of personnel support and integrative skills. Time spent on each task has been calculated on an hourly basis however these are represented as percentage total time in respective task budget justification. Benefits rates are calculated based on current benefits cost as percentage of salaries. Dr. Connon is expected to be appointed Assistant Researcher as of July, 2011; salary and benefits have been adjusted accordingly thereon. A 4% increase in salaries has been applied for subsequent years 2 and 3. *Fringe Benefits*: Fringe benefit rates and annual increases are defined by the University's composite fringe benefit rates for fiscal years 2011-2014.

2. **Operating Expenses Cost: $74,839** plus overheads applied at 25% ($18,710 as required by the University of California at Davis (Total: 93,549).

Inland silverside oligonucleotide microarray development: (Year 1)

***Cost:* $27,763** plus overheads at 25% ($6,941) applied to supplies as required by the University of California at Davis (Total: $34,704).

Supplies: We request funds towards the *Menidia beryllina* transcriptome sequencing and annotation ($22,763), to be sub-contracted to Dr. John Colbourne, Indiana University, and towards general laboratory supplies ($5,000) for sample preparation, molecular reagents for subsequent in-house microarray validation tests, and training. Materials required for sample processing have been factored into the assessment costs.

Genomic assessments of selected contaminants: (Years 2 and 3)

***Cost:* $22,376** plus overheads at 25% ($5,594) applied to supplies as required by the University of California at Davis (Total: $27,970).

Supplies: We request funds towards the purchase of 16 microarray slides from Agilent ($836.00 per slide, total $13,376) as well as necessary molecular reagents for sample preparation, microarray labeling, hybridization and scanning facility costs, and general laboratory operation, and subsequent quantitative PCR assessments and validation ($9,000). Materials required for sample processing have been factored into the assessment costs.

Bioassays and reproductive behavioral responses: (Years 1-3)

***Cost:* $9,900** plus overheads at 25% ($2,475) applied to supplies as required by the University of California at Davis (Total: $12,375**)**.

Supplies: We request funds towards the purchase of commercially available *Menidia beryllina* (3,500 at $0.60 per fish, total $2,100, plus estimated shipping $500), test chemicals purchase ($300), and Environmental Health & Safety waste disposal costs ($5,000). Materials required for sample processing have been factored into the assessment costs. Funds towards concentration analyses of test chemicals are also requested ($2,000).

Cell-based assays and ELISAs: (Years 1-3)

***Cost:* $4,200** plus overheads at 25% ($1,050) applied to supplies as required by the University of California at Davis (Total: $5,250**)**.

Supplies: We request $4,200 towards the purchase of materials and reagents necessary to perform endocrine specific cell assays ($1,800) and choriogenin and vitellogenin ELISAs ($2,400). Human cell lines for the CALUX system will be made available to the project by Dr. M.S. Denison (UC Davis). Yeast cell lines will be provided by Prof. J.P. Sumpter, Brunel University, London, UK. Although these matching funds cannot directly and adequately be priced, they provide a substantial contribution to the success of the project. Materials required for sample processing have been factored into the assessment costs. Antibodies for ELISAs are available from previous projects.

Project Administration: (Years 1-3)

***Cost:* $10,600** plus overheads at 255 ($2,650) applied to supplies and travel, as required by the University of California at Davis (Total: $13,250).

Supplies: We request funds towards office supplies and publication costs ($2,600).

Travel: Funds are requested to support domestic travel ($8,000) over the three year period, including costs of field sample collection ($750), in addition to team member's attendance at scientific conferences ($7,250) to present research findings. Targeted annual conferences include Delta Science Program related conferences and workshops, and the annual meeting of the Society for Environmental Toxicology and Chemistry, California State and National Congress. Respective authorization will be sought for any travel outside the State of California, if deemed appropriate.

3. **Administrative Overhead Cost: $97,282.** The current indirect cost rate for VM:APC is 25% with all California State Agencies (Waiver # 03R-135).

· Class Waiver	No.: **03R-135**	Date Approved: **5/9/2003**	Sponsor Code:
Campus: **OP**	Reason: **C** [A=vital interest; C=sponsor policy]		
Sponsor Name: *CALIFORNIA STATE AGENCIES*			
Project Title: **CALIFORNIA STATE AGENCY AGREEMENTS****			
Waiver Rate: **25.00% MTDC***			
Notes: *UNLESS OTHERWISE SET FORTH IN STATUTE, REGULATION, OR PUBLISH POLICY THAT APPLIES TO ALL RECIPIENTS. C&G MEMO 03-02. SEE OTHER STATE CLASS WAIVERS FOR SPECIFIC PROGRAMS.			

ERP Proposal Application Form

BIOGRAPHICAL SKETCH

Richard E. Connon PhD

Dept. Anatomy, Physiology and Cell Biology
University of California, Davis.
One Shields Avenue, 2160 Haring Hall,
Davis, CA 95616

Telephone: (530) 752-3141
FAX: (530) 752-7690
Email: reconnon@ucdavis.edu

(a) Professional Preparation

Middlesex University, London, UK.	Applied Environmental Sciences (Honors), BSc (1998)
University of Reading, Reading, UK.	Ecotoxicology, PhD (2003)

(b) Appointments

Nov/08-present	**Project Scientist (Asst-II)**, Dept. of Anatomy, Physiology and Cell Biology, UC Davis
Feb/07-Oct/08	**Post-Doctoral Fellow**, Dept. of Anatomy, Physiology and Cell Biology, UC Davis
Jan/03-Aug/06	**Post-Doctoral Fellow**, School of Biological Sciences, University of Reading, UK
Jul/02-Dec/02	**Research Assistant**, School of Biological Sciences, University of Reading, UK
Feb/02-Jun/02	**Research Assistant**, Dept. of Psychology, University of Reading, UK

(c) Publications

i. Five selected publications most relevant to the proposal

CONNON RE, Beggel S, D'Abronzo LS, Geist JP, Pfeiff J, Loguinov AV, Vulpe CD, Werner I. (2010). Linking molecular biomarkers with higher level condition indicators to identify effects of copper exposures on the endangered delta smelt (*Hypomesus transpacificus*). **Environmental Toxicology and Chemistry**, 30(2): 290-300.

Beggel S, Werner I, CONNON RE, Geist JP (2010). Sublethal toxicity of commercial insecticide formulations and their active ingredients to larval fathead minnow (*Pimephales promelas*). **Science of the Total Environment**, 408: 3169-3175.

CONNON RE, Geist J, Pfeiff J, Loguinov AV, D'Abronzo L, Wintz H, Vulpe C, Werner I. (2009). Linking mechanistic and behavioral responses to sublethal esfenvalerate exposure in the endangered delta smelt; *Hypomesus transpacificus* (Fam. Osmeridae). **BMC Genomics**, 10, pp. 608.

Heckmann L-H, Sibly RM, CONNON R, Hooper HL, Hutchinson TH, Maund SJ, Hill CJ, Bouetard A, Callaghan A (2008). Systems biology meets stress ecology: linking molecular and organismal stress responses in *Daphnia magna*. **Genome Biology**, 9(2): R40.

Heckmann LH, Callaghan A, Hooper HL, CONNON R, Hutchinson TH, Maund SJ, Sibly RM (2007). Chronic toxicity of ibuprofen to *Daphnia magna*: Effects on life history traits and population dynamics. **Toxicology Letters**, 172(3): 137-145.

ii. Five additional publications

CONNON R, Hooper HL, Sibly RM, Lim FL, Heckmann LH, Moore DJ, Watanabe H, Soetaert A, Cook K, Maund SJ, Hutchinson TH, Moggs J, De Coen W, Iguchi T, Callaghan A (2008). Linking molecular and population stress responses in *Daphnia magna* exposed to cadmium. **Environmental Science and Technology,** 42(6): 2181-2188.

Heckmann L-H, CONNON R, Hutchinson TH, Maund SJ, Sibly RM, Callaghan A (2006). Expression of target and reference genes in *Daphnia magna* exposed to ibuprofen. **BMC Genomics**, 7: 175.

Hooper HL, CONNON R, Callaghan A, Maund SJ, Leiss M, Duquesne S, Hutchinson TH, Moggs J, Sibly RM (2006). The use of image analysis to estimate population growth rate in *Daphnia magna*. **Journal of Applied Ecology**, 43: 828-834.

CONNON R, Dewhurst RE, Crane M, Callaghan A (2003). Haem peroxidase activity in *Daphnia magna*: a biomarker for sub-lethal toxicity assessments of kerosene-contaminated groundwater. **Ecotoxicology**, 12(5): 387-395.

Amoros I, CONNON R, Garelick H, Alonso JL, Carrasco JM (2000). An assessment of the toxicity of some pesticides and their metabolites affecting a natural aquatic environment using the Microtox system. **Water Science & Technology**, 42(1): 19-24.

(d) Synergistic Activities

- Lecturer on Anatomy Physiology and Cell Biology, Musculoskeletal System. Undergraduate Studies, UC Davis, 2008-2011
- Ecotoxicogenomics Lectures (guest) on UC Davis Graduate Seminar Series, 2007
- Session Co-Chair - Ecotoxicogenomics - SETAC Europe15th Annual Meeting, France, 2005
- Lectures (guest) on Toxicology MSc Program, Royal Holloway University of London, 2005
- Lectures (guest) on Pesticides and Ecotoxicology, School of Agriculture, University of Reading, UK 2005

(e) Collaborators and Other Affiliations

Collaborators and co-editors:

Geist, J., Beggel, S., Unit of Functional Aquatic Ecology and Fish Biology, Department of Animal Science, Technische Universität München, Germany. - Wintz, H., Biorad Laboratories, Life Science Research, Hercules, California. - Loguinov, A.V., Vulpe, C.D., University of California, Berkeley. - Pfeiff, J.M., Array Core Facility, University of California, Davis. - Werner, I. Aquatic Toxicology Laboratory, University of California, Davis. Maund, S.J., Syngenta Crop Protection AG, 4002 Basel, Switzerland. - Hutchinson, T.H., AstraZeneca Global SHE, Environmental Laboratory, Devon, UK. - Iguchi, T., National Institutes of Natural Sciences, Okazaki 444-8787, Japan. - Moggs, J., Lim, F.L., Moore, D., Syngenta Central Toxicology Laboratory, Alderley Park, Cheshire, U.K. - Watanabe, H., Center for Integrative Bioscience, Okazaki National Research Institutes, Japan. - de Coen, W., Soetaert, A., Department of Zoology, University of Antwerp, Belgium. - Callaghan, A., Sibly, R.M., Hooper H.L., Hill, C.J., Bouetard, A., School of Biological Sciences, University of Reading, UK. - Biggs, J. Pond Conservation: The Water Habitats Trust, Oxford Brookes University, Oxford, UK. - Fryer, G., Institute of Environmental and Natural Sciences, University of Lancaster, UK. - Heckmann L-H., Dept. of Terrestrial Ecology, University of Aarhus, Denmark. Loge, F.J., Dept. of Civil and Environmental Engineering, University of California, Davis. - Evans, A.F., Real Time Research Inc., Oregon. - Hostetter, N.J., Roby, D.D., Dept. Fisheries and Wildlife, Oregon State University.

Graduate Advisors and Postdoctoral-Scholar Sponsors:

Graduate Advisor:

- Dr Amanda Callaghan, School of Biological Sciences, University of Reading, Berkshire, UK.

Postdoctoral Advisors:

- Dr Amanda Callaghan, School of Biological Sciences, University of Reading, Berkshire, UK
- Professor Richard M. Sibly, School of Biological Sciences, University of Reading, Berkshire, UK,
- Dr Inge Werner, Department of Anatomy, Physiology and Cell Biology, UC Davis.

Postdoctoral Sponsors:

- Natural Environment research Council (NERC), UK.
- Syngenta Crop Protection, UK and Switzerland.
- AstraZeneca Global SHE, Environmental Laboratory, Devon, UK.
- Inter-Agency Ecological Program, Sacramento, California.

Thesis Advisors and Postgraduate-Scholar Sponsor

Thesis Advisors:

- Dr Jason Weeks, National Centre for Environmental Toxicology, WRc-NSF Ltd. UK.
- Dr Amanda Callaghan, School of Biological Sciences, University of Reading, Berkshire, UK.

Postgraduate-Scholar Sponsor:

- Natural Environment research Council (NERC), UK.

ERP Proposal Application Form

BIOGRAPHICAL SKETCH

Susanne M. Brander

Ph.D. Candidate
University of California, Davis
Dept. Environmental Toxicology
One Shields Avenue
Davis, CA 95616

Adjunct Professor
Department of Biology and Marine Biology
University of North Carolina Wilmington
601 S. College Road
Wilmington, NC 28403

Professional preparation

1999	Elizabethtown College	Business (major), Biology (minor), B.S.
2005	Johns Hopkins University	Environmental Science & Policy, M.S.
2006-present	Univ. California, Davis	Toxicology, Ph.D. (est. completion 6/2011)

Appointments

2011 Adjunct Professor, Dept Biology and Marine Biology, UNCW

Selected publications:

Five most relevant to proposed research:

Brander, S.M. (in prep) Developing *Menidia* spp. as a bioindicator for the detection of estrogenic and androgenic endocrine disruption. Ph.D Dissertation, UC Davis

Brander S.M., I. Werner, J.W. White, L.A. Deavonic. 2009. Toxicity of a pyrethroid mixture to Hyalella azteca at environmentally relevant concentrations. Environmental Toxicology and Chemistry 28: 1493-1499.

Palumbo, A.J., Fojut, T.L., Brander, S.M., and Tjeerdema, R.S. 2010. Methodology for derivation of pesticide water quality criteria for the protection of aquatic life. Phase III: Bifenthrin criteria derivation. Prepared for the Central Valley Regional Water Quality Control Board. Department of Environmental Toxicology, University of California, Davis, CA.

Werner, Inge, Linda Deavonic, Joy Kamphanh, Juergen Geist, Dan Markiewicz, Kevin Reece, Marie Stillway, and *Susanne Brander*. 2007. Pelagic organism decline: acute and chronic invertebrate and fish toxicity testing. Progress Report II. Aquatic Toxicology Laboratory, School of Veterinary Medicine, University of California, Davis, California.

Scott Bodensteiner, Matt Zinkl, Jenner McCloskey, *Susanne Brander*, Larry Grabow, and Paul Sellier. 2008. Effects of desalination brine waste blended with treated wastewater in the aquatic environment of San Francisco. Water Practice 2: 1-9.

Other significant publications:

Brander S., R. Fontana, T. Mata, S. Gravem, A. Hettinger, J. Bean, A. Szoboszlai, M. Marrero, C. Keiper. The Ecotoxicology of Marine Debris. (in review: Journal of the American Biology Teacher)

Brander S.M., J.A. Royle, M. Eames. 2007. Evaluation of the status of anuran populations on a refuge in suburban Maryland. Journal of Herpetology 41: 51-59.

Mosser C.M., J.P. Geist, M.L. Hladik, , S.M. Brander, I. Werner. (in prep) Esfenvalerate toxicity to the cladoceran Ceriodaphnia dubia in the presence of the green algae, Selenastrum capricornutum.

Recent Funding

2010 UC Davis Graduate Student Association Travel Grant ($1,000)

2009 National Science Foundation CAMEOS GK-12 Graduate Fellowship (tuition, stipend)

2009 University of California, Davis Research Scholarship ($5,000)

2009 Sacramento County Sanitation and Wastewater District, Research Grant ($5,000)

2007 CALFED Pre-Doctoral Fellowship (3 years: tuition, stipend, research support $20,000)

2007 University of California, Davis Jastro-Shields Research Scholarship ($3,000)

Synergistic Activities

Invited talks: CA Dept. Pesticide Regulation, SF Estuary Interagency Ecological Program (2010)
NSF GK-12, high school science class mentor (2009-2010)
Society of Environmental Toxicology & Chemistry
- Northern California Student Representative (2009 - 2010)

Society of Toxicology
- Northern California Student Representative (2007 - 2009)
- Northern California K-12 Programs Representative (2007 - 2008)

Advisors

Ph.D.	Gary N. Cherr	UC Davis, Bodega Marine Lab
Masters	J.Andrew Royle, Robin Jung	USGS / Johns Hopkins