PS/SS: Linkage of *In Vitro* Assay Results With *In Vivo* End Points

Estimated Cost: $70,000 for 2013 (Year One). This is proposed as a two-year study; $56,000 will be requested for 2014, pending acceptable progress in Year One.
Oversight Group: Emerging Contaminants Workgroup and Exposure and Effects Workgroup
Proposed by: Nancy Denslow (University of Florida) and Keith Maruya and Steve Bay (SCCWRP)

Proposed Deliverables and Time Line

Deliverable		Completion Date
Task 1:	**Convene focus group and develop actionable plan**	**CSD + 1 month**
Task 2:	**Develop molecular biomarkers for Menidia**	**CSD + 4 months**
Task 3:	**Laboratory tests: Early life stage exposures and in vitro bioassays**	**CSD + 9 months**
Task 4:	**Field-collected sample exposures**	**CSD + 18 months**
Task 5:	**Chemical analysis of CECs**	**CSD + 21 months**
Task 6:	**Reporting**	**Mid-term (Year 1): CSD + 12 months** **Final: CSD + 24 months**

BACKGROUND

A growing number of contaminants of emerging concern (CECs) are found routinely in permitted discharges and their receiving waters. For the few CECs for which analytical methods exist, these methods are still largely in development and only some are routinely performed by commercial services laboratories. As the development and manufacture of chemicals presents an ever changing landscape, those CECs that are produced in high volumes and/or that are capable of being discharged via treated municipal or industrial wastewater effluent or stormwater runoff represent a moving target for environmental quality managers tasked with assessing and/or mitigating their potential for impact.

The CECs of most concern are those which may be potent at trace concentrations (parts per trillion range) and work as endocrine disruptors. Their presence in waterbodies may be harmful to aquatic biota inhabiting these locations. Such endocrine disrupting chemicals can interact directly with soluble hormone receptors or can interfere with the natural synthesis or metabolism of endogenous hormones and thereby impede normal function of these processes in exposed organisms. Most attention has been focused on chemicals which act as estrogens or androgens or their antagonists. Estrogens are important in brain development and programming of tissue differentiation at early time points during development (Feist and Schreck 1996; Lassiter and Linney 2007; Mandiki et al. 2005; Remage-Healey and Bass 2007; Tomy et al. 2009; Vetillard et al. 2006; Zhang et al. 2008).

In our own work, exposure of fathead minnows to concentrations of ethinylestradiol (EE2) at 2 ng/L induced pericardial/yolk sac edema (Johns et al. 2009). The estrogenic mycotoxin zearalenone (exposure range of 2-50 ng/L) also resulted in myocardial edema (Johns et al. 2009). In addition, we analyzed a limited set of gene expression changes including Vtg, which was up-regulated by the two estrogens, steroidogenic acute regulatory (StAR) protein, insulin-like growth factor 1 (IGF-1) and growth hormone (GH) which were also altered. Thus, these genes in target fish species would be viewed as critically important to include in future studies of responses to estrogenic CECs at the molecular level.

Concurrently, novel in vitro methods based on receptor binding or transactivation have been developed that are extremely sensitive to target chemicals acting with the same mode of action, including the potent endocrine disrupting CECs described above. Work is being performed to adapt these in vitro bioassays for water quality assessment and monitoring purposes. Few studies, however, link results from such *in vitro* assays with higher order *in vivo* effects which result in adversity for survival, growth, reproduction, or susceptibility to disease.

The goal of this project is to establish quantitative linkages between the *in vitro* receptor-based assays and traditional endpoints of adversity in a sensitive estuarine fish model, the common silverside (*Menidia beryllina*) which is an established EPA model for estuarine toxicity. As a demonstration, we will focus on estrogenic responses of selected chemicals of interest first in lab exposures (Year 1) followed by exposure to field-collected wastewater treatment plan (WWTP) effluent and estuarine and marine receiving waters (Year 2).

Study Objective and Applicable RMP Management Question

There is considerable concern about the presence of CECs in treated domestic wastewater that is released into the environment and in waters that are reused for irrigation. In California, many WWTPs discharge their treated waters directly into estuaries or into rivers upstream of estuaries. While there are regulations in place for monitoring specific CECs and other regulated chemicals, there is currently not much being done to discover new CECs in water or assess whether the presence of the CECs may adversely affect aquatic biota. If these experiments are successful, we should be able to start to develop methods to integrate results from *in vitro* assays into monitoring programs and determine how they relate to *in vivo* adverse effects. Results from this study will begin to enable managers to determine whether or not additional action is necessary for treated effluents that are discharged into sensitive estuarine environments. This work will not only be important for California, but also for other states that discharge domestic wastewater into marine/estuarine environments.

The objective of this effort is to develop a tool that will assist in the identification of chemicals of emerging concern that are adversely affecting biota. This study would address the following RMP management question (MQ):
MQ1. Are chemical concentrations in the Estuary at levels of potential concern and are associated impacts likely?

A: Which chemicals have the potential to impact humans and aquatic life and should be monitored?

B: What potential for impacts on humans and aquatic life exists due to contaminants in the Estuary ecosystem?

Study Plan

This study will test estrogenic chemicals that were recently recommended for monitoring in California's receiving waters by the State's Science Advisory Panel for CECs (Anderson et al. 2012), e.g. estrone (E1), bisphenol A (BPA), 4-nonylphenol (4-NP) and galaxolide (HHCB). Traditional i*n vivo* endpoints for early life stages of silversides (*M. beryllina)* will include: development, growth, and survival and for juveniles: growth, survival and biochemical endpoints such as plasma vitellogenin and hormone concentrations (Vtg, E2 and T) and hepatic gene expression for at least 5 genes per life stage. We will index estrogen equivalency concentrations required for altering higher order endpoints with biochemical responses within the fish and responses obtained with commercially available estrogen receptor (ER) transactivation assays (see also Task 3). These linkages will enable the use of *in vitro* assays as measures

of both exposure and effect. The concentrations required for both *in vivo* and *in vitro* assays will be quantified to determine reference concentrations above which effects may be expected.

Tasks 1 through 3 will be completed in Year One with 2013 funds. Pending approval from the TRC and ECWG/EEWG, Tasks 4 through 6 will be completed in Year Two with 2014 funds.

Task 1: Initial meeting for project coordination. The study will be initiated by a meeting between the two laboratories to discuss the details of the experimental approach to be used and to set up the time frame for the various experiments.

Task 2: Development of molecular biomarkers for *Menidia beryllina*. There are a few molecular biomarkers already developed for *M. beryllina* including Vtg, ER alpha (esr1), ER beta a (esr3), and androgen receptor (AR), among others (Brander 2011). We will validate these assays with our own samples and also develop additional molecular assays for the following genes: IGF-1; StAR; GH; brain aromatase (cyp19b); and two genes involved in testis differentiation, anti-Mullerian hormone (amh) and doublesex and mab-3 related transcription factor 1 (dmrt1). We will get sequences for these additional genes from a high throughput DNA sequencing experiment, discussed in advance with Drs. Brander and Connon, experts involved in developing a transcriptome for Menidia. We will make all sequences we obtain accessible to these colleagues for their independent studies. The assays that we will develop will be based on quantitative reverse transcriptase polymerase chain reaction (Q-PCR) and involve the quantitative amplification of specific genes from total RNA extracted from early life stage fish or juveniles. These genes have been determined in studies of other fish to be responsive to estrogens *in vivo* (Filby et al. 2007; Ijiri et al. 2008; Johns et al. 2009; Kobayashi et al. 2003). The assays will be validated with standard curves after determining that amplification is proportional to amount of transcript present in the original sample.

Task 3: Laboratory exposures and in vitro bioassays. These experiments will be performed at the University of Florida and SCCWRP. We will divide up the exposures and each will perform a positive control. Exposures will be in the water with at least 5 concentrations of each chemical and a single concentration of 5 ng EE2/L as the positive control. The chemicals that will be tested include E1, 4-NP, BPA, and galaxolide (HHCB). Since Menidia are a sensitive species we will first use a range finding experiment to determine the LC50 and conduct our exposures at or below that level. We will then include two concentrations below and two above this value. The test chemicals will be mixed with a small volume of triethylene glycol (TEG) as a carrier to ensure the chemical gets into the water phase. Dilution water will dechlorinated tap water adjusted to 15 ppt salt (using Instant Ocean) and temperature will be controlled to 20 ^{o}C, following standardized test guidelines for early life testing (EPA, 1995).

Each lab will do two types of exposures: early life stages, where we will buy recently hatched embryos and allow them to grow under the chemical conditions described above. For these assays we will place twenty 10-d old larvae per 200 ml beaker in quadruplicate for each condition, including artificial seawater with and without carrier, the positive control and 5 concentrations of each test chemical. The exposure will extend for 7 days. Fish will be fed newly hatched brine shrimp. End points will be mortality and growth. We will also take at least 5 individuals from each beaker to determine changes in expression for the following genes: IGF-1, CYP19B, GH, dmrt1, amh.

For the Juvenile Test, we will use 50-d old fry and place 10 per 500 ml beaker under the chemical and replicative conditions described above. This period will be just before gonadal differentiation in this species, another time of vulnerability to endocrine disruptors. The fish will be exposed for 10 days in a static renewal system in 15 ppt salinity. Fish will be fed tetramin flakes and supplemented with brine shrimp. The endpoints for this assay will include measurements of whole body homogenate

concentrations of Vtg, E2 and T (if the hormone evaluations are possible at this age) and hepatic measurements of gene expression for Vtg, ERα, ERβ, AR, and StAR using Q-PCR. We will also perform histopathology on the gonads to distinguish males from females and if we can find a sequence for dmrt1, we will correlate its expression with sex of the fish. In some species this gene marks genetically determined sex (Nanda et al., 2002).

For the *in vitro* assays, we will use the same final concentrations as described above, except that the chemicals will be added to the culture as 1/10 volume of a 10X solution, in order not to dilute out the nutrients required for the tissue culture. We will use the same commercial assays for estrogen receptor (ER) transactivation that will be used in a project recently funded by CA (K. Maruya, PI and N. Denslow, CoI, among others) entitled "Evaluating bioanalytical methods as screening tools for monitoring of CECs in California recycled water applications." There will be significant savings in leveraging the funded project as all assay development and validation for the in vitro assays will occur in that project. We will simply apply the assay in this one.

Expectations and Alternative Strategies. We expect that the concentrations of model compounds that we have selected will show higher order effects in survival and growth in the *in vivo* tests (at least at the higher concentrations tested). From past experiments, we are confident that these concentrations will also impact molecular endpoints within the fish resulting in alteration of gene expression. Lastly, the selected concentrations should also be potent enough to change expression of the high throughput assays. *Menidia* are expected to be as sensitive, if not more sensitive that fathead minnows to these chemicals. In the event that they have high mortality in the selected concentrations, we will repeat the experiment at lower concentrations.

Task 4: Exposure to WWTP effluent and receiving water samples and in vitro bioassays

In Year 2, we will test samples of WWTP effluent and receiving waters from two sites, one in southern California and the other in the San Francisco Bay estuary. A sufficiently large volume of treated final effluent and receiving water from each site will be filtered through a sorbing phase (e.g. C18 or Oasis HLB cartridge) to capture organic contaminants and subsequently eluted by organic solvents. A portion of each eluent will be set aside for analytical chemistry and the remainder will be shipped to one or the other of the two participating laboratories where the eluent will be reconstituted to the same proportional volume as the original sample and tested with either an early life stage assay or a juvenile assay.

One sorbing cartridge each will be processed and shipped to SCCWRP and the University of Florida. The cartridges will be eluted and then air dried in order to reconstitute test solutions to 1X, 5X and 10X the concentrations equivalent to what they were at the field site. Each solution will be tested in triplicate and we will use the 5 ng EE2/L as a positive control. We will perform early life stages and juvenile tests as described above. We will also perform in vitro nuclear receptor transactivation assays as described above, but in this case using the concentrates from the field locations.

Expectations and Alternative Strategies.

Based on the results from Task 2 in year 1, we may adjust the concentrations of the positive controls and we may adjust the concentrations of the reconstituted test solutions. If we find that Menidia are more sensitive to the chemicals than we anticipated, all concentrations will be diluted by 10 to get in the range where we do not see more than 10% mortality. Under these conditions, we expect that all endpoints will be viable and that we will be able to compare estrogenic equivalencies across experiments. We may not see much with field waters for androgenic changes, as most of the CECs that target this axis react as anti-androgenic chemicals.

Task 5: Chemical analysis of CECs
Estrone (E1) and EE2 will be measured by ELISA following the methods of Huang and Sedlak (2001). Galaxolide (HHCB), bpA and 4-NP will be measured by GC-MS after extraction and derivatization as described in Ligon et al. (2008). Samples of sufficiently large volume will be collected to ensure the appropriate sensitivity of measurement, based on the range of treatments (lab) and expected receiving water concentrations. An equivalent amount of chemical as evaluated for the in vivo assays will also be assessed for the in vitro assay.

Task 6: Reporting
We plan to submit a mid-term progress report at the end of year 1 and a final report at the end of year 2. We expect that we will be able to derive relationships between the different levels of results, from the molecular high throughput assays to in vivo molecular endpoints and to in vivo higher order changes in survival, growth and development. The estrogenic and androgenic equivalencies that we will derive will help establish the usefulness of high throughput assays as a means to test the quality of estuarine water. We expect this demonstration project to show the usefulness of the approach.

Projected roadmap from this special study to implementation of *in vitro* assays in monitoring.

1. **July 2012 — Development of protocols for commercially available bioanalytical tools.** (sponsored by the State Water Board). Currently there are at least 3 commercial companies that have developed assays for nuclear hormone receptors, including InVitrogen, Biodetection Systems, and SwitchGear. We are in the process of testing the assays and will choose one system for further work. This is part of a project awarded to SCCWRP by the State Water Board (SWB). Initial studies with all three assays indicate that they work well. We will choose a method that has the best chance for implementation by end users in California.
2. **Dec 2012 – Testing of bioanalytical assays for multiple molecular pathways with concentrates extracted from water.** (sponsored by State Water Board). The assays include molecular pathways that are known to be affected by exposures to estrogens, androgens, progesterone and glucocorticoids, among other chemicals. We will use the selected assays to examine extracts of WWTP effluents and compare the results with analytical chemistry of the same extracts. We also plan to participate in a parallel project to use bioanalytical techniques to measure hormone activities in Australian WWTP effluent and surface waters (funded by WERF).
3. **April 2013 — Implement Menidia bioassays following EPA protocols.** (sponsored by RMP) Tasks 1-3 of proposed project. Determine concentrations of E1, 4-NP, BPA, and galaxolide (HHCB) that show effects *in vivo*. Compare these concentrations to those required to change expression of genes *in vivo* and with concentrations required to activate *in vitro* assays showing ER activity.
4. **July 2013 – Start Bight '13 special study utilizing in vitro assays in conjunction with other monitoring tools.** (sponsor TBD). Conduct study using southern California samples that is complementary to concurrent RMP study.
5. **April 2014 — Compare in vivo and in vitro assays for effluent and receiving water**. (sponsored by RMP). Complete tasks 4-5 of proposed project.
6. **September 2014—Integrate results of the proposed RMP *Menidia* project with results from other projects.** (co-sponsored by RMP, SCCWRP and partners, SWB, WERF) This would include other endpoints covered in the other funded studies, e.g. those that are planned in conjunction with Bight 2013, future RMP surveys, and SWAMP special studies.
7. **October 2014 – Workshop to teach bioanalytical methodology.** (sponsor TBD) Transfer of *in vitro* assay technology to public and private laboratories. This would include entities interested in applying the approach in monitoring programs.

8. **January 2015 – Start interlaboratory study to compare bioanalytical methods to current chemical analysis and *in vivo* tests.** (sponsor TBD).
9. **March2016 – Report on interlaboratory study.** If performance is deemed satisfactory, develop guidance to implement bioassays in routine monitoring.
10. **July 2016 – Start pilot implementation study.** Identify partner agencies and facilitate incorporation of in vitro assays into their monitoring programs on a limited basis.
11. **July 2017 – Assess pilot study results.** Review results from pilot study and develop plans and guidance for implementation of assay methods.

Potential partners, funding agencies, or possibilities to leverage funds for future progression of bioanalytical tool development.

1. WERF—applied research
2. NOAA – using Mussel Watch as an environmental effects platform
3. NSF – funding of basic research oriented projects
4. State Water Board – future phases for development and implementation of bioanalytical screening tools
5. Other Water Quality Stakeholders – special studies as part of regional surveys (e.g. Bight 2013; RMP) and specific discharger needs (e.g. outfall maintenance)

Budget

The scope of this study will require two years, with Year 1 devoted to obtaining molecular biomarkers for *Menidia* and defining critical endpoints of adversity in early life stage fish. Year 2 will focus on application of these endpoints to effluent and receiving water samples (see Deliverables and Time Line). We are requesting a total budget of $126,000, with $70,000 for work to be completed in 2012 and the remaining $56,000 for 2013. In addition, SCCWRP will contribute leveraged funds via two projects, one supporting the development of in vitro bioassays (or “transactivation assays”) funded by the State Water Board and scheduled for completion in mid-2013, and from an internally funded project to develop in vivo bioassays using Menidia and other estuarine marine fish species.

Project Budget

Description	Cost per Sample ($)	Total Cost Estimate ($)	Cost Estimate minus match ($)
Yr: 1			
Development of Molecular Biomarkers		22,000	**22,000**
Early life stage assay—model compounds -- 4 (SCCWRP Match – 8,000)	6,000	24,000	**16,000**
Juvenile assay – model compounds -- 4 (SCCWRP Match – 8,000)	6,000	24,000	**16,000**
Transactivation Assays -- 4	3,000	12,000	**12,000**
Analytical chemistry (SCCWRP Match 4,000)	2,000	8,000	**4,000**
Total Yr 1		90,000	**70,000**
Yr:2			
Water extracts ($6,000 in kind SCCWRP)	6,000	6,000	**0**
Early life stage assay – field sites – 4 (SCCWRP Match—8,000)	6,000	24,000	**16,000**
Juvenile assay – field sites -- 4 (SCCWRP Match 8,000)	6,000	24,000	**16,000**
Transactivation assays – 4	3,000	**12,000**	**12,000**
Analytical Chemistry – Field sites –4	3,000	**12,000**	**12,000**
Total yr 2		**78,000**	**56,000**
Total Requested Budget		**168,000**	**$126,000**
SCCWRP contribution	$42,000		

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