

PS/SS: Estrogen receptor *in vitro* assay linkage studies

Estimated Cost: \$45,000 for 2017.

Oversight Group: Emerging Contaminants Workgroup and Exposure and Effects Workgroup

Proposed by: Nancy Denslow (University of Florida)

Proposed Deliverables and Time Line

Deliverable	Completion Date
Task 1: ER dose response linkage <i>in vitro/in vivo</i>	12 months
Task 2: Demonstrate usefulness of assay with environmental samples	12 months
Task 3: Report	12 months

BACKGROUND

There is no longer any question that pharmaceuticals and personal care products are found in surface waters in our environment at concentrations that have biological activities in aquatic organisms (reviewed in (Cooke et al. 2013). Alterations among higher order endpoints have been observed including alterations of gonadal sex differentiation, causing a disproportionate female sex ratio, changes in secondary sex characteristics, reduction in reproduction and growth, and alterations of courting behavior, among others (Matthiessen and Sumpter 1998; Rodgers-Gray et al. 2001; Sarria et al. 2011; Adedeji et al. 2012; Baumann et al. 2014). The most studied are chemicals that either mimic the function of 17 β -estradiol (E2) or interfere with the biosynthesis or metabolism of the endogenous hormone. As might be anticipated, it is now recognized that the activities of multiple estrogen mimics when present together in mixtures in an effluent are additive (Brian et al. 2007), making it problematic to monitor effluents using the “one chemical at a time” approach.

While the gold standard is to measure higher order alterations directly *in vivo*, the experiments are costly in both time and money and require the use of hundreds to thousands of fish and furthermore are impractical to run routinely. Based on the recommendations of the National Academy of Sciences (NRC 2007), toxicologists are turning their attention to high throughput *in vitro* assays that are specific for mechanism of action and which are much more cost effective than *in vivo* assays (Dix et al. 2007; Judson et al. 2009; Martin et al. 2009; Conley et al. 2016). However, before these assays can be used in a regulatory framework, it is important to establish linkages from the *in vitro* assays to *in vivo* end points. While some studies have done this with fresh water fish, studies with estuarine fish are lacking. For the fresh water fish it is now clear that affinity of a chemical for the ligand binding domain of the estrogen receptor is a good predictor of higher order effects (Miyagawa et al. 2014). A strong linkage between the two for estuarine fish would enable managers to monitor routinely for estrogens in San Francisco Bay with a bioanalytical test. The gap in being able to predict *in vivo* endpoints from *in vitro* assays precludes this approach from being widely used. In this project, we will reduce the gap, clearly linking concentrations that are necessary for activity both *in vitro* and *in vivo*. Our preliminary data obtained from the phase 1 application to SFEI, clearly shows this will be

possible to do. In addition, we will pilot a small study to see how the *in vitro* assay would work on both water and contaminated sediments in San Francisco Bay. Recent publications suggest that sediment may be a sink for endocrine active compounds (Sangster et al. 2014; Zhang et al. 2015).

Study Objective and Applicable RMP Management Question

Our first phase study clearly showed that it would be possible to link *in vitro* ER assays to *in vivo* endpoints. Our conclusion was that the concentrations of estrogens needed to be above the EC₅₀ point for the *in vitro* assays to see effects *in vivo* with *Menidia*. However, the dose response curve for the *in vivo* endpoints was broad and we missed several critical doses that would narrow the comparison from *in vitro* to *in vivo*. While the data allows us to extrapolate those values, it would be better to pinpoint the comparison a little more closely and also to repeat the whole study to see if the relationships continue to hold. Further, we intend for this assay to become a standard monitoring tool and will develop a pilot study to measure estrogen equivalencies at 6 locations in the bay, testing both the water and sediments, to determine the usefulness of the approach. Results from this study will begin to enable managers to determine whether or not additional cleanup is necessary for treated effluents that are disposed into sensitive estuarine environments. This work will not only be important for California, but also for other states that border marine environments and which may still be using old technologies for water treatment and discharge. The overall 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?

This targeted study will have two objectives:

- (1) To repeat the *in vivo* portion of the linkage study with tighter concentrations around the likely EC₅₀ for *in vivo* responses.
- (2) To test water and sediment from 6 locations in San Francisco Bay for estrogenic equivalencies as a pilot test for this approach.

Study Plan

In phase 1, we tested both strong and weak estrogens with the *in vitro* estrogen transactivation assay from InVitrogen. We also performed *in vivo* assays with two life stages, larvae (10-17 days post hatch (dph)) and juveniles (30-58 dph) and examined higher order endpoints including for larvae, survival, growth and gene expression and for juveniles, gonadal tissue differentiation, growth and gene expression. For this phase-2 project, we will concentrate our efforts on 17β-estradiol (E2), as a prototypic estrogen for which we will develop the linkage from *in vitro* to *in vivo*. We will use the juvenile life stage of *Menidia beryllina* (inland silversides), as this stage provided the most sensitivity for higher order effects from estrogens. For this assay we will measure gonadal tissue differentiation, growth and hepatic gene expression for two genes, whose expression in juvenile fish depends on the presence of E2. We will index estrogen equivalency concentrations required for altering higher order endpoints with biochemical responses within the fish and responses obtained with the commercially available estrogen receptor (ER) transactivation assay from InVitrogen. These linkages will enable the use of *in vitro* assays as measurements 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.

Task 1: ER dose response linkage in vitro/in vivo

Building on our previous work, we will start with 30 dph *Menidia* and treat them with 7 concentrations of E2, including 2, 10, 20, 40, 80, 200 and 500 ng E2/L compared to control (no E2). The EC₅₀ for the *in vitro* assay is 20 ng E2/L and we expect the curve for *in vivo* higher order effects to be slightly shifted to the right. We know from previous work that 200 ng/L is close to the plateau and by 500 ng/L we have reached the plateau (Fig. 1). 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 be dechlorinated tap water adjusted to 15 ppt salt (using Instant Ocean) and temperature will be controlled to 23 ± 1 °C, following our modifications of the standardized test guidelines for early life testing (US-EPA 1995) (Denslow et al.). Fish will be fed dechlorinated/hatched artemia (E-Z egg) and all exposures will be performed in quadruplicate in tanks containing 6L of water. Exposure solutions will be changed daily at 75% of total volume. Confirmation of exposure concentrations will be performed using an ELISA for E2 (Cayman)(Allinson et al. 2010), as we have done previously (Denslow et al.).

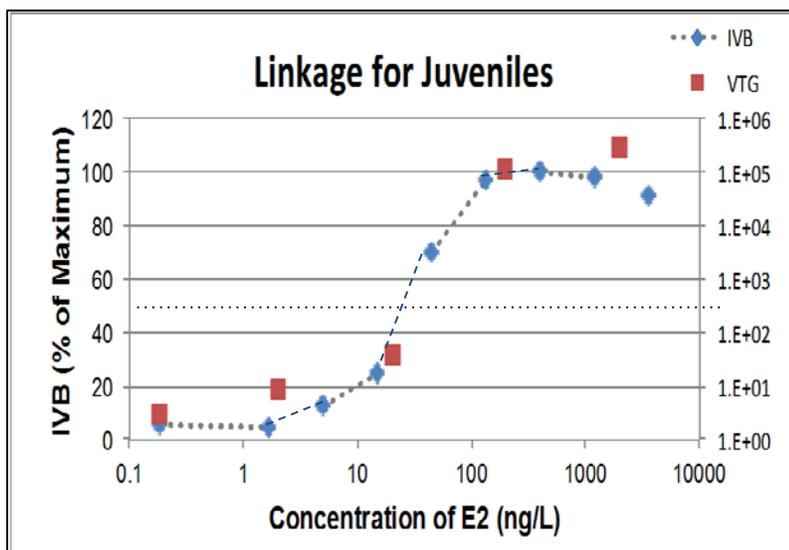


Figure 1. *In vitro* to *in vivo* linkage results from Phase 1. The blue line represents the dose-response curve for the *in vitro* assay, showing an EC₅₀ of about 20 ng/L. The red dots represent hepatic Vtg gene expression after 28 days from *in vivo* exposures. The two assays were superimposed on one graph. We are uncertain about the actual relationship of the two graphs. Feminization of fish occurred at concentrations greater than 200 ng E2/L at which point 80% of the fish were female. At 500 ng E2/L, 100% of the fish were female.

From our phase 1 experiment, we know that female *Menidia* differentiate their gonadal tissue in the 35 to 45 dph time frame, while males differentiate their tissues close to 120 dph. Consequently we will treat the juvenile *Menidia* for 28 days, starting with fish that are 30 dph and going to 58 dph, to capture the window for female tissue differentiation. After this time, some of the fish will be sampled and others will be grown in clean water for two months to capture the male gonadal differentiation period. We will take pictures of the fish at the beginning of the experiment, after 14 days, 28 days and at the end of the grow out period to capture growth.

After the 28-day exposure, some of the fish will be sacrificed and livers will be obtained using a dissecting scope. We will prepare hepatic total RNA and measure expression of vitellogenin (Vtg) and choriogenin (Chg), two genes that are known to be under the control of estradiol. We will use at least two housekeeping genes, ribosomal protein L8 (rpl8) and GAPDH.

For histopathology verification of gonadal sex differentiation, we will use cassettes with 4 compartments and capture the gonadal tissue as a sagittal section, using the methods we used in Phase 1 (Denslow et al.).

Task 2: Demonstrate usefulness of assay with environmental samples

This will be a pilot study to show the utility of the *in vitro* assay in San Francisco Bay. We will work closely with staff at SFEI to collect water (1 L) in triplicate and sediments (50 g) in triplicate from 6 locations of varied condition in the Bay. We will provide HLB cartridges and a protocol to SFEI staff for the water collections, which they will put onto the HLB cartridges and mail them along with the triplicate sediment samples from the same location via FEDEX to the Denslow laboratory. We will elute the HLB cartridges with MEOH once we receive them. Sediments will be extracted with acetone using a sonication method we have developed previously (Dang et al. 2016). Extracts will be tested on the Invitrogen ER transactivation assay along with a full 9-point standard curve in both agonist and antagonist mode with the water and sediment extracts to obtain estrogen equivalencies. Each extract will be tested at 4 concentrations, using a binary dilution scheme, following methods we have previously developed (Escher et al. 2014; Maruya et al. 2015; Mehinto et al. 2015).

After examining the *in vitro* results, two concentrations of the extracts that are quantifiable within the linear range of the activity assay will be injected IP into adult male *Menidia* and these will be held for 48 h to confirm changes in hepatic gene expression for Vtg and Chg. We have determined previously that 48 h is sufficient time to see changes in gene expression for these genes in sheepshead minnow, another estuarine fish with approximately the same sensitivity as *Menidia* (Bowman et al. 2000; Denslow et al. 2001). We will also perform histopathology to confirm the sexes of the fish. These experiments will be performed with at least 8 fish per concentration. Livers will be dissected out for total RNA and gonads will be prepared for histopathology.

Expectations and Alternative Strategies. We expect to see very similar *in vitro* and *in vivo* results with *Menidia*, as we have obtained in the Phase 1 study (Denslow et al.), except that we will have enough concentrations to develop an *in vivo* dose response curve. From past experiments, we are confident that these concentrations will impact molecular endpoints within the fish resulting in alteration of gene expression, alterations of gonadal development and growth. Acceptable mortality in the experiments will be <10% for the quadruplicate tanks. The acceptable variance for duplicate determinations of gene expression by Q-PCR will also be 10%.

Task 3: Reporting

We plan to submit a report at the end of year 1. We expect that we will be able to derive relationships between the different levels of results, from the molecular *in vitro* high throughput assays to *in vivo* molecular endpoints and to *in vivo* higher order changes in growth and gonadal tissue differentiation. We expect this demonstration project to show the usefulness of the approach.

Budget

The scope of this study will require one year. We are requesting a total budget of \$45,314. This project has already had significant leveraging through the completion of phase 1 of the project. Development of the transactivation assays were originally funded by the State of California Water Board in 2013 (\$800,000) and completion of phase 1 of the project required substantial internal funding (on the order of \$50,000), in addition to the funds provided by SFEI. We anticipate that this project will take a full year to complete, but have budgeted time very conservatively.

Project Budget

Description	Cost per unit	Total cost
Task 1: ER dose response linkage		
Supplies: in vivo exposure, gene expression by qPCR, histology, E2 dose verification	\$10,500	\$10,500
Labor -- 2 months	\$12,167	\$12,167
Task 2: Environmental samples from the Bay		
Supplies for 4 samples in triplicate X 3 = 12 samples -- includes 1 Invitrogen kit/4 samples, fish for IP injection, qPCR, histology, SPE columns	\$2,500	\$7,500
Labor -- 1 month	\$6,084	\$6,084
Total direct		\$36,251
IDC at 25%		\$9,063
Total requested from SFEI		\$45,314

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