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Linkage of *In Vitro* Assay Results With *In Vivo* Endpoints Final Report – Phase 1 and Phase 2

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EXECUTIVE SUMMARY

The goal of this project was to determine quantitative linkages between *in vitro* bioanalytical (IVB) assays and higher-order-endpoints that may relate to population level effects in estuarine fish. The fish model chosen for the *in vivo* endpoints was the inland silverside (*Menidia beryllina*), which is an established fish model for estuarine toxicity and for which established USEPA protocols exist. This project focused on estrogenic chemicals commonly found in wastewater effluent including 17β estradiol (E2), estrone (E1) and 4-nonylphenol (NP), which range in potency for the estrogen receptor. We used commercially available *in vitro* assays for human estrogen receptor alpha transactivation to determine the 50% effect concentration (EC_{50}) for the different chemicals. *In vivo* endpoints included alterations in molecular gene expression and higher-order-endpoints normally used in risk assessment, such a gonadal tissue differentiation, growth and survival. Molecular gene expression endpoints were for genes related to brain development (brain aromatase in larvae) and sex differentiation and reproduction (larvae and juveniles). The higher endpoints were survival, growth of larvae, gonadal tissue differentiation, and growth of juveniles for 28 days. A subset of juveniles that were exposed for 28 days to estrogenic chemicals were then transferred to clean water and cultured for an additional 60 days to determine whether the effects observed in the constant exposure for 28 days were reversible. In addition to the individual chemicals, we also exposed larvae and juvenile fish to diluted effluent (5 to 50%) from a wastewater treatment plant that discharges into San Francisco Bay.

EC_{50} values obtained from the *in vitro* assays for the three estrogenic chemicals were 22 ± 2.1 ng E2/L, 81 ± 14 ng E1/L and 20.5 ± 1.6μg NP/L. In the larval assay, we were able to measure changes in gene expression for three genes: brain aromatase b (Cyp19b), vitellogenin (Vtg) and choriogenin L (Chg), at concentrations that exceeded the EC_{50} points for the *in vitro* assays. All three of these genes are known to have estrogen response elements in their promoters in other fish and are likely to have them also in *Menidia.* There were no effects of the chemical on larval survival, except at 200 μg NP/L, which led to 100% mortality. There was a trend to decreased growth with increased concentrations for both E2 and NP but die to the shortness of the exposure (7 days) and variability in growth rate among replicate fish, this was not significant.

Similar gene expression and growth results were obtained for juvenile fish and in addition, we were able to measure effects on gonadal differentiation. The two genes selected for gene expression studies, Vtg and Chg, showed dose response increases in expression starting at the EC_{50} concentrations for the *in vitro* assays. We saw estrogen-dependent changes in gonadal tissue differentiation and for some of the exposures altered growth after 28 days of exposure. After two months of culturing in clean water, some of the organismal effects appeared to be reversed, but others persisted, especially for the higher concentrations of estrogens. For E2, there was significant delay for male gonadal tissue differentiation at doses greater than the EC_{50} even after 2 months in clean water. In addition, females had extensive atresia

of their ovaries. In the case of E1, feminization effects persisted only at 300 ng E1/L. The effects observed at 100 ng E1/L in the 28-day exposure seemed to be reversed. Nonylphenol showed no effects on gonadal tissue differentiation after 28 days but had increased numbers of undifferentiated fish after the two month grow out in the groups that had been originally treated with the higher nonylphenol concentrations, suggesting that male differentiation had been delayed by the 28-day exposure. Effects on growth were less dramatic, with only some of the conditions showing statistically significant effects, and these appeared not to be dose-dependent. It would be prudent to repeat the grow-out phase of the experiment one more time to determine if the observed effects are repeatable.

Our results clearly show that it is possible to establish linkages between the screening level in in vitro assays and higher order endpoints in fish that are influenced by estrogen, such as growth and gonadal sex differentiation. As expected, the *in vitro* responses are the most sensitive endpoints tested and using them as a monitoring tool can provide a margin of safety for aquatic organisms that may be more sensitive to estrogenic chemicals than *Menidia*. Moreover, the margin of safety provided, represented by the ratio of *in vivo* to *in vitro* EC_{50}s ranged from 0.9 to 9 for the potent estrogen E2 to 1.2 for E1 and 2.4 to 3.4 for NP. There were no significant effects on growth, but merely trends, by any of the chemicals tested, suggesting that a higher "n" may be required to see appropriate effects on this endpoint. Using more concentrations for *in vivo* tests can refine these ratios.

Of the endpoints we chose to examine, the most sensitive endpoints were at the molecular level for genes known to contain strong estrogen response elements in their control regions, including Vtg, Chg, and Cyp19b. Expression of the mRNAs for Vtg and Chg is specific to females as shown in previous studies with other fish species. Vtg and Chg are synthesized in the liver and are transported through the blood to developing oocytes in female ovaries. Cyp19b expressed mainly in the preoptic area, mediobasal hypothalamus and telencephalon regions of the brain has been linked to brain sexualization and plasticity during development.

The juvenile fish test was appropriate to assess higher order endpoints such as gonadal tissue differentiation. Larval stages were the least sensitive for growth, probably due to the short duration of the 7-day assay. To capture female tissue differentiation, a sensitive endpoint with good extrapolation possibilities to population level effects, the juveniles required 28 days of exposure. In addition, it was necessary to feed the fish twice a day during the exposure paradigm. While the long exposure periods needed to observe the apical effects might preclude their routine implementation, we have identified opportunities to further streamline the *Menidia* life stage exposure protocols.

Exposure to diluted effluent from a secondary WWTP resulted in little to no observed effects on either life stage. As a first example of how the *in vitro* bioanalytical assay would act in screening mode, the estrogen receptor (ER) transactivation assay response revealed very low equivalent concentrations of estrogenic chemicals (< 5 ng/L) in the diluted effluent used for exposure, confirming its concordance with the molecular and *in vivo* responses observed in either *Menidia* life stage.

This study is among the first to address the linkage between *in vitro* bioassays and higher order effects in estuarine fish in a quantitative way. It also, in a grander scheme, shows the utility of *in vitro* bioassays as monitoring tools for estuarine receiving waters such as those of San Francisco Bay. The project calibrated the *in vitro* bioassays to higher order endpoints in fish showing that the *in vitro* assays were more sensitive than *in vivo* endpoints used for regulatory purposes. EC50 values for the *in vitro* bioassays are likely protective of the environment including more sensitive fish species. As an added benefit, *in vitro* bioassays employed as a

screening element in a comprehensive, tiered monitoring program, are certainly more cost effective compared to performing *in vivo* assays. While estrogens represent a small fraction of the endocrine active chemicals found in receiving waters, this pilot study has shown that it is possible to make linkages with *in vivo* endpoints for specific contaminants. Additional studies with other potentially bioactive contaminants and different *Menidia* life stages and hormonal systems would add to this developing monitoring tool. This project was made possible by funding from SFEI and by leveraging the work with other projects and collaborators.

Full Text

http://ftp.sccwrp.org/pub/download/DOCUMENTS/TechnicalReports/983_LinkageInVitroAssayResultsInVivoEndpoints.pdf