Developing Bioscreening Thresholds for the Glucocorticoid Receptor Cell Assay

Estimated RMP Funds: $175,000 over 3 years **Total Cost of the Project:** $445,000 over 4 years
Oversight Group: Exposure and Effects Workgroup
Principal Investigators: Alvine C. Mehinto (SCCWRP), Nancy D. Denslow (University of Florida)

Statement of the Problem

Contaminants of emerging concern ***(CECs) exerting endocrine disrupting properties present a major concern for the health of coastal ecosystems***. While they are typically found at very low concentrations (picogram to nanogram per liter range), they can act jointly via a common mode of action leading to adverse effects on aquatic organisms. This issue cannot be fully addressed using the current chemical-by-chemical risk assessment approach, which targets known chemicals and relies on chemical-specific toxicity thresholds. Moreover, traditional toxicity endpoints (e.g. growth and survival) do not represent the variety of other relevant sublethal effects that can be induced by prolonged exposure to low levels of CECs, such as impaired tissue development, immune functions, behavior, or reproduction. *In vitro* cell assays have been proposed as rapid ***bioanalytical screening tools to detect and integrate the response of multiple known and unknown CECs***, thus providing the potential for a more comprehensive assessment approach. But before cell assays can be incorporated in monitoring programs, it is essential to establish the quantitative ***linkage between key cellular events and organismal responses, a key component in developing a robust interpretive framework for bioanalytical screening results***. Due to the lack of relevant ecotoxicological data for many CECs, such linkage has only been characterized for a few classes of CECs (e.g. estrogenic chemicals). This project aims to advance the role of cell assays in environmental assessments by developing bioanalytical screening thresholds associated with relevant toxicity endpoints for a group of understudied CECs known as steroidal anti-inflammatory drugs or glucocorticoids (GCs). This bioanalytical interpretive framework will help water quality managers in their task to protect beneficial uses of aquatic resources by identifying and prioritizing CECs that are most likely to impact aquatic life.

Proposed Deliverables and Timeline

Deliverable	Due date
Task 1- Project management and reporting	CSD + 48 months
Task 2- Evaluation of GC impact on the health of *M. beryllina*	CSD + 18 months
Task 3- Development of GR bioscreening thresholds protective of aquatic life	CSD + 36 months
Task 4- Assessment of GCs present in estuarine/marine	CSD + 48 months

Background

The diversity of CECs present in U.S waterways (Kolpin et al. 2002, Vidal-Dorsch et al. 2012, Bradley et al. 2017) and their complex interactions (e.g. additive, synergistic, antagonistic) require an integrative assessment to fully evaluate the ecotoxicological impact of CECs. While current monitoring approaches have generated valuable information on individual chemicals, they do little to address 1) the

occurrence of unknown or non-monitored chemicals, and 2) the potential impact of complex mixtures on aquatic organisms. Thus, complementary *in vitro* cell assays (also known as bioanalytical tools) have been proposed to conduct more comprehensive assessments of environmental CECs (Altenburger et al. 2015, Maruya et al. 2016). ***Cell assays are designed to respond to groups of chemicals acting via a common mode of action by initiating a molecular event*** (e.g. expression of a gene) associated with an adverse outcome pathway. As such, cell assays provide an integrated measure of the response of chemicals in complex mixtures, including those for which analytical methods are not currently available. The U.S. EPA has used a battery of these assays to evaluate the toxicity of individual chemicals as part of their ToxCast and Endocrine Disruptor Screening Programs (Reif et al. 2010). For environmental applications, numerous studies have demonstrated that cell assays can distinguish among samples of varying water quality and guide further chemical and toxicity testing (Snyder et al. 2001, Leusch et al. 2014a, Tang et al. 2014, Mehinto et al. 2017, Maruya et al. 2018). And recently, the California State Water Board has endorsed the utilization of cell assays for water quality assessment.

Effective and comprehensive screening of CECs require the use of a battery of cell assays representing a range of relevant endpoints/toxicity pathways. To date, only one endocrine-related endpoint, the estrogen receptor alpha (ERα) cell assay, has been standardized and vetted for ambient monitoring (Escher et al. 2014, Leusch et al. 2014b, Mehinto et al. 2015, Di Paolo et al. 2016). The linkage between ERα assay and animal responses has also been quantified through a RMP-supported study (Mehinto et al. 2018). Other endocrine-related bioanalytical endpoints have shown promise as monitoring tools, but linkages to adverse effects and/or screening thresholds have not been established. One of these assays is the ***glucocorticoid receptor (GR) cell assay that screens for a class of synthetic hormones called glucocorticoids*** (GCs) (see **Table 1**). These steroidal anti-inflammatory drugs are widely used in human medicine (e.g. to treat asthma, eczema, hay fever, arthritis, etc.) and they can enter the aquatic environment via discharges of treated wastewater effluents. Due to the lack of standardized analytical methods, few monitoring programs have measured individual GCs in receiving waters and the levels of GCs reported in California are typically low or below analytical detection limits (Klosterhaus et al. 2013, Bradley et al. 2017). However, bioanalytical screening surveys have reported GR activity in U.S. wastewater effluents and surface waters (Stavreva et al. 2012, Jia et al. 2016, Conley et al. 2017). The discrepancy between GR assay results and targeted chemical measurements (see **Table 2**) suggest that most environmental GCs are either present at levels below detection limits using conventional analytical methods, or that they not routinely monitored. Thus, the utilization of GR cell assay could improve the ability to monitor environmental GCs and to better investigate their potential impact, especially at sites receiving wastewater effluent discharges and urban runoff.

Table 1. List of synthetic glucocorticoids (GCs) of interest.

GCs	Therapeutic use	Relative potency *
dexamethasone	arthritis, severe allergies	1
betamethasone	asthma	0.5
clobetasol propionate	eczema, skin rash	36
fluticasone propionate	allergies	63
hydrocortisone	insect bite, poison ivy, eczema	0.2

***** Jia et al. 2016; Relative potency expressed relative to dexamethasone using the GR GeneBLAzer assay

Table 2. Occurrence of synthetic glucocorticoids (GCs) and glucocorticoid receptor (GR) bioactivity measurements in the Bay

GC concentrations in surface waters [a] (ng/L)		GR bioactivity of effluents [b] (ng dexamethasone equiv./L)	
betamethasone	< 5		
hydrocortisone	< 593	secondary treated effluent	236
methylprednisolone	< 4		
prednisolone	< 19	tertiary treated effluent	< 23
prednisone	< 45		

[a] Klosterhaus et al. 2013; [b] Mehinto et al. 2016;
Below detection is represented by "< median limit of detection".

Before the GR assay can be incorporated into routine monitoring practices, it is ***critical to establish screening thresholds associated with ecologically relevant adverse effects***. Research has shown that prolonged exposure to GCs can adversely impact fish at different stages of their development (LaLone et al. 2012, Kugathas et al. 2013, Chen et al. 2016). For example, Wilson et al. (2015) showed that zebrafish embryos exposed to GCs altered the size and function of the heart at the adult stage. Preliminary studies conducted by the PIs have also revealed that genes linked to immune functions and metabolism were affected in *Menidia beryllina* larvae exposed to clobetasol propionate, a particularly potent GC (data unpublished). The key adverse outcome pathways induced by exposure to synthetic GCs have been described by Margiotta-Casaluci et al. (2016) and include compromised immune functions, impaired gluconeogenesis and larval development. This project will utilize available toxicological information to identify the most responsive and reliable endpoints for *M. beryllina*, a fish species established by the U.S. EPA for whole effluent toxicity testing that is easily maintained in the laboratory. This species is also related to two smelt species found in California coastal and brackish waters, the topsmelt (*Atherinops affinis*) and the endangered Delta smelt (*Hypomesus transpacificus*). Therefore, *M. beryllina* can serve as a good surrogate to investigate the impact of environmental CECs in the Bay.

This project builds on previous research funded by the RMP, SCCWRP and the State Water Board to develop a comprehensive suite of bioanalytical tools that can be easily interpreted. Results from these efforts have successfully standardized select cell assays for screening of water quality and established a quantifiable linkage for in vitro ERα to in vivo responses. In the proposed four-year project, laboratory-based exposures will be conducted to characterize the linkage among *in vitro* GR bioactivity of GCs occurring in the environment (e.g. clobetasol propionate) and *in vivo* effects at different biological scales (e.g. gene, tissue and organismal changes). The ***results of this project will be used to calculate the relative potency of the GR assay and develop bioanalytical thresholds that are protective of the health of estuarine/marine fish***. The newly developed screening tool and toxicity endpoints will be utilized to assess the potential impact of GCs in estuarine/marine habitats in California.

Study Objectives and Applicable RMP Management Questions

The overall goal of this research is to develop more efficient monitoring tools to screen for known and unknown contaminants and assess their combined effect on estuarine/marine aquatic species. In the San Francisco Bay estuary, chemical occurrence data collected to date suggest that PPCPs such as GCs (see

Table 2) are of low concern, but with the increasing number of emerging contaminants released in the aquatic environment, chemical by chemical monitoring may not be effective or adequate to evaluate their occurrence and impact on aquatic life. Bioanalytical screening tools capable of integrating the response of chemical mixtures present a complementary and efficient method to streamline monitoring and assessment of receiving waters. The 3 specific tasks and objectives identified in this project (see **Table 3** below) will help address two RMP's priority management questions:

1- Which CECs have the potential to adversely impact beneficial uses in San Francisco Bay?

2- Are there any indications of ecological effects caused by exposure to specific chemicals or mixtures of contaminants in the Bay?

Table 3. Study objectives and questions relevant to RMP management questions.

Study objective	Information application
Task 2 aims to identify ecologically relevant toxicity endpoints to assess the impact of GCs.	Novel and sensitive tools will be available to assess the health of fish residing in the Bay.
Task 3 aims to establish bioanalytical monitoring thresholds protective of aquatic life.	The quantitative linkage established will be used to develop an interpretive framework for bioanalytical screening results.
Task 4 aims to determine if levels of GCs present in estuarine/marine can impact fish health.	Bioanalytical data on GC occurrence in estuarine/marine habitats will help determine their potential for impact.

Approach

Task 1- Project management and reporting.

A kickoff meeting will be scheduled where the PIs will develop a detailed experimental design and set a timeline to complete Tasks 2-4. Over the duration of the project, the PIs will meet periodically to discuss results, review the next steps and revise the experimental design if necessary.

Expected outcome: Progress reports will be submitted at the end of Years 1, 2 and 3 (CSD + 12 and 24 months) to present the preliminary findings of the fish exposures and linkage analyses. After completing all lab exposures and analyses, our findings with respect to the GR bioanalytical thresholds will be summarized in a final report (CSD + 48 months) and published as a peer-reviewed manuscript.

Task 2- Evaluation of GC impact on the health of *M. beryllina.*

Objective: Examine a range of parameters and identify the most responsive endpoints for *M. beryllina*.

Methods: Model GCs that exhibit different potencies (e.g. clobetasol propionate, betamethasone; see **Table 1**) will be utilized to conducted single chemical exposures using *M. beryllina* larvae. A minimum of 2 concentrations per GC will be tested in a semi-static exposure system for 14-21 days. For each exposure, fish will be held in glass vessels, with 5 replicate vessels and 10-15 fish per replicate. Test performance and acceptability criteria will be evaluated using U.S. EPA test guidelines (USEPA 2002). Candidate molecular markers measured by qPCR and physiological endpoints that represent known adverse outcome pathways for GCs will be examined (see **Table 4**).

Expected outcome: Results of Task 2 will produce optimized toxicity test parameters (e.g. exposure duration) and a list of molecular and higher order endpoints that are diagnostic of GC effects in fish.

Table 4. Candidate endpoints for *M. beryllina* exposed to model GCs.

Molecular endpoints	Higher order endpoints
glucocorticoid receptor (GR)	growth (length and biomass)
inhibin alpha (INHA)	body deformities
insulin growth factor (IGF-1)	glucose metabolism
kinase adaptor protein 1 (NCK1)	immune response
phosphoenolpyruvate carboxykinase (PEPCK)	survival

Note: Final list of endpoints may change based on availability of new information.

Task 3- Development of GR bioscreening thresholds protective of aquatic life

Objective: Establish a quantitative linkage between GR cell assay response and adverse effects in *M. beryllina* to set conservative screening thresholds.

Methods: The fish toxicity endpoints showing the greatest degree of molecular and physiological changes in Task 2 will be carried over into this task. Fish will be exposed to an expanded series of GC concentrations (at least 5 concentrations per chemical, for three GCs) using the experimental design optimized in Task 2. Concentrations inducing 10% and 50% of the maximum GR assay response (EC_{10} and EC_{50}) and concentrations comparable to the GR activity measured in WWTP effluent samples discharging in the Bay (Mehinto et al. 2016) will be included. Quantification of GC concentrations will be performed using the standardized GR GeneBLAzer transactivation assay (available from Life Technologies), and results will be expressed as a bioanalytical equivalent concentration (BEQ in ng/L) relative to dexamethasone (DEX). The relative potency factor between GR assay responses and in vivo effects will be calculated as in vivo LOEC for a given endpoint divided by the in vitro EC_{10}.

Expected outcome: The linkage between GR assay and key biological endpoints in fish will be characterized, resulting in the development of GR bioscreening thresholds that are protective of aquatic life.

Task 4- Assessment of GCs present in coastal environments

Objective: Determine whether GCs present in estuarine/marine habitats are at levels of concern.

Methods: Water and/or sediment samples (n= 4-8 samples total) will be obtained as part of sampling efforts by the ECWG and/or other monitoring programs. Samples will be processed and analyzed for total glucocorticoid bioactivity using the GR GeneBLAzer assay. Bioanalytical results will be integrated with available targeted measurements to improve our understanding of the occurrence of GCs in estuarine and marine habitats in California.

Expected outcome: A report on GC occurrence and potential impact in coastal habitats will be produced.

RMP Budget Requested and Leveraged Effort

The amount requested from the RMP for this 4-year project is $175,000, with $50,000 requested in Year 1, $75,000 requested in Year 2, $50,000 in Year 3 and no funds requested in Year 4. SCCWRP will

provide salary match in the amount of $50,000 to ensure that all tasks are completed. Detailed budgeting of RMP funds and total cost for the project are provided in **Table 5**.

This project will supplement the proposed ECWG Special Study for 2020 on pharmaceutical occurrence. The ECWG effort will be leveraged to obtain field samples for bioanalytical screening and chemistry data for individual pharmaceuticals including GCs. Other related projects will provide an estimated $220,000 in leveraged funds to support the overall project with:

- $150,000 from the proposed project on bioanalytical tools development and statewide application (PI K. Maruya) (90% likelihood execution in FY18-19).
- $50,000 from proposed project to screen sediment and fish tissues from the Southern California Bight using bioanalytical tools and non-targeted chemical analyses (PI A. Mehinto) (80% likelihood execution in FY18-19).
- $20,000 from the *M. beryllina* project funded by the EPA STAR grant (R835799) to develop relevant toxicity endpoints for endocrine disrupting chemicals (co-PI A. Mehinto).

Table 5. Detailed budget for SCCWRP and UF

	Year 1		Year 2		Year 3		Total RMP cost
Description	**Hours**	**Cost**	**Hours**	**Cost**	**Hours**	**Cost**	
SCCWRP Budget Request = $87,500							
Project staff	172	10,207	260	15,159	140	9,261	34,626
PI A. Mehinto	*60*	*4,210*	*80*	*5,838*	*60*	*4,554*	
Technical staff	*112*	*5,997*	*180*	*9,321*	*80*	*4,707*	
Supplies		5,986		8,763		7,049	21,798
Others		-		500		700	1,200
Total Direct		16,193		24,422		17,010	57,124
Indirect costs		8,807		13,078		7,990	29,876
UF Budget Request = $87,500							
Project staff	182	9,205	202	11,633	182	9,766	30,604
PI N. Denslow	*20*	*2,089*	*40*	*4,303*	*20*	*2,216*	
Technical staff	*162*	*7,116*	*162*	*7,329*	*162*	*7,549*	
Supplies		9,649		16,048		9,088	34,785
Others		-		600		-	600
Total Direct		18,854		28,281		18,854	65,989
Indirect costs		6,146		9,219		6,146	21,511
RMP funds per year		**$50,000**		**$75,000**		**$50,000**	

Total RMP funds requested	$175,000
Total leverage funds	$220,000
SCCWRP salary match	$50,000
Total Project Cost	**$445,000**

Budget justification

Project staff. SCCWRP-matched salary (incl. fringe benefits) is requested for PI A. Mehinto (Senior Molecular Toxicologist at SCCWRP) to manage all aspects of the project, including study design, qPCR and cell-based assays, QA review and data analyses, coordination with UF and report/ manuscripts preparation. SCCWRP-matched salary is also requested for a research technician to process fish/field samples and conduct all the molecular assays.

Funds are requested for PI N. Denslow (Professor in Environmental Toxicology at the University of Florida) who will oversee the work performed in her laboratory and will participate in writing progress reports and manuscripts. Mr. Kevin Kroll (Laboratory manager, technical staff) will conduct the exposures with *M. beryllina,* analyze data for apical endpoints, analyze histological. data, and perform qPCR assays as needed. All labor costs include salaries and fringe benefits.

Equipment. No equipment is requested as SCCWRP and UF are fully equipped to carry out this project.

Supplies. Approximately 30% of the requested funds will be utilized to purchase all supplies needed for the project. These include live *M. beryllina*, live food (Artemia), artificial seawater, water quality test kits/solutions; histological cassettes, fixative/staining solutions; test chemicals, high purity solvents, solid phase extraction cartridges, pure gases; cell assay kits, assay media, sterile 96-well plates, RNA extraction and cDNA kits, Agilent screen tapes, qPCR primers, SYBR Green mix; miscellaneous items such as gloves, pipet tips, cryogenic vials, conical centrifuge tubes, and glassware.

Others. In Years 2 and 3, a total of $1,600 is requested to cover shipping costs of experimental samples using a company that provides tracking numbers and delivery reports. Field sampling costs are not included in the budget, and PI Mehinto will collaborate with the relevant monitoring programs to obtain field samples.

Indirect costs. As a local government agency, SCCWRP's approved negotiated U.S. EPA indirect rate is 86.28% of direct salaries and wages including fringe benefits. Indirect costs charged by the University of Florida are calculated at 32.6% of the total direct costs.

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