PS/SS: Efficient extraction of endocrine disruptors from sediments from San Francisco Bay

Estimated Cost: $18,113 for 2018
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:	**Efficient extraction of sediments**	**12 months**
Task 2:	**Analysis of sediments by in vitro assay**	**12 months**
Task 3:	**Report**	**12 months**

BACKGROUND

Pharmaceuticals and personal care products are found below sewage treatment plants in many parts of the world at concentrations that have biological activities in aquatic organisms (reviewed in (Cooke et al. 2013). The hormone mimics that are most troubling include chemicals that act as estrogens, androgens or glucocorticoid mimics. Chemicals with these activities have been associated with alterations of higher order endpoints including gonadal sex differentiation, which causes 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). Chemicals that have been associated with these activities are generally measured by analytical chemistry including GC MS/MS or LC MS/MS, one chemical at a time. But, it is now recognized that different chemicals that act through the same biochemical pathway when present together in mixtures have additive effects (Brian et al. 2007), suggesting that measuring individual chemicals by analytical chemistry may underestimate the potential for adverse effects at polluted sites.

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). Good methods have been developed for extracting this class of chemicals from waters using OASIS HLB columns that capture a wide variety of chemicals. But fewer methods have been developed for extracting contaminants of emerging concern from sediments, especially sediments from the marine environment and then using the extracts for in vitro assays. This gap in knowledge will be the objective of the current proposal.

In our last project, we may not have efficiently extracted contaminants from sediments from San Francisco Bay. The current project will investigate alternative extraction methods that may work better for polar compounds that elicit endocrine activities. Recent publications suggest that sediment may be a sink for endocrine active compounds (Sangster et al. 2014; Zhang et al. 2015).

Study Objectives and Applicable RMP Management Question

Our Phase 2 study suggested that there was low level of estrogenic activity in San Francisco Bay waters. However, we were not able to clearly determine if sediments were contaminated or

not, as the method employed to extract chemicals from the sediments may not have been the best. The current study will address this gap in our knowledge and to begin to develop a method that can be standardized for adequate monitoring strategies in the bay.

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 method to adequately extract hormone mimics from bay sediments. 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 develop a robust extraction method for endocrine disruptors that may be bound to sediments obtained from San Francisco Bay
(2) To test the extracts by two in vitro assays: InVitrogen estrogen receptor (ER) transactivation assay and glucocorticoid receptor (GR) transactivation assay.

Study Plan

Task 1: Efficient extraction of sediments

In our last study, we extracted 3 g of sediment from various locations using acetone:hexane 1:1 with sonication. We had previously used this method with excellent outcome to extract organochlorine pesticides (OCPs) from high total organic carbon sediments in Lake Apopka (Dang et al., 2016). However, OCPs are more hydrophobic than the EDCs we expect to see in the marine environment, and this method may have not efficiently extracted the contaminants. We also noted that the sediments were fluffy after the treatment and we may not have efficiently separated the fines, making it possible that in two of our extracts sediment fines may have been included into the extract, and then when DMSO was added, this may have solubilized the contaminants, giving rise to what appeared as spikes in our results.

For this task, we will compare extraction efficiencies of two methods and determine the best method by LC MS/MS. We will spike 1 g aliquots of San Francisco Bay sediments suspended in sea water with our standard mixture of heavy isotope labelled hormones (Estradiol d5, ethinylestradiol d4, progesterone d9, testosterone d2, aldosterone d7, and [C13] 17 OH progesterone) at 250 ng of each standard. We will mix the heavy isotope-labeled chemicals with sediments for two weeks by constant stirring. We will monitor the amount of label that is bound by taking aliquots of supernatant after centrifugation at 2, 4, 7, 10 and 14 days to monitor the binding isotherm and to determine the plateau which we expect to achieve by 7 days based on previous work (Dang et al., 2016). This will be the effective amount that we can bind to the sediments. Once we achieve the plateau, we will consider this as the maximum bound and will set our experiments to determine how much of this we can actually extract from the sediments with our different test extraction methods. In addition, we will use sediments that are known to be contaminated naturally with environmental contaminants and this will be a second measure of extractability potential among the methods we propose.

After the two weeks of stirring, we will wash the sediments by centrifugation to remove any unbound hormones before extraction. Sediments will then be freeze dried prior to extraction and weighed.

We will evaluate extractability both by LC MS MS using a targeted approach with MRMs selected for the spiked contaminants and by two transactivation bioanalytical assays for the estrogen receptor and the glucocorticoid receptor. While not all of our spiked contaminants will be bound and not all of the bound will be extracted and some may be degraded in the process, we will be able to compare extractions for the best efficiency overall. The spiking experiment is to ensure that we will see something in our experiment.

The two methods we will employ are developed based on published literature. Both methods will use sonication**.**

Method 1 based on the method of Ribeiro the Souza et al (2015): sediments will be extracted with 5 ml MEOH, followed by 5 ml MEOH:water 1:1, followed by 2 ml acetone, and finally by 3 ml MEOH:water, 1:2, acidified to pH 2 with formic acid. Intermittent sonication will be used for 5 min at each step.

Method 2 based on the paper by Darwano et al (2014). For this method, sediments will be extracted with 5 ml MEOH, followed by 5 ml MEOH/Acetone 3:1; followed by 5 ml Acetonitrile/H20 7:3. Sonication will be used at each of the steps.

For a third experiment, we will compare extractability of a known contaminated sediment with the two methods above to determine which method works best.

The extracts from the different steps will be combined, dried down and further cleaned up either by extraction through an OASIS HLB solid phase cartridge or by extraction with MTBE and then tested by LC MS/MS for recovery of heavy isotope labeled standards. The reason for comparing the cleanup step is that we have had good experience with MTBE for hormones, but it is rather non-polar and it may actually be better to extract through solid phase. We have developed an excellent method for multiplexing hormones using MRM on our AB SCIEX LC MS/MS that works well with steroid hormones extracted from blood or tissues. We will determine the best extraction method for the spiked hormones and also determine if any unlabeled hormones are present in the sediments with our targeted approach.

Task 2: Measurement of activity by InVitrogen Assays

Once we determine the best method for extraction, we will use the method on sediments from three locations in San Francisco Bay (collected by SFEI personnel) to see if they have bioactivity in the Invitrogen ER and GR transactivation assays.

We will work closely with staff at SFEI to collect sediments (10 g) in triplicate from 3 locations of varied condition in the Bay. The triplicate sediment samples from each location will be shipped via FEDEX to the Denslow laboratory. Sediments will be extracted with the best method tested in Task 1. Extracts will be reconstituted in 100 ul of DMSO to improve the sensitivity of the assay. Extracts will be tested on the Invitrogen ER and GR transactivation assays along with a full 9-point standard curve in agonist mode with the sediment extracts to obtain bioanalytical equivalencies (BEQs) for estrogen and glucocorticoid. We will also measure the potential for cytotoxicity and only accept values for the transactivation assay that have less than 20%

cytotoxicity. 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).

Expectations and Alternative Strategies. We expect to obtain a good method for extracting San Francisco Bay sediments and we will use this extraction procedure for three sites of interest in the Bay. We also expect the *in vitro* assay to provide quantitative data from the 3 locations that will be superior to data we obtained in Phase 2.

Task 3: Report

We plan to submit a report at the end of year 1. We expect that we will be able to demonstrate quantitative measurements for sediment extracts and that the extracts function well in bioanalytical assays. 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 $18,113. 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 funding for labor very conservatively.

Project Budget

Description	Cost per unit	Total cost
Task 1: Efficient extraction of sediments		
Supplies: Heavy Isotope standards, solvents	$1,000	
Mass Spectrometry Time	$2,000	
Labor – 1 month	$5,440	$8,440
Task 2: Measurement of activity by InVitrogen Assays		
Supplies: ER and GR Invitrogen Kits	$2,500	
Labor – 1/2 month	$2,720	$5,220
Total direct		$13,660
IDC at 32.6%		$4,453
Total requested from SFEI		$18,113

References:

Adedeji OB, Durhan EJ, Garcia-Reyero N, Kahl MD, Jensen KM, Lalone CA, Makynen EA, Perkins EJ, Thomas L, Villeneuve DL, Ankley GT (2012) Short-term study investigating the estrogenic potency of diethylstilbesterol in the fathead minnow (Pimephales promelas).Environ Sci Technol 46(14): 7826-7835.

Baumann L, Knorr S, Keiter S, Rehberger K, Volz S, Schiller V, Fenske M, Holbech H, Segner H, Braunbeck T (2014) Reversibility of endocrine disruption in zebrafish (Danio rerio) after discontinued exposure to the estrogen 17alpha-ethinylestradiol.Toxicol Appl Pharmacol 278(3): 230-237.

Brian JV, Harris CA, Scholze M, Kortenkamp A, Booy P, Lamoree M, Pojana G, Jonkers N, Marcomini A, Sumpter JP (2007) Evidence of estrogenic mixture effects on the reproductive performance of fish.Environ Sci Technol 41(1): 337-344.

Conley JM, Evans N, Mash H, Rosenblum L, Schenck K, Glassmeyer S, Furlong ET, Kolpin DW, Wilson VS (2016) Comparison of in vitro estrogenic activity and estrogen concentrations in source and treated waters from 25 U.S. drinking water treatment plants.Sci Total Environ.

Cooke PS, Simon L, Denslow ND(2013). Endocrine Disruptors, in Haschek and Rousseaux's Handbook of Toxicologic Pathology,(Haschek, W. M., Rousseaux, C. G. and Wallig, M. A.Eds, Elsevier Inc., Academic Press: 1123–1154.

Dang VD, Kroll KJ, Supowit SD, Halden RU, Denslow ND (2016) Bioaccumulation of Legacy and Emerging Organochlorine Contaminants in Lumbriculus variegatus.Arch Environ Contam Toxicol.

Darwano, H., Duy, S. V., Sauve´, S. (2014) A new protocol for the analysis of pharmaceuticals, pesticides, and hormones in sediments and suspended particulate matter from rivers and municipal wastewaters, Arch. Environ. Contam. Toxicol 66:582-593.

Dix DJ, Houck KA, Martin MT, Richard AM, Setzer RW, Kavlock RJ (2007) The ToxCast program for prioritizing toxicity testing of environmental chemicals.Toxicol Sci 95(1): 5-12.

Escher BI, Allinson M, Altenburger R, Bain PA, Balaguer P, Busch W, Crago J, Denslow ND, Dopp E, Hilscherova K, Humpage AR, Kumar A, Grimaldi M, Jayasinghe BS, Jarosova B, Jia A, Makarov S, Maruya KA, Medvedev A, Mehinto AC, Mendez JE, Poulsen A, Prochazka E, Richard J, Schifferli A, Schlenk D, Scholz S, Shiraishi F, Snyder S, Su G, Tang JY, van der Burg B, van der Linden SC, Werner I, Westerheide SD, Wong CK, Yang M, Yeung BH, Zhang X, Leusch FD (2014) Benchmarking organic micropollutants in wastewater, recycled water and drinking water with in vitro bioassays.Environ Sci Technol 48(3): 1940-1956.

Judson R, Richard A, Dix DJ, Houck K, Martin M, Kavlock R, Dellarco V, Henry T, Holderman T, Sayre P, Tan S, Carpenter T, Smith E (2009) The toxicity data landscape for environmental chemicals.Environ Health Perspect 117(5): 685-695.

Martin MT, Judson RS, Reif DM, Kavlock RJ, Dix DJ (2009) Profiling chemicals based on chronic toxicity results from the U.S. EPA ToxRef Database.Environ Health Perspect 117(3): 392-399.

Maruya KA, Dodder NG, Mehinto AC, Denslow ND, Schlenk D, Snyder SA, Weisberg SB (2015) A tiered, integrated biological and chemical monitoring framework for contaminants of emerging concern in aquatic ecosystems.Integr Environ Assess Manag.

Matthiessen P, Sumpter JP (1998) Effects of estrogenic substances in the aquatic environment.EXS 86: 319-335.

Mehinto AC, Jia A, Snyder SA, Jayasinghe BS, Denslow ND, Crago J, Schlenk D, Menzie C, Westerheide SD, Leusch FD, Maruya KA (2015) Interlaboratory comparison of in vitro

bioassays for screening of endocrine active chemicals in recycled water.Water Res 83: 303-309.
NRC (2007). Toxicity Testing in the 21st Century: . Washington, DC, National Academy Press.
Ribeiro de Sousa, D. N., Grosseli, G. M., Mozeto, A. A., Carneiro, R. L., Fadini, P. S., (2015) Ultrasound-assisted extraction method for the simultaneous determination of emerging contaminants in freshwater sediments, J. Sep. Sci., 38, 3454-3460.
Rodgers-Gray TP, Jobling S, Kelly C, Morris S, Brighty G, Waldock MJ, Sumpter JP, Tyler CR (2001) Exposure of juvenile roach (Rutilus rutilus) to treated sewage effluent induces dose-dependent and persistent disruption in gonadal duct development.Environ Sci Technol 35(3): 462-470.
Sangster JL, Zhang Y, Hernandez R, Garcia YA, Sivils JC, Cox MB, Snow DD, Kolok AS, Bartelt-Hunt SL (2014) Bioavailability and fate of sediment-associated trenbolone and estradiol in aquatic systems.Sci Total Environ 496: 576-584.
Sarria MP, Soares J, Vieira MN, Castro LF, Santos MM, Monteiro NM (2011) Rapid-behaviour responses as a reliable indicator of estrogenic chemical toxicity in zebrafish juveniles.Chemosphere 85(10): 1543-1547.
Zhang Y, Krysl RG, Ali JM, Snow DD, Bartelt-Hunt SL, Kolok AS (2015) Impact of Sediment on Agrichemical Fate and Bioavailability to Adult Female Fathead Minnows: A Field Study.Environ Sci Technol 49(15): 9037-9047.