RMP Emerging Contaminants Workgroup Meeting

April 30th, 2015
San Francisco Estuary Institute
First Floor Conference Room
4911 Central Avenue, Richmond
10:00 am - 4:00 pm

Lunch will be provided

Webex Info
Call-in Toll Number: 1-650-479-3208
Access Code: 626 941 909
URL: https://sfei.webex.com/sfei/j.php?MTID=ma8f4437c7b8713a565afeceb586c4cee

AGENDA

1.	**Introductions, Approval of Minutes, and Goals for Today's Meeting** (Attachment) The goals for today: ● Provide updates on recent and ongoing ECWG activities ● Identify potential new ECWG members ● Recommend which special study proposals should be funded in 2016 and provide advice to enhance those proposals	10:00 Phil Trowbridge
2.	**Information: Update on the CEC Strategy** Review of the RMP prioritization scheme and classification of CECs. Note next steps for each tier in terms of monitoring and how the data will inform management decisions. Briefly describe smaller special studies: ● Annual emerging contaminants support ● Add-on microplastics monitoring in margin sediments	10:05 Rebecca Sutton

3.	**Information: Update on Alternative Flame Retardants Monitoring** The alternative flame retardants project is nearing completion. We are evaluating these constituents in multiple matrices including ambient Bay water, effluent, stormwater, sediment, bivalves, and harbor seal blubber. A brief summary of the results will be presented.	10:35 Rebecca Sutton
4.	**Information: Update on Bioanalytical Tools** (Attachment) The University of Florida and SCCWRP have finished the deliverables for Year 1 and begun work on the deliverables for Year 2. The final Year 1 report is attached. In Year 2 of the study, *in vitro* and *in vivo* bioassays will be performed using San Francisco Bay wastewater effluent and receiving waters. An overview of results to date and the plans for 2015 will be presented.	10:55 Nancy Denslow
5.	**Information: Targeted Metabolomics - Measuring the Effects of Environmental Stressors on Sentinel Species** AXYS has conducted a pro bono metabolomic study of resident Bay species. Metabolomics is the study of cellular response to external stresses such as contaminants.	11:30 Bharat Chandramouli
6.	**Information: Update on Effluent Monitoring - Perfluorochemicals** The priority CECs in effluent project is nearing completion. Wastewater effluents from eight local treatment plants were collected for poly- and perfluoroalkyl substance analysis by AXYS. In addition, a pro bono collaboration with the Department of Toxic Substances Control added a significant component to the study, an oxidation assay to indirectly estimate the total concentration of polyfluorinated compounds. A brief summary of the results will be presented.	11:40 Erika Houtz
7.	**Information: Update on Effluent Monitoring - Fipronil** The priority CECs in effluent project is nearing completion. Wastewater influents and effluents from eight local treatment plants were collected for analysis of fipronil and degradates. A brief summary of the results will be presented.	12:00 Ellen Willis-Norton
	Lunch	12:10

8.	**Information: Benzotriazole UV Stabilizers and Substituted Diphenylamine Antioxidants: Emerging Organic Pollutants in San Francisco Bay** A pro bono collaboration with Environment Canada provides exploratory data on these contaminants in ambient Bay water samples.	12:45 Amila De Silva
9.	**Discussion: Potential New ECWG Members** Dr. David Sedlak will be leaving the ECWG in 2015 after many years of service. The RMP would like to identify researchers who could potentially take his place, taking into account the type of expertise that is needed to complement the other ECWG members. Another option is to maintain the workgroup as it is now. Desired Outcome: List of potential new ECWG members	1:00 Panel
10.	**Proposed Special Study for 2016: Pharmaceuticals** (Attachment) Previous RMP surveys indicate some pharmaceuticals occasionally exceed toxicity thresholds. The RMP CEC Strategy and prioritization methods within the new state CEC guidance suggest a pilot monitoring effort is warranted. In addition, new analytical methods have been developed to target additional pharmaceuticals not previously examined in the Bay. For this agenda item, the Principal Investigator will present the proposed research, followed by questions and comments from the ECWG. The goal is to gather feedback on the merits of the proposal and how it can be improved. A broader discussion of all the proposals will be held in Agenda Item 12. The formal recommendation for funding will be made during Agenda Item 13.	1:15 Rebecca Sutton

11.	**Proposed Special Study for 2016: Non-targeted Analysis of Water-soluble Organic Compounds** (Attachment) The RMP CEC Strategy and new state guidance emphasize non-targeted analysis as an essential means of assuring focus on the contaminants with greatest potential to impact ecosystems. With the completion of the RMP project with NIST to examine fat-soluble contaminants via non-targeted analysis, we are now well-positioned to similarly assess water-soluble contaminants, thus providing a more complete survey of Bay pollution. For this agenda item, the Principal Investigator will present the proposed research, followed by questions and comments from the ECWG. The goal is to gather feedback on the merits of the proposal and how it can be improved. A broader discussion of all the proposals will be held in Agenda Item 12. The formal recommendation for funding will be made during Agenda Item 13.	1:50 Rebecca Sutton
12.	**Discussion: Recommended Studies for 2016** The workgroup will review and critique the proposals presented within the broader context of emerging contaminants and Bay health.	2:25 Phil Trowbridge
13.	**Decision: Recommendations for 2016 Special Studies Funding** RMP Special Studies are identified and funding through a three-step process. Workgroups recommend studies for funding to the Technical Review Committee (TRC). The TRC weighs input from all the workgroups and then recommends a slate of studies to the Steering Committee. The Steering Committee makes the final funding decision. For this agenda item, the ECWG is expected to decide (by consensus) which studies to recommend to the TRC. To avoid an actual or perceived conflict of interest, the Principal Investigators for proposed special studies are expected to leave the room during this agenda item. Desired Outcome: Recommendations from the ECWG to the TRC regarding which special studies should be funded in 2016 and their order of priority.	3:35 Karin North
14.	**Report out on Recommendations**	3:50 Karin North
15.	**Wrap up and adjourn**	4:00

RMP
Emerging Contaminants Workgroup
April 15th, 2014
San Francisco Estuary Institute
Meeting Summary

Attendees:

Tom Mumley (SFB RWQCB)
Karin North (City of Palo Alto)
Mike Connor (EBDA)
Eva Agus (EBMUD)
Simret Yigzaw (City of San Jose)
Derek Muir (Environment Canada)
David Sedlak (UC Berkeley)
Ian Wren (SF Baykeeper)
Lee Ferguson (Duke University)
Luisa Valiela (US EPA)
Eric Dunlavey (City of San Jose)
Kelly Moran (TDC Environmental)
Philip Gschwend (MIT, UC Berkeley)
Denise Greig (The Marine Mammal Center, California Academy of Sciences)
Jay Davis (SFEI)
Becky Sutton (SFEI)
Meg Sedlak (SFEI)
Ellen Willis-Norton (SFEI)
Don Yee (SFEI)
Keith Maruya (SCCWRP)
Richard Grace (AXYS)
Jonathan Benskin (AXYS)
June-Soo Park (DTSC)
Erika Houtz (DTSC)
Heather Peterson (SFPUC)
Nancy Denslow (University of Florida)
Daniel Schlenk (UC Riverside)
Michael Fry (Fish and Wildlife - Hawaii)
Sara Hoover (OEHHA)
Andria Ventura (by phone)

I. Information: Update on Bioanalytical Tools Study [Nancy Denslow]

Nancy Denslow began her presentation by stating that bioanalytical assays are useful if you are unsure what chemicals are affecting aquatic biota. She then listed the year one and year two goals for the San Francisco Bay bioanalytical tools study. Nancy stated that by June 2014 the molecular biomarkers for *Menidia beryllina* will be developed, laboratory tests in early life stage (ELS) and juvenile exposures will be run, and in vitro bioassays will be completed. The model chemicals that will be used in the laboratory exposures include E1, 4-NP, BPA, and galaxolide. Survival, growth, and 5 molecular biomarkers were analyzed for ELS and juveniles during the laboratory exposures. In addition, vitellogenin (Vtg), estrogen, and testosterone were analyzed in juveniles.

High throughput estrogen and androgen receptor assays (InVitrogen assay) were also run for BPA, E1, NP, and bifenthrin. The response curve of the 17-beta estradiol (E2) was compared to the four chemicals to calculate the bioanalytical equivalent concentration. Nancy found that 17-beta estradiol was the most sensitive, followed by E1, 4-NP, and BPA. When the assay is run in antagonist mode, E1 is still a weak estrogen while NP and

bifenthrin act as antagonists at low levels. NP and bifenthrin act as antagonists because they occupy the ligand binding domain and don't let E2 bind to the estrogen receptor. Nancy thinks that galaxolide will also act as antagonist in the high throughput assay.

Nancy discussed the *in vivo* work that has been completed including a 7-day ELS *Menidia* toxicity test using 10-day old *Menidia* larvae. The endpoints for the test were survival and growth. For E2, E1, and NP there were no significant differences for survival and growth as the concentrations increased. Nancy stated that molecular endpoints are more sensitive; therefore, the samples have been preserved for targeted gene expression analysis.

Nancy described the 21-day juvenile *Menidia* toxicity test using 50-day old *Menidia* fry. The endpoints were growth, condition factor, liver RNA, and the carcass to verify sex. Four fish were used as backups to measure Vtg and steroids. The 21-day test has been completed for E2 and E1 and there were no significant differences in weight or length; the test is still running for NP, BPA, and galaxolide. Nancy noted that she is letting some fish grow longer to determine if any changes in sex occur.

Menidia PCR primers were validated for ERa, ERb, AR, Vtg, Growth hormone receptor, doublesex and mab-3 related transcription factor 1 (DMRT1, indicates genetic sex), and others to ensure the primers were working. Subsequently, juvenile E2 exposure was tested. The 10 and 20-day exposed juveniles only had Vtg expression with E2 levels of 100 ng/L; Choriogenin (Chg) appeared to be more sensitive with expression occurring at 30 ng/L E2. Nancy stated that for *Menidia* Chg was a more sensitive biomarker.

Nancy then tested how E2 exposure would induce AR and ERb mRNA. Interestingly, At 100 ng/L AR mRNA increased, but then decreased at 300 ng/L, which Nancy thought could be due to feedback inhibition.

Nancy ended her presentation by describing the field exposure experiment that will occur this coming year. The experiment will include exposing ELS and juvenile *Menidia* to effluent from various sample sites and running assays and identifying molecular biomarkers. The effluent will come from Bay Area WWTPs, collected by SFEI, and from Southern California WWTPs. An initial ER assay was completed using WWTP effluent and with dilutions there was a very clear response; Nancy noted that the same results were not observed with an AR assay.

Discussion:
Lee Ferguson asked about the how bifenthrin acts as an estrogen, if it is through basic binding to the estrogen receptor (ER). Nancy replied that bifenthrin causes Vtg to increase *in vivo*. But, bifenthrin may act on the hypothalamus, rather than directly binding to the ER. Tom Mumley asked if work has been completed on other pyrethroids. Dan Schlenk responded that studies on permethrin have been completed which show that the pyrethroid causes estrogenic activity.

Dan asked if the duration of the exposure was long enough; in his experiments, using a different fish, the fish are dead within a week in 300 ng/L E2. He noted that it would be useful to have a similar endpoint, or threshold, concentration. He agreed with Nancy that Chg is a more sensitive biomarker. Finally, Dan stated that the ability to see DMRT1 was useful in distinguishing phenotypic from genetic sex. Nancy agreed, stating that the *Menidia* she receives comes in two different sizes and it would be useful to determine if the size differences indicate their sex or a difference in age/growth. Dan responded that the tanks should be divided by *Menidia* size class before the exposures are performed.

Derek Muir asked if the "round robin" ER assay that Nancy described at the end will be performed for the four model chemical assay; Nancy responded affirmatively. Derek wondered if there was inter-lab variability; Nancy replied that all of the labs listed the same waters as estrogenic.

David Sedlak asked about how various water quality parameters, such as high ammonia levels, may affect the results. Nancy replied that by changing the pH, ammonia can be removed from the water. David also wondered if *Menidia's* sensitivity to the chemicals changes in saltwater. He stated that there is a known estrogenic response in fish exposed to the model chemicals and wondered about the causative agent if the effluent concentrations do not trigger a response. Nancy replied that all of the treatments were performed in saltwater. She added that *Menidia* is more sensitive than sheepshead minnow but less sensitive than fathead minnow in fresh water. She stated that estuarine type fish may not be as responsive to estrogens and suggested completing a similar experiment with adult *Menidia*.

Denise Greig asked if estrogenicity is expected to increase or decrease growth. Nancy responded that estrogens could do both and she will examine the human growth receptor after exposure to the four chemicals. Michael Fry asked if Nancy could determine the stage of sexual maturity based on the gonads. Nancy replied that her team has performed the histology and the ovaries and oocytes were visible; however, the testes were not. Nancy is planning on taking sagittal sections to view the testes. Meg Sedlak ended the discussion by stating that the ECWG will receive the year one progress report on June 1 and she will ask the workgroup if they support year two of the study.

Action Items:

1. Meg Sedlak will send the year one progress report on June 1 and will ask the workgroup if they support year two of the study.

II. Update on CEC Strategy [Meg Sedlak]

Meg Sedlak provided an update on 2013 CEC activities including the completion of the CEC Synthesis, the CEC Strategy, and the PBDE Summary Report (with a manuscript in progress). Meg noted that there are other CEC activities occurring across the state including statewide recommendations for CEC monitoring in estuaries, an expert panel to advise recycled water use, and the creation of a Green Ribbon Science Panel to advise the Department of Toxic Substance Control on reducing adverse health and environmental

impacts of CECs. Meg noted that Kelly Moran and Becky Sutton are both members of the Green Ribbon Panel.

Meg then briefly reviewed the RMP's CEC Strategy, focusing on the tiered risk based screening approach to monitoring. Meg stated that approach is iterative; there is the potential for removing contaminants from certain tiers with increased information or new management strategies. Meg reviewed the contaminants listed as of moderate concern, Tier III (PFOS, Fipronil, Nonylphenol, and PBDEs), informing the ECWG of ongoing monitoring and potential special studies for each CEC.

PFOS is currently being monitored in bird eggs, sportfish, and sediment. Meg noted that apex predators continue to possess high PFOS concentrations. Today, the 2013 PFC precursor results will be discussed as well as the potential for measuring PFCs in effluent and harbor seal blood. PBDEs will continue to be monitored in sediment and tissue; water sampling will no longer occur because it is not an effective matrix for PBDE monitoring. Meg noted that Nonylphenol and Nonylphenol Ethoxylates (NP/NPE) are not included in S&T, which is consistent with the recommendations of the State CEC panel report. However, they are included as part of the bioanalytical tools project. Today, Becky Sutton will discuss the option for monitoring NP/NPE in WWTP effluent. Fipronil is currently measured in Bay stormwater, sediment, and was measured in ambient Bay water in 2013 (all non-detects). Fipronil monitoring will continue in Bay sediment because there is an increasing trend as well as in stormwater; Bay water monitoring will be discontinued. In the afternoon, Becky will discuss the inclusion of Fipronil in a special study on effluent monitoring.

Meg then noted that Tier II and Tier I contaminants will also be addressed today when 2015 special study proposals are presented including pharmaceuticals and personal care products (Tier II), alternative flame retardants (Tier I), and current use pesticides (Tier I). Outside of the tiered risk framework, the RMP is identifying CECs using bioanalytical tools and NIST broadscan work. Meg was encouraged that not many CECs were identified in the broadscan work.

Meg ended her presentation that the SC supported a placeholder of $100,000 for 2015 CEC special studies. Tom Mumley noted that there are competing priorities in the RMP; therefore, the goal of today's meeting is to review and prioritize the proposed special studies and recommend study designs.

III. Information: Update on Alt Flame Retardant Monitoring [Becky Sutton]
Becky Sutton began her presentation on alternative flame retardant monitoring by stating that the change to TB117 is now in effect, instead of products needing to withstand an open flame, they now just need to withstand a smolder test. Becky noted that another bill, AB127, was recently approved by the Governor requires the California Fire Marshal to review the current flammability standards for insulation material. The Fire Marshal has created a review panel to address the possibility of changing the standard.

Becky then stated that the RMP is monitoring for flame retardants in surface water, stormwater, WWTP effluent, sediment, bivalves, and seal blubber. Da Chen, a professor at Southern Illinois University, has developed methods for phosphate, brominated, and Dechlorane plus-related analytes. He is expanding his phosphate method to include metabolites, and adding a few more target chemicals to the method for brominated flame retardants.

Becky presented general trends in ambient Bay and stormwater alternative flame retardant concentrations. Phosphate flame retardant concentrations were ten times higher in stormwater than in ambient bay water, indicating that stormwater is a source of flame retardants to the Bay. However, the concentration ratios of the various phosphates differed between stormwater and ambient Bay water. Becky ended the presentation by listing the 2014 sampling timeline: effluent is being sampled in April, seals will be sampled in June, sediment in August, and bivalves in September.

Discussion:
Mike Connor asked Becky for an estimated mean concentration for ambient Bay water; Becky replied around 300 ng/L. Derek Muir responded that the concentration she mentioned is globally on the high end. Becky noted that she does not have all of the ambient Bay sample results; therefore, the average concentration may change. Naomi Feger asked if all of the products were flame retardants. Becky replied that the products could also be plasticizers. Lee Ferguson asked if the RMP was measuring tracers (e.g. caffeine) along with the alternative flame retardants. Becky replied that PCBs are being measured, but not in the same 4 L bottle.

Mike Connor stated that the concentrations Becky mentioned would put alternative flame retardants above PBDEs in the tiered risk framework. Becky responded that phosphate flame retardants are metabolized quickly, unlike PBDEs. Derek replied that phosphates should be measured in blood, which is a matrix Becky is considering sampling in seals. June-Soo Park stated that DTSC is considering measuring phosphate flame retardants in human urine samples

IV. Information: Update on AXYS PFC Precursor Pro Bono Study [Jonathan Benskin]
Jonathan Benskin gave the ECWG an update on PFC precursors in San Francisco Bay. Jonathan began by providing background on PFCs, a diverse class of anthropogenic chemicals. He noted that recent studies have found that PFC precursors could be a significant source of PFOS and PFOA, the two most common PFCs, in the environment.

Jonathan stated that in San Francisco Bay, PFOS precursors are sometimes greater than PFOS concentrations in sludge and sediment concentrations. Additionally, precursor concentrations were similar to perfluoroalkyl acid (PFAA) concentrations in stormwater runoff. Based on the evidence that precursor concentrations are elevated in the Bay, Jonathan wondered if elevated levels of PFOS in the Bay can be explained by exposure to precursors. Additionally, if perfluorooctane sulfonyl fluoride (PFOSF) is being phased out, will telomer-based substances become a source of PFAAs in Bay wildlife? The

objective of the study was to measure concentrations of conventional PFAAs, PFCA and PFOS precursors, and emerging phosphorous containing PFAAs in sediment and WWTP effluent in South Bay.

At all three effluent sampling sites, perfluorinated carboxylic acids (PFCA) and perfluorinated sulfonic acids (PFSA) were the dominant classes. However, PFOS and PFOA were not always the main PFCs observed. The PFC profiles differed between all three effluent locations; at sites 1 and 2 the two main contaminants were PFOS and PFOA; at site 3 it was PFPeA and PFHxA. In sediment, the highest concentration of PFOS precursors was observed at Alviso Slough, where the highest concentration of PFOS was also measured. In sediment, diPAP concentrations were an order of magnitude greater than both PFCA and PFSA concentrations. PFCAs were only observed at Cooley Landing.

Discussion:
Derek Muir stated that it would be worthwhile to monitor for precursors in water; one study found high levels of PFOS precursors in the North Sea, indicating they are water soluble. Lee Ferguson found the concentrations of diPAPs in the sediment interesting and asked if it would be useful to also monitor triPAPs. Jonathan responded that the triPAPs are usually not the main ingredients in products and also hydrolyze to diPAPs. Phil Gschwend asked about the production of PAPs over time. Jonathan replied that PAPs became the major surfactant in the food packaging and paper industry starting in 2002; the concentrations in the environment have increased considerably over the past decade. David Sedlak mentioned PAPs' hydrophobicity and Jonathan responded that PAPs partition onto suspended sediments. The concentrations of PAPs are low in effluent, indicating they may be entering the Bay via stormwater runoff.

V. Information: California Safer Consumer Products Regulations and the Green Ribbon Science Panel [Becky Sutton]
Becky Sutton began her presentation by stating that the Green Ribbon Science Panel was formed to help implement the Safer Consumer Products Regulations, which requires alternative assessments for priority products (products that contain a chemical of concern). The regulations will address the question of if a chemical is necessary. RMP advisor Kelly Moran and Becky are both serving on the 15 member Panel and will provide guidance to the Department of Toxic Substance Control (DTSC).

DTSC created an initial candidate chemical list (n=153), which is based on the chemicals' hazard and exposure. An initial priority products list was created based on whether they possessed any of the candidate chemicals. So far, three priority products have been announced: Children's foam-padded sleeping products containing TDCPP; spray polyurethane foam systems containing unreacted diisocyanates; and paint/varnish strippers, surface cleaners containing Methylene Chloride.

Becky's role will include helping DTSC establish means for assessing how the chemicals and associated products may affect ecological health. She noted that the current candidate chemical list is mainly based on human health concerns. She stated that the RMP can

help by encouraging DTSC to consider ecological exposure and toxicity lists; informing DTSC of the CECs the RMP has discovered in the Bay; suggesting that DTSC complete ecological alternatives assessments; helping increase knowledge about products that are in use today; and providing DTSC ideas on potential priority products.

Discussion:
Lee Ferguson stated that many products are imported from China and the chemicals that are in them are not on TSCA and some don't have CAS numbers. Becky responded that DTSC can ask importers to complete the assessment; Lee responded that importers may not know what is in the product. Becky noted that the Panel will begin to address data gaps in the near future. Denise Greig asked if the regulations require increased labelling; Becky replied that required actions will only be determined after the alternatives assessments. The alternative assessments will begin in late 2015. The company that is producing a priority product will first be required to conduct a preliminary, short alternatives assessment report within 180 days of being notified. DTSC will review the preliminary assessment and determine if a longer assessment is needed.

Ian Wren asked why only five products were on the priority products list. Tom Mumley responded that DTSC did not want to take on too many products at the beginning of the program. In the future, more than five products will be included on the list.

Derek Muir wondered if a chemical was only considered hazardous if a study on the chemical had been published. He noted that many high production chemicals have not been studied, but may still be hazardous. Kelly Moran responded that the State was not given the authority to require new data, so chemicals that have not been studied are not included. She added that the State is in the process of developing a three-year workplan for its priority product selection; therefore, it would be timely and help DTSC if the RMP can help advise DTSC on pollutants and/or products that are of concern to Bay biota.

VI. Special Study 2015: Monitoring Wastewater Effluent for CECs [Becky Sutton and Meg Sedlak]
Becky Sutton stated that there are a number of effluent studies the RMP is already completing including evaluating effluent for alternative flame retardants and endocrine disruptor compounds (EDCs; from one WWTP). Becky proposed adding PFOS and PFOS precursors, Fipronil and its degradates, and EDCs to the list of compounds the RMP evaluates in effluent. The study would include collecting grab samples from at least 3 South Bay and Lower South Bay WWTPs, at least 2 Central Bay WWTPs, at least 1 Suisun or San Pablo Bay WWTP, and include 2 WWTPs that discharge to wetlands. The samples would be collected in Fall 2014 and would include a variety of treatment methods. The budget is currently $64,000; however, ECWG members may want to consider also including microplastics and other pharmaceuticals and personal care products in the sampling effort.

Discussion:
Lee Ferguson asked if all the polyethoxylates would be included in monitoring, or just nonylphenol (NP). Nancy Denslow replied that only NP is part of the bioanalytical tools

study. Lee responded that it might be interesting to monitor E1, E2, and E3 polyethoxylates as well as carboxylated NPs. Becky responded that currently the study only includes the essential EDCs, but Keith Maruya is interested in completing a broader screen of EDCs to inform the bioanalytical screening results and to fulfill data gaps identified by the Statewide Expert Panel. David Sedlak noted that he does not consider NP an emerging contaminant and would only suggest monitoring the EDCs in multiple WWTP's effluent if it is critical to the success of the bioanalytical tools study. Derek Muir argued that Environment Canada is worried about hindered phenols, which are structurally related to NP and are highly used. He suggested that Keith create a list of hindered phenols that have not been monitored before and including them in a broader screen of EDCs. Derek agreed to give Keith a list of hindered phenols that would be useful to monitor and added that he would be willing to measure hindered phenols in a few RMP effluent samples.

David stated that he was concerned that the variability in Fipronil concentrations throughout the day will be lost if a grab sample is collected; he suggested collecting Fipronil as a composite sample instead.

Phil Gschwend asked if Becky considered monitoring for inorganics that are associated with the electronics industry. Mike Connor responded that he thought that the RMP has monitored for Osmium in the past. Mike thought it would be useful to have a rough understanding of the inorganics Phil mentioned and supported sampling for them at a few ambient water stations and in WWTP effluent. Naomi Feger asked if influent data would also be necessary; David Sedlak and Eric Dunleavy responded affirmatively. Naomi stated that more research and data gathering is necessary before pursuing a special study.

Action Items:

2. Derek Muir will send Keith Maruya a list of hindered phenols that would be useful to monitor.

VII. Special Study 2015: Pharmaceuticals and Personal Care Products [Rebecca Sutton]

Becky Sutton stated that pharmaceuticals and personal care products (PPCPs) are listed in Tier II (Low Concern) in the CEC Strategy and plasticizers are listed as Tier I (Possible Concern). Despite their inclusion in Tier II there are still many PPCPs for which the level of concern is unknown. Concern for a chemical was evaluated by looking at toxicity thresholds, environmental detections, its chemical properties, and use and loading trends. Becky explained the methodology for identifying high priority PPCPs; high priority PPCPs were defined as chemicals for which environmental concentrations are above the PNEC, or chemicals that do not readily biodegrade and may be harmful for aquatic ecosystems.

Becky listed six PPCPs that were identified as high priorities for monitoring. The first being sulfamethoxazole because three out of 15 detections of sulfamethoxazole in the Bay were above the PNEC. Each sulfamethoxazole sample will cost $535 to $1,910 to

analyze, depending on whether the RMP is interested in analyzing a smaller or larger suite of PPCPs at the same time.

Bisphenol S (BPS) was the next PPCP Becky included as being of high concern. BPS is a replacement for Bisphenol A (BPA) and has not been measured in the Bay. BPS is not likely to degrade and has estrogenic activity and reproductive toxicity. Becky noted that AXYS Analytical Services Ltd. does not analyze BPS; however, Environment Canada does have a method to perform BPS analyses. BPA was the next PPCP on Becky's list because it has high estrogenicity. BPA has been monitored in Bay water and sediment, but was not detected. Keith Maruya noted that in effluent BPA concentrations were around 10-20 ng/L and the PNEC is 60 ng/L.

The fourth chemical Becky described was Butyl benzyl phthalate, a plasticizer. The concentration in Bay sediment was higher than the low apparent effects threshold; however, the Bay water concentrations were 1000 fold below the water PNEC. Becky noted that use of Butyl benzyl phthalate substitutes are increasing and suggested completing AXYS's general screen for phthalates. Mike Connor noted that butyl benzyl phthalate was a priority pollutant and Jay Davis added that the detection limits for the chemical are high.

The next PPCPs on the list were ADBAC and DTDMAC. Becky stated that some river environments contain levels greater than the freshwater PNEC; however, an estuarine sediment PNEC does not exist. The final PPCP was octocrylene, a widely used chemical that is found in many sunscreens. A PNEC does not exist, but there is concern that the chemical is persistent and bioaccumulative. AXYS Analytical does not analyze octocrylene and it has not been monitored in the Bay. Mike Connor thought it would be more interesting to monitor octocrylene in a lake where people swim.

Discussion:
Naomi Feger asked why sulfamethoxazole was listed as low concern if it was detected. Meg Sedlak replied that the values used to be estimates, but the RMP has received more accurate data recently. Derek Muir noted that if a larger suite of PPCPs are analyzed alongside sulfamethoxazole, the detection limits will increase. David Sedlak asked if the PNEC was a legitimate threshold for an estuarine system. If the PNEC is appropriate, then sulfamethoxazole may need to be ranked in a higher tier.

Lee Ferguson stated that ADBAC and DTDMAC are not very bioavailable and will be strongly bound to sediments. Lee noted that ADBAC and DTDMAC have never been measured in stormwater suspended sediment and thought it would be interesting to monitor. Kelly Moran added that ADBAC is a pesticide that could enter stormwater runoff; she will forward the EPA review of ADBAC to Becky. Mike Connor suggested a small monitoring study near AT&T Park and having Bruce Brownawell analyze the samples.

Phil Gschwend stated that he doubts octocrylene is used in mass quantities. He added that many compounds are quickly replaced with alternatives and would suggest looking at

families of compounds. For example, there are a large number of bisphenols that could be analyzed at one time. Becky responded that a complete methodology for analyzing bisphenols has not been developed. Lee noted that the chemicals Becky listed as plasticizers, such as BPA, should be called polymer additives.

Andria Ventura wondered how the effects of the compounds play into what contaminants the RMP chooses to monitor. Becky responded that concentrations are compared to toxicity thresholds when available. Kelly Moran ended the discussion by suggesting monitoring for antimicrobial chemicals that the EPA recently registered, there are clear pathways to the Bay and some level of toxicity data is available. .

Action Items:

3. Kelly Moran added that ADBAC is a pesticide that could enter stormwater runoff; she will forward the EPA review of ADBAC to Becky.

IX. Special Study 2015: Current Use Pesticide (CUPs) [Ellen Willis-Norton and Kelly Moran]

Ellen Willis-Norton began her presentation by stating that the RMP monitors legacy pesticides as part of the Status and Trends (S&T) program. Use of these legacy pesticides ended between 40 and 50 years ago and the RMP has observed a slow decline in concentrations since 1993. As many S&T contaminant concentrations begin to decline or stabilize, the RMP has begun focusing efforts on Contaminants of Emerging Concern (CECs), including current use pesticides (CUPs).

The RMP's CEC Strategy includes ranking the relative risk of CECs to the Bay based on a tiered risk framework. All CUPs are ranked in Tier I (Possible Concern), excluding Fipronil and Pyrethroids (Moderate Concern and Low Concern respectively). However, Ellen noted that CUPs are considered of special concern because they are designed to kill organisms.

CUPs can enter the Bay via stormwater runoff, in bay application, and WWTP effluent. The CEC Strategy suggests screening level monitoring efforts for Tier I contaminants to help determine their concentration in ambient Bay water and sediment, effluent, runoff, and biota.

There are over 1,000 CUPs in existence; therefore, prioritizing which CUPs to monitor in the Bay is essential. The RMP developed a comprehensive monitoring priority list for agricultural CUPs. The list was created using spatially-explicit use data provided by the Department of Pesticide Regulation's California Pesticide Information Portal. Only agricultural pesticides, rather than both urban and agricultural, were included in the list because agricultural use data is reported to the township level. The RMP took the top 50 highest use pesticides within the Region 2 Water Quality Control Board boundary and determined their risk ratio (total use/lowest aquatic life benchmark).

The 20 agricultural pesticides with the highest risk ratio were: Naled, Oxyfluorfen , Flumioxazin, Pyraclostrobin, Mancozeb, 1,3-dichloropropene, Dimethoate, Imidacloprid,

Paraquat Dichloride, Metam-Sodium, Thiophanate-Methyl, Cyprodinil, Trifloxystrobin, Methomyl, Pendimethalin, 2,4-Dichlorophenoxyacetic acid, Diquat Dibromide, Oryzalin, PCNB, and Triflumizole. The use data for all 20 pesticides was mapped to determine where pesticide use was concentrated. The majority of the pesticides were applied in Napa County indicating agricultural pesticide concentrations are likely highest in the Napa River and subsequently San Pablo Bay.

Ellen proposed monitoring the following seven CUPs at three locations within the Napa River in this special study: Oxyfluorfen, Pyraclostrobin, Mancozeb, Imidacloprid, Paraquat Dichloride, Metam-Sodium, Diquat Dibromide. The sediment and water samples will be sent to North Coast Laboratories Ltd., a laboratory with expertise in pesticide analyses.

Discussion:
Mike Connor stated that Diuron has a lot of urban uses and wondered if it should be included in monitoring; Kelly Moran responded that this is an urban contaminant that is being addressed through DPR urban monitoring. Naomi Feger wondered why Naled was not included in the monitoring plan; Kelly replied that Naled should be included since its degradate is of high concern. David Sedlak then noted that he completed a study that demonstrated high estrogenicity in the Napa River. He wondered if the CUPs described in the presentation could be contributing to the estrogenicity.

Kelly Moran suggested timing sampling in the Napa River with pesticide application. She can help retrieve the pesticide application dates to inform monitoring efforts. Kelly added that urban use data was not included because only the total quantity of use is sent to DPR; there is a lack of spatially-explicit urban use data.

Lee Ferguson asked about using passive samplers in addition to collecting grab samples. Phil Gschwend stated that using passive samplers in sediment would be useful. Kelly Moran noted that some of the CUPs are very soluble and may be found in both sediment and water. Nancy Denslow stated that she has been a collaborator on a project that uses a passive sampler in both sediment and water. David Sedlak agreed that the current proposal only gives a narrow view of the CUPs found in the Napa River. He suggested using broadscan techniques or Orbitrap mass spectrometry. Lee Ferguson offered to complete a broadscan screen of some of the samples using his MS/MS. Mike Connor stated that it would be useful to collect both types of samples and to also have Lee run a subset of the samples.

X. Special Study 2015: Microplastics [Ellen Willis-Norton]
Ellen Willis-Norton explained that microplastic is a term used to describe fragments of plastic that are less than 5mm. Microplastics can be pellets that are used as precursors for industrial products, microbeads used in consumer products (e.g. exfoliants), or fragments/fibers of plastics that are the breakdown products of larger plastic materials. Microplastics can enter the aquatic environment through wind, stormwater runoff, or effluent. It is important to note that both California and New York have proposed bans on microplastics found in cosmetics and many companies have already have pledged to

phase out the use of microbeads in their skin cleansers. Therefore, the concentrations entering wastewater may decrease in the future.

Studies have found that microplastics are also to adsorb to organisms, blocking their feeding appendages. Ingestion of microplastics can block the digestive tract, reduce growth rates, block enzyme production, lower steroid hormone levels, affect reproduction, and cause the adsorption of toxins. The potential for ingesting toxins occurs because microplastics readily accumulate hydrophobic organic compounds, due to their high surface area to volume ratio.

Ellen stated that multiple regions have monitored for microplastic pollution including in Chesapeake Bay, Puget Sound, the Los Angeles River, Santa Monica Bay, and the Great Lakes. Ellen noted that the study in the Great Lakes is on-going and the researchers, including the project lead Sherri Mason (SUNY Fredonia), are currently considering adding effluent sampling to the monitoring effort.

Ellen noted that microplastics were sampled in San Francisco Bay surface waters in 2011. The study determined the mass of microplastic at sites in Central Bay that were suspected to be most influenced by trash. The concentration of microplastics was similar to the concentration range observed in Puget Sound and the San Gabriel River. However, the study only measured the mass of the microplastics, rather than the abundance and composition. Additionally, effluent has not yet been monitored in San Francisco Bay.

Ellen recommended sampling for microplastics at 10 S&T ambient water and sediment sites as well as sampling effluent to help identify whether personal care products were a significant source of microplastic pollution in the Bay. Ellen stated the samples would be analyzed be Dr. Sherri Mason, the project lead for the Great Lakes microplastic study, and the study would cost approximately $5,000 to complete.

Discussion:
Lee Ferguson asked if chemical composition was included in the analyses. Ellen responded that she will check with Dr. Sherri Mason. Ian Wren noted that the study could be separated into two different studies based on the plastic fragments size; microbeads are likely found mainly in effluent while other fragments would be primarily found in stormwater. Ian wondered if this study should focus on microbeads. Kelly Moran suggested partnering with a student of Dr. Swee Teh at UC Davis, who is analyzing the effects of microbead ingestion on fish. Jay Davis responded that because the cost to complete the study is so low it may be easier to complete the study without a partnership with UC Davis.

Action Items:

4. Ellen Willis-Norton will ask Dr. Sherri Mason if chemical composition was included in the analyses of microplastics.

Linkage of *In Vitro* Assay Results With *In Vivo* End Points
Final Report – Phase 1
June 2, 2014

University of Florida: Sumith Jayasinghe, Kevin Kroll, Olanike Adeyemo, Candice Lavelle and Nancy Denslow

SCCWRP: Alvina Mehinto, Steve Bay and Keith Maruya

Linkage of *In Vitro* Assay Results With *In Vivo* End Points
Final Report – Phase 1

June 2, 2014

The goal of this project is to establish quantitative linkages between the *in vitro* receptor-based assays and traditional endpoints of adversity in an estuarine fish model, the common silverside (*Menidia beryllina*), which is an established EPA model for estuarine toxicity. To work out the method for this type of linkage analysis, we decided to concentrate on chemicals that are found in wastewaters that behave as weak estrogens. We are in the midst of our analyses, which we should complete in the next 3-4 months for work promised for year 1. So far we have had substantial success with our approach and a few problems that we are in the process of solving. This report is organized around the milestones set up in our proposal.

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**

Task 1 Convene focus group and develop actionable plan

Researchers from the Denslow Lab at the University of Florida and from SCCWRP met at the start of the project to plan how the project would be approached. In addition we have had several conference calls to coordinate experimental approaches and we have emailed each other with specific protocols to get input from all sides. We decided to use *Menidia beryllina* as the test species as this fish is reported to be sensitive to contaminants, inhabits estuarine locations in CA and the San Francisco Bay area and is used by EPA as a test organism **(Figure 1)** (Chapman et al. 1995). Drs. Connon and Susanne Brander are also using this fish as a model for the San Francisco Bay area and we agreed to collaborate with them on aspects of this project. They have agreed to make available to us gene sequences they have obtained from a transcriptomics project. This task was completed.

Figure 1. *Menidia beryllina* as a test organism

Task 2. Develop molecular biomarkers for *Menidia*

For this task we agreed to develop quantitative PCR (Q-PCR) assays to evaluate at least 10 different genes for their expression *in vivo*. Five of the genes were for evaluation in early life stage (ELS) and five for evaluation of critical genes in juvenile fish. These gene expression measurements are important to set up the linkage of the *in vitro* assays to responses *in vivo*. Detailed descriptions of the methods used are in Appendix A.

While we promised only ten assays for genes by Q-PCR, we have actually prepared 13 assays. We validated 7 assays that had been previously developed by Susanne Brander for *Menidia*, as part of the Ph.D. dissertation (Brander 2011). These assays were for Vitellogenin (Vtg), estrogen receptor alpha (ERα), estrogen receptor beta a (ERβa), androgen receptor (AR), Choriogenin L (Chg), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and cytochrome P450 A1 (Cyp1A) **(Appendix B, Supplementary Figure 1)**.

We also developed and validated assays for an additional 6 genes: insulin growth factor 1 (IGF-1); steroidogenic acute regulatory protein (StAR); growth hormone receptor (GHR); brain aromatase (cyp19b); anti-Mullerian hormone (amh); and doublesex and mab-3 related transcription factor 1 (DMRT1), and two more housekeeping genes ribosomal protein L8 (rpL8) and 18S ribosomal RNA (18S rRNA) **(Appendix B, Supplementary Figure 1)**. As expected, Vtg and ERα were expressed predominantly in the liver of females. We were hopeful that DMRT1 would be related to sex and be expressed exclusively in males and serve as a male biomarker, but we found that it was expressed in the gonads of both males and females. Expression levels were higher in males than in females, but it would be difficult to use this gene as a biomarker of genetic sex since it is expressed in both sexes. DMRT1 serves as a biomarker of sex determination in medaka, but not in many other fish species (Guo et al. 2005; Johnsen and Andersen 2012; Hattori et al. 2013).

We optimized the QPCR assays for each of the genes (Appendix B, Supplementary Figure 2). The amplicons were specific for the genes of interest, only one product was seen in melting experiments and the efficiency of amplification was between 95-105%. All RNA samples passed quality control standards with high A260/A280 ratios and good RNA integrity numbers. All total RNA samples were treated with DNase to remove traces of contaminating DNA. The assays were deemed of good quality to assess relative changes in gene expression with exposures.

Dr. Richard Connon (UC Davis) shared sequences for *Menidia beryllina* that he obtained from a transcriptome project funded by another source. We will complete RNA-Seq experiments in collaboration with Drs. Connon and Susanne Brander in phase 2 of this project. The scope of this collaboration has been focused to include exposures of early life stages to 17β-estradiol (E2), nonylphenol (NP), bifenthrin (BF) and vehicle control.

The original deliverables for this task have been completed.

Task 3. Laboratory tests: Early life stage and juvenile exposures and *in vitro* bioassays

There were three parts for this task; (1) development of the *in vitro* assays to determine EC50's for each of the estrogens; and performance with (2) *in vivo* assays with early life stage fish; and (3) *in vivo* assays with juveniles undergoing gonadal tissue differentiation. All of these deliverables have been completed.

A. *In vitro* Bioassays (UF)

We used InVitrogen GeneBlazer assays to derive estrogen equivalence relationships among the test substances: E2, E1, 4-NP, and BPA. We also tested bifenthrin and galaxolide. All chemicals were purchased from Sigma Chemical Co, with the exception of galaxolide, which was custom synthesized by Dr. John Rimoldi (University of Mississippi), a colleague of Dr. Dan Schlenk. Consequently all work with galaxolide will be in collaboration with Drs. Rimoldi and Schlenk.

The InVitrogen assays are cell-based estrogen receptor (ER) transactivation assays. They depend on a human cell line that normally does not express ERs. To make this cell line, the ligand-binding domain of human ER alpha was attached to the GAL4 DNA binding domain of a yeast factor and this construct was stably transfected into the human cell line. In addition, a reporter gene that codes for the beta lactamase protein under the control of 5 estrogen response elements was also stably transfected into the same cell line. When estrogen or an estrogen mimic come into the cells, they bind to the ligand-binding domain of the ER, alter the conformation of the receptor allowing it to bind to the promoter region (control region) of the reporter gene. This causes the beta lactamase mRNA to be transcribed, and then translated into protein. To confirm that this protein has been expressed and is active, the detection system uses a substrate that the beta lactamase can specifically cleave, thereby causing a signal to be emitted. This is a very sensitive assay for estrogen activation of its receptor.

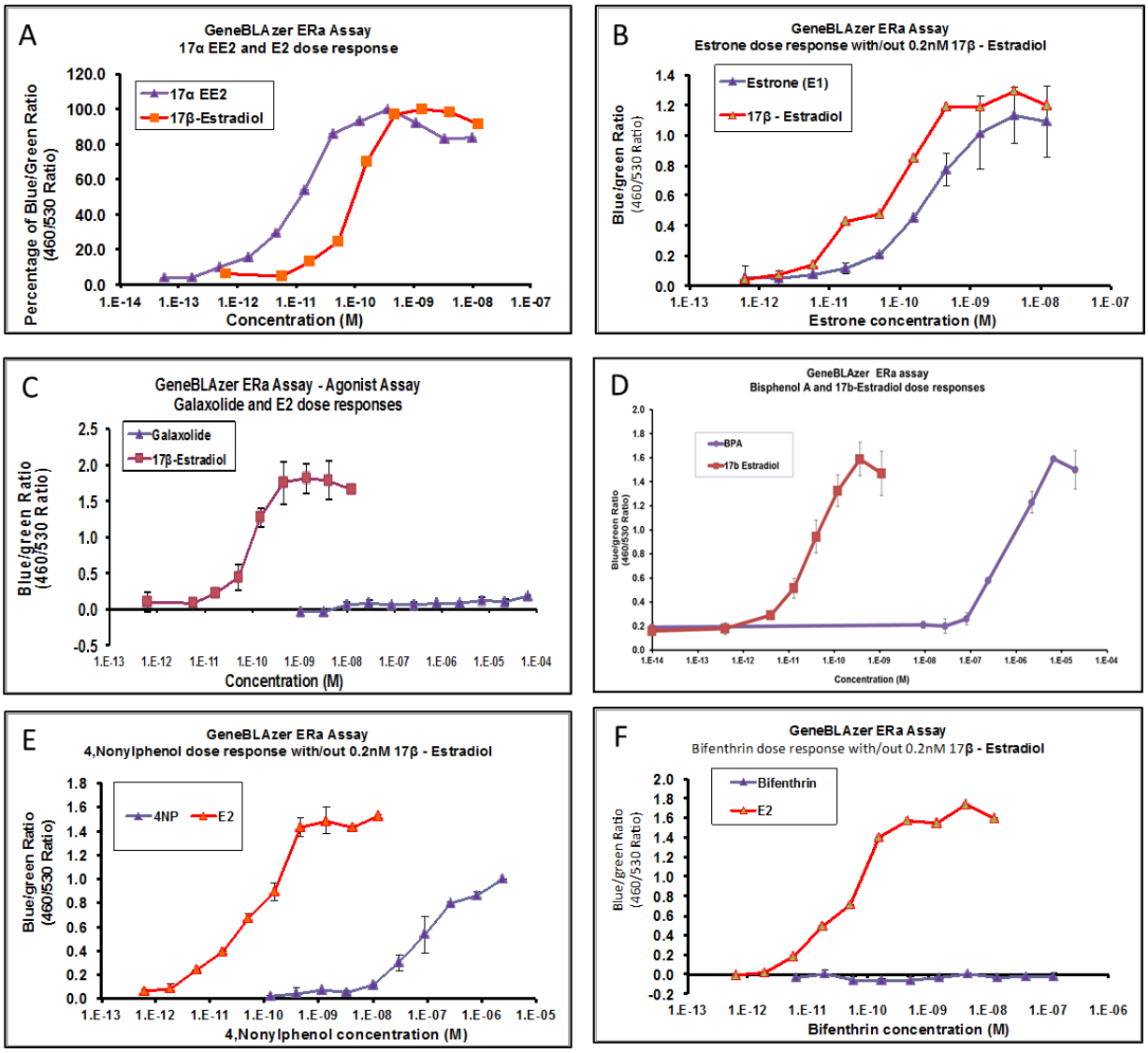


Figure 2: Dose response of InVitrogen ERα GripTite Division Arrested cells to strong and weak ER agonists. Cells were plated in triplicate in 96-well clear bottom plates and dosed with strong and weak ER agonists for 18 h in the presence of 0.5% DMSO, loaded with LiveBLAzer™-FRET B/G substrate (2 h), and fluorescence emission was recorded at 460 and 530 nm using a BioTek Synergy H1 Hybrid Reader.

All InVitrogen assays were performed in agonist and antagonist modes for all the chemicals. For the agonist mode we used at least 9 different concentrations of the test chemical, at half log intervals and a negative control. A positive control (E2) was performed, as well, in order to compare its response with the weaker estrogens. We saw positive signals for 17α–ethinylestradiol (EE2), E2, estrone (E1), 4-nonylhenol (4NP) and bisphenol A (BPA). There was no signal in agonist mode for bifenthrin (BF) and an extremely weak signal for galaxolide (GAL) (**Figure 2).** All specific methods for this assay are described in detail in **Appendix A.** We calculated EC50's for EE2, E2, E1, 4NP and BPA **(Table 1).**

Table 1. EC50 values for tested chemical

Chemical	EC50 (M)
17a-ethinyl estradiol (EE2)	1.11E-11
17b-estradiol (E2)	3.96E-11
Estrone (E1)	2.52E-10
4-Nonylphenol (4NP)	8.57E-8
Bisphenol A (BPA)	4.7E-7

We also performed the assay in antagonist mode in the presence of 0.2 nM E2, a concentration that should produce about 80% of the maximum signal (**Figure 3**). When we added the test chemicals to these assays, we saw a small amount of antagonism for E1 and NP at the lower concentrations, a phenomenon that has been described before (Kim et al. 2002). These chemicals bind to the ligand-binding domain of the receptor but at very low concentrations they do not transactivate the receptor. But, because the ligands are present, E2 is less efficient at binding and thus there is a little bit of competition.

In the case of galaxolide and bifenthrin, the antagonism is very pronounced at the lower concentrations. Bifenthrin appears to be an antagonist also at the higher concentrations. The molecular mechanisms by which bifenthrin acts on fish is still debated in the literature (Brander et al. 2012; Riar et al. 2013). It is possible that bifenthrin is metabolized to a more active metabolite such as to 4-hydroxy bifenthrin and that this activates estrogen receptors. In our hands this metabolite does not activate the human ERα in the Invitrogen Assays, but apparently this metabolite is quite potent on fish ERβ's (Brander, personal communication). Another possibility is that bifenthrin or a metabolite may act at a different point on the HPG axis, resulting in overall estrogenic activity *in vivo* (Riar et al. 2013).

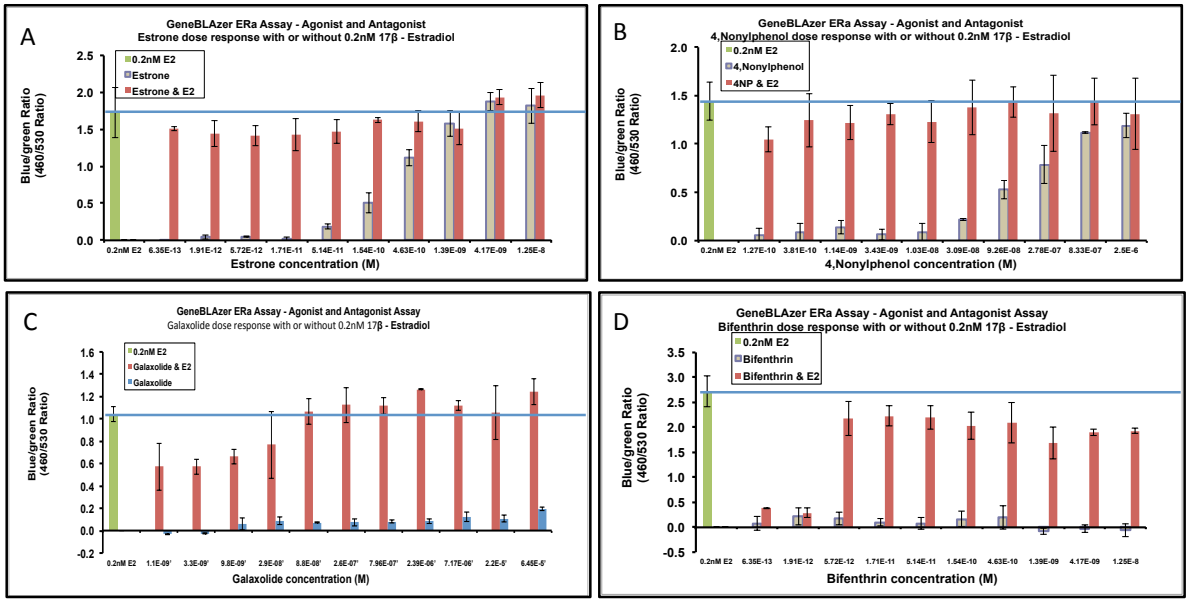


Figure 3. Antagonist mode for the InVitrogen ERα assay. Cells were plated in triplicate in 96-well clear bottom plates and dosed a mixture of E2 (0.2 nM E2 final concentration in wells) with respective concentrations of those chemicals for 18 h in the presence of 0.5% DMSO, loaded with LiveBLAzer™ FRET B/G substrate (2 h), and fluorescence emission was recorded at 460

and 530 nm using a BioTek Synergy H1 Hybrid Reader. The Blue/Green ratio of 0.2 nM E2 alone is given for the comparison.

B. *In vivo* early life stage assays (SCCWRP)

Early life stage (ELS) assays were conducted using 10-day-old *Menidia beryllina* larvae following the EPA protocol. The laboratory set up is shown below for the exposures in beakers (**Figure 4**). The specific methods that were employed for the assay are found in Appendix A. Table 2 contains the nominal concentrations of chemicals that were used.

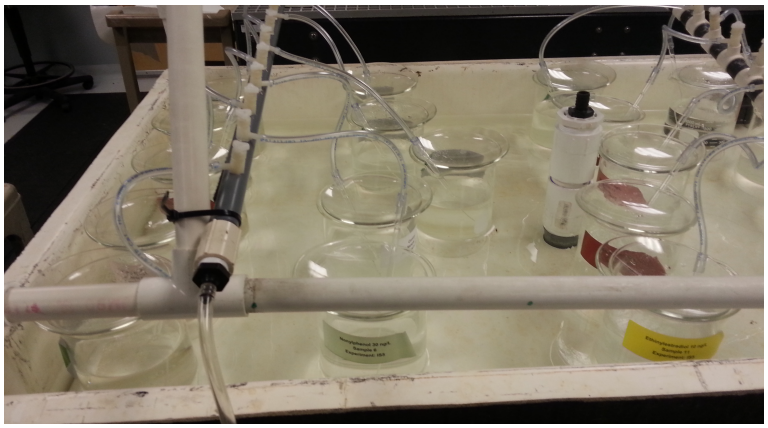


Figure 4. Experimental set up for testing early life stages of *Menidia beryllina* at SCCWRP.

The first experiment was an exposure of 10 day old *Menidia* larvae to E2 following the EPA protocol. A 7-day exposure was conducted with seawater (control), 0.02% methanol (solvent control), 10, 30, 100 and 300 ng E2/L and 10 ng EE2/L as positive control. Exposure concentrations for E2 were selected based on observations from exposure of juveniles conducted at UF. The endpoints of the ELS assay were growth (measured as dry weight) and survival. On day 0, a subsample of fish was used to calculate the average dry weight per larvae. On day 7, the surviving larvae were preserved in liquid nitrogen for subsequent Q-PCR analyses. Fish subsamples were used to estimate the mean dry weight per larvae for each treatment.

Experimental results: Exposure to E2 had no significant effects on survival or growth (**Table 2** and **Figure 5**). Similar exposure experiments were performed with E1, 4NP, BPA and GAL using the concentrations described in **Table 3**.

Table 2: Summary data for 7-day exposure of *Menidia* larvae to various concentrations of E2

Treatment	Seawater control	Methanol control	10 ng/L 17β-estradiol	30 ng/L 17β-estradiol	100 ng/L 17β-estradiol	300 ng/L 17β-estradiol	10 ng/L ethinylestradiol
Survival (%)	87.8	92.7	89.7	89.8	90.1	87.0	93.6
Sig diff from control (one-way ANOVA)	No	No	No	No	No	No	No
Mean dry wt/larvae (mg) ±SD	0.64 ± 0.17	0.68 ± 0.17	0.62 ± 0.21	0.65 ± 0.18	0.67 ± 0.10	0.80 ± 0.07	0.77 ± 0.06
Sig diff from control (one-way ANOVA)	No	No	No	No	No	No	No
Mean temp. (°C)	25.1	25.1	25.0	25.1	25.1	25.1	25.0
Mean salinity (ppt)	15.2	15.1	15.2	15.1	15.1	15.1	15.1
Mean DO (mg/L)	7.19	7.00	6.91	7.22	6.90	7.10	6.93
Average pH	8.29	8.18	8.11	8.19	8.20	8.20	8.17

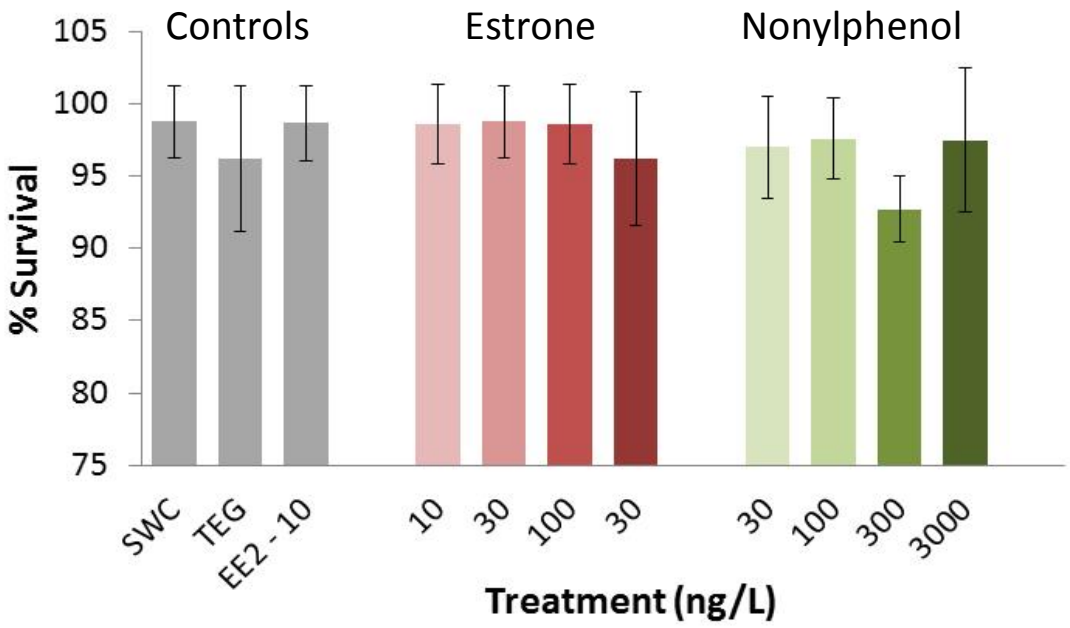


Figure 5. Effect of estrone and nonylphenol exposures on survival of *Menidia* larvae exposed for seven days.

Table 3: *Menidia beryllina* were exposed to the following treatments for seven days.

Treatment	**Nominal concentration**
Seawater control (artificial seawater)	
Vehicle control (TEG)	50 µL/L
EE2 (positive control)	10 ng/L
E1	10, 30, 100, 300 ng/L
4NP	30, 100, 300, 3,000 ng/L
BPA	300, 1,000, 3,000, 30,000 ng/L
Galaxolide	300, 1,000, 3,000, 30,000 ng/L

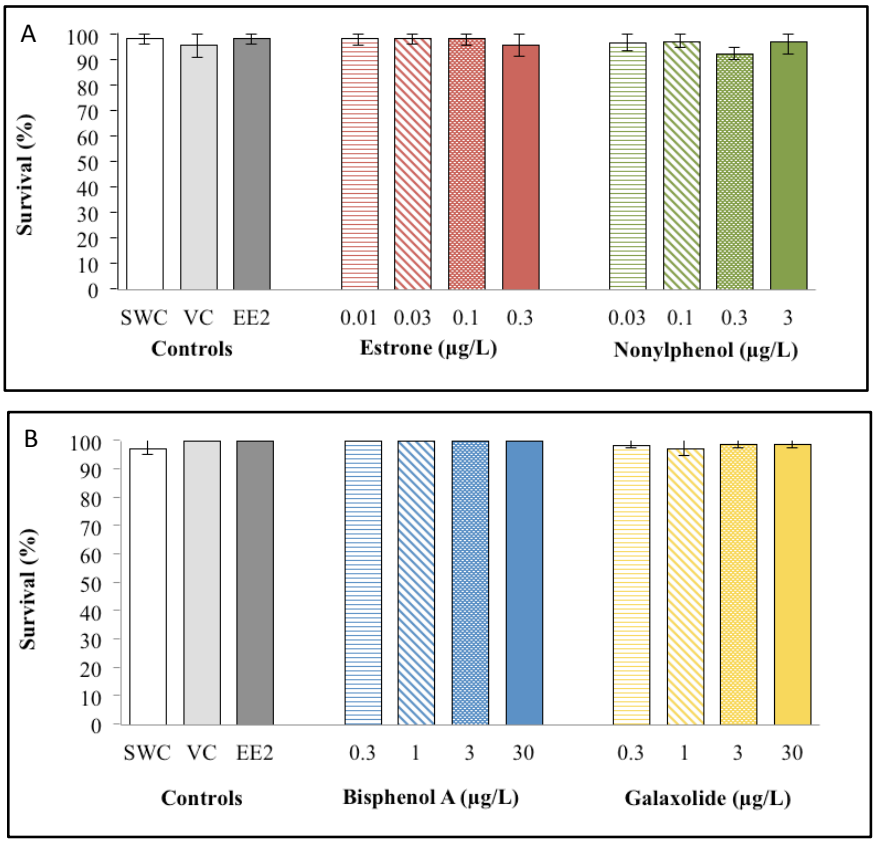


Figure 6: Mean survival (%) for *Menidia beryllina* larvae 7-day exposure to test chemicals. Error bars represent standard deviation (20 fish/replicate, 4 replicates/treatment) and (*) denotes a significant difference compared to the seawater control (SWC). A) Experiment 1- *Menidia* larvae were exposed to seawater only (SWC), a vehicle control (0.005% TEG; VC), a positive control (EE2), and four concentrations of E1 and 4NP. B) Experiment 2- *Menidia* larvae were exposed to SWC, VC, EE2 and four concentrations of BPA and GAL.

Exposure of *Menidia* larvae to test concentrations of E1, 4NP, BPA or galaxolide had no significant effect on survival. In both sets of experiments, the mean survival was greater than 95% for all treatments (**Figure 6)**. It was observed that the growth rate was highly variable among larvae. No significant differences were found in the mean dry weight of exposed larvae compared to larvae in the seawater and/or vehicle controls (**Figure 7**).

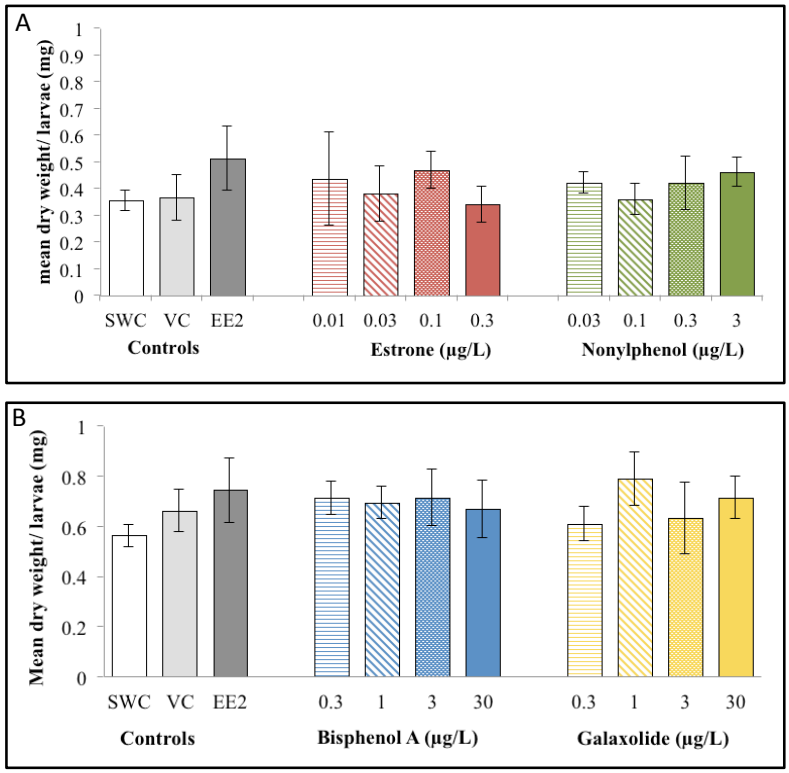


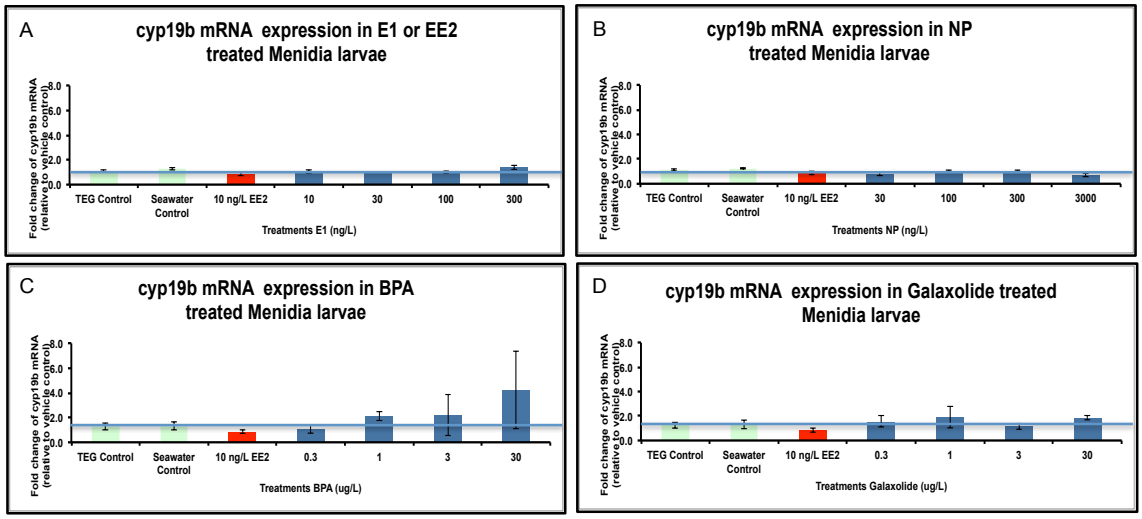
Figure 7: Effects of A) E1, 4NP, B) BPA and galaxolide on the mean dry weight of *Menidia* larvae after 7 days of exposure. There were no differences in growth among the chemical exposed larvae and those in seawater (SWC), vehicle control (VC) and EE2. Error bars represent standard deviation (5 larvae/replicate, 4 replicates/treatment) and (*) denotes a significant difference compared to SWC.

Gene expression studies for *Menidia* larvae

We performed Q-PCR for 5 genes that were expected to relate to effects from estrogen exposure and to higher order apical endpoints. Two of the genes were associated with expected responses to E2, cyp19b (brain aromatase) and StAR (steroidogenic acute regulatory protein) **(Figure 8**). Cyp19b has been shown to have estrogen response elements in its promoter in several teleost species (Callard et al. 2001; Chang et al. 2005; Le Page et al. 2008). StAR is a protein that controls the rate-limiting step for the initiation of steroidogenesis as it shuttles cholesterol into mitochondria for transformation into sex steroids (Chen et al. 2014).

BPA was the only test chemical to show a dose-dependent increase in Cyp19b and an increase in expression of StAR mRNA in larvae. This appeared to be a non-monotic effect with a larger increase at 1 ug/L than at higher concentrations. BPA effects on StAR are known from mammalian systems and fish (Zhou et al. 2008; Liu et al. 2012). GAL showed a trend toward increases in StAR in a dose-responsive manner. It is clear from the literature that GAL can affect steroidogenesis by altering expression of several of the genes in the pathway, but not StAR (Li et al. 2013). However, this study was performed with H295R cells and they may not reflect the *in vivo* actions of GAL for early life stage fish.

Cyp 19 B



StAR

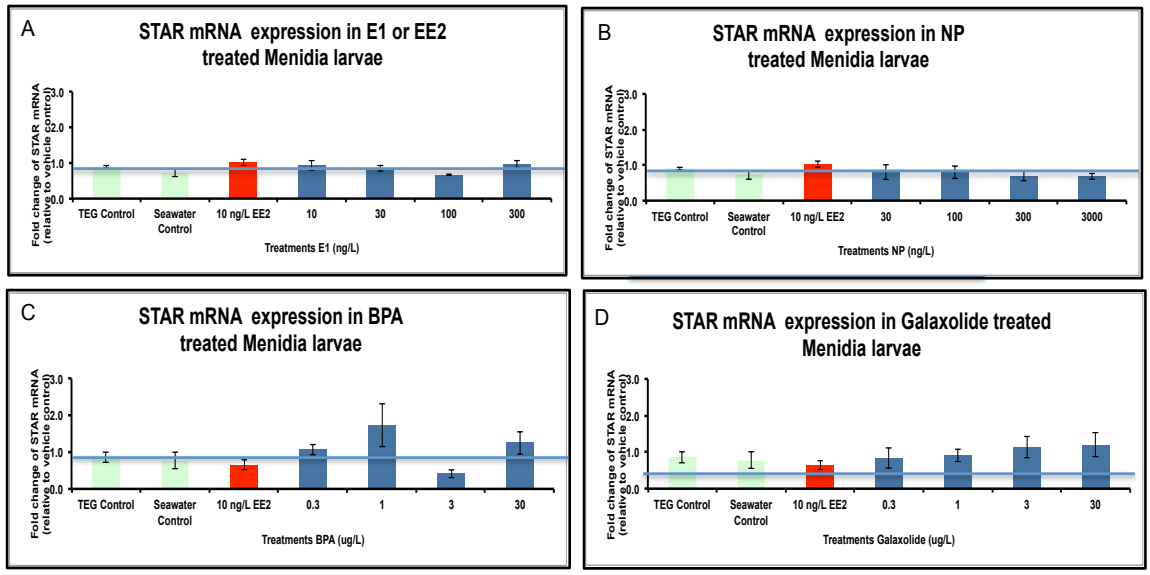
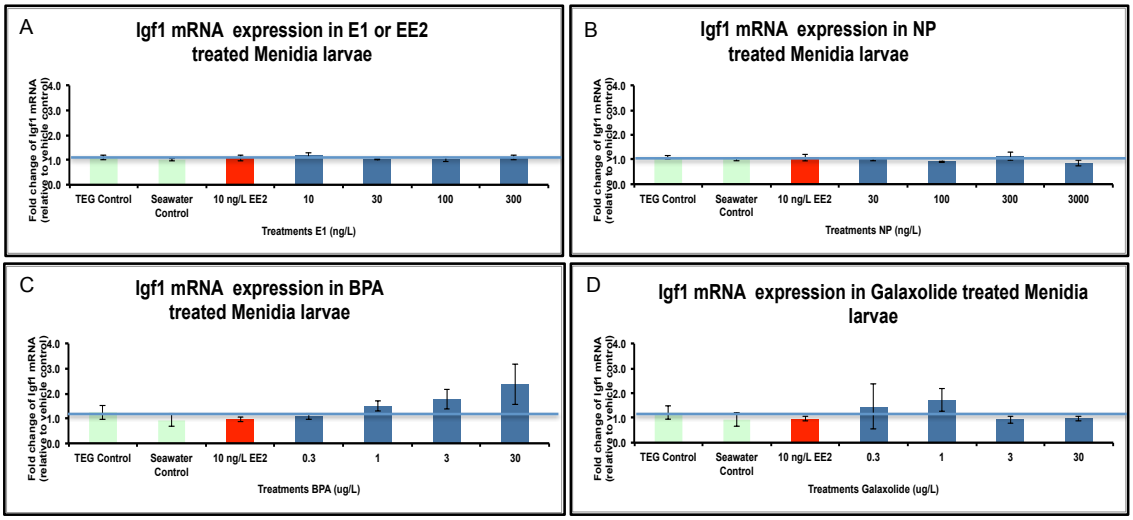


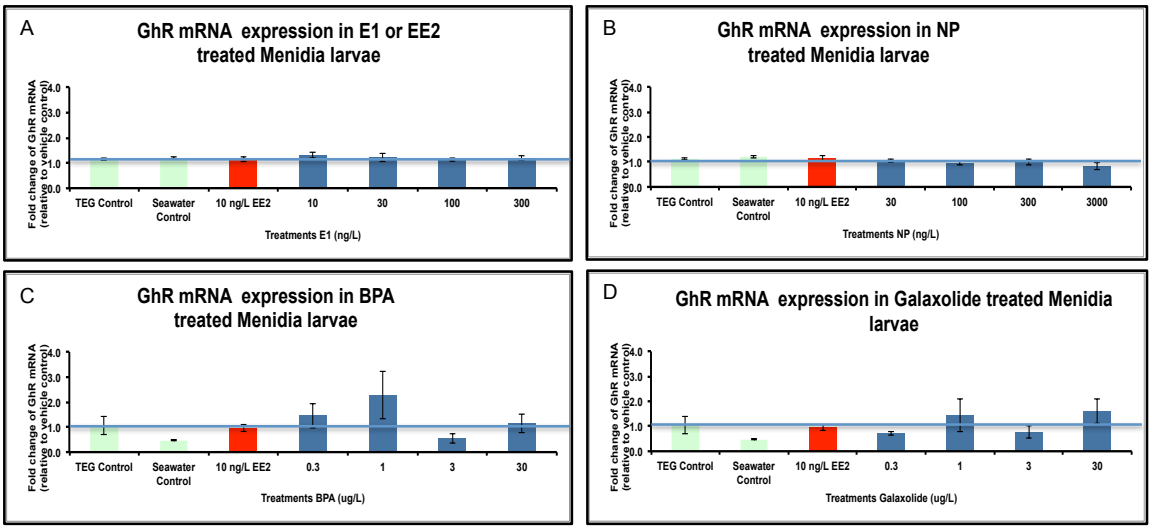
Figure 8. QPCR analysis of Cyp19b and StAR in *Menidia* larvae.

Other genes chosen to evaluate embryos were related to growth and sex. These included IgF1 (insulin like growth hormone 1); ghr (growth hormone receptor) (Filby et al. 2006; Beckman 2011; Fuentes et al. 2013) and antimullerian hormone (amh) (Schulz et al. 2007; Hattori et al. 2013) (**Figure 9).**

Igf1


A
Igf1 mRNA expression in E1 or EE2 treated Menidia larvae
Fold change of Igf1 mRNA (relative to vehicle control)
TEG Control
Seawater Control
10 ng/L EE2
10
30
100
300
Treatments E1 (ng/L)
B
Igf1 mRNA expression in NP treated Menidia larvae
Fold change of Igf1 mRNA (relative to vehicle control)
TEG Control
Seawater Control
10 ng/L EE2
30
100
300
3000
Treatments NP (ng/L)
C
Igf1 mRNA expression in BPA treated Menidia larvae
Fold change of Igf1 mRNA (relative to vehicle control)
TEG Control
Seawater Control
10 ng/L EE2
0.3
1
3
30
Treatments BPA (ug/L)
D
Igf1 mRNA expression in Galaxolide treated Menidia larvae
Fold change of Igf1 mRNA (relative to vehicle control)
TEG Control
Seawater Control
10 ng/L EE2
0.3
1
3
30
Treatments Galaxolide (ug/L)

GhR


A
GhR mRNA expression in E1 or EE2 treated Menidia larvae
Fold change of GhR mRNA (relative to vehicle control)
TEG Control
Seawater Control
10 ng/L EE2
10
30
100
300
Treatments E1 (ng/L)
B
GhR mRNA expression in NP treated Menidia larvae
Fold change of GhR mRNA (relative to vehicle control)
TEG Control
Seawater Control
10 ng/L EE2
30
100
300
3000
Treatments NP (ng/L)
C
GhR mRNA expression in BPA treated Menidia larvae
Fold change of GhR mRNA (relative to vehicle control)
TEG Control
Seawater Control
10 ng/L EE2
0.3
1
3
30
Treatments BPA (ug/L)
D
GhR mRNA expression in Galaxolide treated Menidia larvae
Fold change of GhR mRNA (relative to vehicle control)
TEG Control
Seawater Control
10 ng/L EE2
0.3
1
3
30
Treatments Galaxolide (ug/L)

Amh

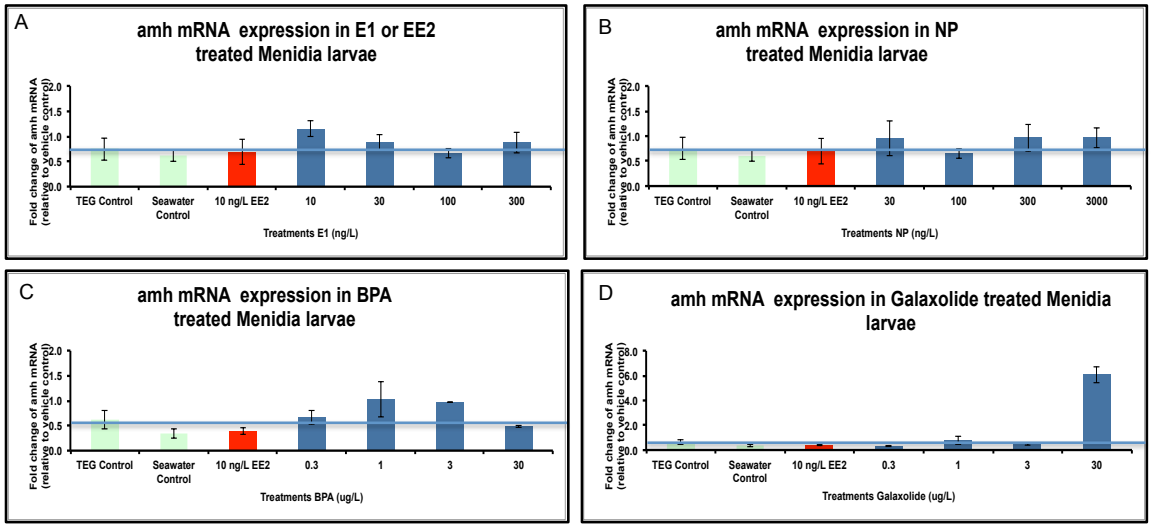


Figure 9. QPCR analyses for Igf1, GHR and Amh for *Menidia* larvae. The Y axis for amh in response to GAL is different than for the other contaminants.

E1 and NP showed effects only on amh, with a higher increase in mRNA steady state levels at the lower concentration of 10 ng/L E1 and 30 ng/L NP. But, these effects were not large and not significant. BPA on the other hand showed a dose-dependent response on IgF1 and non-monotonic effects on ghr and amh, with 1 ug/L showing maximal response. GAL showed a non-monotic dose response for igf1 (maximal response at 0.3 and 1 ug/L (concentrations that were antiestrogenic in the Invitrogen assay). The response for GAL was variable for ghr but showed a significant increase in amh at 30 ug/L.

We also tested DMRT1, hoping that it would be able to distinguish genetic males from genetic females. While initial tests looked promising, we found that DMRT1 was expressed more in adult male gonads than in female gonads, as reported for other fish by others (Guo et al. 2005). However, because it was expressed in both sexes, it did not work as a good biomarker of genetic sex. Nevertheless we tested to see if its expression could be altered by estrogens in *Menidia* embryos (**Figure 10**). The main effect we saw was a reduction in expression by the strongest estrogen EE2 at a concentration of 10 ng/L.

DMRT1

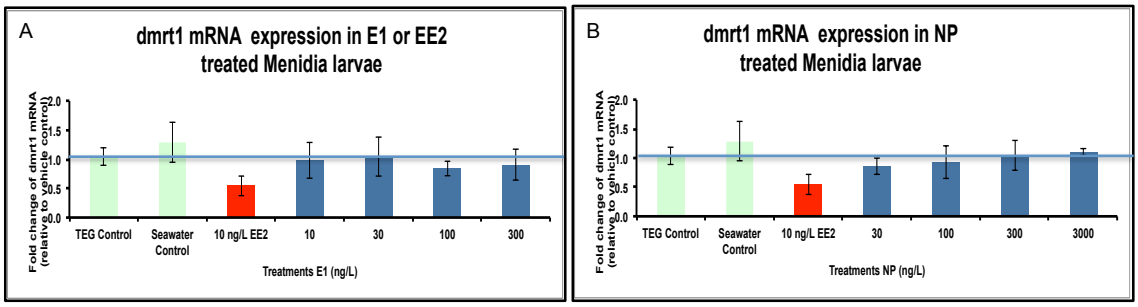


Figure 10. QPCR analysis of dmrt1 in *Menidia* embryos in response to EE2, E1 and NP.

C. Juvenile assays (UF) – Exposure procedures for juvenile fish were developed at the University of Florida. A full description of this assay is found in the **Appendix A**. The initial plan was to expose juvenile fish for 10 days over the period of gonadal differentiation, which we had expected to occur between day 50 and 60 in *Menidia* but the livers were too small to dissect out. In addition, sex determination is temperature dependent (absent exogenous contaminants) and occurs after a fish has reached 20-35 mm in length (Conover and Fleisher 1986). Our first pilot test was with E2 at 4 concentrations half log apart (3, 10, 30 and 100 ng/L) (**Figure 11**). We observed a high degree of variability in fish size at day 10, preventing us from separating livers from all fish. Thus, we used whole fish for Q-PCR analysis.

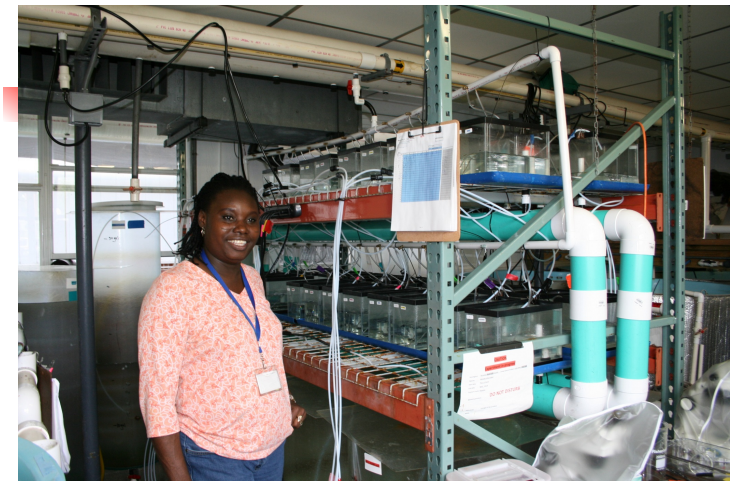


Figure. 11. Experimental set up for juvenile *Menidia beryllina* at the University of Florida.

In the initial experiment, we were able to see Vtg increase in whole fish but only at the 100 ng/L concentration (**Figure 12)**. Interestingly, when we conducted Q-PCR for Chg we observed elevated Chg levels in whole fish at much lower concentrations of E2, starting at 3, 30 and 100 ng/L compared to vehicle control. This was reported previously by Brander (Brander 2011), suggesting that chg is more sensitive than Vtg.

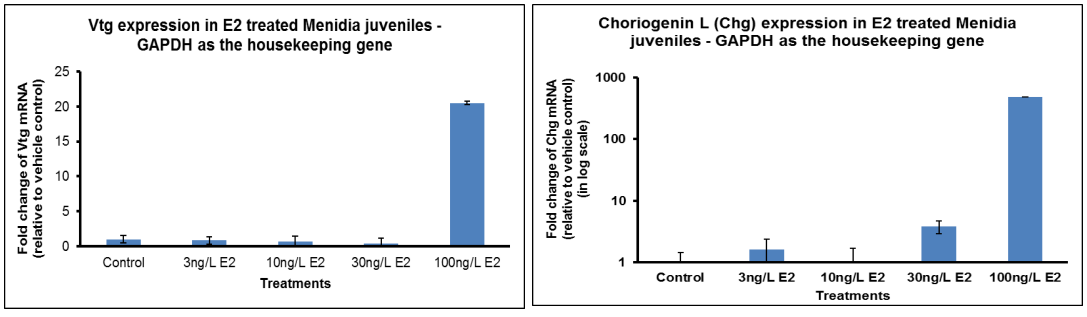


Figure 12. Figure 12. Treatment of *Menidia* juveniles with different levels of E2 resulted in elevated levels of vitellogenin (vtg) and Choriogenin L (Chg). *Menidia* juveniles (~ 50 days old) were exposed to E2 (3, 10, 30 and 100ng/L) for 10 days (50% daily static renewal) for 10 days. Total RNA was extracted from whole-body homogenates and, following reverse transcription, Vtg and Chg were PCR-amplified from cDNA template using Q-PCR. GAPDH was

used as an internal control. Fold change data are mean ± standard deviation relative to vehicle control.

The experiment was repeated, this time allowing fish to be exposed for 21 days to 71 days post hatch (dph). By the end of these longer exposures, fish size was indeed larger, allowing for excision of livers from all fish as well as identification of differentiated gonads. We used this experimental paradigm for the remaining test chemicals (E1, NP, BPA and GAL, Table 3). We used 10 ng/L EE2 as a positive control. Endpoints measured were length and condition factor, histopathology of the gonad and gene expression changes for 5 genes: ERα, ERβ, AR, Chg and Vtg.

Table 3. Nominal and actual concentrations for juvenile *Menidia* 21- to 71-day exposures to estrogenic test chemicals. Actual concentrations were determined by ELISAs specific to each chemical, as described in Appendix A.

Exp I

17β-estradiol ng/L	
nominal	actual
0	0
10	11
30	29
100	95
300	206

Exp II

Nonylphenol ng/L	
nominal	actual
0	tbd
30	tbd
100	tbd
300	tbd
3000	tbd
10,000	tbd

Ethinyl estradiol ng/L	
nominal	actual
10	2.1

Exp III

Estrone ng/L	
nominal	actual
0	0
10	7
30	40
100	131
300	303

Exp IV

Bisphenol A ng/L	
nominal	actual
0	0
300	383
1000	2077
3000	4531
30000	31674

[a]The ELISA assay for NP was back ordered, so we have not been able to confirm the actual concentrations used.

Length and condition factor:
We saw no effects on growth or condition factor; again this is probably due to the great variation in size of the fry at the beginning of the experiment. Data for this endpoint is found in Appendix A, Supplemental Figure 3.

Sex differentiation of the gonads determined by histology:
Fish were fixed in formalin and then trimmed under a dissecting microscope to generate mid-sections of gonadal tissue. This was done by removing the tail about 1 mm post cloaca and the upper part of the body posterior to the heart. The fist mid-section was then embedded in paraffin to the tail pointed up and sliced sagittally at several levels to ensure capture of gonadal tissue. Details of these methods are in the appendix. The sex of each fish was verified by visual inspection using a compound microscope at 20 and 40X. Figure 13 shows typical histological sections at 40X and 100X.

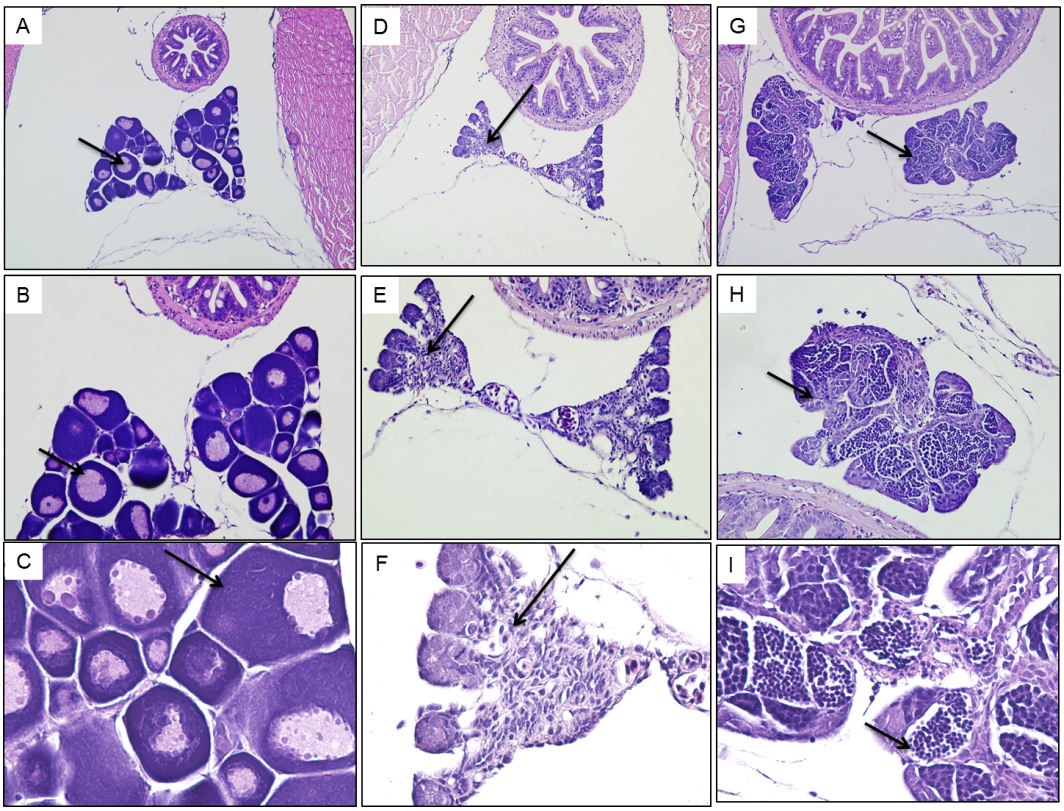


Figure 13. Histological sections of 71-day old *Menidia* stained with Hematoxylin and Eosin stain (H&E stain). Typical sections showing (A-C) oogonia; typical in females; (D-F) undifferentiated gonadal tissue (gonia) and (G-H)spermatogonia, typical in males. Photomicrograph of sex differentiation top row is 20X, middle row is 40X and bottom row is 60X.

Sex differentiation in *Menidia* is controlled by temperature and length of fish (Conover and Fleisher 1986). Our results suggest that full gonadal differentiation may require a longer window, as many of the gonads were undifferentiated. As noted above, there was substantial size difference among the fry, and this may have contributed to the variance seen in sexual differentiation of gonadal tissue, but we did not set out to test the idea that size and sexual differentiation were correlated and thus we lack data to confirm that hypothesis. We also did not perform Q-PCR for DMRT1 in these fish as a possible measure of genetic sex, but as indicated above, this marker is not fool proof for *Menidia*.

We had expected that gonadal tissue differentiation would have been completed by 71 dph (Conover and Fleisher 1986). Our data suggests that female ovarian tissue differentiates within this time frame but male gonadal tissue differentiation may take longer. For groups with at least 8 fish with detectable gonads, we saw mostly either females or undifferentiated tissue. We cannot comment on whether gonads observed would subsequently differentiate into male tissue.

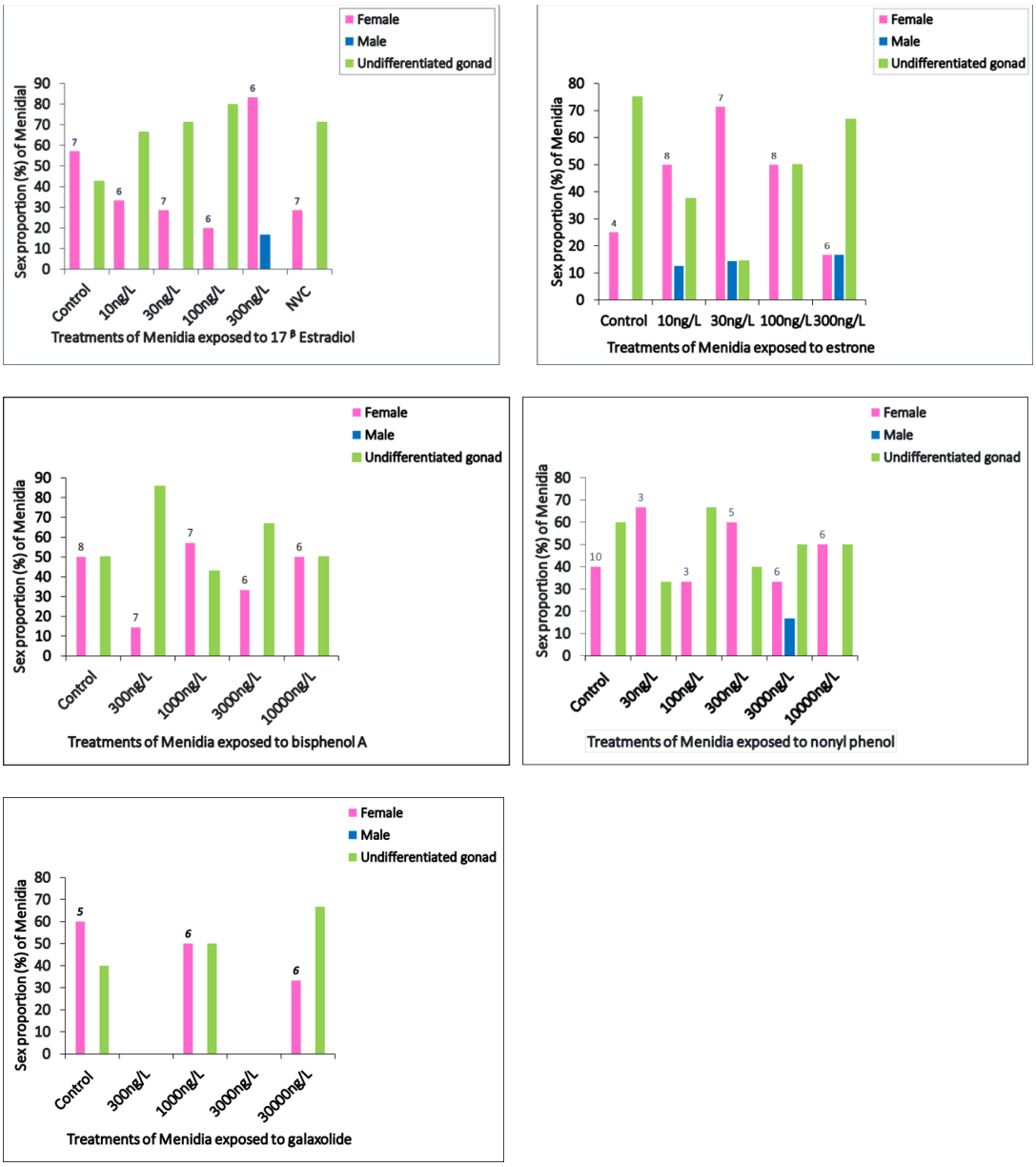


Figure 14. Proportion of females (pink), males (blue) and undifferentiated tissue (green) in *Menidia* after 21 days of treatment and at 71 days of age. The number above each pink columns is the number of fish per group that were analyzed, which was dependent on our ability to identify gonadal tissue in a given specimen. Very few males were identified.

Other than for E2 at 300 ng/L, the proportion of females based on gonadal tissue observations did not seem to differ from controls. For E1, there seemed to be an increased proportion of females with increasing concentration up to 100 ng/L, with, in contrast, a drop at the highest concentration (300 ng/L) where there seemed to be a higher proportion of undifferentiated gonads. This suggested a delay in gonadal maturation due to the high concentration of chemical; however, the power of the experiment was low and this should be repeated. There was no apparent or obvious effect on the proportion of females due to BPA, 4NP or GAL.

Size influence on sexual differentiation: As mentioned above, we had a wide variety of sizes of fish in the experiment, and it was possible that gonadal differentiation occurs at a specific fish size. Generally as seen below, fish with male phenotypes were bigger than the females and the undifferentiated ones. Statistical significance could not be established because in all the cases (signified by the lack of standard deviation) the number of males corresponded to a single fish. Undifferentiated fish were about the same size as females.

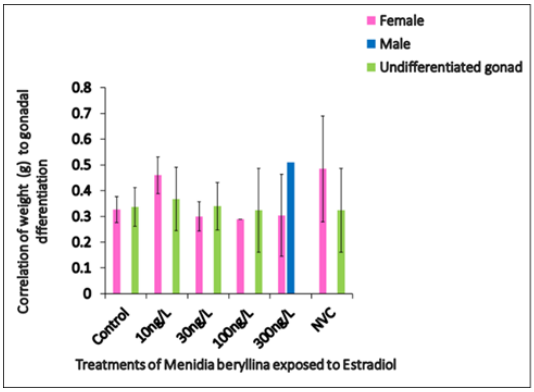
The variation in length was not dose dependent. No male was identified in the control in all treatments despite the fact that they had comparable weight and length. Additionally in control for all exposures, females were identified at varying lengths from a low of 16 mm to a high of 28 mm. In fish exposed to contaminants, females were also identified from a low length of 16 mm (E1 and GAL) to a high of 28 mm (BPA) and 29 mm (E2). Males were 25 mm (E1, E2), 20 mm and 28 mm (4NP); while undifferentiated fish also ranged widely, from the lowest at 16 mm (E1, GAL) to the highest at 28 mm (E2) and 29 mm (BPA). Sex was determinable with a compound microscope at 20X magnification for differentiated males and females, while undifferentiated ones could only be confirmed at 40x magnification.

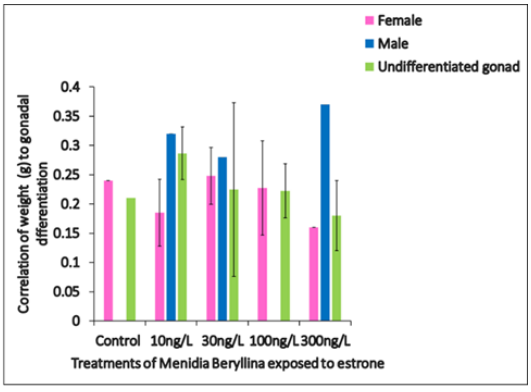
Table 4. Weight and length of fish by sex determination.

Treatment	**Female fish[a] weight (g) & Length(mm)**	**Male fish[a] Weight (g) & Length(mm)**	**Undifferentiated fish[a] Weight (g) & Length(mm)**
17β estradiol, 300 ng/L	0.3±0.15; 21±0.09	0.51; 25	none
Estrone, 10 ng/L	0.19±0.05; 19±0.2	0.32; 22	0.29±0.04; 22±0.06
Estrone, 30 ng/L	0.24±0.05; 22±0.1	0.28; 22	0.23±0.1; 20±02
Estrone, 3,000 ng/L	0.16; 18	0.37; 25	0.18±0.06; 18.5±0.2
NP, 3,000 ng/L	0.5; 25	0.48; 28	0.41; 25

[a]Entries without standard deviations are examples of a single fish.

Correlation of weight to sex proportion: We also examined if overall weight of the fish had an influence on sexual differentiation of the gonad (**Figure 15**). Although not dose dependent, generally in most of the treatments the mean weight of differentiated fish were higher than the undifferentiated. The general exception was with the controls, where the undifferentiated fish had higher mean weight than differentiated fish, but this varied with the set examined. This is probably due to the high variance in fish size at the beginning of the experiment of the 50 dph fish.





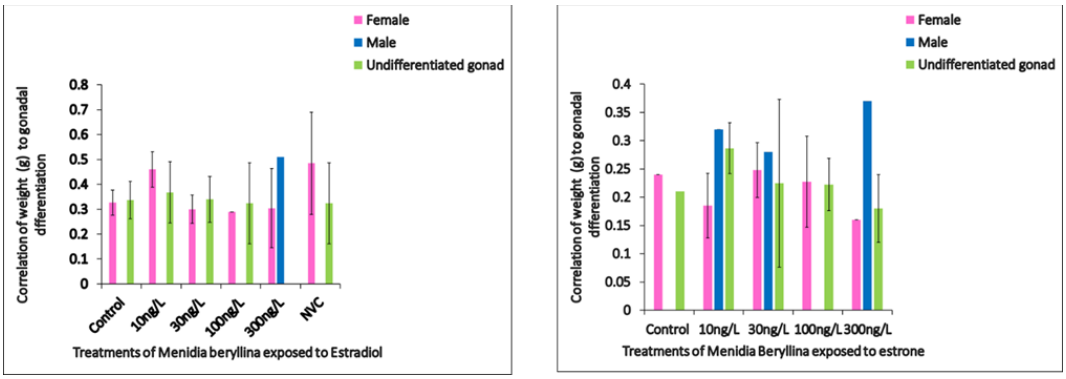


Figure 15. Correlation of weight of fish to sex identification.

Temperature

(Strussmann et al. 2010) reported that family Atherinopsidae to which *Menidia beryllina* belong show temperature-dependent sex determination (TSD) which might also make them prone to dysfunctions such as highly skewed sex ratios. In the present study, mean exposure temperatures during the 21 day period were 22.8 ± 1.5°C (E2), 22.6 ± 1.1°C (E1), 21.2 ± 1.9 °C (BPA), 22.8 ± 0.98°C (4NP) and 22.7 ± 1.0°C (GAL). The maximum mean temperature did not exceed 22.8 ± 1.5°C and the minimum temperature range did not fall below 19°C during the period of exposure. According to Duffy et al, (Duffy et al. 2010) these temperatures fall within an intermediate sex ratio-producing temperature (21°C) as opposed to temperatures that feminize (15°C) and masculinize (28°C) reported for Atlantic silversides, *Menidia menidia*.

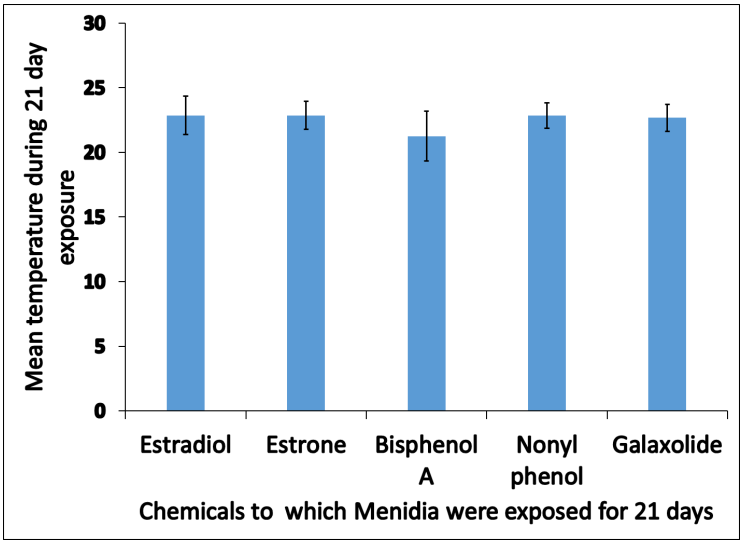


Figure 16. Mean temperature during the 21 day exposures

Influence of contaminants on growth

It was difficult to get a clear understanding of the effects of the different contaminants on growth of the juveniles. The 45-day old fish that were received were different sizes when they arrived and we distributed them randomly to the test tanks. We did not separate them out by size. We did notice that placing them in contaminant tanks increased the variability tremendously of the sizes of the fish and this was not dependent on sexual differentiation. In **Figure 17**, we plotted the overall weight of the fish for those that we checked for sexual differentiation as a function of their contaminant concentration for two of the contaminants, a relatively strong estrogenic contaminant, E1, and a weaker estrogen, NP. As can be seen from these graphs, the controls appear to have less variance in their size than the contaminant treated fish. We get a similar plot for fish length, but with a less pronounced effect. For other exposures, there was no difference in the variance of control and exposed fish. More work will need to be done to determine if this is a real phenotypic change, or just a random selection of fish, since our n is small.

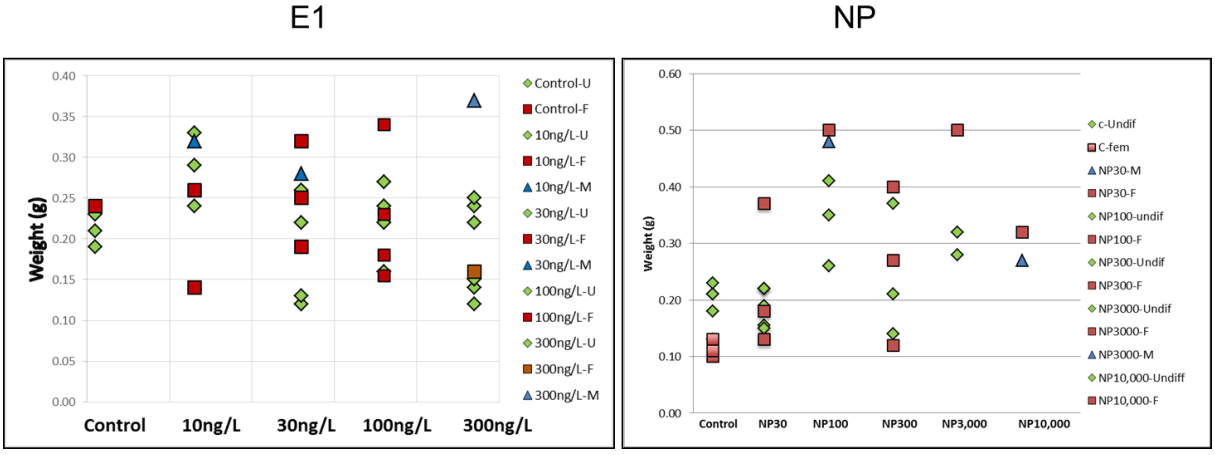
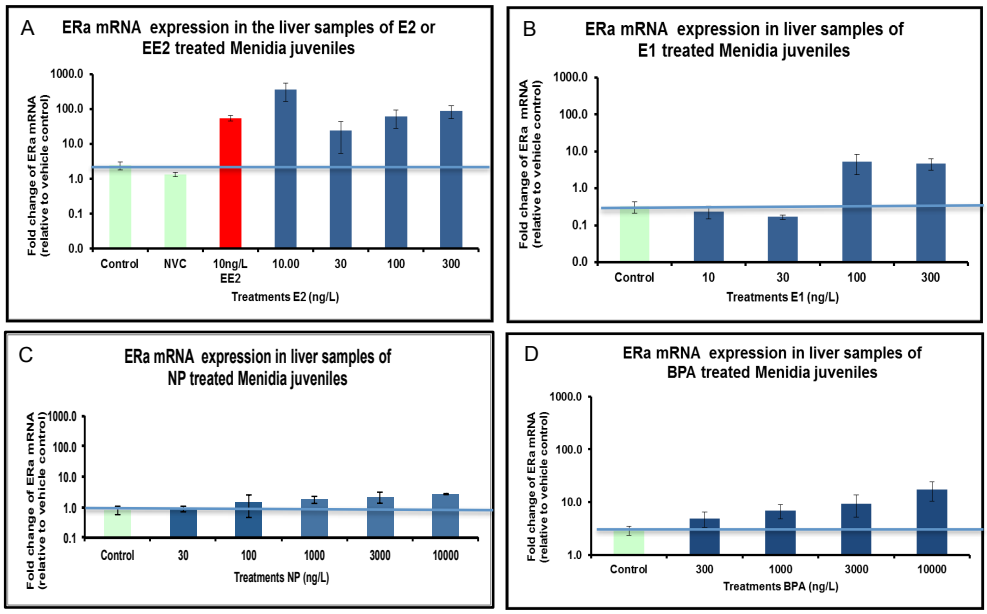


Figure 17. The effect of estrogenic contaminants on weight of fish. This represents only those fish that were used for sex determination by histology. Red squares, females; blue triangles, males; and green diamonds, undifferentiated gonadal tissues.

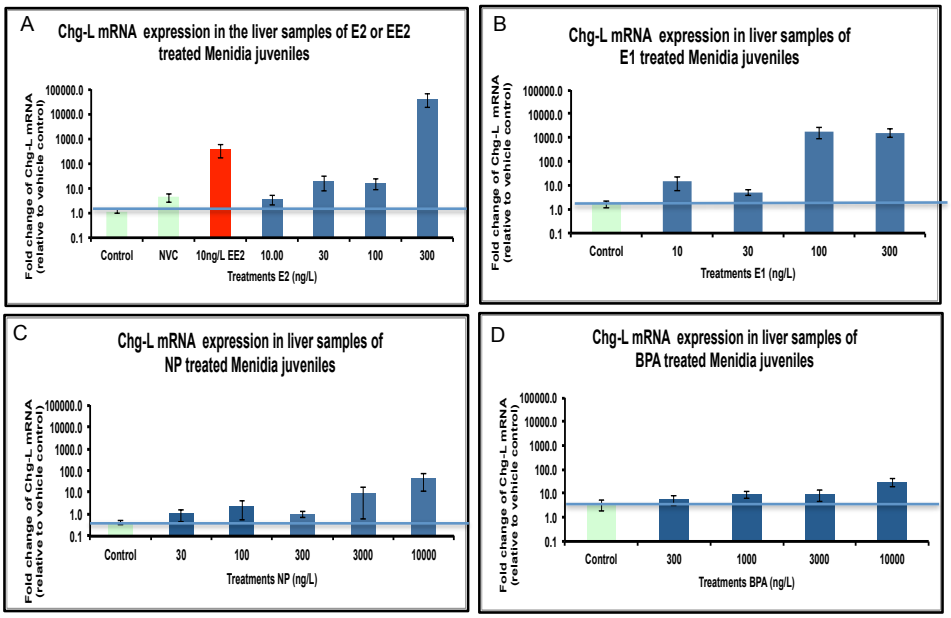
Molecular biomarkers for juvenile *Menidia* exposed to contaminants.

We tested the livers from exposed juvenile *Menidia* for differential expression of 5 genes that could be related to endocrine disruption: estrogen receptor alpha (ERa), Vitellogenin (vtg), choriogenin (chg), androgen receptor (AR), and estrogen receptor beta (ERb). The different treatments resulted in dose-dependent increases in ERa, vtg and chg, in consonance with other studies in fish (Sabo-Attwood et al. 2004; Yu et al. 2006; Chen et al. 2008) (**Fig 18**). The effects on AR and ERβ differed by treatment. The two relatively potent estrogens, E2 and E1, appeared to have a dampening effect on the expression of the two genes by almost two fold. We observed a dampening of ERβ by relatively strong estrogens previously in other fish species (Sabo-Attwood et al. 2004). On the other hand, 4NP and BPA seemed to have a dose-dependent increase of expression (**Figue 19**). This has also been seen for ERβ in other fish (Chandrasekar et al. 2010; Palermo et al. 2012). It is known that these two chemicals have other endocrine activities besides activating the soluble ER. They both can function as an anti-estrogen at low concentrations, as demonstrated by the *in vitro* assays and both also function as anti-androgens. BPA also affects the thyroid hormone axis. Thus, their effects on these two other genes may be due to other activities.

ERa


A
ERa mRNA expression in the liver samples of E2 or EE2 treated Menidia juveniles
Fold change of ERa mRNA (relative to vehicle control)
1000.0
100.0
10.0
1.0
0.1
0.0
Control
NVC
10ng/L EE2
10.00
30
100
300
Treatments E2 (ng/L)
B
ERa mRNA expression in liver samples of E1 treated Menidia juveniles
Fold change of ERa mRNA (relative to vehicle control)
1000.0
100.0
10.0
1.0
0.1
0.0
Control
10
30
100
300
Treatments E1 (ng/L)
C
ERa mRNA expression in liver samples of NP treated Menidia juveniles
Fold change of ERa mRNA (relative to vehicle control)
1000.0
100.0
10.0
1.0
0.1
Control
30
100
1000
3000
10000
Treatments NP (ng/L)
D
ERa mRNA expression in liver samples of BPA treated Menidia juveniles
Fold change of ERa mRNA (relative to vehicle control)
1000.0
100.0
10.0
1.0
Control
300
1000
3000
10000
Treatments BPA (ng/L)

Chg


A
Chg-L mRNA expression in the liver samples of E2 or EE2 treated Menidia juveniles
Fold change of Chg-L mRNA (relative to vehicle control)
100000.0
10000.0
1000.0
100.0
10.0
1.0
0.1
Control
NVC
10ng/L EE2
10.00
30
100
300
Treatments E2 (ng/L)
B
Chg-L mRNA expression in liver samples of E1 treated Menidia juveniles
Fold change of Chg-L mRNA (relative to vehicle control)
100000.0
10000.0
1000.0
100.0
10.0
1.0
0.1
Control
10
30
100
300
Treatments E1 (ng/L)
C
Chg-L mRNA expression in liver samples of NP treated Menidia juveniles
Fold change of Chg-L mRNA (relative to vehicle control)
100000.0
10000.0
1000.0
100.0
10.0
1.0
0.1
Control
30
100
300
3000
10000
Treatments NP (ng/L)
D
Chg-L mRNA expression in liver samples of BPA treated Menidia juveniles
Fold change of Chg-L mRNA (relative to vehicle control)
100000.0
10000.0
1000.0
100.0
10.0
1.0
0.1
Control
300
1000
3000
10000
Treatments BPA (ng/L)

Vtg

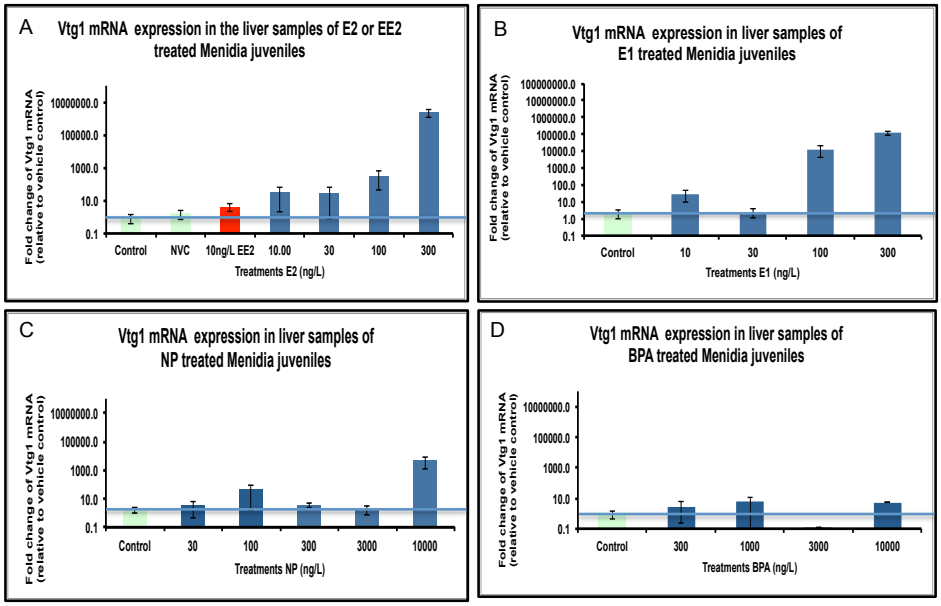
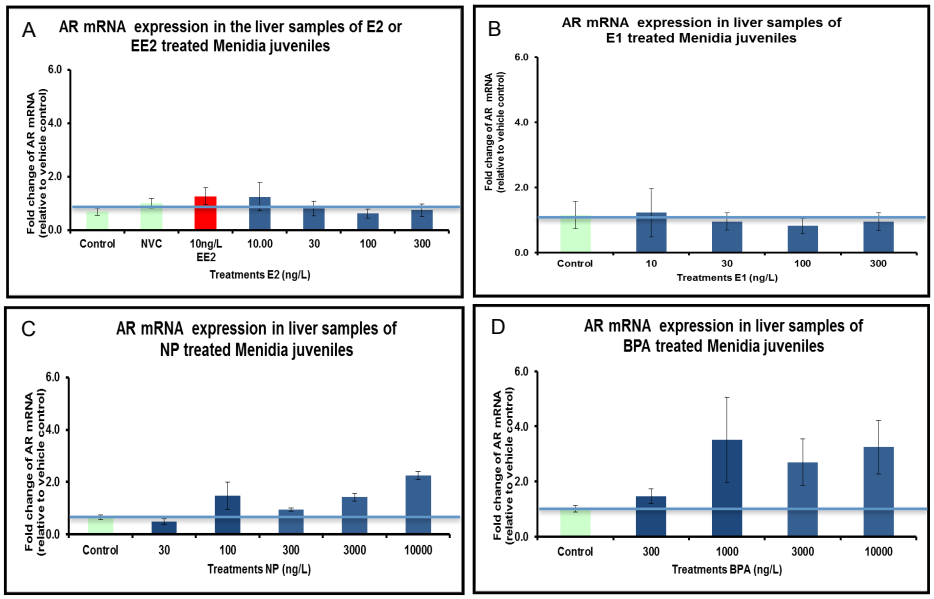


Figure 18. Q-PCR results for ERa, Chg and Vtg on juvenile *Menidia* exposed to E1, E2, 4NP and BPA for 21 days. GAPDH was used as an internal control. Fold change data are mean ± standard error relative to vehicle control. The horizontal line indicates the level of the control.

AR



ERβ

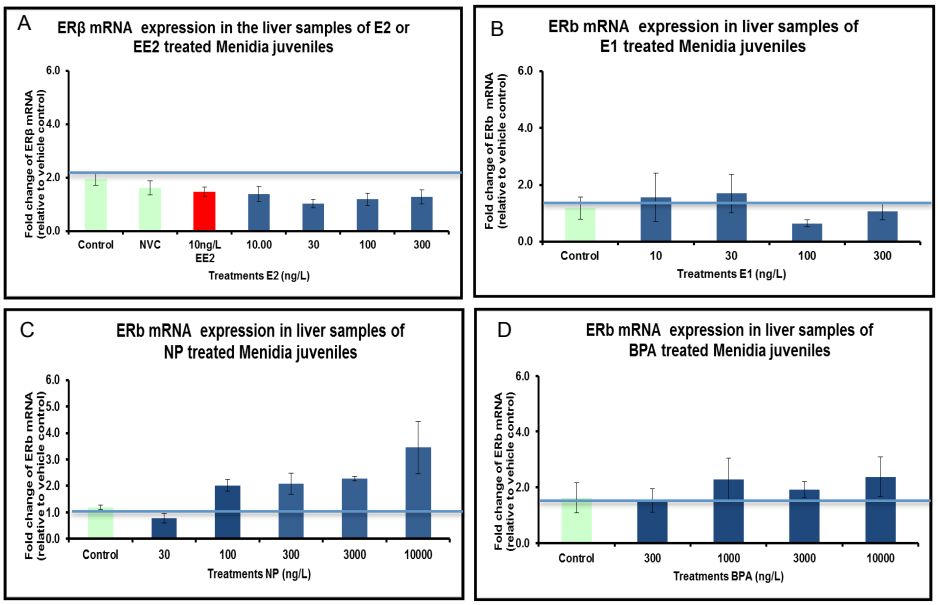


Figure 19. Q-PCR results for AR and ERβ on juvenile *Menidia* exposed to E1, E2, 4NP and BPA for 21 days. GAPDH was used as an internal control. Fold change data are mean ± standard error relative to vehicle control. The horizontal line indicates the level of the control.

Conclusions:

1. Several molecular biomarkers for gene-specific expression were developed for *Menidia beryllina* using Q-PCR.

2. The *in vitro* response of a commercially available estrogen receptor transactivation assay was characterized for E1, E2, 4NP, BPA, GAL and bifenthrin, referenced to the strong agonist EE2. The potency of our test estrogens was as follows:
   E2 > E1 > 4NP > BPA >> GAL, bifenthrin

3. Survival and growth of *Menidia* larvae were not affected by nominal exposure concentrations as high as 300 ng/L of E1; 3000 ng/L of E2; 3 ug/L of 4NP and 30 ug/L for BPA and GAL. Actual exposure concentrations for this series of experiments needed to more completely interpret these observations will be determined in Year 2.

4. Gene expression studies for *Menidia* (larvae) indicated different activities of the estrogenic compounds. The exposures for the larvae were only for seven days possibly insufficient time for a robust transcriptional effect. We have not yet measured the actual concentrations for the exposures.

   a. We had expected to see increases in Cyp19b with all estrogenic chemicals and not GAL because promoters for Cyp19b in fish are known to have estrogen response elements (EREs). To our surprise, only BPA showed a positive dose-dependent response. It is possible that we misidentified the gene sequence, something we will work on more in the next period.

b. *Menidia* larvae-- StAR gene. Only GAL showed a linear dose response, but BPA showed what appeared to be an inverted U shape curve for this gene. This is the main regulator of stroidogenesis.
c. *Menidia* larvae – IgF1 gene is associated with growth. Only BPA produced a linear dose responsive association, despite not being able to observe actual growth in the larvae.
d. *Menidia* larvae – GhR is also associated with growth. Only BPA showed a response, but this was inverted U shaped curve with a maximum effect at 1 ug/L
e. *Menidia* larvae – Amh is associated with being male. BPA showed an inverted dose response curve and GAL showed a high induction but only at the highest concentration of 30 ug/L.
f. *Menidia* larvae – DMRT1 is associated in some fish with maleness. In other fish it is expressed both in males and females, but at much higher levels in males. The only notable effect was seen with ethinylestradiol at 10 ng/L where we saw a distinct depression of expression of this gene.

5. Gene expression studies in juveniles. Strong and weak estrogens behaved as anticipated with biomarkers known to chart estrogenic effects, including Era, Chg and Vtg. Effects on AR and ERb by some of the weak estrogens are probably more related to their other activities, for example it is known that both NP and BPA can act as antiandrogens and that BPA also can suppress transcription of the thyroid hormone receptor (Rostkowski et al. 2011; Sheng et al. 2012).
    a. *Menidia* juveniles – ERa strong dose response for all of the chemicals tested. E2 reached a plateau at low concentrations as seen in o0ther studies. NP was the weakest of the responses.
    b. *Menidia* juveniles – Chg -- Nice dose responses for all the chemicals tested. BPA was weaker than NP.
    c. *Menidia* juveniles – Vtg – Nice dose responses for all the chemicals tested. BPA was weaker than NP
    d. *Menidia* juveniles – AR – We expected no response from pure estrogens and that was the case for E2 and E1, but very strong response for NP and BPA
    e. *Menidia* juveniles – ERb – in other studies, pure estrogens tend to downregulate this gene. We saw that effect with E2 and E1, but NP and BPA upregulated this gene.

6. Gonadal tissue developed during 21 to 71 day *Menidia* exposures was disproportionately female and/or undifferentiated. To put the role of chemical exposure in perspective, the development of males (based on gonadal tissue development) needs to be further investigated.

7. *Menidia* size is critical to allow for excision of gonadal and liver tissue for determination of sex and biomarkers of sexual reproductive status (Chg, Vtg). Our initial experiments suggested that at 21 days, ovarian tissue has differentiated but not testicular tissue, suggesting that to capture this tissue we would need to treat the fish for a longer period of time at our temperature and water conditions. We will perform an additional experiment to verify the time frame for testicular differentiation.

8. Initial observations indicate that we should get a better handle on effects on growth by separating out fish by size at both the larvae and juvenile stages and that we should better understand the time frame for testicular differentiation.

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Appendix A: Methods

A. *In vitro* bioassays for ERa and EEQ calculation (UF)

Exposure solution extracts were made up in DMSO and were stored at -80^{o}C until bioanalysis. ERa- GripTite DA cells plated with ~50,000 cells per well in a 96-well clear bottom plate. Cells were stimulated with different concentrations of the reference chemical (E2) or estrogen mimic in the presence of 0.5% DMSO overnight. The following day, cells were loaded with LiveBLAzer™-FRET B/G Substrate and incubated in the dark for 2 hrs. Fluorescence emission values at 460 nm and 530 nm were obtained using a standard fluorescence plate reader (BioTek Synergy H1 Hybrid Reader) and the calculated Blue/ Green Ratios plotted against the indicated concentrations of the chemical (EE2, E2, E1, BPA or NP).

To calculate EEQs in the exposure extracts a previously described (Escher et al. 2014) was followed. Samples were analyzed on the same plate as a standard curve of E2 for the ERα assay and then used to calculate bioanalytical equivalent concentrations (BEQs). To calculate the EEQs of any exposure solution extract, EC_{10} or $ECIR_{1.5}$ values of the exposure solution extract and the reference chemical (E2) were calculated first. Then, the EC_{10} or $ECIR_{1.5}$ value of the reference chemical (E2) was divided by the respective value of the exposure solution extract.

B. Fish larval exposures (SCCWRP)

Inland silverside (*Menidia beryllina*) were purchased from Aquatic BioSystems. Nine-day old larvae were acclimated in 1 L glass beakers containing 800 mL of artificial seawater (Instant Ocean) at 15 parts per thousand (ppt) for 24 h. The following day, the animals were inspected and replaced when necessary to ensure that each beaker contained 20 larvae at the beginning of the exposures. Larvae were fed newly hatched brine shrimp throughout the exposure until 1 day before the end of the exposures. For this study, larvae were exposed for 7 days to four concentrations of the following endocrine disrupting compounds (EDCs): estrone and nonylphenol (experiment 1), and bisphenol A and galaxolide (experiment 2). Each experiment also included a seawater control, a vehicle control (0.005% triethylene glycol; TEG), and a positive control (17α-ethinylestradiol). Table 1 describes the different treatments and concentrations used in this study. Each treatment consisted of four replicate beakers.

The exposures were conducted using a static system. Test solutions were prepared daily and used to change 75% of the water in each beaker. Water quality parameters were routinely measured and maintained throughout the exposures within the following range: temperature of 24 ± 1 ^{o}C, salinity of 15 ± 1 ppt, dissolved oxygen > 6.5 mg/L, pH 7.95 ± 0.20 and ammonia <0.2 mg/L. The nominal concentrations are found in Table 2 of the main report.

Water chemistry

At day 0, 1, 3, 5, and 7, composite water samples (from all 4 replicates per treatment) were collected for chemical analyses. Samples were preserved with 5 mL of methanol, pH adjusted to 7 using 1M hydrochloric acid and solid phase extracted using Oasis HLB 6cc cartridges.

Apical and molecular endpoints

The number of dead larvae was recorded daily and used to calculate the percent survival for each treatment. Effects of EDCs on growth were examined by measuring the biomass. Five fish per replicate beaker were placed in small pre-weighed aluminum pans and dried for 24 h at 60^{o}C. The following day, the pans were weighed and the average weight per fish was estimated.

The rest of the larvae (12-15) were flash frozen in liquid nitrogen and preserved at -80^{o}C. The samples were sent to University of Florida for RNA extraction using RNA Stat-60 and cDNA synthesis.

Statistical analyses
The effects of EDCs on percent survival and mean dry weight per larvae (mg) were determined by one-way analysis of variance (ANOVA) using the statistical software package R. Level of significance was set at $p < 0.05$.

C. Juvenile fish exposures – UF
Lab reared *menidia* (45 day post hatch) were purchased from a bioassay supplier, Aquatic Biosystems (Ft Collins CO), and acclimated for 5 days before exposure. Upon arrival and during the experiments, the fish were fed live brine shrimp nauplii (BSN) (2-3 days post hatch) daily. Feeding rates were maintained for each aquarium by washing (15 ppt seawater) and concentrating live brine shrimp using a 150um filter, and pipetting an equal volume of the live feed to each tank. Feeding rates were increased and verified every few days. Water quality (dissolved oxygen, pH, ammonia) was verified weekly or as needed.

We attempted to use artificial diets, but were not successful. In a pilot study, we realized that Menidia appear to only ingest feeds in the water column. If food is uneaten, it goes to the bottom of the tank where it quickly compromised the water quality and was difficult to remove. BSN remain alive and swimming for several days in the test water. However, un-hatched brine shrimp eggs appear also to be ingested by the fish, accumulate in the gut, and can cause mortality in 1-2 weeks. It is difficult to remove all the unhatched cysts from the live brine shrimp due to their size and buoyance. In the future, we will use chemically de-chorionated brine shrimp eggs which can be digested and minimize mortalities due to feeding.

Chemicals
All chemicals were initially dissolved in 95% ethanol with the exception of Galaxolide, which was an ethanol/DMSO (1:1) combination in a sealed GC container to prevent volatilization. Dilutions of the dissolved chemical stock solutions (10 mg/ml) were further diluted in triethylene glycol (TEG) to create individual spiking solutions for each dose. The final concentration of TEG (containing the test chemical) was maintained at 50µl/ liter of test water. The nominal and actual concentrations of the test solutions are in Table 3 of the full report.

Exposure Solutions
City water used for these experiments was carbon filtered to remove chlorine and potential hydrophobic contaminants. Salt water (15 ppt) was prepared using Instant Ocean in a 400 gallon fiberglass tank with heavy aeration. Prepared saltwater was pumped thru a 25 micron filter to remove any fine debris.

Exposure solutions were stored in a 50 gallon fiberglass tank that was continually mixed by mild aeration. The water in each tank was changed daily (50%) by partially draining each aquarium. Fresh solutions were then pumped into each aquarium using Chemfluor tubing. This tubing has been used and validated by the EPA to be low or non-binding for chemicals. Fifteen 50-day post hatch *Menidia* were exposed to the test solutions for 21 days in 2.5 gallon glass aquaria, containing 4 liters of test water, and aerated with a glass pipette. All exposures were run in quadruplicate. One liter water samples from each of the bulk water holding tanks was collected for chemical analysis.

Concentrations of E1, E2, EE2, NP, BPA, and control solutions were verified using ELISA kits (Abraxis). One liter of each exposure solution was collected at the end of the experiment from the bulk holding tanks and stored at 4°C. E1, E2, and EE2 were concentrated down to 1.0 ml using C18 solid phase extraction cartridges (AccuBOND II ODS-C18, Agilent) and eluted with methanol. NP and BPA SPE concentration utilized a Nexus matrix (BondElut, Agilent). The remaining portion was evaporated with nitrogen and reconstituted in distilled water containing 10% methanol.

Tissue collection

The fish were anesthetized using MS-222 (100 mg/ml). The total weight (to 0.01 g) and lengths (to 0.1 mm) of each fish were recorded. The liver was removed using a dissecting microscope by making a small incision in the chest, and then flash frozen using liquid nitrogen. The remaining carcass for each fish was preserved in 10% buffered formalin for histological verification of sex and reproductive stage. Whole fish were anesthetized, flash frozen, and stored at -80°C as a “back-up” for RNA quantification. A total of 4 livers, and 4 whole fish were collected from each aquarium at the end of the experiment.

Histology

In order to ensure capture of the gonadal tissue during sectioning, the fish were trimmed under a dissecting microscope after formalin fixation. The tail was severed 1mm post the cloaca and then posterior to the heart. The resulting mid-sections were imbedded in paraffin so the tail pointed up and then sliced sagitally at several levels posterior the cloaca to ensure capture of gonad tissue. Histological processing was conducted by Histological Tech Services (Gainesville, FL) and stained by H&E. The sex of each fish was verified by visual inspection using a compound microscope at 20X, 40X and 60X.

Appendix B: Validation of QPCR assays for Menidia beryllina

A) Verification of primer design for QPCR for various genes involved in reproduction. For this set of experiments, liver tissues were obtained from Menidia and then extracted for total RNA. This RNA sample was then evaluated for purity (A260/A280 ratio with the NanoDrop spectrophotomer). Primers were designed for the genes listed below (Table S1). Other primers were from Susanne Brander (Brander 2011). All primers were first verified by regular PCR and migrated into a gel (Fig. S1) and then by Q-PCR to check the linearity of the amplification (Fig. S2).

Table S1. Menidia primers designed and validated for PCR and qPCR

Transcript name	Name of the Primer	Primer sequence
Menidia berulina- insulin-like growth factor i	MB-Igf1-*Fwd*	CGATGTGCTGTATCTCCT
	MB-Igf1-*Rev*	CTCTCTCTCCACAGACAAA
Menidia - STAR	MB-StAR- *Fwd*	GCCAGGACACGATGATTA
	MB-StAR- *Rev*	CTATACAGGTAGGCCCATTC
Menidia - GhR	MB-GhR- *Fwd*	AGCCAGTAGAGACCAAAC
	MB-GhR- *Rev*	GTTGAGGAGCAGACTATGA
Menidia – Brain Aromatase	MB-cyp19b- *Fwd*	GCAGGATGTGATGGAGAA
	MB-cyp19b- *Rev*	CACTGCCTGACGTTATCT
Menidia – anti-mullerian hormone	MB-AMH- *Fwd*	TCCTGATTGGTGGAGAAC
	MB-AMH-*Rev*	CTCAGCTCACACAGGAAC
Menidia- dmrt1	MB-dmrt1-*Fwd*	GACTGTCAATGCCCAAAG
	MB-dmrt1-*Rev*	GCCACAGGACTACAAATC

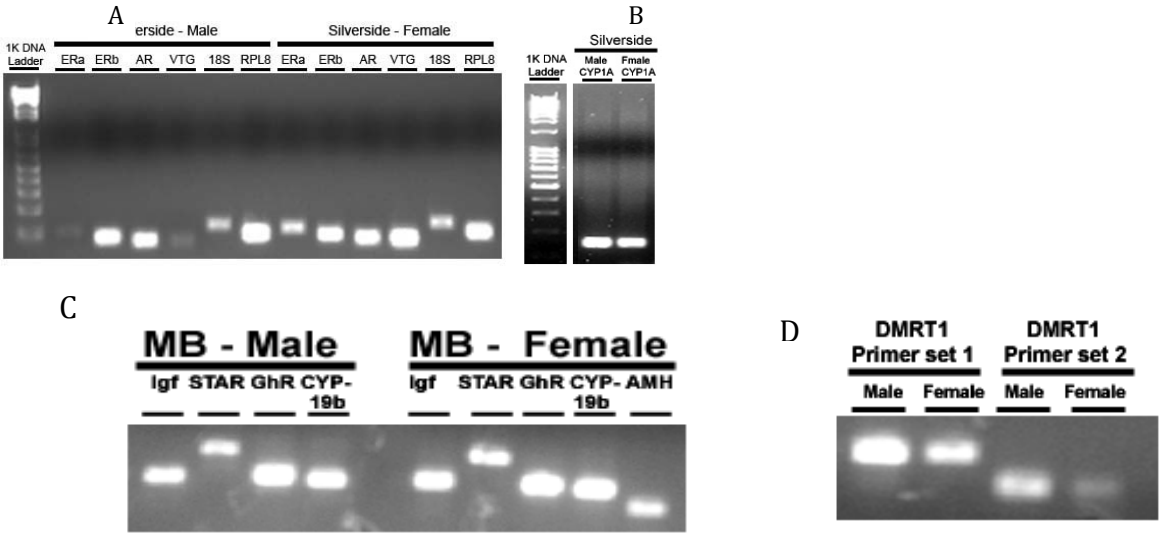
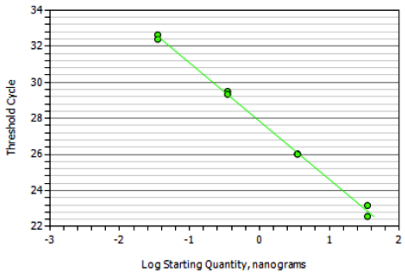


Figure S1: PCR verification of primers for (A) Vtg, ERa, ERb, AR, 18S rRNA and rpl8; (B) CYP1A; (C) Igf, StAR, GhR, Cyp19b, amh and (D) DMRT1 in adult male and female Menidia. Total RNA was extracted from adult Menidia liver tissues and amplified with primers specific for the amplified sequences. Abbreviations: Vtg, Vitellogenin; ERa, estrogen receptor

alpha; ERb, estrogen receptor beta; AR, androgen receptor; 18S rRNA, 18S ribosomal RNA, rpl8, ribosomal protein L8; CYP1A, cytochrome P450 A1; IgF, insulin like growth factor, StAR, steroidogenic acute regulatory protein; GhR, growth hormone receptor; CYP19b, brain aromatase; amh, anti-mullerian hormone; DMRT1, doublesex and mab-3 related transcription factor 1.

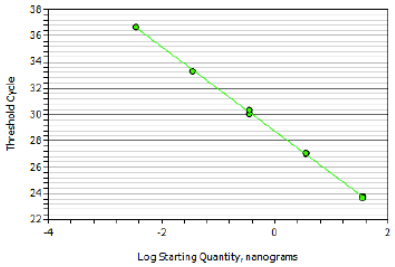
B) Amplification efficiency for each of the primers. Dilution curves were prepared for each of the primers to verify the amplification efficiency. All primer pairs were between 95 and 105 % efficient.

ER alpha



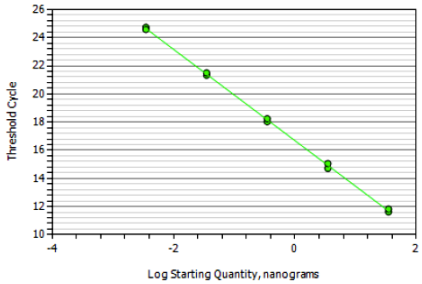
Fluor	PCR Efficiency(%)	R Squared	Slope	y-Intercept
FAM	103.9	0.997	-3.233	27.845

ER beta



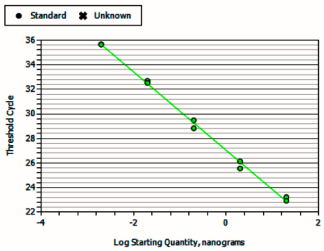
Fluor	PCR Efficiency(%)	R Squared	Slope	y-Intercept
FAM	104.8	1.000	-3.211	28.739

VTG1



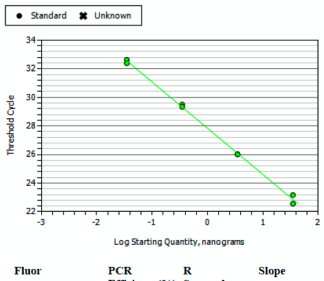
Fluor	PCR Efficiency(%)	R Squared	Slope	y-Intercept
FAM	103.5	0.999	-3.240	16.685

Chg-L



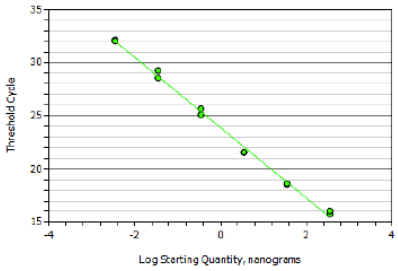
Fluor	PCR Efficiency(%)	R Squared	Slope	y-Intercept
FAM	105.7	0.997	-3.192	27.040

AR



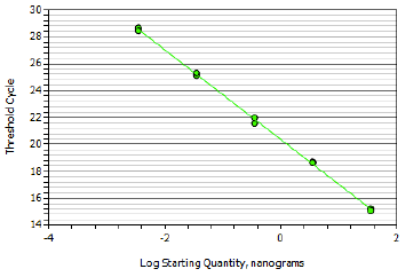
Fluor	PCR Efficiency(%)	R Squared	Slope	y-Intercept
FAM	104.9	0.993	-3.223	28.232

GAPDH



Fluor	PCR Efficiency(%)	R Squared	Slope	y-Intercept
FAM	100.6	0.996	-3.307	23.889

RLP8

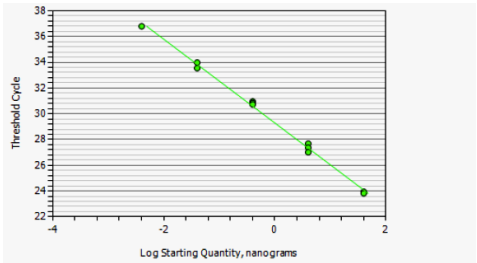


Fluor	PCR Efficiency(%)	R Squared	Slope	y-Intercept
FAM	99.5	0.999	-3.333	20.359

Figure S2. Q-PCR assays validation for ERα, ERβ, Chg, AR, Vtg1 and two housekeeping genes, RLP8 and GAPDH for juvenile *Menidia* and of GhR, Cyp19b, IgF1, StAR, amh, & DMRT1 for larval *Menidia*. Efficiency of the reaction should be between 95% and 105% to be useable for measuring changes in gene expression.

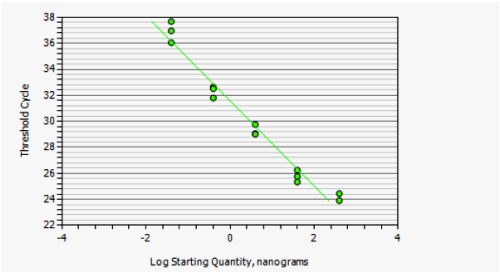
Validation of primers for q-PCR for ELS Menidia.

GhR



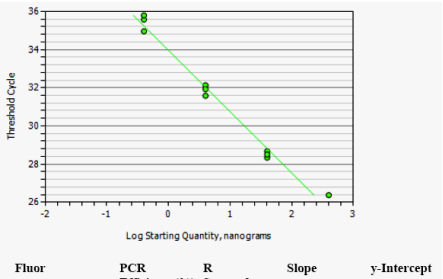
Fluor	PCR Efficiency(%)	R Squared	Slope	y-Intercept
FAM	103.1	0.996	-3.250	29.290

Cyp 19b



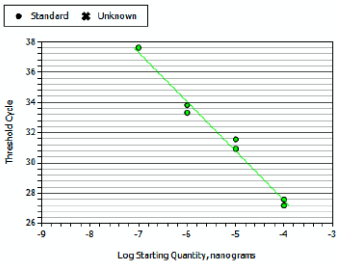
Fluor	PCR Efficiency(%)	R Squared	Slope	y-Intercept
FAM	102.1	0.968	-3.273	31.584

IgF1



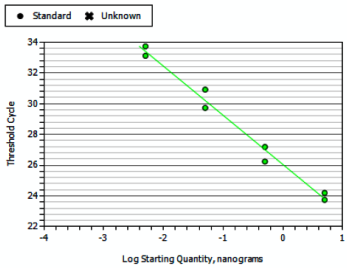
Fluor	PCR Efficiency(%)	R Squared	Slope	y-Intercept
FAM	104.9	0.984	-3.211	33.961

StAR



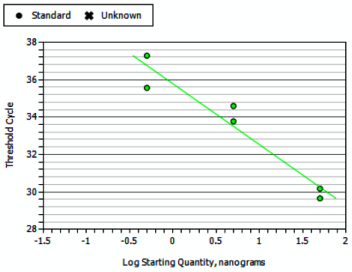
Fluor	PCR Efficiency(%)	R Squared	Slope	y-Intercept
FAM	102.8	0.983	-3.258	14.491

Amh



Fluor	PCR Efficiency(%)	R Squared	Slope	y-Intercept
FAM	105.4	0.984	-3.199	26.046

DMRT1



Fluor	PCR Efficiency(%)	R Squared	Slope	y-Intercept
FAM	103.1	0.927	-3.249	35.781

Special Study Proposal: Characterization of Pharmaceutical Contamination in Ambient Bay Water, Margin Sediment, and Wastewater

Summary: Pharmaceutical pollution is widely detected in the Bay, and earlier pilot studies indicate key pharmaceutical contaminants can approach levels of concern for wildlife. This study will monitor ambient Bay water and margin sediment for pharmaceutical pollution, providing data essential to a current evaluation of the potential risks of ~150 pharmaceutical contaminants via the RMP's Tiered CEC Risk and Management Framework. In addition, this study will monitor treated wastewater for pharmaceuticals, providing information useful for studying the loading rates and fate of pharmaceuticals discharged to the Bay.

Estimated Cost: $91,375

Oversight Group: ECWG

Proposed by: Rebecca Sutton (SFEI)

PROPOSED DELIVERABLES AND TIMELINE

Deliverable	***Due Date***
Task 1. Project Management (write and manage sub-contracts, track budgets)	Winter 2015 – Spring 2017
Task 2. Develop detailed sampling plan	Spring 2016
Task 3. Field Sampling	Summer 2016
Task 4. Lab analysis	Fall 2016
Task 5. QA/QC and data management	Winter 2016
Task 6. Final report	3/31/2017

Background

Pharmaceuticals are detected frequently in U.S. waterways, creating concern for their potential to impact wildlife as well as humans. Laboratory studies indicate fish exposed to antidepressant medications at environmentally relevant doses exhibit behavioral changes that affect survival and reproduction (e.g., Weinberger and Klaper 2014; Brodin et al. 2013). Antibiotic medications, designed specifically to kill organisms, may disrupt bacterial communities and essential functions (e.g., Näslund et al. 2008), impart broader antibiotic resistance (e.g., Rizzo et al. 2013), and are often toxic to algal species (e.g., Ferrari et al. 2004). Other pharmaceutical compounds have significant endocrine disrupting effects on aquatic species (e.g., Kolodziej et al. 2013). Pharmaceuticals typically enter the wastestream through excretion and flushing of unused medicines, suggesting the primary pathway for Bay contamination is via treated wastewater.

An increasing focus on proper pharmaceutical prescription, use, and disposal is occurring at federal, state, and local levels, and suggests the need to evaluate the level of concern associated with pharmaceutical pollution in the Bay. Current policy actions are largely motivated by concerns other than pollution (e.g., antibiotic resistance in infectious bacteria, drug abuse and accidental poisoning), meaning reduced Bay contamination may be an incidental result. Recent management actions include:

- Obama administration's National Action Plan for Combating Antibiotic-Resistant Bacteria, released March 2015, which lists activities such as "implementation of healthcare policies and antibiotic stewardship programs that improve patient outcomes, and efforts to minimize the development of resistance by ensuring that each patient receives the right antibiotic at the right time at the right dose for the right duration."
- Increased emphasis on drug takeback programs that prevent down-the-drain disposal:
    - Locally, the Alameda County ordinance requiring drug manufacturers fund stewardship and disposal costs has survived legal challenges to date;
    - San Francisco has just passed a similar stewardship program, and Marin may be next;
    - A 2014 bill to create a similar program statewide (SB 1014) passed the State Senate but died in the Assembly;
    - The federal DEA made significant changes to disposal rules to aid voluntary drug takeback programs.

Given this growing policy focus on pharmaceuticals, it would be appropriate at this time for the RMP to gather new data to evaluate the level of concern that should be associated with the presence of these contaminants in the Bay. Findings could suggest the need for targeted management actions, or could suggest existing activities are sufficient to protect wildlife from harm.

The RMP has assessed Bay pharmaceutical pollution in two previous special studies involving samples collected in 2006 (Harrold et al. 2009) and 2009-2010 (Klosterhaus et al. 2013a). The results of these monitoring efforts indicate that the following specific pharmaceutical compounds merit further monitoring:

Ciprofloxacin – Meets state guidance criteria for monitoring in sediment.[1] This widely prescribed antibiotic was detected in Bay sediment at concentrations up to 678 ng/g dry weight (Klosterhaus et al. 2013b). The highest measured concentration exceeds both a lowest observable effect concentration, or LOEC, for effects on bacterial community structure (100 ng/g dry weight) and a half maximal effective concentration, or EC_{50}, for pyrene degradation (400 ng/g dry weight; Näslund et al. 2008). Current levels of contamination may be a concern for both bacterial diversity and an essential ecosystem

[1] Recent state guidance regarding contaminants of emerging concern (CECs) in California's aquatic ecosystems outlines an objective means of prioritizing monitoring activities through calculation of monitoring trigger levels (MTLs) using available toxicity thresholds, appropriate safety factors, and measured or predicted environmental concentrations (Anderson et al. 2012; Dodder et al. 2015).

service these organisms may perform in Bay sediment.

Sulfamethoxazole – Intermittent detection above a toxicity threshold.[2] This antibiotic was detected in ambient Bay water at concentrations up to 1,060 ng/L (Klosterhaus et al. 2013b). A PNEC calculated using standard methods endorsed by the EMEA (2006), and using an assessment factor (AF) of 50 as directed by the European Chemicals Bureau (European Communities 2003), has been calculated as 118 ng/L by Grung et al. (2008). Intermittent detection above a PNEC is insufficient grounds to classify a contaminant as a moderate concern (Tier III) contaminant according to the RMP's Tiered CEC Risk and Management Framework, but suggests the need for further monitoring. Should exceedances prove to be more common than limited previous data suggest, reclassification as a moderate concern contaminant may be indicated.

Erythromycin – Intermittent detection above a toxicity threshold.[2] This antibiotic was detected in ambient Bay water at concentrations up to 41.6 ng/L (Klosterhaus et al. 2013b). The highest Bay measurement exceeds an algal PNEC of 22 ng/L (back-calculated from molar value provided by Gonzalez-Pleiter 2013). As for sulfamethoxazole, intermittent detection of erythromycin above a PNEC in previous pilot studies suggests the need for further monitoring to evaluate how frequently exceedances occur, and whether this contaminant merits classification as a moderate concern for the Bay.

Previous studies of pharmaceutical contamination in the Bay evaluated ~100 different contaminants; over 3,000 pharmaceuticals are currently registered for use in the U.S. (Howard and Muir 2011). Continuing method development provides the ability to target important pharmaceuticals classified by Howard and Muir (2011) as high priorities for environmental monitoring, such as:

- Bupropion hydrochloride (Wellbutrin XL; antidepressant; CAS 31677-93-7)
- Irbesartan (Avapro; blood pressure medication; CAS 138402-11-6)
- Trazadone (Oleptro; antidepressant; CAS 19794-93-5)

Analytical methods for these particular compounds are expected to be available in May 2015, as part of a new list of pharmaceutical targets offered by AXYS Analytical. Approximately 50 additional pharmaceuticals for which no Bay data exist can be measured using the full suite of AXYS pharmaceutical analyses.

Study Objectives and Applicable RMP Management Questions

This study will provide data essential to determining the level of concern associated with pharmaceutical pollution in the Bay. Currently available data suggest the need for further monitoring of three antibiotics: ciprofloxacin, sulfamethoxazole, and erithromycin. Should

[2] According to the RMP's Tiered CEC Risk and Management Framework, a Tier III or "moderate concern" chemical is typically one where there is "…frequent detection at concentrations greater than the PNEC or NOEC but less than EC_{10}, the effect concentration where 10% of the population exhibit a response, or another low level effects threshold…" Sutton et al. 2013).

new monitoring show levels of these pharmaceuticals frequently exceed toxicity thresholds, reclassification as moderate concern (Tier III) contaminants may be appropriate.

An expanding array of pharmaceutical targets available via AXYS Analytical also means the RMP can now collect data on new analytes that have been specifically identified by Howard and Muir (2011) as priority contaminants for environmental monitoring. In addition, up to 50 pharmaceutical analytes for which no Bay data are yet available can be assessed via the full suite of AXYS analyses.

Comparison of contaminant levels in the pathway of WWTP effluent with Bay water and sediment levels can provide preliminary information as to pharmaceutical loadings and fate in the Bay. These comparisons may suggest that specific compounds are especially persistent in the environment and may require special attention, perhaps in the form of additional, targeted management actions.

Management questions to be addressed by monitoring pharmaceuticals in WWTP effluent and Bay water and sediment are the same as those of the overall RMP program, as shown in Table 1.

Table 1: Study objectives and questions relevant to RMP management questions

Management Question	Study Objective	Example Information Application
1) Are chemical concentrations in the Estuary at levels of potential concern and are associated impacts likely?	Monitor over 150 pharmaceuticals in Bay water and sediment. Compare measured concentrations to toxicity thresholds to determine levels of concern associated with each according to the Tiered CEC Risk Framework.	Do target pharmaceuticals have the potential to cause impacts to Bay wildlife? Do data indicate a need for management actions?
2) What are the concentrations and masses of contaminants in the Estuary and its segments? 2.1 Are there particular regions of concern?	Compare levels measured in different embayments.	Are expectations of higher levels of contamination in the Lower South Bay substantiated?
3) What are the sources, pathways, loadings, and processes leading to contaminant-related impacts in the Estuary? 3.1. Which sources, pathways, etc. contribute most to impacts?	Obtain information on pharmaceutical contamination in treated wastewater and ambient Bay water and margin sediment.	Are relative distributions of pharmaceutical contaminants in effluents versus Bay water and sediment consistent with our expectations for various contaminant processes?
4) Have the concentrations, masses, and associated impacts of contaminants in the Estuary increased or decreased? 4.1. What are the effects of management actions on concentrations and mass?	Review new results alongside available data from previous RMP studies for indications of trends in pharmaceutical contamination over time.	Are pharmaceuticals for which we have previous measurements found at increasing or decreasing levels in Bay media?
5) What are the projected concentrations, masses, and associated impacts of contaminants in the Estuary?	Review measured results alongside available projections of population growth and age as well as anticipated changes to pharmaceutical prescribing and other relevant actions.	Which anticipated changes or actions are likely to have the greatest impact on pharmaceutical pollution? Are additional/different actions needed?

This monitoring effort would most directly address questions 1, 2, and 3, characterizing pharmaceutical contamination and its potential for impacts at the current time. Inferences regarding past or future levels of contamination would involve digestion of the data within the context of changes to the Bay Area population (size and age distribution), patterns in prescribed medications, and wastewater treatment technologies, all of which may play a role in addressing questions 4 and 5. This additional research is not part of this proposal but could be completed as a second phase of this study.

In addition, the study will address the emerging contaminants priority question: What emerging contaminants have the potential to adversely impact beneficial uses of the Bay?

Approach

Effluent Sampling

Effluent samples provide essential information on the major pathway for pharmaceutical contaminants to enter the Bay. The state guidance on CECs directs agencies to include sampling WWTP effluent when screening for emerging contaminants (Dodder et al. 2015).

24-hour composite samples of WWTP effluent (up to 4 L HDPE) voluntarily provided by two to four high volume Bay Area dischargers will be characterized. Participants will include a WWTP employing secondary treatment, as well as one using more advanced measures. Sampling will occur in the summer of 2016, when inflow and infiltration are insignificant. A total of up to five samples will be analyzed, up to four effluent samples and a blank designed to capture airborne pharmaceuticals with the potential to contaminate samples.

One discharger has agreed to participate and contribute in-kind services to collect samples but is not specifically named here, as dischargers will have the option to keep their identities confidential in subsequent reporting of the data. Measurements for each discharger will be reported individually.

Ambient Bay Water Sampling

Bay water sample collection will take place in Central, South, and Lower South Bays in the summer of 2016. Previous study of Lower South Bay has revealed elevated levels of some pharmaceuticals (Harrold et al. 2009), a finding consistent with the greater influence of treated wastewater and reduced levels of dilution, particularly in the dry season.

Grab samples of ambient Bay water (up to 4 L HDPE) will be collected at up to nine Bay sites. A field duplicate will also be collected at one site; a blank collected at a wastewater facility will be used to assess the likelihood of contamination with airborne pharmaceuticals (e.g., asthma medications). To collect samples, SFEI staff will collaborate with existing sampling cruises conducted by other agencies; initial exploration of these opportunities is already underway. As such, equipment and rental costs are likely to be low.

Bay Margin Sediment Sampling

Sediment sample collection will occur in margin locations near treated wastewater discharges associated with participating WWTPs. Samples (up to 4 L HDPE) will be conducted at up to four margin sites in the summer of 2016. A field duplicate will also be collected, for a total of five samples.

Analytical Methods

Samples will be analyzed by AXYS Analytical (Sidney, BC, Canada) for pharmaceuticals in Lists 1-7 (Lists 1-6, AXYS Method MLA-075, currently available; List 7, AXYS Method MLA-104, to be released May 2015) using liquid chromatography tandem mass spectrometry (LC-MS/MS). AXYS Analytical was selected to provide analytical services for this study because they have unique qualifications for analyzing pharmaceuticals in environmental media. They test for more different pharmaceutical compounds than any other commercial laboratory in North America. Target analytes for List 7 in particular were selected following consultation with health and environmental agencies regarding pharmaceutical compounds of greatest potential concern for ecological health.

Analytes targeted via Lists 1-6 are provided in Table 2, along with initial information as to extraction and LC-MS/MS mode needed for each. Potential analytes for List 7 are provided in Table 3. This method is expected to be available in May 2015.

Previous studies in the Bay have utilized Lists 1, 3, 4, and 5 only.

Table 2. Pharmaceutical analytes in Lists 1-6 (AXYS Analytical). Superscripts indicate analytes for which only estimates of concentration are available.

List 1 - Acid Extraction in Positive Ionization	***List 4 - Basic Extraction in Positive Ionization***
Acetaminophen	Albuterol
Azithromycin	Amphetamine
Caffeine	Atenolol
Carbadox	Atorvastatin
Carbamazapine	Cimetidine
Cefotaxime	Clonidine
Ciprofloxacin	Codeine
Clarithromycin	Cotinine
Clinafloxacin	Enalapril
Cloxacillin [1]	Hydrocodone
Dehydronifedipine	Metformin
Digoxigenin	Oxycodone
Digoxin	Ranitidine
Diltiazem	Triamterene
	List 5 - Acid Extraction in Positive Ionization
1,7-Dimethylxanthine	
Diphenhydramine	Alprazolam
Enrofloxacin	Amitriptyline
Erythromycin-H20	Amlodipine
Flumequine	Benzoylecgonine
Fluoxetine	Benztropine
Lincomycin	Betamethasone
Lomefloxacin	Cocaine
Miconazole	DEET
Norfloxacin	Desmethyldiltiazem
Norgestimate	Diazepam
Ofloxacin	Fluocinonide
Ormetoprim	Fluticasone propionate
Oxacillin [1]	Hydrocortisone
Oxolinic acid	10-hydroxy-amitriptyline
Penicillin G [1]	Meprobamate
Penicillin V	Methylprednisolone
Roxithromycin	Metoprolol
Sarafloxacin	Norfluoxetine
Sulfachloropyridazine	Norverapamil
Sulfadiazine	Paroxetine
Sulfadimethoxine	Prednisolone
Sulfamerazine	Prednisone
Sulfamethazine	Promethazine

Sulfamethizole
Sulfamethoxazole
Sulfanilamide
Sulfathiazole
Thiabendazole
Trimethoprim
Tylosin
Virginiamycin

List 2 - Tetracyclines in Positive Ionization

Anhydrochlortetracycline
Anhydrotetracycline
Chlortetracycline
Demeclocycline
Doxycycline
4-Epianhydrochlortetracycline
4-Epianhydrotetracycline
4-Epichlortetracycline
4-Epioxytetracycline
4-Epitetracycline
Isochlortetracycline [2]
Minocycline
Oxytetracycline
Tetracycline

List 3 - Acid Extraction in Negative Ionization

Bisphenol A
Furosemide
Gemfibrozil
Glipizide
Glyburide
Hydroclorothiazide
2-hydroxy-ibuprofen
Ibuprofen
Naproxen
Triclocarban
Triclosan
Warfarin
Propoxyphene
Propranolol
Sertraline
Simvastatin
Theophylline
Trenbolone
Trenbolone acetate
Valsartan
Verapamil

List 6 - Acid Extraction in Positive Ionization

Amsacrine
Azathioprine
Busulfan
Citalopram
Clotrimazole
Colchicine
Cyclophosphamide
Daunorubicin
Diatrizoic acid
Doxorubicin
Drospirenone
Etoposide
Iopamidol
Medroxyprogesterone acetate
Melphalan
Metronidazole
Moxifloxacin [3]
Oxazepam
Rosuvastatin
Tamoxifen
Teniposide
Venlafaxine
Zidovudine

Table 3. Possible pharmaceutical analytes in List 7 (AXYS Analytical), expected May 2015.

Bupropion hydrochloride (31677-93-7)
Cefazolin sodium (27164-46-1)
Cefprozil (92665-29-7)
Clopidogrel - clopidogrel carboxylic acid
Clopidogrel, Clopidogrel bisulfate (113665-84-2; 120202-66-6)
Eprosartan (13304-01-4)
Fenofibrate (49562-28-9)
Fenofibrate metabolite: Fenofibric acid
Gabapentin (60142-96-3)
Irbesartan (138402-11-6)
Lamotrigine (84057-84-1)
Lamotrigine metabolite: Lamotrigine 2-N-glucuronide
Mycophenolate Mofetil (128794-94-5)
Mycophenolate Mofetil metabolite: Mycophenolic acid
Pravastatin sodium (81131-70-6)
Quetiapine, Quetiapine fumurate (111974-69-7; 111974-72-2)
Quetiapine metabolite: Norquetiapine
Ramipril (87333-19-5)
Ramipril metabolite: ramiprilate
Telmisartan (144701-48-4)
Topiramate (97240-79-4)
Trazadone (19794-93-5)
Trazadone metabolite: m-chlorophenylpiperazine
Decoquinate (CAS# 18507-89-6)
Hygromycin B (CAS# 31282-04-9)
Nicarbazin (CAS# 330-95-0)
Melengestrol Acetate (CAS 2919-66-6)
Iopromide (CAS# 73334-07-3)
Tilmicosin

Budget

The following budget represents estimated costs for this proposed special study (Table 4). Efforts and costs can be scaled up or down by changing the types of analyses (e.g., Lists 1-7) and the number and type of samples.

Table 4. Pharmaceuticals Characterization: Proposed Budget.

Expense	Estimated Hours	Estimated Cost ($)
Labor		
Project Staff	220	30000
Senior Management Review	16	3200
Project Management	0*	
Contract Management	0*	
Data Technical Services		13000
GIS Services	12	975
Creative Services	20	1600
IT Services	0	
Communications	0	
Operations	0	
Subtotal		
Subcontracts		
Name of contractor		
AXYS		42000
Direct Costs		
Equipment		0
Travel		200
Printing		0
Shipping		400
Other		0
Grand Total		
		91375

*services included in the base RMP funding

Budget Justification

Field Costs

Field costs will be low as a result of strategic study design, as well as the collaborative nature of the Bay science and management community. Wastewater agencies that choose to participate in the study will receive sample collection kits with instructions to allow them to provide crucial in-kind services to collect and ship samples themselves, minimizing SFEI staff time needed for sample collection. We expect to find ready accommodation on pre-existing water sampling cruises conducted by other agencies, limiting the cost of ambient water sample collection to staff labor hours spent on the Bay. Sediment samples will be collected from readily accessible margin sites near WWTP discharges, and will not require additional funds apart from staff time and shipping.

Laboratory Costs

Analytical costs per sample for pharmaceuticals (Lists 1-7) are expected to be $2,300 per water or wastewater sample and $2,400 per sediment sample. For 13 water samples and 5 sediment samples (including duplicates and blanks), the analytical costs are expected to be $42,000.

Data Management Costs

Standard data management procedures and costs will be used for this project.

Reporting

Bay water and sediment data will be reported via RMP web tools (e.g., CEDEN). Results will be reported to the RMP committees in the form of a draft manuscript for publication in a peer-reviewed journal by 3/31/17.[3]

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Special Study Proposal: Non-targeted Analysis of Water-soluble Compounds in Ambient Bay Water and Wastewater to Identify Emerging Contaminants

Summary: Non-targeted analysis, a key element of the RMP's CEC strategy and recent state CEC guidance, can help to provide a measure of assurance that the RMP is not missing unexpected yet potentially harmful contaminants simply because of failures to predict their occurrence based on use or exposure prioritization criteria. The RMP has completed non-targeted analysis of fat-soluble compounds in bivalve tissue and seal blubber, but another major class of chemicals, water-soluble (polar) organic contaminants, has not been evaluated. This proposed study will fill this data gap by conducting a broad screen of ambient Bay water (passive and grab samples) and wastewater (composite samples) for polar organic compounds such as: detergents and other surfactants, pesticide and pharmaceutical breakdown products, and plastic additives. This type of non-targeted study will lay the foundation for future targeted CEC monitoring by helping to identify new potential contaminants of concern without *a priori* knowledge of their occurrence.

Estimated Cost: $52,000

Oversight Group: ECWG

Proposed by: Rebecca Sutton (SFEI), Lee Ferguson (Duke University)

PROPOSED DELIVERABLES AND TIMELINE

Deliverable	***Due Date***
Task 1. Project Management (write and manage sub-contracts, track budgets)	Winter 2015 – Spring 2017
Task 2. Develop detailed sampling plan	Spring 2016
Task 3. Field Sampling	Summer 2016
Task 4. Lab analysis	Fall 2016
Task 5. QA/QC and contaminant risk review	Winter 2016
Task 6. Draft report and fact sheet	3/31/2017
Task 7. Final report and fact sheet	6/30/2017

Background

The RMP has developed a pro-active emerging contaminants program, and conducts policy-relevant monitoring via Special Studies to help identify and address problematic, unregulated contaminants before they cause significant harm to the Bay. The RMP has established a unified emerging contaminants strategy (Sutton et al. 2013) with three elements: 1) targeted chemical monitoring and relative risk evaluation using a tiered risk and management action framework; 2) review of the scientific literature and other aquatic monitoring programs as a means of identifying new emerging contaminants for which no Bay occurrence data yet exist; and 3) non-targeted analysis to create inventories of unanticipated contaminants in tissues, sediment, or water that can be used to direct targeted chemical monitoring or toxicity identification evaluations.

Recently completed state guidance on emerging contaminants in aquatic ecosystems echoes many aspects of the RMP strategy (Dodder et al. 2015). In particular, non-targeted analysis plays a key role in the comprehensive CEC management framework (see pg 40 Dodder et al. 2015). Non-targeted analysis is an essential means of assuring focus on the contaminants with greatest potential to impact an ecosystem, by seeking to remove a "knowledge bias" on previously identified problem chemicals. One form of non-targeted analysis specifically recommended by the state guidance document is development of bioanalytical tools; the RMP has commissioned one such study from scientists at the Southern California Coastal Water Resources Project (SCCWRP) and the University of Florida, which is nearing completion.

Other non-targeted methods highlighted by the state guidance are those "designed to screen for new or unexpected contaminants; i.e., unknown CECs" (pg 29, Dodder et al. 2015). The RMP, in collaboration with the National Institute of Standards and Technology (NIST), recently completed a non-targeted analysis of Bay harbor seal blubber and mussel tissues, which focused on persistent, fat-soluble (nonpolar), chlorine and bromine-rich chemicals (Sutton and Kucklick 2015). This investigation brought to light five contaminants not previously identified in Bay wildlife, and for which toxicity is largely unknown. However, most of the Bay chemical contamination was from high priority contaminants that the RMP already monitors, or closely related compounds. More polar, water-soluble organic compounds were not covered by this recent non-targeted tissue analysis. Polar organic contaminants are of significant concern to the water quality of the San Francisco Bay, as they may exhibit meso-range transport, be difficult to remove through treatment strategies, and cause effects on wildlife through endocrine disruption and other mechanisms. The following monitoring proposal would fill this important data gap. Detergents, plastics, and medications are examples of products that can contain such water-soluble, polar organic contaminants.

Study Objectives and Applicable RMP Management Questions

Given the increased burden on the RMP from multiple areas of interest to stakeholders, it is imperative that the RMP focus on those CECs that are the highest priority. Traditional, targeted contaminant monitoring focuses on specific lists of chemicals already identified as potentially problematic through either expert judgement, anticipation of high toxicity, use-

based prioritization, or other *a priori* methods. Through non-targeted monitoring, we can provide a measure of assurance that the RMP is not missing unexpected, potentially harmful contaminants in the Bay water simply because of failures to predict their occurrence based on use or exposure prioritization criteria.

Non-targeted analysis is an essential element of the RMP's CEC Strategy (Sutton et al. 2013). The RMP recently completed a non-targeted analysis focusing on fat-soluble (hydrophobic) compounds in tissue samples (Sutton and Kucklick 2015). This study identified a few unexpected contaminants, but the good news is that the majority of chemical contamination was from high priority contaminants that the RMP already monitors, or closely related compounds.

The current proposal is to use non-targeted analysis to scan for more water-soluble (polar) organic contaminants in the Bay (grab and passive samples) as well as in treated wastewater effluent, which is anticipated to be a major and important source of these compounds to the Bay. A special study on water-soluble contaminants would provide data on those contaminants that were not part of the study of fat-soluble compounds, essentially filling a major data gap in characterizing possible contaminant chemistries in the Bay. This would make the Bay the first ecosystem to be studied via non-targeted methods for both water- and fat-soluble contaminants.

Using the proposed non-targeted analytical strategies outlined below, Dr. Lee Ferguson at Duke University has tentatively identified 52 water-soluble compounds from seven functional classes including pharmaceuticals, flame retardants, pesticides, and consumer product chemicals in wastewater effluent discharged to surface waters in central North Carolina (Ferguson et al., in prep). Nine of these compounds have not been detected in the environment previously. Examples include ZPCA (a transformation product of the sleep-aide zolpidem [Ambien]), raltegravir (HIV treatment), and Atorvastatin lactone (transformation product of atorvastatin [Lipitor]).

Should a non-targeted study of the Bay identify unexpected water-soluble contaminants such as these, the information could indicate a need for a follow-up RMP Special Study designed to specifically assess the new "candidate" CECs on a quantitative basis. It could also point to ecotoxicity data gaps or suggest new management priorities. Thus, we anticipate that positive identifications resulting from the proposed study would be potentially very high in impact.

In contrast, because of the comprehensive nature of the non-targeted methods proposed herein, should few unexpected contaminants be identified, the RMP would then have considerable evidence that existing polar organic CEC monitoring is indeed already focusing on the highest priority contaminants for the Bay.

Table 1: Study objectives and questions relevant to RMP management questions

Management Question	Study Objective	Example Information Application
1) Are chemical concentrations in the Estuary at levels of potential concern and are associated impacts likely?	Identify water-soluble contaminants not yet characterized by targeted monitoring efforts. Evaluate future monitoring needs and toxicity data gaps.	Have previous targeted monitoring efforts focused on contaminants with the highest relative risk to the Bay? Which newly identified contaminants merit further monitoring?
2) What are the concentrations and masses of contaminants in the Estuary and its segments? 2.1 Are there particular regions of concern?	Initial comparison of specific embayments with respect to detection.	Are there regional differences in presence of newly identified contaminants?
3) What are the sources, pathways, loadings, and processes leading to contaminant-related impacts in the Estuary? 3.1. Which sources, pathways, etc. contribute most to impacts?	Gain an unbiased inventory of water-soluble (polar) organic contaminants in key, high-volume wastewater discharges. Allow an initial exploration of differences between secondary and advanced wastewater treatment with respect to contaminant removal.	Are any newly identified contaminants in wastewater also detected in the Bay? Do differences in detection for wastewater and ambient Bay water suggest persistence, degradation, or additional pathways (e.g., stormwater) for specific contaminants?
4) Have the concentrations, masses, and associated impacts of contaminants in the Estuary increased or decreased? 4.1. What are the effects of management actions on concentrations and mass?	Establish a baseline for future studies.	
5) What are the projected concentrations, masses, and associated impacts of contaminants in the Estuary?	Identify sources of newly identified contaminants to evaluate effects of current management actions on potential discharges and project trends with likely changes in use and wastewater treatment technology.	Are relevant management actions having the intended effect? Will newly identified contaminants suggest the need for additional or different management actions?

This monitoring effort would most directly address questions 1, 2, and 3, identifying water-soluble contaminants not yet characterized by targeted monitoring efforts, and providing information useful to initial comparisons with respect to contaminants in different embayments and discharged from secondary versus more advanced water treatment facilities. This proposal does not include an examination of potential sources of newly identified contaminants. Such a study could be completed in future years and would provide information useful in addressing questions 4 and 5, concerning likely past and future trends.

In addition, the study will directly and explicitly address the emerging contaminants priority question: What emerging contaminants have the potential to adversely impact beneficial uses of the Bay?

Approach

Ambient Bay Water Sampling

Bay water sampling will be conducted using both grab samples and passive sampling devices called Polar Organic Chemical Integrative Sampler (POCIS, see Figure 1; Environmental Sampling Technologies, St. Joseph, MO). Grab samples have the advantage of providing analytical data for polar organic contaminants that is less convoluted by sampling bias and more representative of actual water conditions, but also has the disadvantage of providing only a snapshot of the pollutants in a particular location at a particular time, rather than more broadly integrated information. Passive samplers, while semi-quantitative at best, can be used to provide an integrated assessment of the pollutants present (or absent) in a location over a longer time span (e.g., 28 days). The lengthy time of deployment also means contaminants at trace levels are more likely to be detected, provided they have favorable uptake dynamics into the sampler.

Three POCIS canisters will be deployed, one each in the Lower South Bay, Central Bay, and North Bay (Figure 2). Site selection and deployment will be conducted in collaboration with nutrients researchers at SFEI and elsewhere, as they have deployed and are monitoring and servicing a number of moored nutrient sensors throughout the Bay. Deployment will occur in the summer of 2016, when WWTP-derived contaminant levels are often highest due to low river inflow and POTW-system infiltration/inflow. Each POCIS holder will be deployed for a maximum of 28 days. The POCIS samplers contain a solid phase sorbent (Waters Oasis HLB) that is widely used for sampling a large range of water-soluble organic chemicals from water.

Each POCIS canister will contain three POCIS samplers to provide triplicate measurements at each location; however, only two of the three will be analyzed using RMP funds. The third POCIS from each site will be kept in reserve and would be analyzed at no additional cost to the RMP if unusual variability is observed in the first two POCIS. A total of seven POCIS samples will be analyzed using RMP funds, two from each of three sites and a single blank.

Grab samples (4 L glass) will be collected in the same locations on deployment and retrieval of the POCIS, to provide a snapshot, non-integrated picture of polar organic contaminant loadings in water at each location. A total of eight grab samples will be analyzed, two from

each of three sites, along with a field duplicate and a blank. Each grab sample will be shipped (on ice) to Dr. Ferguson's laboratory at Duke University (NC) after collection for immediate extraction and analysis as described below.

Figure 1. Deployment holder featuring one POCIS holder containing three POCIS. Dimensions 15 cm high x 16 cm wide. Environmental Sampling Technologies, est-lab.com

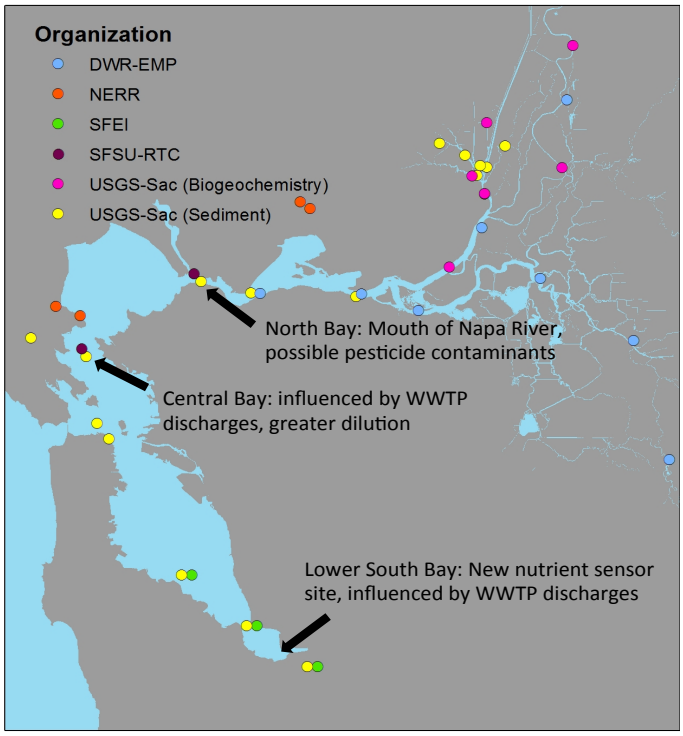

Figure 2. Suggested sites for grab and passive sampling of ambient Bay water. Suggested sites are marked with arrows. Other potential locations featuring moored nutrient sensors are marked with circles, the color of which signifies which agency is responsible for the sensor.

Effluent Sampling

Effluent samples provide essential information on a major pathway for polar organic contaminants to enter the Bay. The state guidance on CECs directs agencies to include sampling wastewater treatment plant (WWTP) effluent when screening for emerging contaminants (Dodder et al. 2015). Compounds that persist in treated effluent at significant levels are likely to be polar and water-soluble rather than fat-soluble, making the focus of this proposed study particularly useful to the wastewater community.

24-hour composite samples of WWTP effluent (4 L glass) voluntarily provided by two to four high volume Bay Area dischargers will be characterized. Participants will include a WWTP employing secondary treatment, as well as one using more advanced measures. Sampling will occur in the summer of 2016, when inflow and infiltration are insignificant. A total of five samples will be analyzed, up to four effluent samples and a blank. As with water samples described above, these will be shipped (on ice) to Dr. Ferguson's laboratory at Duke University (NC) immediately after collection for extraction and analysis as described below.

One local discharger has agreed to participate and contribute in-kind services for sample collection but is not specifically named here, as dischargers will have the option to keep their identities confidential in subsequent reporting of the data. Measurements for each discharger will be reported individually.

Analytical Methods

Non-targeted analysis of 20 samples will be conducted by Dr. Ferguson's Lab (Duke University) using cutting-edge Orbitrap liquid chromatography high resolution mass spectrometry (LC-HRMS). POCIS samples (shipped directly from SFEI to Duke University) will be processed as recommended by the vendor (e.g., elution with methanol/MTBE prior to evaporation and reconstitution in HPLC-MS mobile phase). Water samples will be immediately filtered (< 0.45μm GF/F) for particle removal and processed for solid-phase extraction using an automated SPE system (Dionex Autotrace 280) fitted with custom layered-bed extraction cartridges (containing cation exchange, anion exchange, hydrophobic, and amphiphilic resins) and eluted with sequential basic and acidic methanol/MTBE solvent systems prior to combination and concentration of the extracts.

Extracts will be separated using UHPLC (Thermo Hypersil Gold column, 1.9 μm particle size, 2.1 x 100 cm) over a 70 minute gradient prior to introduction into the mass spectrometer. The LTQ-Orbitrap MS/MS will be operated at 100,000 resolution to achieve < 2 ppm mass accuracy across the mass range of interest. Sample extracts will be spiked with internal mass calibration/quantitation standards (chosen from a set of stable-isotope labeled compounds available in the PI's laboratory) immediately prior to injection. Ionization will be performed by either electrospray in either positive or negative polarity mode, depending on the analyte. High resolution detection of analytes in MS mode will be performed by the Orbitrap analyzer, while simultaneous data-dependent MS/MS will be performed in the LTQ Velos module before the Orbitrap. Ions for MS/MS analysis (10 per Orbitrap scan) will be dynamically chosen on a per-scan basis, with priority given to accurate mass values corresponding to compounds in compiled "suspect" lists (already compiled based on

production volume, toxicity, and/or literature reports), with secondary priority given to "non-target" analytes in order of decreasing intensity. These MS/MS data will provide important information to aid in identification of non-target analytes.

Data generated through these approaches will be applied to both commercially-available (ThermoFisher Scientific TraceFinder, Compound Discoverer, and MassFrontier) and custom-written processing software designed to aid in identifying polar organic compounds based on HRMS/MS data. Final validation of tentative identities will be made based on authentic standard match wherever possible.

The Ferguson laboratory has extensive experience in use of accurate mass MS and MS/MS for identifying non-target compounds in complex mixtures (Benotti et al. 2003; Eichhorn et al. 2005; Cui et al. 2009; Stapleton et al. 2011), and this strategy has proved successful for identifying emerging contaminants in wastewater (preliminary work as described above), as well as in coastal surface waters impacted by water reuse activities (e.g., on Kiawah Island, SC). These new identifications include several micropollutants that have not, to our knowledge, been previously reported to occur in environmental media such as wastewater or surface water. Dr. Ferguson's laboratory was chosen for this work because it is uniquely qualified and experienced to undertake the experiments described. The Ferguson Lab has also agreed to contribute up to $10,000 of in-kind services to the project (e.g., technician and PI effort) because of the high priority and potential for high-impact results to be generated from the work.

Budget

The following budget represents estimated costs for this proposal. Efforts and costs can be adjusted by changing the number of matrices explored or the number of samples evaluated.

Table 2. Budget summary.

Expense	Estimated Hours	Estimated Cost ($)
Labor		
Project Staff	135	19000
Senior Management Review	21	4200
Project Management	0*	
Contract Management	0*	
Data Technical Services	0	
GIS Services	8	650
Creative Services	25	2000
IT Services	0	0
Communications	0	0
Operations	0	0
Subtotal		
Subcontracts		
Name of contractor		
Lee Ferguson		20000
Linda W.		3000
Direct Costs		
Equipment		2000
Travel		400
Printing		250
Shipping		500
Other		
		52000

*Not needed because core RMP funding provides this service.

Budget Justification

Field Costs

Details concerning passive sampling equipment:

POCIS: $65/each x 3/site x 3 sites + 1 blank = $260
POCIS holder (rental): $220 x 3 sites = $660
Total POCIS equipment costs ~$1,000

Reporting Costs

Preparation of a draft manuscript for publication in a peer-reviewed journal would be the responsibility of the analytical partner, and will require relatively little RMP staff time. RMP staff will produce a 2-page fact sheet to describe the results and their implications for RMP stakeholders and the general public. This fact sheet would be a companion to one recently completed for non-targeted analysis of fat-soluble compounds (Sutton and Kucklick 2015).

Laboratory Costs

The RMP can benefit from a significant discount in laboratory costs currently available due to outside funding of the Ferguson Lab. This discount will *not* be available in the future. For non-targeted analyses conducted in 2016, the estimated cost is $1,000/sample; in the future, the cost will be at least $1,500/sample.

Data Management Costs

No data management is needed for this proposed project, as it is not targeted, analyte-specific analysis.

Reporting

Deliverables will include: a) a draft manuscript[1] that serves as an RMP technical report due by 3/31/2017; b) a plain language RMP fact sheet describing the results and their implications due by 3/31/2017; and c) additions to other RMP publications such as the Pulse.

[1] The draft manuscript will be distributed by email, not published on the website, so as to not jeopardize publication of the manuscript in a peer-reviewed journal.

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Special Study Proposal: Monitoring Microplastics in the Margin

Summary: Building upon the RMP Special Study for 2015 to characterize microplastics in Bay Area effluent and ambient Bay sediment and water, this study seeks to augment the planned 2015 Bay Margins Sediment Study by including microplastics monitoring in the study design. Microplastics are well known to accumulate in sediments from densely urban areas. This study will provide a characterization of microplastics in surface sediments in the shallow Central Bay margin areas, thereby addressing an important data gap.

Estimated Cost: $14,325

Oversight Group: ECWG

Proposed by: Rebecca Sutton (SFEI)

PROPOSED DELIVERABLES AND TIMELINE

Deliverable	***Due Date***
Task 1. Project Management (write and manage sub-contract, track budgets)	May-December 2015
Task 2. Select sites and conduct field sampling (part of margins study)	Summer 2015
Task 3. Laboratory analysis; QA/QC	Fall/winter 2015-2016
Task 4. Draft/final factsheet	March 2016

Background

General Background:
Microplastic is a term used to describe fragments of plastic that are less than 5 mm (Wright et al., 2012). Microplastics can be pellets that are used as precursors for industrial products, microbeads used in consumer products (e.g., exfoliants), or fragments/fibers of plastics that are the breakdown products of larger plastic materials. Microplastics can enter the aquatic environment through wind, stormwater runoff, or illegal dumping of plastic materials (Eriksen et al., 2013). Additionally, both microbeads from cosmetic products and plastic fibers (e.g., polyester and acrylic) from clothing can be washed down the drain and enter wastewater treatment plants (European Commission 2012). Microplastics may not be captured by wastewater treatment plants because they are buoyant and do not flocculate; therefore, they can be released in wastewater (Hogue, 2013).

Microplastics are found in surface waters, the water column, and sediment because of the varying density of plastic particles. They can also be found in the gut and circulatory system of aquatic organisms that ingest the particles. Studies have found that microplastics are also able to adsorb to organisms, blocking their feeding appendages

(Wright et al., 2012). Ingestion of microplastics can block the digestive tract, reduce growth rates, block enzyme production, lower steroid hormone levels, affect reproduction, and cause the adsorption of toxicants (Wright et al., 2012). The potential for ingesting toxicants occurs because microplastics readily accumulate hydrophobic organic compounds, due to their high surface area to volume ratio (Teuten et al., 2007). In fact, the sorption of persistent organic pollutants (POPs) to microplastics exceeds sorption to sediments by two orders of magnitude (Mato et al., 2001); in one study, the concentration of POPs on microplastics was six orders of magnitude higher than the concentration in the surrounding water column (Teuten et al., 2007). Therefore, the ingestion of microplastics by organisms can increase the exposure of aquatic life to toxic pollutants.

Microplastic Monitoring Studies

Plastic pollution has increased over the past several decades and is often the dominant type of pollution in aquatic environments (Eriksen et al., 2013). Both industrial and densely populated coastal areas have been identified as microplastic hotspots (Wright et al., 2012). Most studies on plastic pollution in the United States have focused on macro-plastics (Ryan et al., 2010). However, there are a growing number of microplastic monitoring efforts in the United States, including a study in Santa Monica Bay, the Los Angeles River, and an on-going study in the Great Lakes.

The Santa Monica Bay study was completed in 2001 and was a partnership between the Algalita Marine Research Foundation and the Southern California Coastal Water Research Project. The study was noteworthy because it was the first microplastic monitoring effort that not only measured the abundance in the surface layer, but also at mid-depth and at the sediment-water interface (Lattin et al., 2004). The study monitored microplastics at varying depths because only 46% of microplastics are positively buoyant. The study observed microplastics at all depths and found that the abundance increased considerably after a storm event. Another microplastic study is just beginning in the Los Angeles area; Dr. Marcus Eriksen is monitoring microplastics in the Los Angeles River. The study will help determine if microplastics are entering Los Angeles' coastal waters through the urban watershed.

Microplastic pollution is also currently being measured in the surface waters of the Laurentian Great Lakes. The study found that microplastic pollution was greatest in Lake Erie, most likely because it is the most populated region (Eriksen et al., 2013). Unlike the Santa Monica Bay study, the microplastics were analyzed using scanning electron microscopy. Therefore, both abundance and the chemical composition of the particles were analyzed. The study is on-going and the researchers, including the project lead Dr. Sherri Mason (SUNY Fredonia), are currently considering adding effluent sampling to the monitoring effort.

The RMP has undertaken a small special study evaluating microplastics in effluent, as well as ambient Bay water and sediment. Funding for this 2015 study was released early to allow sample collection beginning in 2014. Microplastics at two different sizes were collected from the treated effluent of 8 Bay Area wastewater treatment facilities. Ten

ambient Bay sediment samples were collected as part of the 2014 RMP Status and Trends sediment summer sampling cruise: Central Bay (4 samples), Lower South Bay (2), and South Bay (4). Although four samples have been collected in the Central Bay, they were not collected in close proximity to the margins, where we hypothesize the highest concentrations of microplastics are likely to exist. RMP staff, working in collaboration with non-profits San Francisco Bay Keeper and 5 Gyres, were able to collect 9 ambient Bay surface water trawl samples near the sediment sites. All Bay Area effluent, sediment, and water samples have been submitted to Dr. Sherri Mason at SUNY Fredonia for sample processing, visual sorting, and abundance analyses. Results are expected in the summer of 2015.

This study would address an important data gap by providing an estimate of microplastics in the margins of the Central Bay, an area that is ecologically quite productive and at the same time known as area that is highly contaminated, particularly by plastic trash. Sediment in densely populated areas can be heavily contaminated with microplastics (Wright et al., 2012); a statistically significant relationship between population and microplastic abundance has been identified (Brown et al., 2011).

Given the widespread detection of microplastics in the environment and the potential conduit these particles serve introducing POPs into the food chain, several state legislatures have begun proposing bans on the use of microplastics in certain industries. A bill to ban microplastics in cosmetics was introduced in the California assembly in 2014; however, it failed by one vote. A number of similar bills prohibiting microplastics in personal care products have been introduced in the other states such as the Great Lakes states (Council of State Governments, 2014). Illinois and New York states passed bans in 2014 (Council of State Governments, 2014). In addition, Johnson & Johnson, L'Oréal, Colgate-Palmolive, and Procter & Gamble have pledged to phase out the use of microbeads in their skin cleansers (Hogue 2013).

Study Objectives and Applicable RMP Management Questions

This study will provide an initial characterization of microplastics in the surface sediment in the shallow Central Bay margin areas. These data will help us better understand the distribution of microplastics in the Bay and the potential for uptake into the food web. The study will complement a 2015 special study on microplastics that measured concentrations in ambient water, ambient sediments, and wastewater effluent. The study will address two RMP Management Questions:

1) Are chemical concentrations in the Estuary at levels of potential concern and are associated impacts likely?
2) What are the concentrations and masses of contaminants in the Estuary and its segments?
    2.1 Are there particular regions of concern?

In addition, the study will address the emerging contaminants priority question: What emerging contaminants have the potential to adversely impact beneficial uses of the Bay?

Approach

Two size fractions of microplastics will be sampled, 5-mm-0.355-mm (the size fraction that is characteristic of personal care product microbeads) and 0.125-0.355-mm (the size fraction that is characteristic of microfibers), in Bay sediment. Sediment sampling will occur as part of the margins sampling study in the summer of 2015. Ten sediment samples will be collected using a modified van Veen grab or hand scooped from exposed intertidal sediment. The 10 stations will be a subset of the 40 stations sampled during the margins sediment monitoring. Station selection will be informed by available data on plastic trash abundance.

After collection, the sediment samples will be sent to Dr. Sherri Mason at SUNY Fredonia for sample processing, visual sorting, and abundance measurements. This laboratory was selected to ensure consistency because it is doing the analyses for the 2015 RMP sediment samples.

Budget

The proposed budget for the study is $14,325. This includes staff time to manage the project, coordinate collection and shipping of samples, and write a fact sheet that will include all RMP microplastics data (2015 and 2016 special studies).

Sample collection costs will be minimal, as samples will be collected as part of the existing margin sediment special study. Analytical costs are also low, at $100/sample.

Table 1. Budget summary.

Expense	Estimated Hours	Estimated Cost ($)
Labor		
Project Staff	50	7,050
Senior Management Review	4	800
Project Management	0*	
Contract Management	0*	
Data Technical Services	0	
GIS Services	4	325
Creative Services	18	1,500
IT Services	0	0
Communications	0	0
Operations	0	0
Subtotal		

Subcontracts	
Name of contractor	
SUNY	1,000
Graphic Design contractor	2,450
Direct Costs	
Equipment	500
Travel	100
Printing	100
Shipping	500
Other	
	14,325

*Not needed because core RMP funding provides this service.

Reporting

A draft fact sheet summarizing the approach, analyses and results of the study will be submitted to the ECWG and TRC. Upon receipt and incorporation of comments, a final factsheet will be issued.

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Special Study Proposal: Emerging Contaminants Strategy

Summary: Increasing engagement on emerging contaminants issues by the San Francisco Bay Regional Water Board, RMP stakeholders, and the general public is reflected in headline news as well as policy actions at local, state, and federal levels. Work to advance the RMP's Emerging Contaminants Strategy has increased significantly in the last year, driven by increased demand for independent information on key contaminants. Critical new deliverables, such as assisting the Water Board as the agency prepares emerging contaminants action plans for the Bay, have been added to the primary deliverables of this strategy: Tracking new information regarding contaminant occurrence and toxicity and updating the RMP's tiered risk and management action framework for emerging contaminants in San Francisco Bay (see Sutton et al. 2013). For this reason, this proposal requests an additional $5,000 for strategic emerging contaminants tasks.

New developments like the recently disseminated state CEC guidance (Dodder et al. 2015), along with the completion of critical RMP studies on non-targeted analysis, indicate the need to formally revise the RMP CEC strategy document (Sutton et al. 2013). This proposal requests an additional $12,000 to create a fully updated strategy document as a key deliverable for the 2016 Emerging Contaminants Strategy Special Study.

Estimated Cost: $37,000
Oversight Group: ECWG
Proposed by: Rebecca Sutton (SFEI)

PROPOSED DELIVERABLES AND TIMELINE

Deliverable	***Due Date***
Task 1. Information gathering from a variety of sources throughout the year, including presentations at scientific conferences	2016
Task 2. Assist Water Board and other stakeholders with science summaries relating to policy including emerging contaminants action plans and comment letters regarding proposed actions of other agencies	12/31/2016
Task 3. Present an update of emerging contaminants strategy, ongoing or completed special and pro bono studies, and new studies to the Steering Committee	12/31/2016
Task 4. Review tiered monitoring and management risk framework, present findings to the Water Board	9/30/2016
Task 5. Complete update of RMP CEC strategy document, including discussion of state CEC guidance, conclusions of non-targeted studies (broad scan, bioanalytical tools), revised tiered framework tables	3/31/2017

Background

The science and management of contaminants of emerging concern (CECs) is an area of dynamic recent development. Competing Senate bills introduced this year to reform the federal Toxic Substances Control Act are a clear sign of the growing concern surrounding the widespread introduction of thousands of chemicals into commerce without significant testing to establish safety for humans and wildlife. The general public has become increasingly engaged on issues of chemical safety and potential environmental harm, informed by headlines in major newspapers across the country. The RMP's recent study documenting declines in flame retardant contamination in San Francisco Bay (Sutton et al. 2015) made the front page of the San Francisco Chronicle, and and was broadcast widely via local print, radio, and television news, as well as in major publications like Scientific American.

The RMP, a global leader on contaminants of emerging concern (CECs), stays ahead of the curve by identifying problem pollutants *before* they can harm wildlife. The RMP has completed a strategy document outlining a comprehensive, forward-looking approach to addressing CECs in San Francisco Bay (Sutton et al. 2013). The RMP's CECs strategy consists of three major elements. First, for contaminants known to occur in the Bay, the RMP evaluates relative risk using a tiered risk and management action framework. This risk-based framework guides future monitoring proposals for each of these contaminants. The second element of the strategy involves review of scientific literature and other aquatic monitoring programs to identify new contaminants for which no Bay data yet exist. Finally, the third element of the strategy consists of non-targeted monitoring, including broadscan analyses and development of bioanalytical tools.

For the RMP's CECs strategy to remain relevant and timely, it needs to be regularly updated with new information on analytical methods and study findings from the RMP and others. Funds are needed to review new results, track relevant work being conducted elsewhere, and keep stakeholders apprised of findings. At the same time, it is important for the RMP to provide relevant, objective science to inform the growing number of policy actions concerning emerging contaminants, an increasing demand on staff time. In the last six months, RMP emerging contaminants experts have responded to a Water Board information request concerning the state of science surrounding perfluorochemicals as it relates to developing emerging contaminant action plans, and provided necessary scientific support for Water Board comment letters regarding two USEPA proposed significant new use rules concerning nonylphenol ethoxylates and perfluorochemicals.

By the end of 2015, a number of new developments will necessitate a thorough revision of the RMP CEC strategy document to assure it evolves with the latest science. These new developments include: 1) a state-wide guidance document concerning CEC monitoring in aquatic environments; 2) completion of an RMP special study consisting of non-targeted broad scan analysis of Bay tissue samples to identify CECs not yet monitored; and 3) completion of an RMP study to develop bioanalytical tools to identify estrogenicity due to contaminants. The potential impact of these larger scale developments on the RMP's CEC strategy requires full revision of the strategy document, as opposed to the revision of specific tables considered emerging contaminants strategy deliverables for 2015.

Study Objectives and Applicable RMP Management Questions

Through this Special Study, the RMP has traditionally funded updates to the tiered risk and management framework (element one of the RMP CEC strategy), review of the state of the science concerning CECs and interaction with other monitoring groups (element two), and interpretation of the findings of non-targeted analysis (element three) to determine new monitoring priorities.

Additional demands now placed on the RMP's emerging contaminants team include: a) scientific assistance to the Water Board as agency staff prepare action plans for priority CECs; b) increased engagement with stakeholders (e.g., briefings for the Water Board and the RMP Steering Committee); and c) scientific advisory support for the Water Board and other stakeholders concerning relevant policy proposals and actions at the local, state, and federal levels (e.g., USEPA proposed significant new use rules). To assure that the RMP is able to provide cost-effective expertise to address these demands, this proposal requests a higher level of funding for 2016 to assure that the policies that are developed are based on sound science.

As described above, key developments with the potential to impact the core RMP CEC strategy make revision of the strategy document in 2016 a high priority. Periodic revision was anticipated as necessary to maintain the relevance of this document in the face of an evolving science and policy landscape.

Table 1: Study objectives and questions relevant to RMP management questions

Management Question	Study Objective	Example Information Application
1) Are chemical concentrations in the Estuary at levels of potential concern and are associated impacts likely?	Compare existing occurrence data with new toxicity information reported in the scientific literature. Evaluate future monitoring needs and toxicity data gaps.	Does the latest science suggest a reprioritization of chemicals as we learn more about them? Which newly identified contaminants merit further monitoring?
2) What are the concentrations and masses of contaminants in the Estuary and its segments? 2.1 Are there particular regions of concern?	Does new knowledge including recently published toxicity data and/or source/pathway information suggest different relative risks for any of the five subembayments?	What are the key regional influences on different subembayments that impact concentrations, masses, and potential risk of emerging contaminants?
3) What are the sources, pathways, loadings, and processes leading to contaminant-related impacts in the Estuary? 3.1. Which sources, pathways, etc. contribute most to impacts?	Does new research in other regions provide insight as to key sources, pathways, loadings, and processes that affect impacts of emerging contaminants?	Are relative levels of contaminants in different matrices or subembayments consistent with our expectations for various contaminant processes?
4) Have the concentrations, masses, and associated impacts of contaminants in the Estuary increased or decreased? 4.1. What are the effects of management actions on concentrations and mass?	Does trend data from other regions suggest likely trends in the Bay? Which new management actions are likely to impact contaminant levels?	Are additional or different actions needed to reduce levels below aquatic toxicity thresholds?
5) What are the projected concentrations, masses, and associated impacts of contaminants in the Estuary?	Do data on production, use, and source trends in the scientific and trade literature provide a means of prioritizing relative risk of Bay contaminants?	Do production, use, and source trends suggest likely changes in the relative risk of specific emerging contaminants?

Emerging contaminants strategy work most directly addresses questions 1, 3, and 5, by assuring that all manner of relevant new information is brought to bear in evaluating the relative risk of emerging contaminants to Bay wildlife. For example, a new study identifying a lower toxicity threshold for a particular contaminant might suggest that the relative risk tier in which that contaminant had been placed should be revised.

In addition, the study will address the emerging contaminants priority question: What emerging contaminants have the potential to adversely impact beneficial uses of the Bay?

By providing funding for the emerging contaminants strategy, the RMP can be assured it is getting "the most bang for its buck," targeting the highest priority contaminants among the many thousands in commerce and potentially discharged to the Bay. The RMP is a global leader in CEC monitoring, yet it must be efficient and pragmatic in the face of finite

resources. A modest increase in funding for this task will allow for strategic thinking using the latest science, so that the RMP can continue to generate the information water managers need to effectively address emerging contaminants in the Bay.

Approach

Base funding ($20,000) for this effort has supported the review of key information sources throughout the year. These sources include:

- Abstracts of newly published articles in key peer-reviewed journals (e.g., Environmental Science and Technology, Environmental Toxicology and Chemistry, Environment International)
- Documents produced by other programs (e.g., USEPA, Environment Canada, European Chemicals Agency, Great Lakes CEC Program)
- Abstracts and proceedings from relevant conferences (e.g., Society of Environmental Toxicology and Chemistry, International Symposium on Brominated Flame Retardants)

Additional funding ($5,000) would support staff to provide additional services, such as:

- Additional presentations, briefings, and stakeholder interactions
- Scientific assistance to the Water Board as the agency prepares emerging contaminant action plans
- Scientific assistance to stakeholders engaged in emerging contaminants policy

Finally, a major emerging contaminants deliverable proposed for 2016 is full revision of the RMP CEC Strategy document (Sutton et al. 2013). The estimated cost for this task is $12,000. A number of critical developments have occurred since its original publication in 2013, as detailed previously, and the RMP's overall strategy should evolve to encompass new science and policy. Updates to the tiered risk-management action framework for San Francisco Bay would be included within this larger deliverable.

Budget

The following budget represents estimated costs for 2016 Emerging Contaminants Strategy, including additional deliverables not included in the proposals from previous years.

Table 2. 2016 Emerging Contaminants Strategy budget (see Appendix for more detail)

Deliverables	**Funds**
Tasks 1-4: Information gathering from a variety of sources throughout the year, including presentations at scientific conferences; Assist Water Board and other stakeholders with science summaries relating to policy including emerging contaminants action plans and comment letters regarding proposed actions of other agencies; Present an update of emerging contaminants strategy, ongoing or completed special and pro bono studies, and new studies to the Steering Committee; Review tiered monitoring and management risk framework, brief the Water Board	$25,000
Task 5: Update RMP CEC Strategy document	$12,000
Total	$37,000

Budget Justification

Essential Emerging Contaminants Strategy Deliverables

In past years, a strategy fund of $20,000 has covered a number of essential tasks to assure that the RMP's monitoring of CECs remains relevant and timely, as described previously. New demands placed on CEC staff indicate a need for a discrete increase in these funds to $25,000. For example, developing a single memo for the Water Board describing the state of science and policy for a particular contaminant for which an action plan is being developed may require 20 hours of senior staff time @ $150/hr, resulting in an expenditure of $3,000.

RMP CEC Strategy document update

To produce a revised CEC strategy document, we estimate 60 hours of senior staff time @ $150/hr ($9,000), 15 hours of junior staff time @ $70/hr ($1,050), and 15 hours of design staff time @ $115/hr ($1,725).

Reporting

Emerging contaminants strategy work would be captured in the updated RMP CEC Strategy document proposed as a major deliverable. A number of RMP CEC Strategy presentations (Emerging Contaminants Workgroup, Steering Committee, and Annual Meeting) and briefings (Water Board, others as needed) provide further opportunities to report on this work.

References

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Sutton R, Sedlak M, Davis J. 2013. Contaminants of Emerging Concern in San Francisco Bay: A Strategy for Future Investigations. SFEI Contribution 700. San Francisco Estuary Institute, Richmond, CA. http://www.sfei.org/documents/contaminants-emerging-concern-san-francisco-bay-strategy-future-investigations

Sutton R, Sedlak MD, Yee D, Davis JA, Crane D, Grace R, Arsem N. 2015. Declines in polybrominated diphenyl ether contamination of San Francisco Bay following production phase-outs and bans. Environmental Science and Technology. 49:777-784.