Contaminants of Emerging Concern in Puget Sound English sole (*Parophrys vetulus*): Exposure to and Effects of Selected Estrogenic Chemicals and Pharmaceuticals

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June 2016

Washington Department of FISH and WILDLIFE

WDFW Report Number FPT 20-10

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This study is funded in part by the United States Environmental Protection Agency (EPA) National Estuary Program (NEP), under Puget Sound Ecosystem Restoration and Protection Cooperative Agreement grant G1400206 with Washington State Department of Ecology (Ecology).

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ACRONYMS & ABBREVIATIONS

Acronyms and abbreviations used frequently in this report are listed below, those used infrequently are excluded.

BPA	bisphenol A
cDNA	complementary DNA
CECs	contaminants of emerging concern
CSO	combined sewer overflow
Ct	cycle threshold
DNA	deoxyribonucleic acid
e.g.	for example
Ecology	Washington State Department of Ecology
E1	estrone
E2	17β-estradiol
E3	estriol
EE2	17α-ethynylestradiol
EC	estrogenic chemical
EDC	endocrine disrupting chemical
EPA	U.S. Environmental Protection Agency
et al.	and others
GC/MS	gas chromatography/mass spectrometry
i.e.	in other words
LC-MS/MS	liquid chromatography-tandem mass spectrometry
LOQ	limit of quantitation
mRNA	messenger ribonucleic acid
NP	nonylphenol
NTC	no template control
OP	octylphenol
PSEMP	Puget Sound Ecosystem Monitoring Program
QAPP	Quality Assurance Project Plan
qPCR	quantitative polymerase chain reaction
RT-PCR	reverse transcriptase polymerase chain reaction
RNA	ribonucleic acid
SOP	standard operating procedures
SSRI	selective serotonin reuptake inhibitors
tOP	tert-octylphenol
VTG	vitellogenin
WWTP	waste water treatment plant
WDFW	Washington Department of Fish and Wildlife

UNITS OF MEASUREMENT

ft	feet
MG	million gallons
MLLW	mean lower low water
mm	millimeter
ng/g	nanograms per gram (parts per billion)
ng/L	nanograms per liter (parts per trillion)
ng/mL	nanograms per milliliters(parts per billion)

EXECUTIVE SUMMARY

This project provided a Sound-wide assessment of the presence and biological impact of selected Contaminants of Emerging Concern (CECs) in English sole (*Parophrys vetulus*), a long standing, key indicator species for toxics monitoring in Puget Sound. This project leveraged field and laboratory assets from an ongoing, long-term toxics monitoring program (Puget Sound Ecosystem Monitoring Program) with regional laboratories developing cutting-edge ecotoxicology techniques (NOAA's Northwest Fisheries Science Center and Battelle's Pacific Northwest National Laboratory) to (a) develop analytical methods for, and provide a current evaluation of the extent and magnitude of CEC contamination in English sole and (b) develop cost-effective bioeffects endpoints for these CECs.

Two major classes of CECs were evaluated, estrogenic chemicals (ECs), including three natural estrogens (17β-estradiol, estrone, and estriol), one synthetic estrogen (17α ethynyl estradiol), and three nonsteroidal xenoestrogenic compounds (bisphenol A, nonylphenol and octylphenol); and three selective serotonin reuptake inhibitors (SSRIs; fluoxetine, sertraline and citalopram) that may amplify the effects of ECs. The EC bioeffect endpoint was a method for measuring vitellogenin (VTG) induction, a widely accepted biological indicator of EC exposure.

The project successfully achieved its goals of developing new tools for exposure metrics for ECs in fish bile, a bioeffects metric for exposure to ECs, and measuring SSRIs in English sole. These tools may be applied in upcoming 2017 English sole surveys, and will be further refined as we incorporate them into WDFW/PSEMP's biennial English sole contaminant survey. We also combined these new methods with existing methods on English sole tissue samples collected in 2011 and 2013 to identify:

- continued altered reproductive timing in female fish from Seattle Waterfront in Elliott Bay, likely from exposure to ECs,
- relatively high concentrations of ECs in sole from highly-developed urbanized habitats, especially Seattle Waterfront and Sinclair Inlet,
- widespread VTG induction in male sole, with highest values primarily observed in highly developed urbanized habitats, especially Tacoma Waterway and Seattle Waterfront,
- little or no recent exposure of English sole to SSRIs, likely because sole did not occur near enough to, or forage long enough near, putative SSRI sources (such as wastewater treatment plants).

Although ECs appeared concentrated in highly developed areas and we observed the greatest EC effects in urban areas, the correlation between EC exposure-and-effects was less clear on an individual fish basis. The reasons for this could be:

  - a mismatch in timing of our sampling between exposure and effects,
  - differences in kinetics and metabolism of various ECs and VTG mRNA synthesis
  - insufficient sample sizes to achieve enough power to detect relationships, if they existed, because of the highly variable EC and VTG values measured in individual fish within a station, and

- the presence of androgenic or anti-estrogenic chemicals that may mask or alter the effects of ECs.

These results highlight the need for continued work to refine these tools for monitoring indicator species such as English sole. Although effects from exposure to ECs seemed clear in some cases, high variability in response metrics precluded unambiguous conclusions in some cases. Power analysis of existing samples will help to define the sample sizes needed to identify spatial and temporal differences for upcoming monitoring efforts. Evaluation of multiple metrics (histological examination of gonads, hepatic VTG induction, and measurement of biliary ECs) is still appropriate and necessary to understand and track the health effects of these chemicals. Controlled dosing studies in the lab may help to elucidate health effects thresholds, which are needed to evaluate the measurements made in wild English sole, and to elucidate the level of normal VTG mRNA synthesis in natural, unexposed male English sole, if one exists. Finally, continued evaluation of additional exposure and health metrics, including assessment of VTG proteins in plasma, is warranted as they become available, and more cost effective.

Establishing VTG induction as a monitoring tool for English sole fills a recognized, important gap in the Puget Sound Partnership's *Toxics in Fish Vital Sign*. Combining results from this project with existing PSEMP efforts to monitor a wide range of other contaminants will provide a balanced approach for prioritizing contaminant-related recovery efforts in Puget Sound.

INTRODUCTION

Puget Sound is a semi-enclosed glacial fjord, subdivided into five distinct hydrologic basins (North Puget Sound, the Main Basin, Whidbey Basin, South Puget Sound, and Hood Canal), which differ in chemical, physical, and biological properties. Over the last 100 years, Puget Sound has been altered dramatically by anthropogenic activities, including over-fishing, habitat loss and inputs of toxic chemicals (Ruckelshaus and McClure, 2007). Contaminant inputs to Puget Sound, including Contaminants of Emerging Concern (CECs), are a special concern due to its semi-enclosed and increasingly urbanized hydrological system, which receives about 2 million m^3 of Waste Water Treatment Plant (WWTP) effluent per day (WDOE, 2008), plus billions of liters of mixed storm water runoff containing untreated effluent from Combined Sewer Overflows (CSOs; King County, 2009).

Currently, Puget Sound's shorelines and watersheds range from highly developed urbanized or industrialized to nearly pristine conditions. Numerous studies have documented that marine species in the most heavily urbanized and industrialized areas, such as the Seattle Waterfront in Elliot Bay, Duwamish Waterway, Sinclair Inlet, Tacoma City Waterway in Commencement Bay, and Eagle Harbor, all located in the Main Basin, are exposed to concentrations of toxics chemicals often at levels high enough to impair their health (Essington et al., 2011). In contrast, levels of contaminant exposure are generally lower in marine species sampled from the least developed, relatively rural basin of Hood Canal, and parts the North Puget Sound (e.g., Strait of Georgia, and the Gulf of Bellingham - Vendovi lsland) and the South Puget Sound (e.g., Nisqually River reach).

Endocrine Disrupting Chemicals (EDCs) have emerged as contaminants of high concern because of their widespread presence in aquatic environments and their potentially far reaching effects on hormone-mediated physiological functions including growth, development, behavior, and reproduction. Priority EDCs emerging internationally as threats to ecological and human health are Estrogenic Chemicals (ECs, including natural estrogens from exogenous and endogenous sources, such as 17β-estradiol (E2), estrone (E1) and estriol (E3); the synthetic hormone 17α-ethynylestradiol (EE2); and non-steroidal xenoestrogenic compounds such as bisphenol A (BPA), nonylphenol (NP), octylphenol (OP) (Pettersson et al., 2007; Suter 2001 IARC 2007, Routledge et al., 2008; Chapin et al., 2008). These CECs are commonly and widely detected in water and sediments, and can disrupt hormonal and metabolic processes even at relatively low concentrations. Data on exposure concentrations and toxic effects of BPA, NP, OP, EE2 and natural estrogens are limited for marine ecosystems (Scott et al. 2006, Scott et al. 2007), including Puget Sound, but are necessary to assess the risk that they pose for the health of fish and other biota in the ecosystem.

Pharmaceuticals, including some drugs that act as endocrine disruptors, represent another class of CECs for the Puget Sound region and elsewhere. Of the many pharmaceuticals released into the environment, anti-depressants such as Selective Serotonin Reuptake Inhibitors (SSRIs) are of high concern due to their environmental persistence and effects on aquatic animals (Lister et al., 2009; Kreke and Dietrich 2008; Brooks et al., 2005; Schultz et al., 2009). Lubliner et al. (2010) concluded that advanced nutrient removal systems in Puget Sound's WWTPs failed to remove SSRIs such as fluoxetine. Indeed, in a 2012 U.S. Environmental Protection Agency (EPA)-funded study, Battelle Pacific Northwest National

Laboratory staff detected several SSRIs at moderately high concentrations (from >200 to 1000 ng/L) in effluent from eight Puget Sound WWTPs (Harding et al. 2016). Levels of SSRIs detected in Puget Sound WWTP effluent are within the range of concentrations associated with alterations to reproduction, growth, and development (Corcoran et al., 2010), but the extent to which Puget Sound biota are exposed to and affected by SSRIs is unknown. The combination of documented releases of SSRIs into Puget Sound and their known adverse effects on fish health constitute a risk to biota and are a high priority gap for CEC status and trend monitoring.

As a member of the Puget Sound Ecosystem Monitoring Program (PSEMP), the Washington Department of Fish and Wildlife (WDFW) assesses status of and trends in the health of Puget Sound fishes and macro-invertebrates related to their exposure to toxic contaminants. Although some contaminants currently monitored by PSEMP's Toxics in Biota unit exhibit hormone-disrupting properties, EDCs as a class represent a clear gap in status and trends monitoring of CECs in Puget Sound. Additionally, EDCs have been identified as a key component of the Puget Sound Partnership's *Toxics in Fish Vital Sign*, indicators being used to track the recovery of Puget Sound. In particular, the *Toxics in Fish Vital Sign* is intended to document the status and trends of EDCs, especially ECs, in Puget Sound biota, incorporating clear metrics related to EDC effects on fish health. Pharmaceuticals, such as the widely prescribed SSRI antidepressants, are also of interest because they may amplify the effects of ECs (reviewed in Corcoran et al. 2010). The EDC component of the *Toxics in Fish Vital Sign* is being used to establish and evaluate recovery targets as they relate to biota health and EDC exposure.

The goals of this study were twofold: (a) develop methods to analyze and estimate the extent and magnitude of exposure of adult English sole (*Parophrys vetulus*) to selected endocrine-disrupting CECs, and (b) evaluate the extent to which these EDCs adversely affect the reproductive health of English sole.

Our study objectives were to: (1) develop and apply a rigorous detection and quantitation method to provide data for English sole on two classes of currently used CECs with EDC properties: ECs (measured in bile) and SSRIs (measured in liver), via WDFW's long standing Puget Sound-wide reconnaissance surveys, and (2) develop and apply a rigorous detection and quantitation method for Vitellogenin (VTG) induction in male fish, (a widely accepted biological indicator of EC-effects) for English sole, a key contaminant indicator species previously documented to have exhibited exposure to environmental source of ECs.

The VTG induction metric was developed specifically for use in long-term monitoring of Puget Sound biota, and will augment existing toxics monitoring of biological indicators in Puget Sound by adding bio-effect metrics to complement bile and tissue residue measurements of CECs for English sole. Moreover the exposure and effects metrics that were developed for ECs and SSRIs will be assessed in combination with other metrics currently monitored by PSEMP, including gonadal condition, liver disease, and lipid content, as well as tissue residues of persistent, bioaccumulative, toxic chemicals. This comprehensive approach will document exposure and effects of multiple CECs across a wide range of potential environmental contaminant conditions in Puget Sound. The biological indicators of EDC exposure and effects will close a critical gap in PSEMP toxics monitoring and the *Toxics in Fish Vital Sign*, and provide a major step towards prioritizing new CECs for assessing Puget Sound recovery.

MATERIALS AND METHODS

Study Design

This project was designed to (1) develop methods for analyzing ECs and selected pharmaceuticals in English sole, a key indicator species for toxic contaminants in Puget Sound, (2) provide a Puget Sound-wide assessment of the presence of ECs and pharmaceuticals in this species, and (3) measure health effects in English sole related to exposure to these chemicals. We focused primarily on seven ECs (EE2, E2, E1, E3, BPA, NP, and OP) which can potentially cause feminization of male English sole, and alter reproductive activities of females, and secondarily on three SSRIs, fluoxetine, sertraline and citalopram. SSRIs are a class of environmentally persistent pharmaceuticals (commonly prescribed as anti-depressants), which recent studies suggest can amplify effects of ECs (Corcoran et al. 2010). Additionally we measured VTG production in adult English sole by specific quantification of VTG messenger Ribonucleic Acid (mRNA) levels in liver by Reverse Transcriptase quantitative Polymerase Chain Reaction (RT-qPCR), a common approach due to its high sensitivity and rapid induction by estrogens compared to detectable VTG protein in blood (García-Reyero et al., 2004; Hemmer et al., 2002; Yamaguchi et al., 2009).

Detailed sampling and analytical methods followed standard operating procedures originally proposed for this study in its Quality Assurance Project Plan (QAPP; O'Neill et al. 2014). These methods are summarized below along with additional pertinent sampling details.

Study Area

The project study area was focused on the foraging habitat of adult English sole in Puget Sound. It included ten index sites from which English sole have been monitored for over 20 years for chemical contamination by PSEMP (Figure 1). Collectively, the ten sampling locations encompass a range of contaminant sources and levels, from relatively rural, low-development areas such as the Hood Canal, Strait of Georgia, and the Gulf of Bellingham (near Vendovi Island) and the Nisqually Reach, to the moderately developed Port Gardner Bay and Eagle Harbor, and to Puget Sound's most highly urbanized and industrialized areas including Seattle Waterfront, the Duwamish Waterway, Sinclair Inlet, and Tacoma's City Waterway.

The ten sampling locations were assigned to a "Site Type" (Table 1) of low, moderate, or high development based on a combination of two factors; 1) distance to the nearest upland watershed catchment area, called an Assessment Unit (AU), and 2) the "percent developed imperviousness" of that AU. The AUs were originally developed by Ecology (Stanley et al., 2012) and were determined to be of an appropriate size for this study (median area of 8.8 km^2 or 3.4 $mile^2$). We used "percent developed imperviousness" measures from the National Land Cover Database 2006 (Fry et al., 2011; Wickham et al., 2013), with a spatial resolution of 30 meters, to calculate the mean percent impervious surface within each AU along the Puget Sound shoreline. Using this combination of factors the sampling locations ranged from:

- High development - within 500 meters (m) of land with >50% impervious surface, to

- Moderate development - within 500 m of land with >25% impervious surface or within 2000 m of land with >50% impervious surface, to
- Low development - more than 2000 meters (m) away from land with >25% impervious surface.

Fish Collections

Analyses were conducted on English sole tissue samples collected in anticipation of this study, from previous trawl surveys conducted by WDFW in 2011 and 2013. Up to 120 adult male and female English sole (greater than 230 mm in total length) were targeted for collection at each of 10 sites in each year. The trawl surveys followed the WDFW's PSEMP Standard Operating Procedures (SOP) detailed in WDFW-PSEMP (2013).

Tissues Sampled

Three tissue matrices from adult English sole were targeted in this study, (1) gonads for histological analysis of the stage of reproductive development, (2) bile for analysis of ECs and (3) liver tissue for analysis of SSRIs and for VTG gene expression. All tissue samples of bile, liver and gonads were collected from a subset of the English sole collected by WDFW in the 2011 and 2013 trawl surveys. In brief, in both 2011 and 2013 at each site, samples of bile, liver (all samples for VTG gene expression and a few of the samples for SSRIs) and gonad were removed from up to 60 recently euthanized fish aboard the fishing vessel. In 2013, additional liver samples for SSRI analyses were collected from up to 60 sole at each site that were frozen and transported to the WDFW laboratory for necropsy.

Table 1 depicts the number of samples collected and processed for each type of analysis. Generally, sample sizes and locations were selected to maximize statistical power for discriminating a wide range of expected exposure scenarios. Liver samples for VTG gene expression analyses primarily focused on males and was often limited by the number of male fish collected at a site. Where possible, a minimum of 10 males at each of ten sites were sampled for VTG gene expression. Analyses of VTG gene expression on were conducted on 11 female fish from two high development sites and ten females from a low development site as a positive control. The number of bile samples that were available for analysis of ECs was limited by the volume of bile collected from individual fish. Where possible, we collected paired samples for analyses of ECs in bile and liver VTG gene expression. Although the individual bile and liver samples were not always collected from the same fish, the sample size of paired samples was large, and occurred across a wide range of potential exposures, which *a priori* should have provided sufficient pairings to model the relationship between exposure and effects. Histological analyses of gonad samples to determine the reproductive stage of the fish was completed for all fish that were also analyzed for ECs in bile and liver VTG gene expression. Most of the fish that that were analyzed for SSRIs were not also analyzed for VTG gene expression, ECs or stage of reproductive development.

Tissue resections generally followed Washington Department of Ecology's SOP for whole bodies and body parts (Ecology, 2010) and a detailed description of tissue sample collection methods used in this study is available in the study QAPP (O'Neill et al. 2014). Further details on tissue treatments are discussed below.

Figure 1. Location of ten long-term monitoring stations for the PSEMP English sole surveys.

Table 1. Sample site characteristics and number of English sole samples collected for analysis of estrogenic chemicals (ECs), vitellogenin (VTG) gene expression, and selective serotonin reuptake inhibitors (SSRIs).

								Males Sampled (N)			Females Sampled (N)			
Site Name	Location	Site Type (Development)	Mean Latitude (deg)	Mean Longitude (deg)	Sample Dates	Mean Depth (m)	Fish Sampled for Gonadal Development (N)	ECs in Bile	VTG mRNA in Liver	Paired EC & VTG	ECs in Bile	VTG mRNA in Liver	Paired EC & VTG	Fish Sampled for SSRIs in Liver[c]
Strait of Georgia	southwest of Point Roberts	Low	48.86	-122.96	4/27/11 5/17/13	216	120	4	7	3	11	0	0	20
Vendovi Island	southwest of Lummi Island, Gulf of Bellingham	Low	48.65	-122.64	4/28/11 4/16/13	53	120	3	9	3[a]	8	0	0	22
Port Gardner	adjacent to Everett Harbor	Moderate	47.99	-122.25	4/25/11 4/18/13	37	120	13	21	13[b]	7	0	0	20
Hood Canal	southeast of Hood Canal Bridge	Low	47.83	-122.64	4/26/11 4/15/13	45	119	16	10	7[b]	8	10	7[a]	22
Eagle Harbor	adjacent to Winslow Ferry Terminal	Moderate	47.62	-122.51	4/20/11 4/19/13	11	120	10	10	6	7	0	0	12
Seattle Waterfront	east side of Elliot Bay	High	47.61	-122.35	5/17/11 4/22/13	40	120	22	14	12[a]	5	7	5	20
Duwamish River	Kellogg Island	High	47.56	-122.34	5/16/11 4/23/13	8	113	11	11	6[a]	7	0	0	22
Sinclair Inlet	Bremerton Waterfront	High	47.55	-122.64	4/20/11 4/24/13	11	120	10	10	6	12	0	0	13
Tacoma City Waterway[d]	southern end of Commencement Bay	High	47.26	-122.44	4/19/11 4/26/13	11	103	10	10	2[a]	5	4	0	18
Nisqually Reach	East Anderson Island	Low	47.16	-122.67	4/18/11 4/25/13	136	120	5	10	5[a]	8	0	0	14

[a] One composite VTG sample was created to match a composite bile sample. Values of VTG composite is a calculated mean of individual sample values used to create the composite.
[b] Two composite VTG samples were created to match two composite bile samples. Values of VTG composites are calculated means of individual sample values used to create the composites.
[c] The majority of SSRI analysis was performed on livers collected from fish that were not analyzed for gonadal development.
[d] This site is also referred to as Thea Foss Waterway

Stage of Gonadal Development

Gonads from all the English sole collected in this study were analyzed histologically to determine stage of gonadal development according to the methods described by Johnson et al. 1991, Sol et al. 1998 and Johnson et al. 2008. Briefly, gonadal tissues were embedded in paraffin, sectioned, stained and examined microscopically. Ovaries were classified into 6 developmental stages: regressed, previtellogenic, vitellogenic, ripe/hydrated, spawning, and spent. Testes were classified into 5 developmental stages: regressed, early spermatogenesis (referred to as recrudescence by Sol et al. 1998 and Johnson et al. 2008), late spermatogenesis (referred to as spermiogenesis by Sol et al. 1998 and Johnson et al. 2008), spawning, and spent.

Chemical Analyses

Target ECs analyzed in this study included three natural estrogens, E2, E1 and E3, the synthetic estrogen hormone EE2, and three nonsteroidal xenoestrogens, BPA, NP [measured as n-nonylphenol (n-NP)], and OP [measured as tert-octylphenol (t-OP) and n-octylphenol (n-OP)] (Table 2).

Estrogenic Chemicals

Bile samples of adult English sole were collected from freshly euthanized fish collected in 2011 and 2013. All bile samples were immediately frozen after collection for later analysis. Sufficient bile volume was collected from 219 fish to create 183 bile samples for analyses of ECs, comprising 159 samples from individual fish and 24 composite samples of bile from 2-5 fish each. The bile was analyzed for three natural estrogens (E1, E2, and E3), the synthetic estrogen hormone (EE2) and three nonsteroidal xenoestrogens (BPA, NPs, and OPs) using a Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS) method developed specifically for this purpose (de Silva et al., 2013). The analytical method comprised three steps: (a) protein precipitation and enzymatic hydrolysis, (b) solid-phase extraction (SPE), and (c) quantitative analysis by LC-MS/MS. Full details of this analytical method are available in the QAPP for this study (O'Neill et al. 2014).

Table 2. Contaminants of emerging concern measured in this study.

Chemical Class	Individual Chemical	CAS Number
Estrogenic Chemicals (ECs)	17β-estradiol (E2)	50-28-2
	Estrone (E1)	53-16-7
	17β-estriol (E3)	50-27-1
	bisphenol A (BPA),	80-05-7
	n-nonylphenol (n-NP)	104-40-5
	tert-octylphenol (t-OP)	140-66-9
	n-octylphenol (n-OP)	1806-26-4
	17α-ethynylestradiol (EE2)	57-63-6
Selective Serotonin Reuptake Inhibitors (SSRIs)	Fluoxetine	54910-89-3
	Sertraline	79617-96-2
	Citalopram	59729-33-8

Selective Serotonin Reuptake Inhibitors

Liver snips of 206 adult English sole (approximately 20 each from the ten index sites) were taken from freshly euthanized or recently frozen fish collected in 2013. The liver snips were placed in jars and then frozen at -20°C until subsequent Selective Serotonin Reuptake Inhibitors (SSRI) analysis. From the 206 liver snips, sufficient weight of liver tissue was available to create 183 samples for SSRI analyses, 171 samples (Table 1) from individual fish and 12 composite samples of liver from 2-3 fish each.

The liver tissue samples were analyzed for fluoxetine, sertraline, and citalopram using Gas Chromatography Mass Spectrometry (GC-MS), using the method described by Wille (2008) and Wille et al. (2008). This method comprised four steps: (a) extraction, (b) cleanup (using a liquid-liquid cleanup method based on Eap et al., 1996), (c) derivatization with heptafluoro-butyrylimidazole (HFBI), and (d) quantification by GCMS with selected-ion monitoring. Full details of the SSRI analytical method are available in the QAPP for this study (O'Neill et al. 2014).

SSRI Laboratory Exposure Study

A laboratory exposure study was completed to 1) validate that the analytical method we used was able to detect SSRIs in English sole tissues, 2) estimate the bio-concentration factors for individual SSRIs, and 3) assess which of three tissue matrices was the most suitable for analysis. Nineteen English sole collected from Seattle Waterfront in April 2015 were transported to the Battelle Pacific Northwest National Laboratory. The fish were weighed to the nearest gram and randomly assigned to one of three 370 L exposure tanks. Fish were not fed during the study. After an acclimation period of three days the fish were exposed to a mixture of three SSRIs for 12 and 23 days. Two exposure levels were tested that represented one and ten times (1X and 10X) the median concentrations of citalopram, fluoxetine, and sertraline measured in a separate study of eight WWTP effluents in 2012 (Schultz, unpublished data). The low dose median exposure treatment (i.e., 1X effluent dose) water concentrations were 557 ng/L citalopram, 36 ng/L fluoxetine and 55 ng/L sertraline. These concentrations are supported by Meador et al. (2016) who detected at least two SSRI compounds in two WWTP Puget Sound effluents, with concentrations ranging from 57-60 ng/L (fluoxetine) and 89-116 ng/L (sertraline -- they did not report citalopram). The high dose median exposure treatment (i.e., 10X effluent dose) water concentrations were 5740 ng/L citalopram, 380 ng/L fluoxetine and 331 ng/L sertraline. The in-flow rate of SSRI stock solution was maintained throughout the exposure treatments, except for a slight increase in the low dose treatment after the ninth day of exposure.

Vitellogenin Gene Expression Analysis

Pieces of liver for VTG gene expression analysis were taken from individual freshly euthanized fish collected in 2011 and 2013. Each snip was placed immediately into RNALater®, incubated at room temperature or in a refrigerator for up to 2 weeks, and then frozen at -80°C until subsequent analysis. Transcripts for VTG were measured in 133 liver samples (112 males and 21 females). A minimum of ten male fish from each of the ten sites was analyzed for VTG gene expression, except for the Strait of Georgia and Vendovi Island sites where only seven and nine male fish were collected, respectively. To establish the baseline level of VTG induced by endogenous estrogen in female English sole, female fish were also analyzed for VTG gene expression: seven from the Seattle Waterfront, ten from Hood Canal, and four from Tacoma City Waterway. Because there was no assay available to quantify VTG gene

expression in English sole, we developed for the first time a specific real-time quantitative Polymerase Chain Reaction (qPCR) for this purpose. This process comprised four steps: 1) isolation of liver total Ribonucleic Acid (RNA) isolation and synthesis of complementary deoxyribonucleic acid (cDNA); 2) cloning of partial cDNAs encoding VTGs and candidate housekeeping genes (also referred to as reference genes) for development of standards; 3) selection of the VTG and housekeeping genes for further assay development; and 4) development of qPCRs (Figure 2). Full details of the VTG gene expression analytical methods are described in the QAPP for this study (O'Neill et al. 2014).

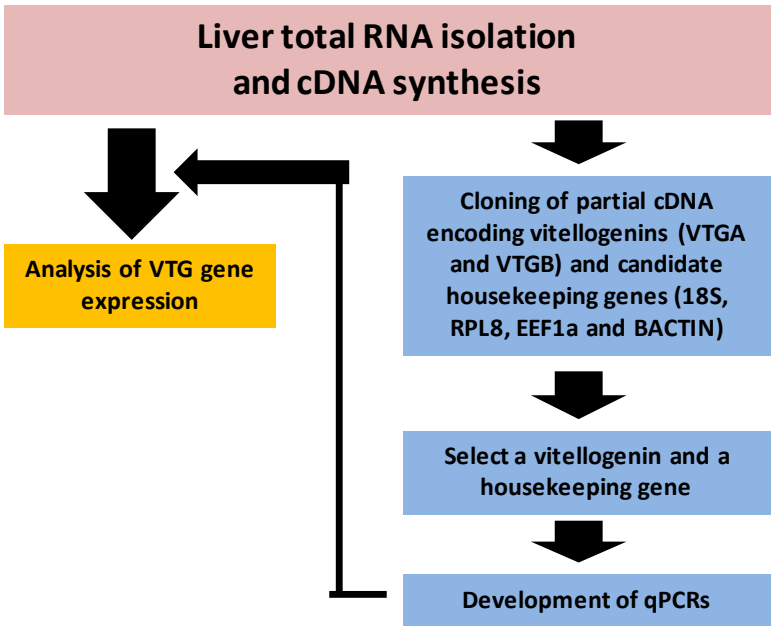

Figure 2. Schematic representation of the major steps used in the VTG gene expression assay development in English sole.

Teleost fish have multiple VTG genes due to genome duplication events (Finn and Kristoffersen, 2007). At least two distinct VTG genes, VTGA (also known as VTG1 or VTGAa) and VTGB (also known as VTG2 or VTGAb), have been reported in most fish species (Ferreira et al., 2013; Hiramatsu et al., 2002; Sawaguchi et al., 2005), including the flatfish European plaice (*Pleuronectes platessa*; Brown et al., 2004). A number of recent studies have reported differences in the regulation of transcription for the distinct hepatic VTG genes in the presence of ECs. In general, it seems that VTGA gene is more sensitive to the presence of ECs than VTGB, as found in plaice (Brown et al., 2004), zebrafish (*Danio rerio*) (Wang et al., 2005), and *Lipophrys pholis* (Ferreira et al., 2009). However the opposite scenario was recently reported for *Channa punctatus*, where the VTGB gene had a higher sensitivity to ECs (Rawat et al., 2013) and medaka *Orizias latipes* (Yamaguchi et al., 2005; Ishibashi et al., 2016). Therefore, the sensitivity of each VTG gene to ECs must be characterized in a specific sentinel fish species in order to improve the use of this biomarker as an index of estrogen exposure for environmental monitoring research.

We developed qPCRs to determine liver transcript levels of English sole VTGA and VTGB, and compared their sensitivity to ECs. Development of this assay was aided by the known VTG sequences from

numerous teleosts available in Genbank, a public database of genomic and proteomic information including several flatfish species. In addition, we also had extensive experience in cloning, sequencing and developing qPCRs for numerous genes from fish tissues (Campbell et al., 2006; Guzmán et al., 2013; Luckenbach et al., 2011; Smith et al., 2013).

Quantitative analysis of gene expression using qPCR typically requires the use of a constitutively expressed 'housekeeping gene' as an internal control to normalize for differences in starting the cDNA template between samples (Bustin, 2002). The fundamental requirement for validation of the expression stability of an internal control gene prior to its use in the system being studied is also well-defined. Nevertheless, in contrast to the situation for many mammalian experimental systems (Morse et al., 2005), studies investigating the effects of environmental ECs on gene expression in non-mammalian vertebrates have used housekeeping genes more or less randomly as internal controls, and without any validation of their expression stability in the system being studied, which may have serious implications for the interpretation of the data for the gene(s) of interest. This study, therefore, set out to assess different housekeeping genes for their potential use as internal controls to normalize the expression of VTG mRNA in English sole liver samples. Four housekeeping genes were assessed, including 18S ribosomal RNA (18S), ribosomal protein L8 (RPL8), elongation factor 1 alpha (EEF1a) and beta actin (BACTIN). These genes were selected based on a study where several housekeeping genes were evaluated for use in gene expression analyses related to effects of environmental estrogens in fish (Filby and Tyler, 2007).

Most qPCR assays for fish VTGs reported in the literature use relative expression normalized to a housekeeping gene. Since we wanted to develop a quantitative assay that could be used in a monitoring program where year-to-year comparisons are important, we developed standards for VTG and housekeeping genes for calculation of copies of VTG per copy housekeeping gene transcripts.

Data Analysis

Non-detected analytes were censored with a “less than limit of quantitation” (<LOQ) or “U” qualifier for ECs and SSRIs. For statistical analyses, the value reported for non-detected analytes was 0.5x LOQ. The bias associated with substituting 0.5x LOQ is small when the frequency of non-detects is low (Baccarelli et al. 2005), as was the case for this study.

Analysis and presentation of contaminant (ECs and SSRIs) and VTG gene expression data collected for this study were conducted using non-parametric ANOVA (Kruskal-Wallis) to compare spatial and temporal differences in contaminant exposure and gene expression. Dunn’s post hoc multiple range tests were used to make pairwise comparisons for all significant differences in Kruskal-Wallis.

We used linear regression to model the relationships between specific ECs in male and female English sole and among ECs within a sex to help determine whether the EC were derived from exogenous and/or endogenous sources. Linear regression was also used to evaluate the degree to which EC exposure in English sole could predict the degree of VTG induction in that species. For all regression analyses, the data were log-transformed to meet the assumptions of normality and equal variance.

RESULTS & DISCUSSION

Stage of Gonadal Development

The dominant condition of English sole ovaries was “regressed” and testes were ”spent”, reflecting the typical pattern of spawn timing for English sole in Puget Sound. In Puget Sound, gonadal recrudescence in English sole typically begins in the late summer or early fall, with females entering vitellogenesis and males beginning sperm production at this time (Johnson et al., 1991; Sol et al., 1998). Throughout the winter months, gonadal development proceeds and peak spawning typically occurs in February and March. By April or early May, when our sampling occurred, females have generally completed spawning, and typically exhibit a spent or regressed condition. Males may continue to exhibit a spermatogenic or spawning condition beyond May. In the present study, English sole conformed to this typical pattern of reproductive cycling at all sites except the Seattle Waterfront site (Figure 3).

Female English sole, but not males, from the Seattle Waterfront exhibited an atypical pattern of sexual maturity. While 79–100% of females from nine sites had completed spawning, and their ovaries were in a spent or regressed to pre-vitellogenic state, only 50% of female sole were spent or regressed and the remaining 50% were in spawning condition at the Seattle Waterfront site (Figure 3A). At nine of the ten sites, the majority of male sole had also completed spawning and their testes were in a spent or regressed state (50-97%) and few fish were in spawning condition (3-20%). At the Seattle Waterfront site, 57% of the male sole had completed spawning and 38% were in spawning condition (Figure 3B).

The atypical pattern of sexual maturity demonstrated by female sole sampled from Seattle Waterfront in 2011 and 2013 appears to be a consistent phenomenon at this location. A previous study of gonadal development in English sole, from 16 Puget Sound sites sampled from 1997 to 2001, documented relatively high proportions of female and male sole that were in an advanced state of gonadal development or in spawning condition for three sampling sites in Elliot Bay, including the Seattle Waterfront site (Johnson et al. 2008).

An analysis of gonadal development in English sole from 1997-2013 further documents that female sole from Seattle Waterfront in April and May routinely had a higher proportion of fish that were in an advanced state of gonadal development or were actively spawning compared to other sites, although the proportion has declined somewhat in recent years (Figure 4A). From 1997 – 2001, the average proportion of female sole that were in an advanced state of gonadal development or were actively spawning was 64%, but between 2005 and 2013, the average proportion declined to 50%. Additional years of monitoring data are needed to confirm whether this decrease in recent years is a significant decline or a sampling anomaly. In contrast, for male sole, this analysis of gonadal development suggests that their pattern of sexual maturity in Seattle Waterfront is not as atypical as the data from Johnson et al (2008) suggested (Figure 4B). Male fish from the highly developed Seattle Waterfront site, as well as those from three low development sites in the northern regions of Puget Sound (Hood Canal, near Vendovi Island and the Strait of Georgia), frequently had years in which greater than 40% of the fish collected were in spawning condition or with an advanced stage of gonadal development when the late spring PSEMP surveys were conducted.

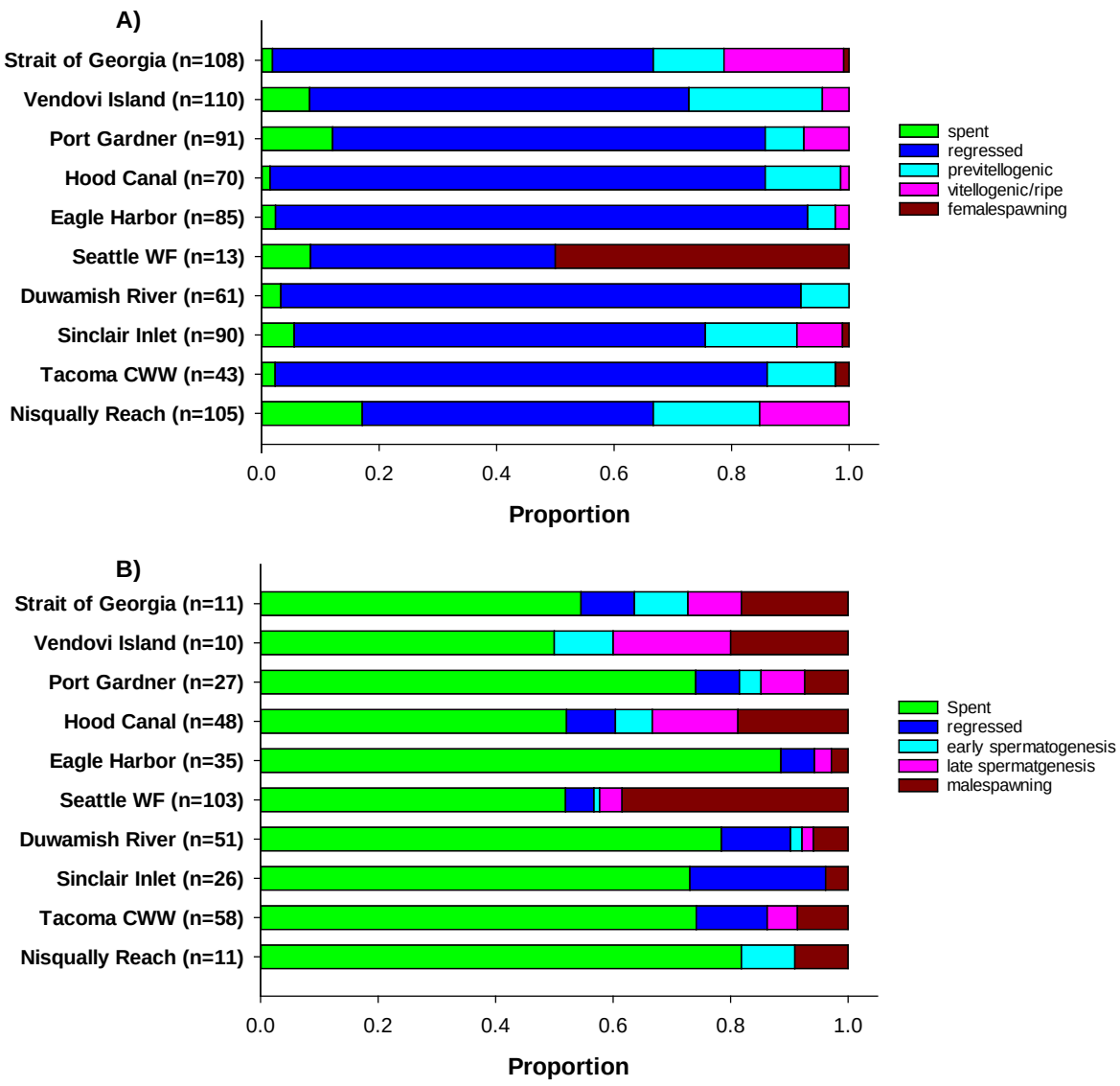

Figure 3. Proportion of female (A) and male (B) English sole in different stages of reproductive development at Puget Sound sites sampled in April - May of 2011 and 2013. WF = Waterfront, CWW = City Waterway

At the Seattle Waterfront, recent trend in the proportion of female sole that were in an advanced state of gonadal development or were actively spawning is not related to changes in the size and ages of fish sampled. First, fish size and age differences among sampling sites did not account for the atypical pattern of sexual maturity observed for female sole from Seattle Waterfront. The ten sites monitored over time included sites that had fish of similar sizes and ages to those sampled at Seattle Waterfront (e.g. Sinclair Inlet and the Strait of Georgia) as well as sites with smaller/younger fish (e.g., Vendovi Island). Second, within a site, the proportion of female sole that were in an advanced state of gonadal development or were actively spawning was not correlated with the size/age of the fish that were sampled (data not shown for brevity). For example, the average age of sole sampled at Seattle Waterfront from 1997 to 2013 ranged from six to eight years, except for 2001 when the average age was ten years.

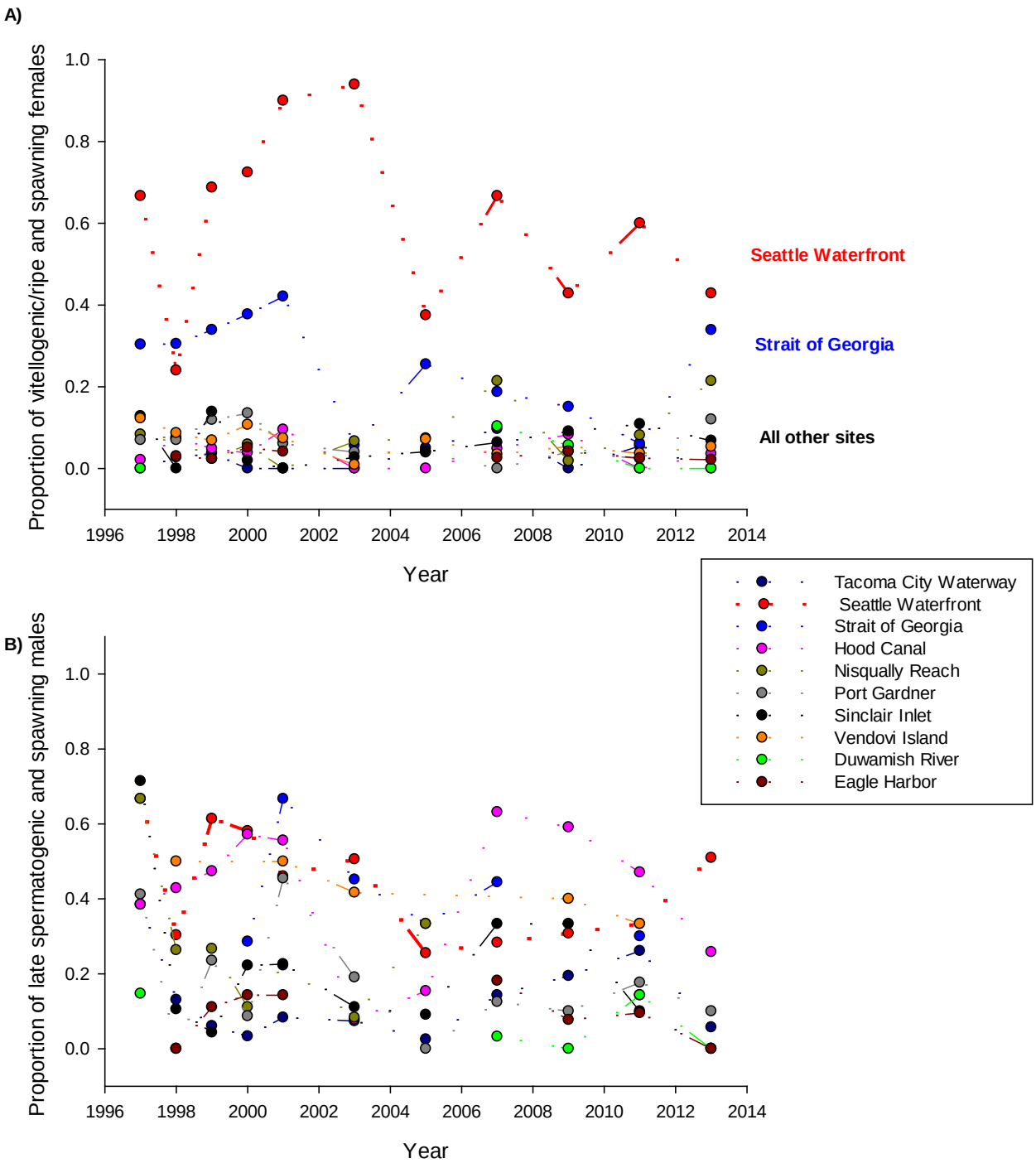

Figure 4. Time trends in the proportion of female (A) and male (B) English sole that were at an advanced stage of gonadal development or spawning in April and May. Fish with an advanced stage of gonadal development included females with ovaries that were vitellogenic or had ripe/hydrated eggs and males with testes in late spermatogenesis. Reproductive stage was determined by histological examination of the gonads as detailed in the methods. Data for years with fewer than five male or female sole collected at a site were excluded.

Chemical Analyses

Estrogenic Chemicals

Development and Validation of Method to Measure ECs in bile

We developed a LC-MS/MS standard operating procedure to analyze ECs in English sole bile that is a cost-effective method sufficient to evaluate the target analytes within acceptable limits for precision, accuracy, sensitivity, comparability, representativeness, and completeness. Following the methods outlined above we completed three steps to analyze for the presence of ECs in bile samples: 1) protein precipitation and enzymatic hydrolysis, 2) solid-phase extraction and 3) quantitative analysis by liquid-chromatography/tandem mass spectrometer (LC-MS/MS).

Within each batch of field samples, a method blank (water), a spiked blank and a spiked matrix (uncontaminated bile) were also extracted in order to assess extraction quality and accuracy. Surrogated standards were added in each field samples to calculate the targeted ECs and these standards recovery were measured to evaluate extraction efficiency for each sample. Several field sample replicates were analyzed. Within each batch injected in the LC-MS/MS, a calibration curve was run before the field samples. A continuing calibration solution was also injected at the beginning, between approximately 10-12 injections and at the end of the sequence to assess instrument stability during the analysis. As detailed in Appendix A, the EC data quality met the criteria for analysis of target analytes in bile samples outlined in the QAPP for this project (O'Neill et al. 2014) except for minor deviations that did not compromise the usability of the results. Below is a brief summary of the quality control check performed.

Calibrations

Continuing calibration verification standards were analyzed at the start, middle and end of the LC-MS/MS analytical sequence for each sample set and the results met our continuing calibration criteria

Method Blank Analysis

A method blank was analyzed for ECs with each sample set. Laboratory criteria for method blanks were met for each sample set.

Internal Standards and Surrogate Recoveries

Recoveries of internal standards and surrogates for the English sole bile samples and all quality assurance samples [method blank, spiked blank and matrix spike] associated with the analyses of these samples were within our laboratory criteria except a few analytes that were sometime recovered just outside the acceptable criteria range.

Sample Replicates

Bile samples from eleven English sole were analyzed in duplicate in the associated sample sets. Laboratory criteria for sample replicates were met for these replicated samples except for sample a sample from the Duwamish (the relative percent difference for BPA was 63, just about the $\leq$ 50% criteria.

Spiked Solvent Blanks and Matrix Spike Blanks

The percent recoveries of the EC and xenoestrogens in the coho salmon bile matrix spike ranged from 36 – 129%, with lower recoveries of E3 (ranging from 36 – 64%) in the matrix spike compared to the other analytes. The percent recoveries ranged from 51 – 132% in the solvent spiked blanks, with lower recoveries of E3 (ranging from 51 – 90%) compared to the other analytes.

Measures of Estrogenic Chemicals in Bile of English Sole Samples

The three natural estrogenic hormones were widely detected in bile of female and male English sole throughout Puget Sound. E2 and E1 were detected in 100% of the female and male English sole samples but E3 was detected less frequently, occurring in 0-75% and 0-100% of the female and male sole from each site (Table 3). The nonsteroidal xenoestrogens BPA and tOP were detected in English sole but less frequently than the natural estrogenic hormones; however, nOP and nNP were never detected. Although the natural hormones were widely detected, the synthetic hormone EE2 was never detected (< 0.68 - < 2.5 ng/mL bile). EE2 has a relatively high octanol/water partition coefficient and low water solubility compared to the naturally occurring estrogens (Kai et al. 2000) and has been hypothesized to partition more readily into the particulate and sludge component during sewage treatment (Vega-Morales et al. 2013). Consequently, EE2 is often only detected in wild fish in close proximity to concentrated effluent. For example, Fenlon et al. detected EE2 in bile of caged roach (*Rutilus rutilus*) held downstream, but in close proximity to WWTP effluent whereas EE2 was not detected or detected at a lower concentration (< 0.4 – 1.7 ng/mL) in wild roach in the same river.

Overall, the greatest concentrations of natural estrogens were measured in female English sole bile from the Seattle Waterfront. The range of biliary concentrations of natural estrogens in females ranged from 3 -26000 ng/mL for E2, considerably higher than E1 (4.4 – 2400 ng/mL) and E3 (<LOQ – 550 ng/mL) and the highest maximum concentration was observed at the Seattle Waterfront. This is likely due, at least in part, to the more advanced reproductive stage of females at the Seattle Waterfront since endogenous estrogen levels peak during vitellogenesis and remain relatively high until spawning. In contrast, the maximum concentrations measured in female English sole from other sites were 660 ng/mL for E2 (Sinclair Inlet), 62 ng/mL for E1 (Sinclair Inlet), and 5.6 ng/mL for E3 (Nisqually Reach; Table 3). The median concentration of these hormones varied significantly among sites except for E3 in females ($p < 0.0001$ for all comparisons), with concentration of E2 and E1 in female sole from the Seattle Waterfront, which is adjacent to Puget Sound's most highly developed watershed, statistically higher than those measured at in female sole in the Duwamish and Tacoma City Waterway. However, most sites had statistically similar concentrations of natural estrogens (Table 3, Figure 5 A-C). For example, while the greatest median concentration was exhibited by female English sole at the Seattle Waterfront location, almost 54 times greater than Nisqually Reach, the site with the next highest concentration, the difference proved to be not statistically significant based on a Kruskal-Wallis ANOVA of ranked concentrations ($p>0.05$) because of the high degree of variability among samples. Likewise, median concentrations of E1 and E3 in female sole from Seattle Waterfront were also 7 and 10 times those measured at Nisqually Reach, but not significantly different (Figure 5A-C).

Biliary concentrations of natural estrogens were also highest in male English sole from the Seattle Waterfront. E2 and E1 in male sole ranged from 1.0 – 1400 and 1.0 – 870 ng/mL, similar to each other but higher than the concentrations of E3 (<LOQ – 260 ng/mL; Table 3) and for each of these ECs, the

Table 3. Summary statistics for estrogenic chemicals (ECs) measured in the bile of English sole (ng/mL bile) collected in 2011 and 2013 from ten Puget Sound sites with varying levels of development. EE2 was never detected and therefore not shown in this table. Chemicals are arranged in order of decreasing estrogenicity per Vega-Morales et al. (2013).

			Low Development Site				*Moderate Devlopment Site*		*High Development Site*			
	EC (sex)	*Summary Statistic*[a]	Strait of Georgia	Vendovi Island	Hood Canal	Nisqually Reach	Port Gardner	Eagle Harbor	Seattle Waterfront	Duwamish River	Sinclair Inlet	Tacoma City Waterway
Decreasing Estrogenicity	E2 (male)	N	4	3	16	5	13	10	21	11	10	10
		% Detected	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%
		Median	***13***	***12***	***4.5***	***29***	***12***	***6.4***	***380***	***4.1***	***15***	***4.7***
		25th Percentile	4.8	11	3.1	14	4.6	5.4	74	2.6	5.9	2.8
		75th Percentile	25	40	6.6	296	24	9.0	860	8.4	18	6.3
		Mean	14	21	5.0	130	20	7.3	481	19.5	14	5.3
		Minimum	2.7	11	1.4	12	1.0	4.9	15	1.8	3.4	1.5
		Maximum	28	40	10	560	90	12	1400	170	26	15
	E2 (female)	N	11	8	8	8	7	7	5	7	12	5
		% Detected	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%
		Median	***28***	***39***	***14***	***55***	***6.1***	***11***	***3000***	***6.7***	***32***	***4.6***
		25th Percentile	15	21	11	29	4.9	7.3	24	5.4	17	2.1
		75th Percentile	67	50	24	69	20	18	16950	9.1	56	7.5
		Mean	76	45	17	52	12	12	7390	8.5	84	4.8
		Minimum	7.0	13	10	19	3.0	5.1	12	5.1	9.5	1.3
		Maximum	390	130	30	99	25	20	26000	18	660	9.5
	E1 (male)	N	4	3	16	5	13	10	22	11	10	10
		% Detected	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%
		Median	***7.3***	***14***	***5.0***	***18***	***5.9***	***6.2***	***235***	***6.1***	***15***	***4.3***
		25th Percentile	4.5	11	3.8	9.4	3.2	4.3	91	4.5	6.3	2.7
		75th Percentile	11	32	7.6	195	16	8.0	573	8.7	23	6.0
		Mean	7.7	19	5.5	85.3	9.7	6.4	329	19	15.4	4.4
		Minimum	4.0	11	1.8	6.7	1.0	3.3	7.7	3.0	5.4	1.4
		Maximum	12	32	12	360	28	11	870	150	28	8.6
	E1 (female)	N	11	8	8	8	7	7	5	7	12	5
		% Detected	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%
		Median	***6.1***	***13***	***5.3***	***19.5***	***3.7***	***5.4***	***130***	***4.4***	***16***	***2.5***
		25th Percentile	3.8	7.5	3.9	8.4	1.8	4.8	7.1	2.7	12	1.5
		75th Percentile	18	17	8.0	21	6.9	9.8	1555	6.3	22	4.3
		Mean	9.4	15	6.1	16	4.4	6.5	651	4.5	22	2.8
		Minimum	3.3	4.4	3.8	5.8	1.7	4.2	4.1	1.4	11	1.1
		Maximum	26	43	11	24	8.5	10	2400	8.0	62	4.4

Continued…

Table 3. continued.

	EC (sex)	Summary Statistic [a]	Low Development Site: Strait of Georgia	Vendovi Island	Hood Canal	Nisqually Reach	Moderate Devlopment Site: Port Gardner	Eagle Harbor	High Development Site: Seattle Waterfront	Duwamish River	Sinclair Inlet	Tacoma City Waterway
Decreasing Estrogenicity	E3 (male)	N	4	3	16	5	13	10	21	11	10	10
		% Detected	25%	100%	6%	60%	46%	10%	95%	9%	40%	0%
		Median	***0.63***	***2.1***	***0.37***	***2.1***	***0.86***	***0.58***	***22***	***0.50***	***0.65***	***nc***
		25th Percentile	0.49	1.9	0.37	0.66	0.42	0.42	7.9	0.41	0.37	nc
		75th Percentile	2.4	2.2	0.47	17	2.0	0.85	61	0.65	1.4	nc
		Mean	1.2	2.1	0.48	7.3	1.2	0.62	47	2.0	1.1	nc
		Minimum	<0.47[b]	1.9	<0.37	<0.37	<0.37	<0.37	<1.1	<0.37	<0.37	<0.34
		Maximum	3.0	2.2	1.4	29	2.7	1.0	260	17	4.7	<0.47
	E3 (female)	N	11	8	8	8	7	7	5	7	12	5
		% Detected	27%	75%	38%	75%	14%	14%	60%	0%	58%	0%
		Median	***0.37***	***2.1***	***0.70***	***2.9***	***0.55***	***0.50***	***30.0***	***nc***	***1.2***	***nc***
		25th Percentile	0.37	0.84	0.38	1.0	0.37	0.37	0.57	nc	0.50	nc
		75th Percentile	1.2	3.2	1.1	5.1	0.55	0.85	307	nc	2.6	nc
		Mean	1.2	2.1	1.0	2.9	0.7	0.63	129	nc	1.8	nc
		Minimum	<0.37	<0.47	<0.37	<0.37	<0.37	<0.34	<0.44	<0.37	<0.37	<0.37
		Maximum	5.0	3.8	3.4	5.6	2.0	1.2	550	<0.70	4.9	<0.70
	bisphenol A (male & female)	N	16	11	24	13	20	17	27	18	22	15
		% Detected	27%	27%	8%	46%	40%	53%	89%	100%	82%	87%
		Median	***3.6***	***2.8***	***2.3***	***5.5***	***3.6***	***6.0***	***12***	***36***	***19***	***12***
		25th Percentile	2.3	2.3	2.3	2.7	2.7	2.5	8.0	20	7.8	7.1
		75th Percentile	5.9	9.2	3.9	11	7.6	12	17	66	49	36
		Mean	5.0	5.5	3.3	6.5	5.4	7.5	13	47	37	21
		Minimum	<2.3	<2.3	<2.3	<2.3	<2.3	<2.0	<2.3	8.2	<2.3	<2.3
		Maximum	13	17	7.5	13	13	17	28	140	160	69
	tOP (male & female)	N	10	6	19	7	12	8	26	10	15	13
		% Detected	0%	0%	0%	15%	5%	6%	74%	17%	23%	13%
		Median	***nc***	***nc***	***nc***	***15***	***15***	***15***	***59***	***16***	***15***	***15***
		25th Percentile	nc	nc	nc	15	15	15	36	15	15	15
		75th Percentile	nc	nc	nc	41	17	19	92	34	37	17
		Mean	nc	nc	nc	28	19	18	70	36	24	20
		Minimum	<15	<15	<15	<15	<15	<13	<15	<15	<15	<13
		Maximum	<19	<19	<19	79	52	33	220	180	61	57

[a] Half LOQs were used as the non-detected values to calculate summary statistics.

[b] < indicates the value was not detected. The number listed is half the LOQ value.

nc Not calculated

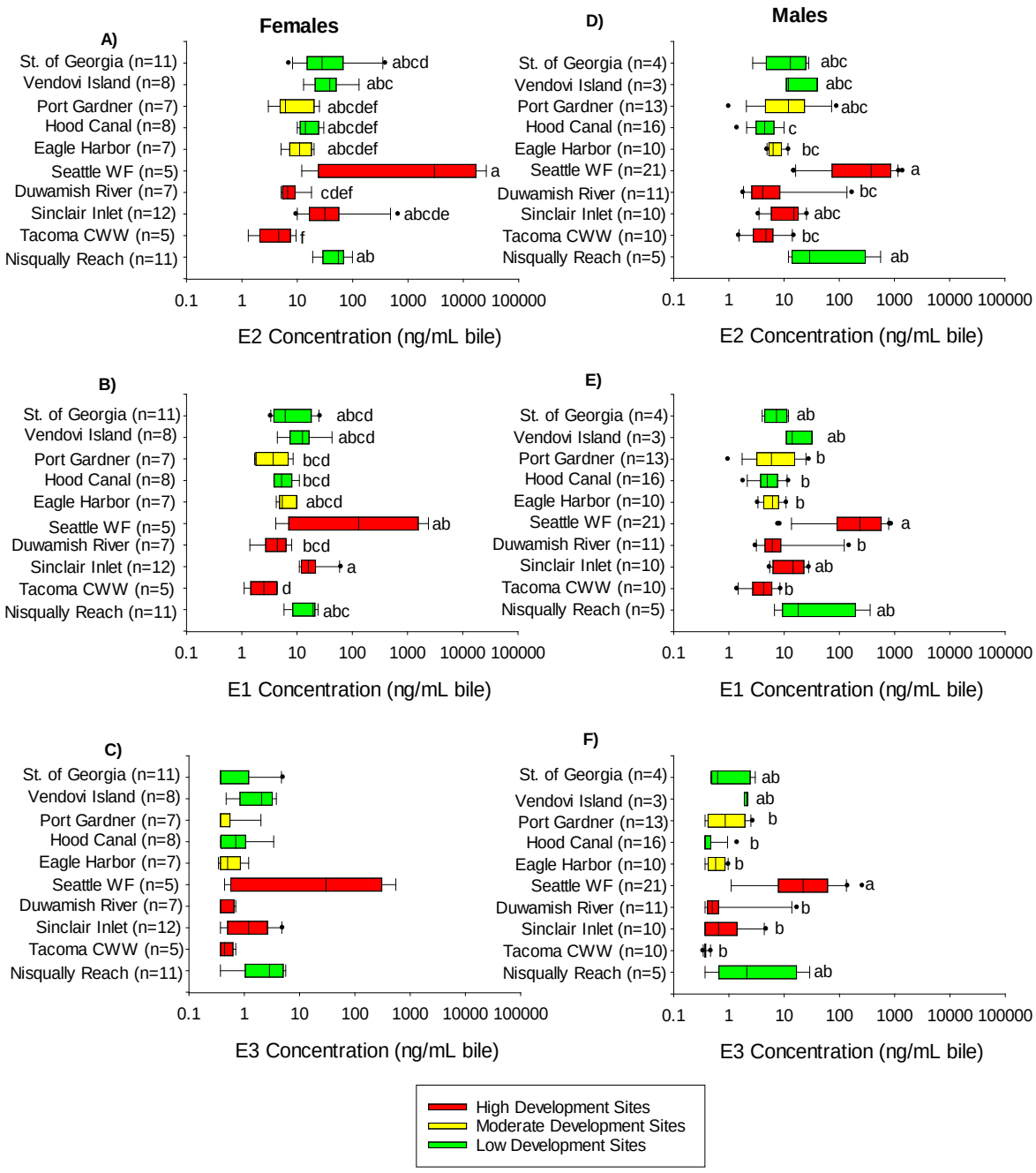

Figure 5. Concentrations of three natural estrogens measured in bile of female (A - C) and male (D - F) English sole collected in 2011 and 2013 from ten Puget Sound sites with varying levels of development. Within sex for each EC, similar lower case letters signify no significant difference among sites ($p > 0.05$). Note log scale on horizontal axis. WF = Waterfront, CWW = City Waterway.

highest maximum concentrations were observed at the Seattle Waterfront. As was observed for females, male sole from Seattle Waterfront had higher median concentrations of E1, E2, and E3 than most other sites, although not always significantly higher (Figure 5D-F). For example, median concentrations of E1, E2, and E3 in male sole from Seattle Waterfront were 10 - 13 times higher than male fish at Nisqually Reach, the site with the next highest median concentrations (Figure 5C-E), but the two sites were statistically indistinguishable ($p>0.05$) due to the high degree of variability among samples.

English sole from high-development sites as a group tended to have elevated levels of natural estrogens relative to moderate and low-development sites, however, this pattern was more pronounced for males than females. Among males, median concentrations of natural estrogen at the pooled high-development sites as a group were almost double those measured at the moderate and low development sites, however, this difference was only statistically significant for E1 (Kruskal-Wallis ANOVA post-hoc test; $p < 0.05$, Table 4). Among females, median concentrations of E1 were almost twice that measured at moderate and low-development sites, but not statistically different among sites ($p= 0.053$). Levels of E2 and E3 were not elevated in female English sole from pooled high development sites compared to moderate and low-development sites (Table 4). Furthermore, females from the low development sites as a group had significantly higher concentrations of E2 than females from moderate and high development sites (Table 4).

In contrast to the natural estrogens, levels of BPA in English sole were not elevated at the Seattle Waterfront, but were typically higher in sole collected from other high-development sites (i.e., the Duwamish Waterway, Sinclair Inlet, and Tacoma City Waterway). Biliary concentrations BPA in English sole ranged from, <LOQ - 160 ng/mL (Table 3). Median concentrations of BPA in sole were highest in fish from the Duwamish, but not significantly higher than the sole from other high-development sites (Figure 6A). Among high-development sites, BPA was detected at higher frequencies and the median BPA concentrations ranged from 12 - 36 ng/mL, but were less than $\leq$ 6.0 ng/mL at sites with moderate and low development (Table 3). Highly developed sites pooled as a group had a statistically higher median biliary concentration of BPA than pooled moderate and low-development sites (17, 5 and 3 ng/mL respectively; Table 4, Figure 7A).

Similar to natural estrogens, English sole from the Seattle Waterfront were also exposed to higher concentrations of tOP than sole from other locations. Biliary concentrations of tOP in English sole ranged from, <LOQ -220 ng/mL (Table 3). The highest median tOP concentration was observed in fish collected from the Seattle Waterfront (59 ng/mL), but was not significantly higher than the median tOP concentration in fish from the Nisqually Reach, the only low development sites with detectable concentrations of tOP. Fish from high-development sites had detectable tOP more frequently and the fish from high- development sites when pooled as a group also had higher median biliary concentrations of tOP than fish from low-development sites when pooled as a group, but not significantly higher than levels in fish from pooled moderate-development sites (Figure 7B).

Overall, levels of biliary natural estrogens and synthetic non-steroidal ECs in English sole exhibited high variability within sites and within sexes. This, combined with highly skewed concentrations

necessitating the use of less sensitive statistical models (ANOVAs of ranked data), resulted in low power overall. This points to the need for higher sample sizes, to better understand and model the distribution of the data, and to overcome what appears to be inherently highly variable contamination condition.

Table 4. Summary statistics for concentrations of estrogenic chemicals (ECs) measured in bile of English sole (ng/mL bile) collected in 2011 and 2013 from Puget Sound sites, pooled according to level of nearby watershed development. EC analytes are listed in order of decreasing estrogenicity (Vega-Morales et al., 2013) and all results are grouped by sex. Within sex for each EC, similar lower case letters signify no significant difference (p > 0.05) among site-development type.

	EC (gender)	*Summary Statistic* [1]	Low Development Sites	Moderate Devlopment Sites	High Development Sites
Decreasing Estrogenicity	E2 (male)	N	28	23	52
		% Detected	100%	100%	100%
		Median	***7.3 a***	***8.5 a***	***15 a***
		25th Percentile	3.4	5.2	4.4
		75th Percentile	14	13	248
		Mean	30	14	202
		Minimum	1.4	0.99	1.5
		Maximum	560	90	1400
	E2 (female)	N	35	14	29
		% Detected	100%	100%	100%
		Median	***29 a***	***10 b***	***14 b***
		25th Percentile	15	5.8	6.7
		75th Percentile	55	19	36
		Mean	50	12	1312
		Minimum	7.0	3.0	1.3
		Maximum	390	25	26000
	E1 (male)	N	28	23	53
		% Detected	100%	100%	100%
		Median	***6.4 b***	***5.9 b***	***11 a***
		25th Percentile	4.1	3.6	5.6
		75th Percentile	12	11	200
		Mean	22	8.3	144
		Minimum	1.8	0.96	1.4
		Maximum	360	28	870
	E1 (female)	N	35	14	29
		% Detected	100%	100%	100%
		Median	***7.4 a***	***5.2 a***	***11 a***
		25th Percentile	4.4	3.4	4.2
		75th Percentile	18	7.3	18.5
		Mean	12	5.4	123
		Minimum	3.3	1.7	1.1
		Maximum	43	10	2400

Continued…

Table 4 (continued)

	EC (gender)	*Summary Statistic* [1]	Low Development Sites	Moderate Devlopment Sites	High Development Sites
Decreasing Estrogenicity	E3 (male)	N	28	23	52
		% Detected	29%	30%	50%
		Median	***0.47 a***	***0.6 a***	***0.88 a***
		25th Percentile	0.37	0.44	0.37
		75th Percentile	1.8	1.2	19
		Mean	2.0	0.92	20
		Minimum	<0.37[2]	<0.37	<0.34
		Maximum	29	2.7	260
	E3 (female)	N	35	14	29
		% Detected	51%	14%	34%
		Median	***0.92 a***	***0.53 a***	***0.65 a***
		25th Percentile	0.37	0.37	0.37
		75th Percentile	2.9	0.78	2.3
		Mean	1.7	0.66	23
		Minimum	<0.37	<0.34	<0.37
		Maximum	5.6	2.0	550
	bisphenol A (male & female)	N	64	37	82
		% Detected	25%	46%	89%
		Median	***3.0 b***	***5.0 b***	***17 a***
		25th Percentile	2.3	2.6	8.9
		75th Percentile	6.0	9.3	35
		Mean	4.7	6.4	28
		Minimum	<2.3	<2.0	<2.3
		Maximum	17	17	160
	tOP (male & female)	N	42	20	64
		% Detected	5%	10%	47%
		Median	***15 b***	***15 a***	***17 a***
		25th Percentile	15	15	15
		75th Percentile	15	17	58
		Mean	18	18	44
		Minimum	<15	<13	<13
		Maximum	79	52	220

[1] Half LOQs were used as the non-detected values to calculate summary statistics.

[2] < indicates the value was not detected. The number listed is half the LOQ value.

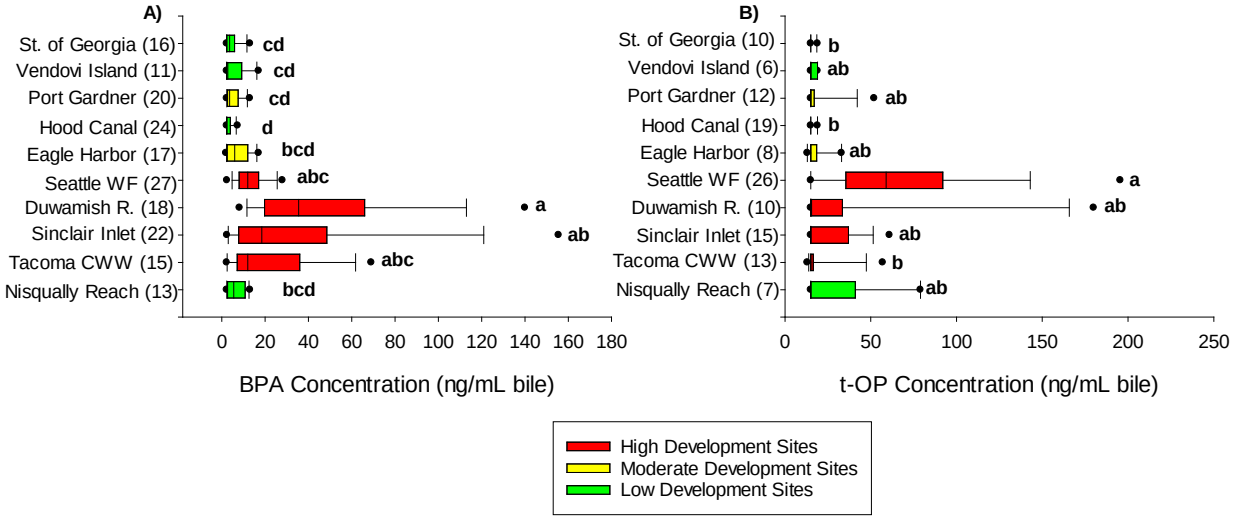

Figure 6. Comparison of (A) bisphenol A (BPA) and (B) tert-octylphenol (tOP) measured in bile of English sole (females and males combined) collected in 2011 and 2013 from ten Puget Sound sites with varying levels of development. For each chemical, similar lower case letters signify no significant difference among sites (p > 0.05).

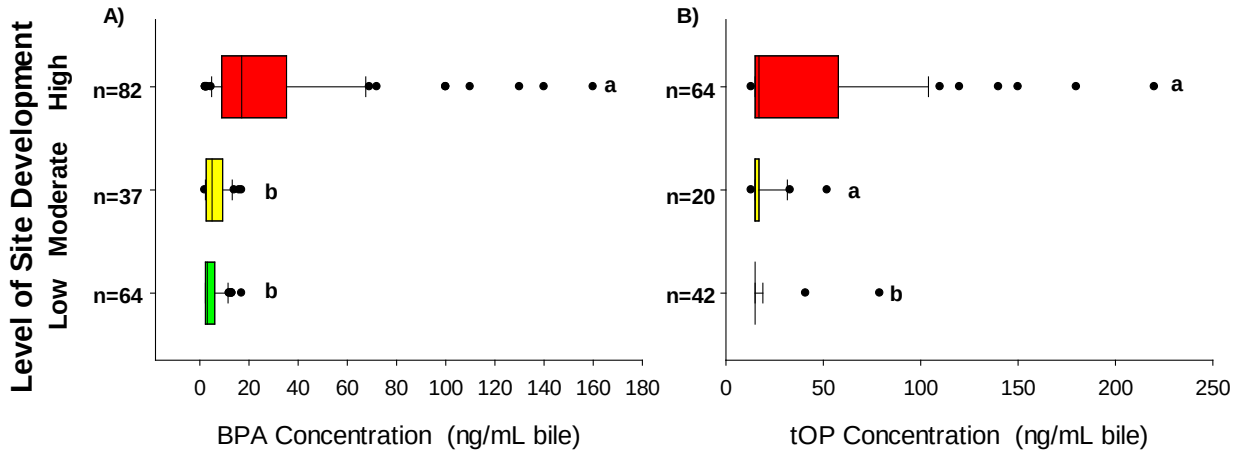

Figure 7. Comparison of BPA and tOP biliary concentration measured in English sole (females and males combined) collected in 2011 and 2013 from Puget Sound sites organized by high, moderate and low levels of site development. For each chemical, similar lower case letters signify no significant difference among sites (p > 0.05).

Circulating estrone (E1) has been reported in plasma of females of several fish species, including European flounder (*Platichthys flesus*; Budzinski et al. 2006), yellow perch (*Perca fluviatilis*; Noaksson et al. 2004), rainbow trout (*Oncorhynchys mykiss*; von Bohemen and Lambert 1981) and catfish (*Clarias batrachus*; Singh and Singh 1987) especially in early stages of vitellogenesis (van Den Belt et al. 2004), but levels are generally lower than levels of E2. Estriol (E3) production has also been reported in female fish (e.g., Ponthier et al. 1998), but the identity of this steroid was not confirmed (identity classified as 'probable') in this study and levels were extremely low. Hence the presence of E1 and possibly E3 may

be expected in wild, free-ranging female fish, albeit at low levels compared to E2. Although the median concentration of these three estrogens generally followed this pattern for all levels of site development, maximum E2 concentrations were observed in three female sole from one high-development site (Seattle Waterfront – Table 4), approximately 67x greater than the maximum E2 in sole from sites with lower development. Natural production of E2 has been reported in plasma of male fish, although at very low concentrations (e.g. Mayer et al. 1989), however the production of these estrogens in male fish are well below concentrations in female fish (Fostier et al. 1983; Pankhurst and Carragher, 1991; Kime 1993; Houtman et al. 2004; Sisneros et al. 2004).

The patterns of EC concentration measured in English sole support a conclusion that the majority of ECs were derived from exogenous (environmental) sources. For synthetic, non-steroidal ECs, which are always from an exogenous source, the concentration of BPA was highly correlated between female and male across all sites with a slope near one, indicating similar exposure between the sexes (Figure 8, Table 5). The range of tOP concentrations was too low to identify an unambiguous slope, however the male:female tOP relationship appeared to follow the same pattern as BPA.

For the natural hormones, E1 and E3, in both male and female English sole and E2 in male fish were also likely derived from exogenous sources, whereas E2 in females are likely from a mixture of exogenous and endogenous sources. Similarly to the BPA, we observed a high correlation of E1 and E3 between male and female English sole with a slope near to 1, indicating an unexpected parity of these two natural estrogens between the sexes. The simplest explanation for this condition is a common exogenous environmental source for these chemicals (Figure 8, Table 5). Data on endogenous concentrations of E1 and E2 are very limited in male fish, but are generally very low, well below concentrations in female fish (e.g. Pettersson et al. 2007, Fenlon et al. 2010). For example, Fenlon et al. (2010) documented that the biliary concentrations of E1 in male roach reared in dechlorinated tap water, free of exogenous natural estrogens, ranged from 2.8 - 9.1 ng/mL bile, two to five times lower than the concentrations of 15.3 ng/mL measured in female roach.

The correlation of E2 between the sexes exhibited a slope significantly lower than 1 across all site-development types (i.e., 0.53, Table 5), almost half of that observed between the sexes for E1 and E3, indicating much higher E2 concentration in female than male fish – a much different pattern than E1 and E3. We assumed that much of the E2 in male English sole in our study were was from an exogenous source as the concentrations we measured in bile were generally lower than those observed for male roach reared in dechlorinated tap water free of exogenous source of natural hormones (Fenlon et al. 2010). Moreover, the concentrations of E2 measured in male fish in our study were more similar or higher than the concentrations observed in caged roach exposed to WWTP effluent (Fenlon et al. 2010). Based on the modeled relationship between E2 in female and male English sole in our study (Table 5), at sites where the female fish had 1000 ng/mL of E2, males would have approximately 110 ng/mL, well above levels typically observed in male fish not exposed to exogenous sources of natural estrogens. However, for females, a combination of endogenous and exogenous sources likely contributed to the observed levels of E2, especially when samples included prespawning females.

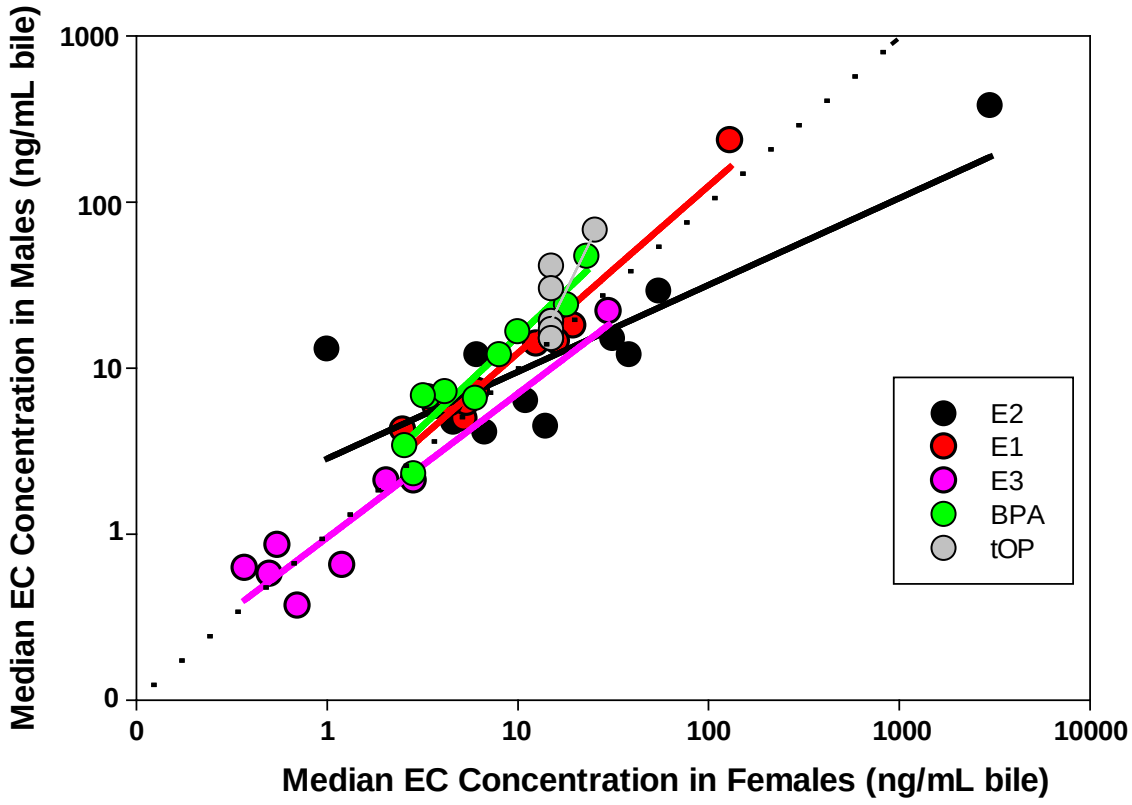

Figure 8. Comparison of median concentrations of estrogenic chemicals measured in female and male English sole from the same site. Regression coefficients are listed in Table 5.

Table 5. Result for the linear regression comparing concentrations of estrogenic chemicals (ECs) in female and male English sole from the same site.

EC Regression Model				Estimated			
[log EC male = a(log EC female) +y0]	N	r^2	Factor	Coefficient	Std. Error	t-value	p-value
E2	10	0.66	slope	0.523	0.122	4.274	**0.003**
			intercept	0.456	0.190	2.403	**0.043**
E1	10	0.95	slope	1.005	0.079	12.660	**<0.001**
			intercept	0.086	0.085	1.019	0.338
E3	8	0.89	slope	0.868	0.116	7.480	**<0.001**
			intercept	-0.020	0.069	-0.293	0.780
BPA	10	0.88	slope	1.085	0.134	8.074	**<0.001**
			intercept	0.109	0.114	0.962	0.364
tOP	10	0.53	slope	2.340	0.704	3.329	**0.010**
			intercept	-1.462	0.845	-1.730	0.122

Although the levels of ECs were highly correlated between male and female English sole across sites, exposure to BPA appears to be more variable than that for the natural hormones and tOP, possibly due to different environmental sources. Within each sex, the concentrations of E1, E3, and tOP were correlated with E2 concentrations, but the levels of BPA were not (Figure 9, Table 6.), indicating a potentially different environmental source for BPA. Puget Sound receives both untreated and treated sewage and moreover, WWTPs in the region use varying processes to treat sewage effluent (Lubliner et al. 2010). Depending on the wastewater treatment process used in a particular location, there could be considerable variation in the amount of BPA, naturally produced hormones, and tOP removed from sewage effluent. Overall, the high correlation between E2 and, both E1 and E3 within female (r^2 = 0.873 and 0.762) and within male (r^2 = 0.936 and 0.860) English sole across all locations suggests that these ECs are consistently discharged from WWTPs. In contrast, BPA is not correlated with levels of E2 in female (r^2 = 0.013) or male fish (r^2 = 0.015), suggesting that the BPA is not consistently discharged from WWTPs.

Collectively, the overall estrogenic activity of all EC combined, estimated as total E2 equivalent concentrations (EEQs) from the combined concentrations of natural hormones and nonsteroidal xenoestrogens, indicate English sole from Seattle Waterfront experienced the highest estrogen exposure. EEQs were estimated for each fish by summing the E2 equivalent factors (EEFs) for each EC measured in the bile. The EEFs for each EC were based on average values reported by Vega-Morales et al. (2013). The median EEQ in female and male sole from Seattle Waterfront were 3,000 and 410 ng/mL bile, 52 and 12 times those measured in female and male sole from Nisqually, the site with the next highest median EEQs (Figure 10). The majority of EEQ estrogenicity was accounted for by E2, which has an EEF of 1, the highest EEF of any compound we detected. Estrogenicity of E1 and E3 were reported as roughly one-tenth of E2 (both with an EEF of 0.11); tOP and BPA contributed little to the overall EEQ with EEFs of 0.00021 and 0.00039.

The higher EEQs in sole from the Seattle Waterfront were likely associated with higher exposure to sewage effluent. As discussed previously, the majority of the E1 and E3 and some of the E2 measured in female sole, and the majority of E1, E2, and E3 measured in male English sole were likely from an exogenous source. Although major sources of the naturally occurring hormones include wastewater and animal waste (Ying et al. 2002), large agricultural facilities that produce animal waste do not operate near or drain into Elliott Bay, where the Seattle Waterfront site is located. However, multiple outfalls deliver treated and untreated sewage to Elliott Bay from the Seattle landscape, the largest metropolis in the Puget Sound region. Naturally produced hormones have been shown to be the major contributors to the estrogenic activity observed in sewage effluents and receiving water (Desbrow et al. 1998, Rodgers et al. 2000, and Aerni et al., 2004).

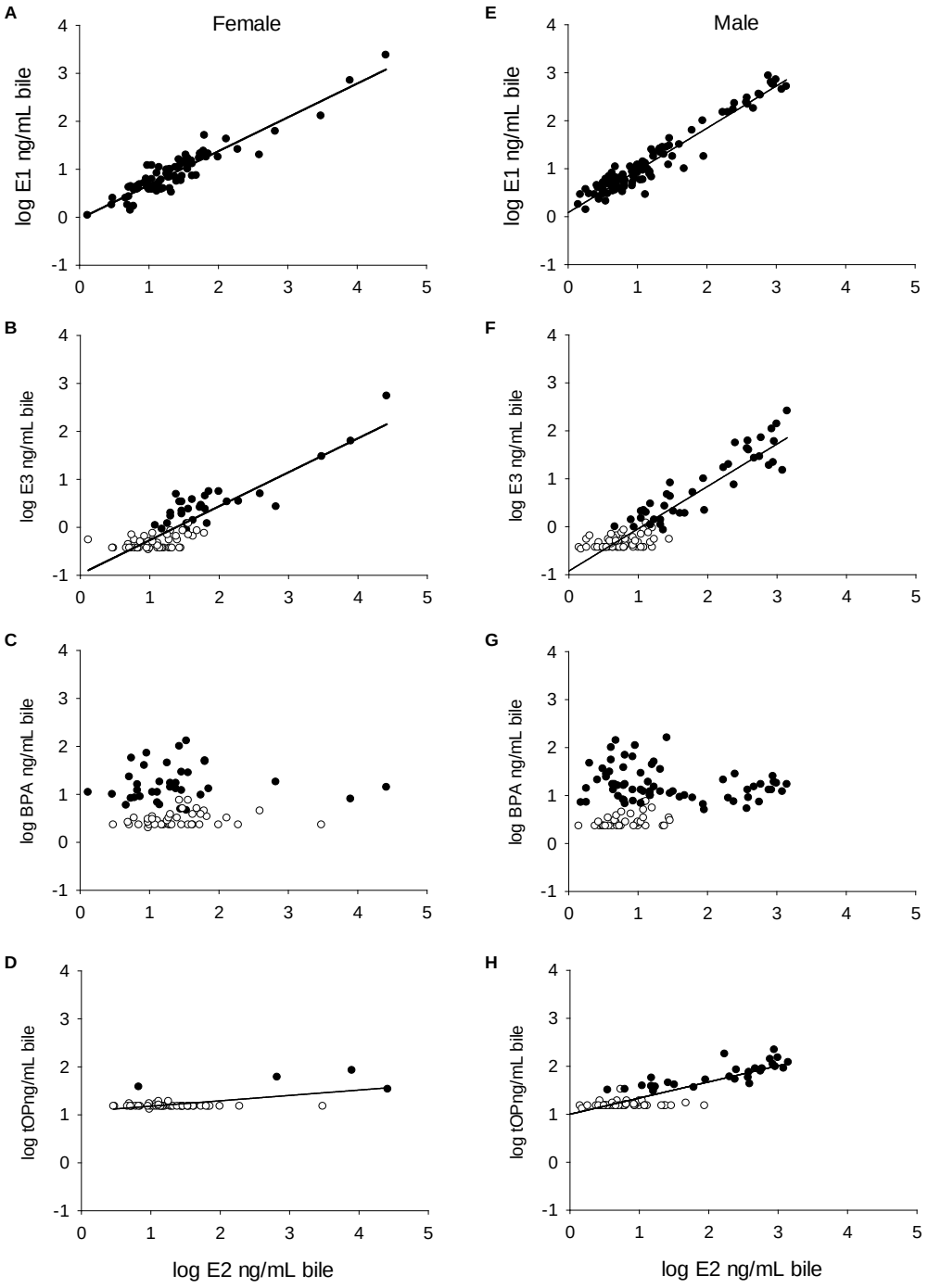

Figure 9. Relationship between estradiol (E2) and other estrogenic chemicals (ECs) in female (A-D) and male (E-H) English sole. Open circles were used for non-detected ECs, with 0.5 LOQ values. Regression coefficients are listed in Table 6.

Table 6. Result for the linear regression comparing concentrations of estrogenic chemicals (ECs) in female and male English sole from the same site.

EC Regression Mode				Estimated			
[log EC = a(log E2) +y0]	N	r^2	Factor	Coefficient	std-err	t-value	p-value
E1 in female sole	78	**0.873**	slope	0.705	0.031	23.057	**<0.001**
			intercept	-0.032	0.048	-0.667	0.507
E3 in female sole	78	**0.762**	slope	0.708	0.045	15.724	**<0.001**
			intercept	-0.973	0.070	-13.929	**<0.001**
BPA in female sole	78	-0.013	slope	0.007	0.077	0.092	0.927
			intercept	0.805	0.120	6.716	**<0.001**
tOP in female sole	50	0.328	slope	0.112	0.023	4.994	**<0.001**
			intercept	1.067	0.036	29.458	**<0.001**
E1 in male sole	103	0.936	slope	0.880	0.023	38.762	**<0.001**
			intercept	0.086	0.032	2.655	**0.009**
E3 in male sole	103	0.860	slope	0.883	0.035	25.041	**<0.001**
			intercept	-0.920	0.050	-18.301	**<0.001**
BPA in male sole	103	0.015	slope	0.094	0.059	1.609	0.111
			intercept	0.856	0.084	10.225	**<0.001**
tOP in male sole	74	0.747	slope	0.337	0.023	14.704	**<0.001**
			intercept	1.001	0.036	27.865	**<0.001**

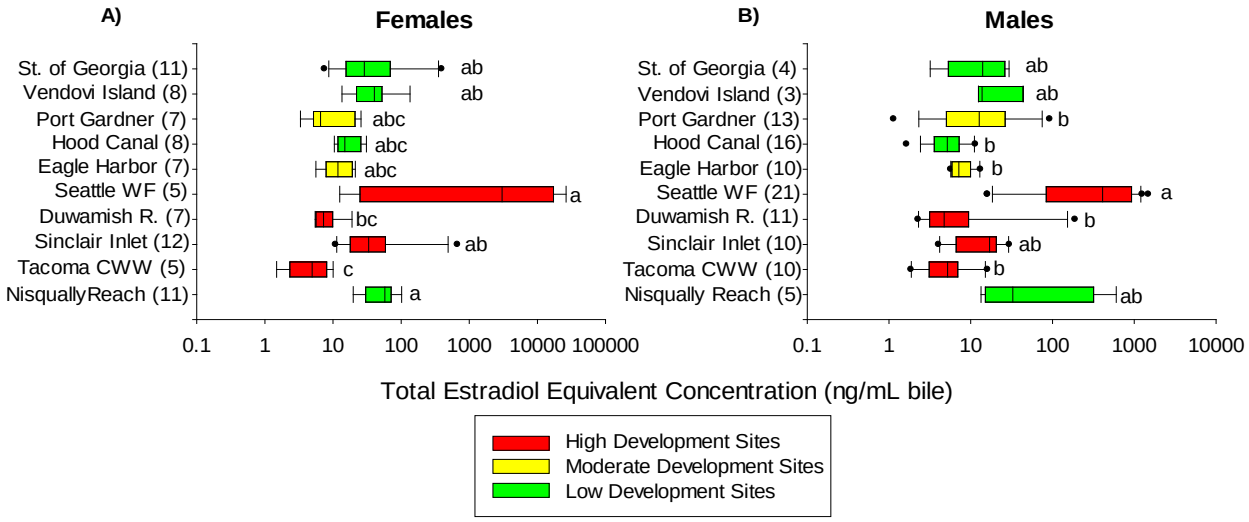

Figure 10. Comparison of Total Estradiol Equivalent Concentration (ng/mL bile) measured in female (A) and male (B) English sole from Puget Sound sites with varying degrees of development in 2011 and 2013. Within sex similar lower case letters signify no significant difference among sites (p > 0.05). WF = Waterfront, CWW = City Waterway

From the mid-2000s to 2013, levels of E2 in English sole from the Seattle Waterfront and Nisqually Reach, two of the three sites where statistical comparisons were possible, appeared to have increased (Figure 11). In contrast, biliary BPA concentrations measured in male sole from all five sites were not significantly different between the first sampling in 2005 (da Silva et al. 2013) and the current sampling in 2011-2013. However, normal fluctuations of E2 and BPA on a smaller time scale have not been measured yet; more observations are required to draw conclusions regarding time trends for these chemicals.

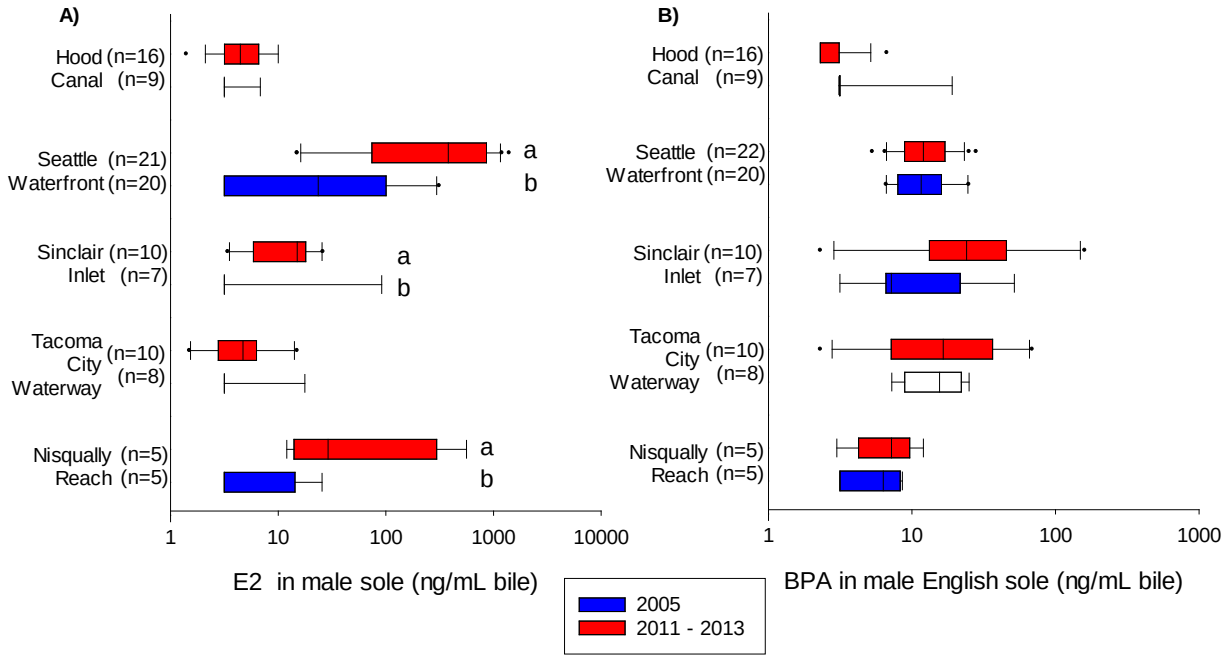

Figure 11. Temporal comparison of 17β- estradiol (E2) and bisphenol A (BPA) concentrations measured in bile of male English sole collected in 2005 (from de Silva et al. 2013) compared to 2011-2013. Letters ("a" and "b") are used to denote significantly different concentrations of E2 in bile of English sole at a site between two sampling times ($p < 0.05$). Biliary BPA concentrations at individual sites did not vary significantly between two sampling times ($p > 0.05$).

Selective Serotonin Reuptake Inhibitors

Development and Validation of Method to Measure of SSRIs in English sole

We defined a cost-effective method sufficient to evaluate the target analytes (i.e., fluoxetine, sertraline and citalopram) within acceptable limits for precision, accuracy, sensitivity, comparability, representativeness, and completeness. Following the methods outlined above we completed four steps to analyses for the presence of SRRI in liver tissue: 1) extraction, (2), cleanup (using a liquid-liquid cleanup method based on Eap et al., 1996), (3) derivatization with heptafluoro-butyrylimidazole and (4) quantification by GCMS with selected-ion monitoring. As an internal standard, hexadeuterated paroxetine (d6-paroxetine) was added during the extraction step. As detailed in Appendix A, the SSRI data quality met the criteria for analysis of target analytes in liver tissue outlined in the QAPP for this project (O'Neill et al. 2014) except for minor deviations that did not compromise the usability of the results. Below is a brief summary of the quality control results.

Calibration and Matrix Spiked Blanks

Initial matrix free calibration of the instrument for retention time was made using verified fluoxetine, sertraline, and citalopram standards, derivatized exactly as was done for sample extracts. Subsequent calibration of the instrument during sample analysis was done using actual English sole liver homogenate that was spiked with different amounts of the analytes. Thus, for this effort no distinction is made between "calibration standards" and "matrix spike standards" because they are the same. This approach to calibration was used because no suitable surrogate matrix for English sole liver tissue could be found (see Appendix A for details). Analyte concentrations were calculated using point-to-point calibration with at least four concentration levels of calibration standards.

Method Blank

All analytes met acceptance criteria for blanks.

Internal Standard and Surrogate Recoveries

Recoveries of the internal standard (hexa-deuterated paroxetine; d6-paroxetine) for the English sole liver samples were generally within laboratory criteria (recoveries between 50-150%). Most samples were also reconstituted in 100 µl of solvent, however, some samples (such as those from the Duwamish site and select samples from other sites) were intentionally reconstituted in a smaller volume (25-50 µl) to improve sensitivity to the analytes. Thus, for these samples, recoveries exceeded 150%. A few isolated samples (13 or 7% of total analyzed samples), were flagged as "AF" for analytical failure due to excessive moisture in the vials which affected the % recovery.

Sample Replicates

Sample replicates were not performed due to limited sample size for nearly all samples and the complete lack of detection of the analytes in the samples assayed.

Laboratory SSRI Study

The laboratory dosing study confirmed the method for this study was cost-effective and adequate to detect and measure SSRIs in English sole exposed to SSRIs in concentrations they might experience near WWTP outfalls in Puget Sound. Concentration of the three SSRI compounds, fluoxetine sertraline, and citalopram, and one metabolite (des-methyl sertraline) measured at the 12 and 23 day treatments were similar to each other for each of the four compounds, three tissue matrices and two exposure doses (Figure 12), indicating the sole reached equilibrium SSRI concentrations during the treatment. For example, fluoxetine concentrations measured in liver tissue of sole in the low dose experiment were similar for fish exposed for 12 and 23 days, (31 and 29 ng/g; Figure 12a). Likewise fluoxetine concentrations measured in liver tissue of sole in the high dose experiment were similar for fish exposed for 12 and 23 days (89 and 65 ng/; Figure 12 a). Two exceptions to this were high-exposure sertraline in liver and high-exposure des-methyl sertraline in trunk kidney, both of which exhibited higher concentrations in the longer 23 day exposure period.

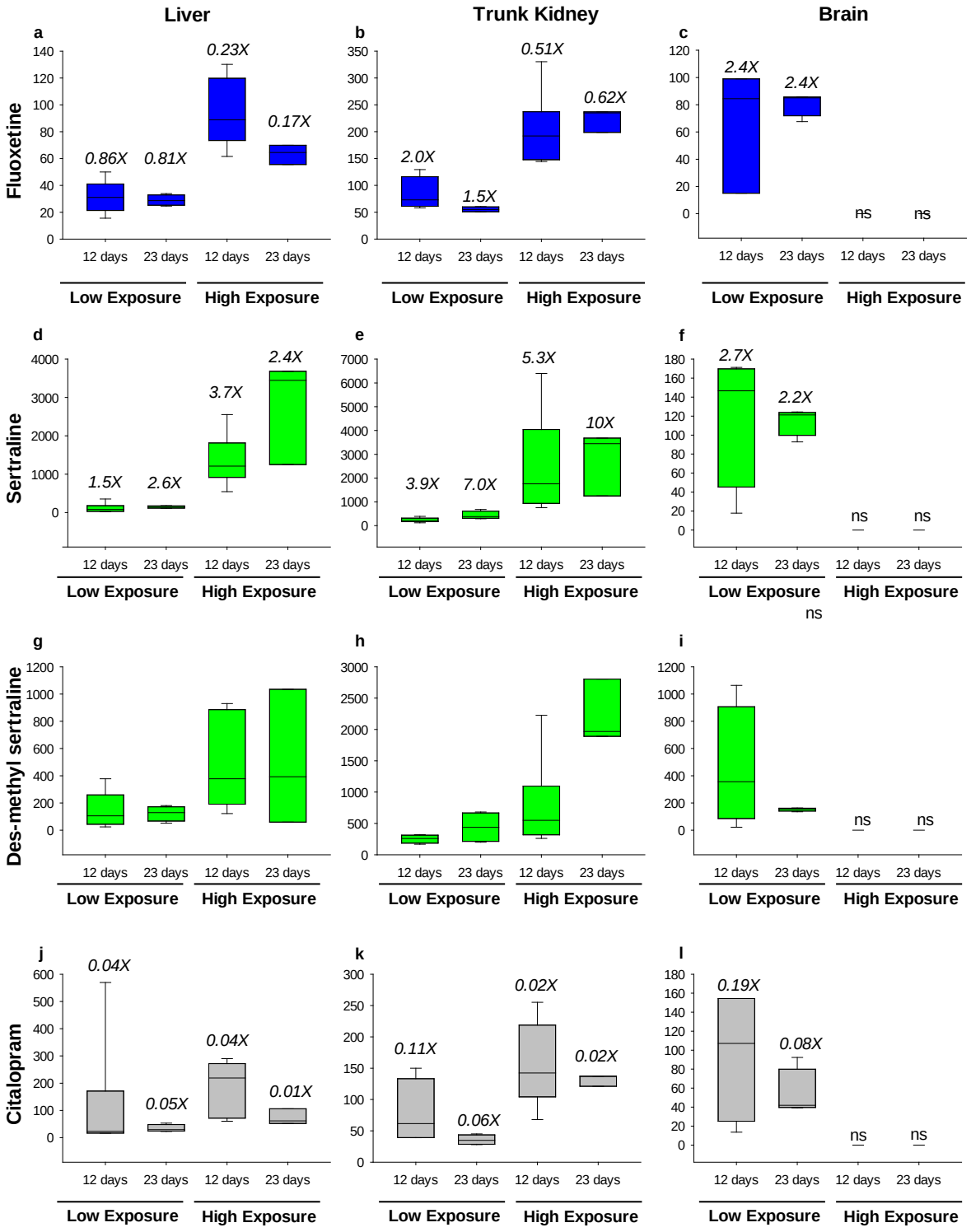

Figure 12. Comparison of selective serotonin reuptake inhibitors (SSRIs) accumulated in English sole tissue (concentration in ng/g wet wt) after a 12 and 23 day exposure to low and high water concentrations of SSRIs. Low and high concentrations approximate 1X and 10X the median concentration measured in effluent from waste water treatment plants in Puget Sound (see text for details). Numbers intalics above box plots are calculated biocentrations factors based on median concentrations in water and tissue in exposure treatments.

Results of the laboratory exposure study validated that the SSRI analytical methods used for this study was effective at detecting SSRIs in English sole because all three SSRIs were detected in liver, kidney and brain tissues for both the low and high exposure treatments (1X and 10X median effluent does; Figure 12). Overall, concentrations of the SSRIs measured in tissues of English sole were lowest for fluoxetine, intermediate for citalopram and highest for sertraline (Figure 12). Median concentrations of fluoxetine, citalopram and sertraline in sole tissues ranged 31 – 85, 23 - 110, and 80 – 380 ng/g, respectively, for the low dose exposure treatment and 65 – 240, 62 – 220, and 800 – 3500 ng/g, respectively, for the high dose exposure treatment.

Furthermore, SSRI concentrations detected in the high dose exposure treatment were always higher than those measured in the low dose treatment, further validating the method could detect SSRIs over an environmentally relevant and wide concentration gradient. Median concentrations of fluoxetine in the high dose treatment were 2 – 4 times higher than those measured in sole from the low dose treatment (Figure 12 a-c). Similarly, median citalopram concentrations in sole tissues were also 2 – 10 times in high dose than the low dose treatment (Figure 12j-l). The greatest difference in SSRI concentrations between the low and high dose treatments was observed for sertraline, with median concentration in the high dose treatment 6 – 15 times higher (Figure 12d-f).

For a given water concentrations, higher sertraline concentrations were accumulated in English sole tissue than fluoxetine and citalopram. Bio-concentration factors (BCFs), the ratio of a contaminant concentration in biota to its concentration in the surrounding water, for sertraline ranged 1.5 to 7X for the various tissue matrices and exposure treatments, based on median concentrations measured in tissues and waters (Figure 12, BCFs indicated above box plots). In contrast, the BCFs for fluoxetine and citalopram ranged from 0.17 – 2.4X and 0.01 – 0.19X. Sertraline is the most lipophilic of the three SSRIs we tested, a factor that likely contributed to its higher BCF. Consequently, sertraline was well absorbed compared to the other SSRIs. Median sertraline concentrations in sole tissues were considerably higher than the fluoxetine concentrations (1.5 to 7 times and 9 – 15 times for the low and high dose exposures), although the sole were exposed to similar median water concentrations of both SSRIs (55 vs. 36, and 330 vs. 380 ng/L for the low and high dose exposures). Citalopram was the least absorbed by English sole. Despite being exposed to median water concentrations of citalopram that were approximately 10 – 17 times higher than the sertraline (560 and 5700 ng/L for the low and high dose treatments), median citalopram concentrations in sole were generally about a tenth to a third of the sertraline concentrations for similar matrices and treatment exposures.

The exposure study confirmed all three tissues were suitable for measuring exposure to SSRIs, although the trunk kidney generally exhibited higher concentrations than brain or liver across most of the dosing scenarios. Citalopram was more variable across tissues, especially for dosing scenarios resulting in low citalopram concentration. Given that mass of brain tissue in these fish is small, liver and trunk kidney would likely be more desirable as monitoring tissues. Fluoxetine, sertraline, and citalopram concentrations in liver and trunk kidney were positively correlated between liver and trunk kidney (Figure 13, Table 7).

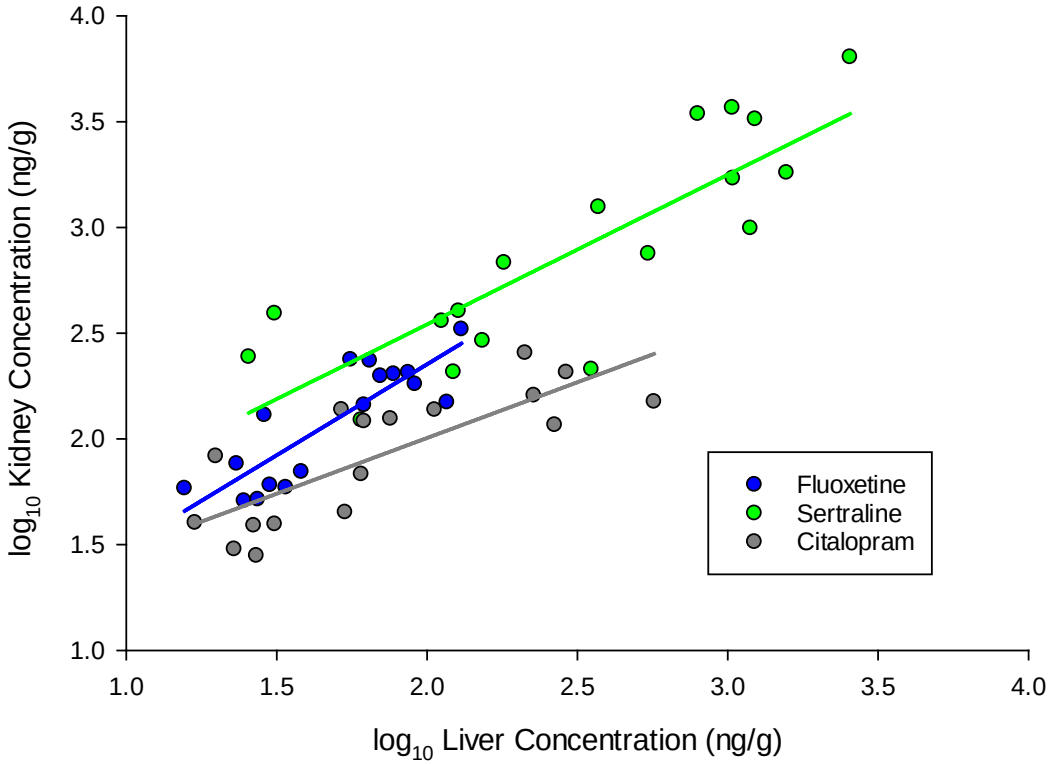

Figure 13. Comparison of selective serotonin reuptake inhibitor (SSRI) concentration accumulated in liver vs. kidney tissue of English sole after waterborne exposure to SSRIs.

Table 7. Result for the linear regression comparing concentrations of selective serotonin reuptake inhibitors (SSRIs) in liver and kidney tissue of laboratory exposed individual English sole. For each SSRI, data were pooled for sole in low and high exposure treatments.

SSRI Regression Model log [SSRI kidney] = (a*log [SSRI liver]) +y0]	N	r^2	Factor	Estimated Coefficient	std-err	t-value	p-value
Sertraline	18	0.70	slope	0.707	0.031	6.096	<0.001
			intercept	1.128	0.048	3.792	0.002
Fluoxetine	17	0.72	slope	0.861	0.138	6.232	<0.001
			intercept	0.631	0.235	2.680	0.02
Citalopram	17	0.64	slope	0.527	0.102	4.144	<0.001
			intercept	0.950	0.195	4.864	<0.001

Measures of SSRI in English sole Field Samples

No SSRI compound was detected in any English sole liver from any of the ten sites sampled. Limits of quantitation for fluoxetine, sertraline and citalopram in sole livers were 1.6, 1.9, and 9.2 ng/g,

respectively. Given the relatively high BCF we observed for sertraline we might have expected sertraline as the most likely SSRI to be detected in English sole, assuming similar exposure concentrations in Puget Sound. For example, based on the median concentrations in the 23-day low-dose laboratory exposure results for sertraline (55 ng/L), which approximated the median dose in WWTP, and assuming a 50X dilution of WWTP effluent near the outfall, the predicted environmental exposure to sertraline of 1-ng/L of seawater should be detected in liver tissue at a concentration of 2.8 ng/g. Comparable exposures to fluoxetine would also likely produce detectable levels due to better analytical sensitivity. Citalopram may be the most difficult SSRI to detect because of its low BCF, despite its higher environmental occurrence. Thus, our analytical method should have been able to detect fluoxetine and sertraline if the fish were close to WWTP outfalls and CSOs, but may have been less suitable for detecting SSRIs in fish outside the immediate dilution zone.

Meador et al. (2016) reported fluoxetine and sertraline in whole body juvenile Chinook salmon (*Oncorhynchus tshawytscha*) and sertraline in whole body staghorn sculpin (*Leptocottus armatus*) collected near WTTP effluent in two estuaries in concentrations, (4.9, 17 ng/g, and 0.20 ng/g, respectively). The SSRI LOQs reported in our lab and field measurements for English sole were lower than the SSRI concentrations detected in salmon but above the concentration detected in sculpins. Our English sole were collected approximately 3,000 to 4,000m from the same WWTPs sampled by Meador et al. (2016) for the two locations where salmon, sculpins and English sole were both collected. Based on their results, it is likely that the English sole we sampled were too far from WTTP effluent or spent insufficient time near WWTP effluent to record exposure to SSRIs originating from these WTTPs, or were exposed at a concentration below our LOQ. Additionally, species-specific metabolic differences, ventilation and ingestion rates could affect the degrees to which SSRIs are accumulated in these species.

Vitellogenin Gene Expression

Development and Validation of Method to Measure Vitellogenin Gene Expression via qPCR

We successfully developed for the first time a cost-effective specific qPCR to detect and quantify VTG gene expression in English sole and demonstrated the applicability of the method using previously collected English sole samples. Following the steps outlined in the methods above, we successfully 1) isolated liver total RNA and synthesized cDNA; 2) cloned partial cDNA encoding VTGs and candidate housekeeping genes; 3) selected VTG and housekeeping genes; and 4) developed qPCRs. As detailed in Appendix A, the gene expression data quality met the criteria for analysis of English sole liver VTG gene expression by qPCR outlined in the QAPP for this project (O'Neill et al. 2014) except for minor deviations that did not compromise the usability of the results. Below is a brief summary of the quality control results.

RNA isolation, DNase treatment of RNA and cDNA synthesis methods

Methods for RNA isolation, DNase treatment of RNA and cDNA synthesis were conducted according to Guzmán et al., 2013. RNA quality met the laboratory criterion for all samples. Genomic DNA contamination was effectively eliminated by DNase treatment and PCR carryover contamination was within the acceptable range.

Cloning partial cDNAs encoding VTGs and Selection of VTG housekeeping genes

As mentioned previously, to determine whether one type of VTG gene, type A (VTGA) or B (VTGB), is more sensitive than the other to ECs in English sole, expression of both genes was compared in a random subset of liver samples representing samples from all collection sites (i.e. high, moderate and low development sites at which English sole may be exposed to a wide range in magnitude of ECs). For this, we used the relative abundance of VTGA vs. VTGB mRNAs as a proxy for sensitivity to ECs. In addition, because levels of VTG mRNAs have not been reported in English sole at these sampling locations, we used liver samples from English sole males treated with E2 as a positive control (Guzmán et al., 2008).

The comparison of the relative abundance of VTGA and VTGB transcripts in liver samples from males treated with E2 or sampled at different locations in Puget Sound was conducted using semi-quantitative PCR (semi-qPCR). Briefly, liver total RNA was isolated, DNase-treated and reverse transcribed as described previously (Luckenbach et al., 2011). The semi-qPCRs for VTGA and VTGB had a total volume of 25 µl and consisted of 1X GoTaq Green Master Mix (Promega), 0.4 µM of gene-specific forward and reverse primers and 0.5 ng of the cDNA template based on the amount of total RNA loaded in reverse transcription (RT) reactions. Semi-qPCRs were run on an iCycler Thermocycler (BioRad) using standard cycling conditions (Guzmán et al., 2015). Finally, all semi-qPCR products were electrophoresed on a 2% E-gel and produced a single product, which was assessed for size, clarity and relative intensity (Smith et al., 2013).

As expected, treatment with E2 induced a massive increase of VTGA and VTGB mRNAs in English sole liver (see Figure 14, lanes A and B). Interestingly, transcripts for both VTGA and VTGB genes were amplified by semi-qPCR in all liver samples, regardless of the site of sampling (i.e., high, moderate and low development, see Figure 14, lanes C to H). However, VTGB was more highly expressed than VTGA in all three locations (see Figure 14, C vs. D, E vs. F, G vs. H). Considering that the same amount of cDNA was loaded in each reaction (0.5 ng based on RNA concentration), these results suggested that VTGB is expressed at a higher level and might give a more robust response to potential ECs than VTGA in English sole as found for medaka (Ishibashi et al. 2016).

Of the four housekeeping or reference genes assessed for their potential use in this study (18S ribosomal RNA (18S), ribosomal protein L8, β-actin, and elongation factor-1 α.), 18S was selected as the best for use as an internal control to normalize the levels of VTG mRNA in English sole liver samples. The selection of one of these genes as our housekeeping gene for subsequent data normalization was based on expression stability among samples from collection sites in the study (i.e. high, moderate and low development sites that may expose English sole to a wide range in magnitude of ECs). In addition, in order to maximize the representativeness of all samples scheduled to be analyzed in the study, we also included samples from both sexes (females and males) and years (2011 and 2013).

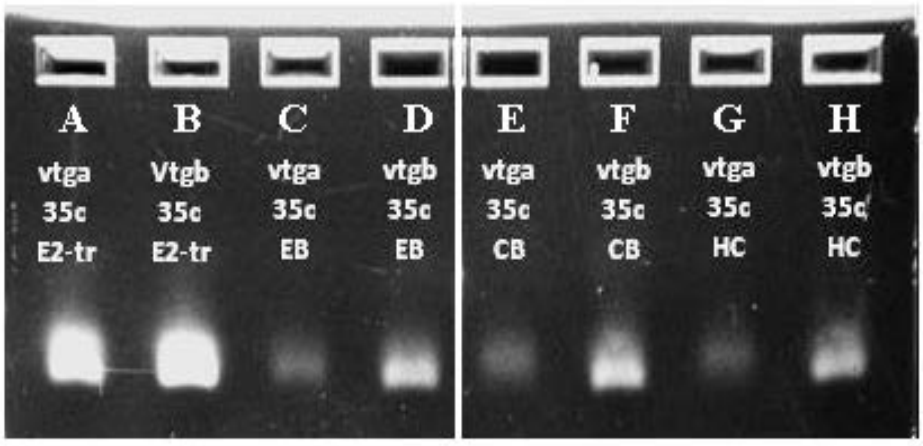

Figure 14. Agarose (2%) gel electrophoresis of semi-qPCR products of VTGA and VTGB cDNAs in liver samples of male English sole. Fish were treated with E2 (E2-tr, positive control), or sampled at several location sites in Puget Sound: Seattle Waterfront in Elliot Bay (EB, high development site), Tacoma City Waterway in Commencement Bay (CB, moderate development site) and Hood Canal (HC, low-development site). Semi-quantitative PCRs were run for 35 cycles (35c). For all samples, 0.5 ng of cDNA (based on RNA concentration) was loaded per reaction.

The stability of candidate housekeeping gene expression in liver samples from males and females sampled at different location in Puget Sound was conducted using quantitative PCR (qPCR). Briefly, liver total RNA was isolated, DNase-treated and reverse transcribed as described above. Briefly, the qPCRs for housekeeping gene candidates had a total volume of 12.5 µl and consisted of 1X Power SYBR Green master mix (Applied Biosystem, Life Technologies), 150 nM of the forward and reverse primer, and 0.5 ng of the cDNA template, based on the amount of total RNA loaded in the RT reaction. Quantitative PCR assays were run on an ABI 7900HT Fast Real Time PCR System (Life Technologies) in 384-well plates using standard cycling conditions (Guzmán et al., 2015).

Raw expression of the four housekeeping gene candidates (18S, ribosomal protein L8, β-actin, and elongation factor-1 α) is shown in Figure 15. Among the four candidates, levels for both β-actin, and elongation factor-1 α mRNAs were consistently lower in those fish sampled in 2011 compared to 2013 (ANOVA $p<0.05$). The same pattern was observed for ribosomal protein L8, albeit differences were not significant. In contrast, although some minor oscillations were found for 18s, this gene showed a stable expression pattern among samples based on sex, location and year. These results indicated that 18S was a suitable housekeeper to use in liver gene expression analyses related to ECs in English sole.

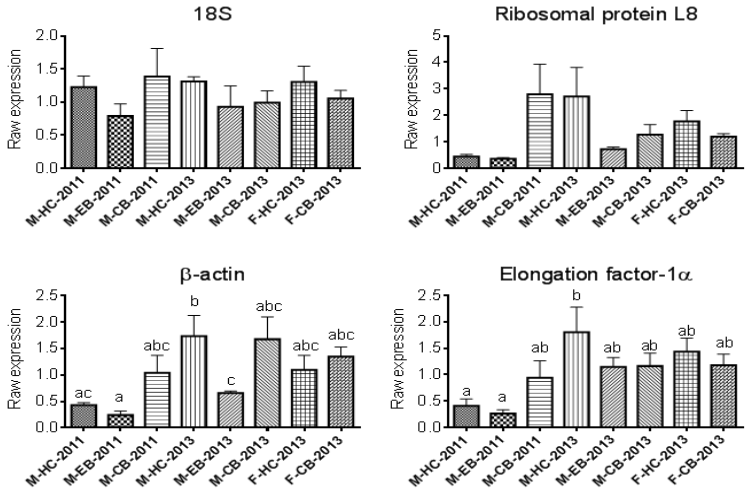

Figure 15. Expression of candidate housekeeping genes in English sole liver. Female (F) and male (M) fish were sampled in Puget Sound: Seattle Waterfront in Elliot Bay (EB, high development site), Tacoma City Waterway in Commencement Bay (CB, moderate development site) and Hood Canal (HC, low development site) in 2011 and 2013.

qPCR Methods and Performance

Development of qPCRs for VTGB and 18s followed protocols for other genes developed in our lab (Luckenbach et al., 2011), while standards generated from cDNA to quantify copy number were developed as described in Guzmán et al., 2009. The qPCR methods and performance were within acceptable ranges with calculated efficiencies of 103.8% for VTGB qPCR and 107.5% for 18S. Likewise the qPCR specificity and reproducibility was met for all samples. The requirements for qPCR sensitivity were also met and all experimental samples were above the limit of detection for both VTGB and 18s.

VTG Gene Expression in English Sole Field Samples

Using the VTGB qPCR assay, 100% of the English sole liver samples had detectable levels of VTG gene transcripts (expressed as copies VTGB/copy 18S). VTG gene expression varied widely among individual fish ranging from 12 to 100,887 VTGB:18S in females with lower levels in males ranging from 0.09 – 583 VTGB:18S (Table 8). As expected, VTG levels were higher (two to seven times) in females than males from for the three sites where both female and male fish were sampled (Table 8, Figure 16). Because normally vitellogenic females were expected in all locations, these results support a conclusion that the method is appropriate in sensitivity and range to capture normal synthesis of VTG in female fish.

Among female sole, the highest median VTG gene expression was measured in fish from the Seattle Waterfront (51 VTGB:18S), followed by Hood Canal (44 VTGB:18S) and Tacoma City Waterway (38 VTGB:18S), although variability was too high to detect significant differences among these three sites (ANOVA on ranked values, $p = 0.379$; Figure 16A). Pooling female sole across the two high-development sites did not affect this result; VTG levels in female sole from the combined Seattle Waterfront and Tacoma City Waterway (high development) sites were not significantly different from the single non-urban site sampled (Figure 17 A; Mann-Whitney U Statistic= 53.000, $p = 0.916$; Table 9).

Although VTG was statistically homogeneous across sites, three female English sole from the Seattle Waterfront (high-development) site exhibited both unusually high VTG (Figure 16A) and high natural ECs (Figure 5a-c). Histological examination of their ovaries indicated these three fish were also in a spawning condition (Figure 18A). We were unable to statistically distinguish this condition across sites, however this highly unusual condition was unique to the Seattle Waterfront, supporting the Johnson et al. (2008) conclusions that English sole from this site exhibited altered reproductive timing, likely attributable to exogenous ECs.

Among male English sole, we observed a generally increasing pattern of VTG from low- to high-development sites, albeit with highly variable VTGB:18S within sites (Figure 16B); fish from one high-development site (Tacoma City Waterway) had significantly higher VTG levels than those collected from the two low-development sites (Nisqually Reach and Vendovi Island) with all other sites exhibiting intermediate levels (ANOVA on ranked values, $p = <0.001$; Figure 16B). Unlike female English sole, males did not show any relationship between spawning condition and the level of VTG induction (Figure 18b). Pooling male English sole by the type of site development better highlighted the differences in VTG induction with degree of development. The highly developed sites had significantly higher median levels of VTG induction than those from pooled moderate-development and pooled low-development sites (ANOVA on ranked values; $p = <0.001$; Figure 17B), but the moderate and low-development sites were not significantly different from each other. The high within-site VTG variability for male fish reduced our ability for detecting true differences, if more existed than we were able to discriminate.

Although we lacked statistical power to fully discriminate our VTG levels across each site type, the pattern of VTG induction in male sole observed in 2011 – 2013 is generally consistent with results reported for VTG induction based on VTG in plasma by ELISA that were measure in 1997 - 2001 for male sole for many of the same sites (Johnson et al. 2008). In the male fish sampled from 1997-2001 (Johnson et al. 2008), plasma VTG levels were also relatively low in comparison with those in maturing females, even in males from the most urbanized sites. However, both studies showed significantly higher VTG induction, whether measured by gene expression with qPCR, or as VTG in plasma, in male sole from high-development sites. Moreover, both studies observed vitellogenin in male English sole from low-development sites, which had been originally selected as a clean reference area. These studies combined suggest that although male English sole from urbanized or highly developed habitats in Puget Sound likely experience the greatest level of exposure to exogenous ECs, the phenomenon is more widespread, and not restricted to these habitats.

There were some differences between the current study and the Johnson et al. (2008) study in relationships at specific sites. For example, in the 1997-2001 sampling, plasma VTG levels were highest in male sole from Seattle Waterfront, while in the 2011-2013 study, the highest levels VTG gene expression were seen in sole from Tacoma City Waterway. This suggests a possible change in exposure at these sites, and perhaps a decline in exposure in Seattle Waterfront, which would be consistent with the change in reproductive stage seen in female English sole. However, it is difficult to compare plasma VTG levels from the Johnson et al. 2008 study with VTG gene expression from the current study, because we do not as yet have information on the relationship between the two methods in English sole.

Additionally, in the present study, some degree of VTG gene expression was seen in 100% of the samples analyzed. Detection of low levels of hepatic VTG mRNA in male fish using qPCR has been reported in a number of studies, including lab studies where control animals had no history of exposure to ECs (e.g. Ishibashi et al. 2016). However, in the 1997-2001 sampling, the percentage of male fish with detectable VTG in plasma ranged from 0% at Outer Sinclair Inlet, South Port Orchard, Eagle Harbor, and Nisqually Reach to 46.9% at the Myrtle Edwards site in Seattle Waterfront. This difference is likely attributable to differences in VTG assay between the two studies. All of the VTG mRNA (measured by qPCR method used in the current study) would not necessarily be translated to circulating protein in the plasma of the fish. Thus, the qPCR assay is more sensitive, and may detect differences in gene expression that would not necessarily be reflected in plasma protein levels measured by the ELISA, or that, in the ELISA, could not be differentiated from background.

Other methodological differences in VTG assay between the current study and the Johnson et al. (2008) study could account for other inconsistent or variable results between the two studies. While VTG mRNA increases more rapidly than VTG protein in plasma in response to exposure to ECs, the response is also more transient, at least following a single exposure to an EC (Lim et al. 1991; Korte et al. 2002; Bowman et al. 2002; Scholz et al. 2004; Hemmer et al. 2002). In a study with fathead minnow injected with a low dose of E2 (Korte et al. 2002), VTG mRNA reached a peak in 4 hours, then declined markedly and was no longer detectable by day 6. Even with a high dose it was no longer detectable by day 12. Similar time courses for VTG mRNA induction have been observed in tilapia injected with E2 (Lim et al. 1991) and rainbow trout exposed to the xenoestrogen, nonylphenol (Lech et al. 1996). In contrast, VTG protein concentrations in plasma generally increase less rapidly, but remain elevated for much longer. In fathead minnow, Korte et al. (2002) reports that plasma VTG was detectable 16 hours following treatment and remained elevated for at least 18 days after E2 treatment. The delayed increase and longer persistence of VTG from plasma is also reported in male tilapia (Lim et al. 1991), medaka (Scholz et al. 2004), largemouth bass (Bowman et al. 2002), and sheepshead minnow (Hemmer et al. 2002, Bowman et al. 2002). Thus the effects of a transient exposure to ECs might be detectable for a longer period as VTG in plasma than as liver VTG gene expression.

On the other hand, VTG gene expression may increase at EC exposure levels where plasma VTG is unaffected. For example, in a laboratory exposure to E2, Emmersen et al. 1979 found that liver VTG mRNA increased with doses as low as 5 μg, while at this dose plasma VTG did not change. Arukwe et al. 2001 reported similar results in juvenile rainbow trout exposed to nonylphenol (NP); increased VTG mRNA was observed at a low dose of NP at which there was no change in VTG protein. Similarly, in sheepshead minnow, Folmar et al. 2000 reported increased VTG mRNA expression at E2 exposure concentrations of 100 ng/L and above, but increased plasma VTG only at concentrations of 200 ng/L and above. Thus, the two assays differ in sensitivity and kinetics of the response, and this must be considered when interpreting field data.

Table 8. Summary statistics for VTG gene expression in liver of male and female English sole from ten index sites in Puget Sound. Data are expressed as a ratio of VTGB to housekeeping gene 18S (VTGB:18S). Sites are grouped according to the degree of land development in watersheds adjacent to sites.

		Low Development				*Moderate Development*		*High Development*			
Sex	*Summary Statistic*	Strait of Georgia	Vendovi Island	Hood Canal	Nisqually Reach	Port Gardner	Eagle Harbor	Seattle Waterfront	Duwamish River	Sinclair Inlet	Tacoma City Waterway
Male	N	7.0	9.0	10.0	10.0	21.0	10.0	14.0	11.0	10.0	10.0
	% Detected	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
	Median	***1.8***	***1.4***	***6.6***	***1.4***	***1.9***	***3.2***	***10.0***	***3.8***	***7.0***	***18.5***
	25%	0.5	0.9	2.9	0.3	1.0	1.7	3.6	1.8	2.7	9.7
	75%	6.7	4.0	12.4	3.5	4.1	26.0	22.3	12.8	15.7	30.3
	Mean	5.1	7.0	9.3	59.8	9.0	16.6	16.2	6.8	9.3	25.5
	Min	0.3	0.4	1.6	0.1	0.3	0.4	1.3	1.2	2.2	4.2
	Max	23.4	47.9	31.8	582.6	72.1	75.8	73.4	19.7	21.8	92.1
Female	N	--	--	10.0	--	--	--	7.0	--	--	4.0
	% Detected	--	--	1.0	--	--	--	1.0	--	--	1.0
	Median	--	--	***44.5***	--	--	--	***51.0***	--	--	***38.2***
	25%	--	--	28.5	--	--	--	29.0	--	--	18.3
	75%	--	--	177.3	--	--	--	92,722.3	--	--	46.2
	Mean	--	--	107.4	--	--	--	38,125.5	--	--	34.2
	Min	--	--	18.7	--	--	--	12.3	--	--	13.7
	Max	--	--	422.1	--	--	--	100,887.8	--	--	46.9

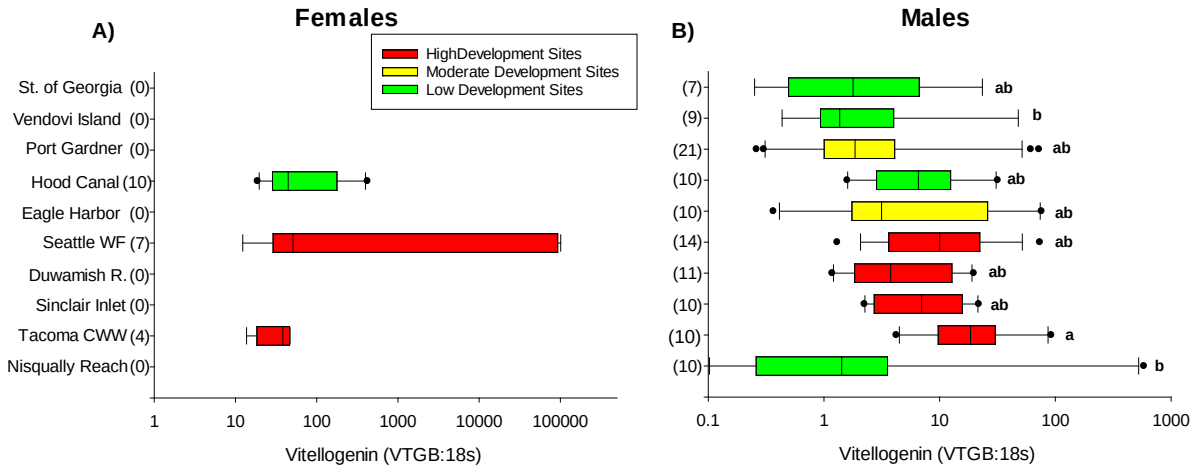

Figure 16. Comparison of VTG gene expression data in liver of male and female English sole by sample site (sample size in parentheses). Data are expressed as a ratio of VTGB to housekeeping gene 18s (VTGB:18S). Similar lower case letters signify no significant difference among sites (p > 0.05).

Table 9. Comparison of VTG gene expression data in liver of male and female English sole with site data pooled according to level of land development. Data are expressed as a ratio of copies VTGB per copy housekeeping gene (VTGB:18S).

Sex	*Summary Statistic*	*Low Development Sites*	*Moderate Development Sites*	*High Development Sites*
Male	N	36	31	45
	% Detected	100%	100%	100%
	Median	***2.3***	***2.7***	***8.5***
	25%	1.0	1.2	3.5
	75%	6.6	4.1	19
	Mean	22	11	14
	Min	0.09	0.26	1.2
	Max	583	76	92
Female	N	10	--	11
	% Detected	100%	--	100%
	Median	***44***	--	***47***
	25%	29	--	29
	75%	177	--	73,126
	Mean	107	--	24,274
	Min	19	--	12
	Max	422	--	100,888

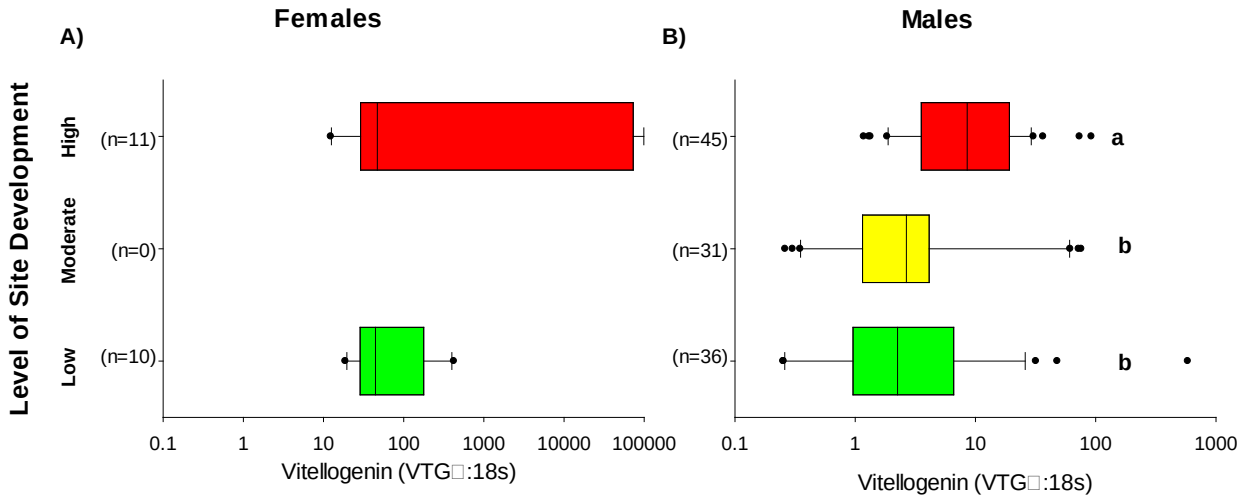

Figure 17. Comparison of VTG gene expression in liver of male and female English sole from low, moderate and high development sites. Data are expressed as a ratio of copies VTGB per copy housekeeping gene, 18S (VTGB:18S). Similar lower case letters signify no significant difference (p > 0.05).

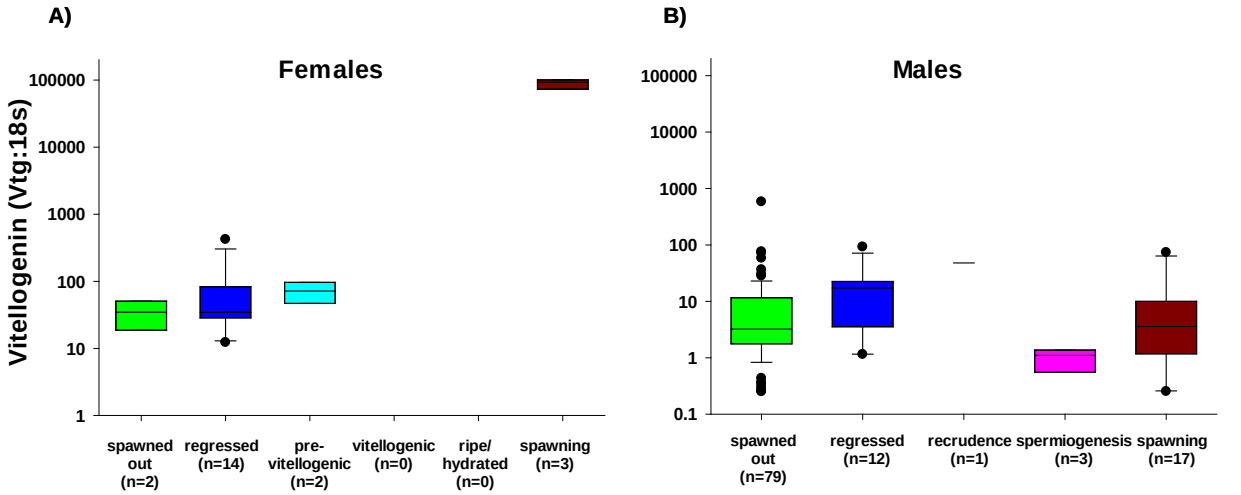

Figure 18. Reproductive developmental stage in female and male English sole, also analyzed for VTGB (from Figures 16 and 17). Note the log scale on the vertical axis, with different values for males and females, and the three spawning stage females in the upper right of the A) panel.

Effects of CEC Exposure on English sole

Following up on the study by Johnson et al. (2008), da Silva et al. (2013) documented that the likely cause of the VTG induction in male English sole from Puget Sound was environmental sources of ECs -- chemicals which were subsequently detected in bile of this species in concentrations ranging from the Limit of Quantitation (LOQ; 6-12 ng/mL bile) to 52 and 310 ng/mL bile for BPA and E2, respectively. The main source of some of the most estrogenic of these chemicals in the environment is widely attributed

to wastewater (see review by Hotchkiss et al., 2008). Peck et al. (2011) also documented VTG induction in field-caught juvenile Chinook salmon from Seattle Waterfront and other Puget Sound sites in 2006 and 2007, indicating that wild juvenile salmon can be affected by ECs as they transition from freshwater to marine environments in certain Puget Sound watersheds. Such biological indicators of exposure and effects hint at potentially wide-spread effects of ECs in Puget Sound fish.

Although ECs appeared concentrated in high-development sites, especially the Seattle Waterfront, and we observed the greatest VTG induction in high-development areas, the correlation between EC exposure-and-effects was less clear on an individual fish basis. VTG gene expression in female sole was highly correlated with the concentration of natural ECs measured in the bile of the same fish, although linear regression models of these relationships were highly leveraged by three female fish from the Seattle Waterfront. These three female English sole were classified as "spawning" individuals with ripe, hydrated eggs based on ovarian histology and exhibited the highest levels of E2, E1, and E3 and elevated levels of VTG gene expression (Figure 19 A, C, and E). Although these three fish also exhibited the greatest concentration of biliary BPA and tOP, we observed no correlation of VTG with BPA overall in females (Figure 19G), and a weak correlation of VTG with tOP (based on only six detections of tOP; Figure 19I). This pattern was similar for VTG compared with EEQ (Figure 20); the positive correlation between VTG and EEQ in females was driven by the three individuals from the Seattle Waterfront (mentioned above).

VTG gene expression in male English sole was not significantly correlated with any of the ECs (Figure 19 B, D, F, H and J). Likewise, there was no significant relationship between VTG gene expression in male fish and EEQ (Figure 20). The lack of a correlation between ECs in bile (an estimate of exposure) and VTG in male fish (an estimate of the effect of exposure to ECs) was somewhat surprising, especially so for the Seattle Waterfront site that had the greatest biliary ECs in both males and female English sole, and altered reproductive timing in females. We had expected to see a positive correlation between the putative exogenous ECs and VTG mRNA activity.

However, there are several reasons why such a correlation might be difficult to detect. First, VTG gene expression and biliary EC concentrations reflect relatively short-term exposure to ECs – on the order of days – however, VTG gene expression may not match (in time) the biliary EC concentrations or other effects of chronic exposure to environmental estrogens. Additional laboratory exposure studies are needed to understand the exposure timeline represented by biliary EC concentrations and VTG gene expression. Second, within-site variability of ECs and VTG was high, which could be affected by episodic or variable wastewater discharges and CSO events, the fish's proximity to the discharges or other potential sources, and movement patterns and foraging behavior of the fish. Foraging behavior in particular, may greatly affect the variability in the levels of EC metabolites measured in bile at a site. Concentrations of EC metabolites vary with the gut fullness – fish with full stomach have much lower EC metabolite concentrations than fish with empty stomachs (Brumley et al. 1998). Fish for this study were collected from at various times of the day and were held in a live tank until they could be necropsied to remove their bile. The fish were not fed while in the live tank. At any one site, some fish would have

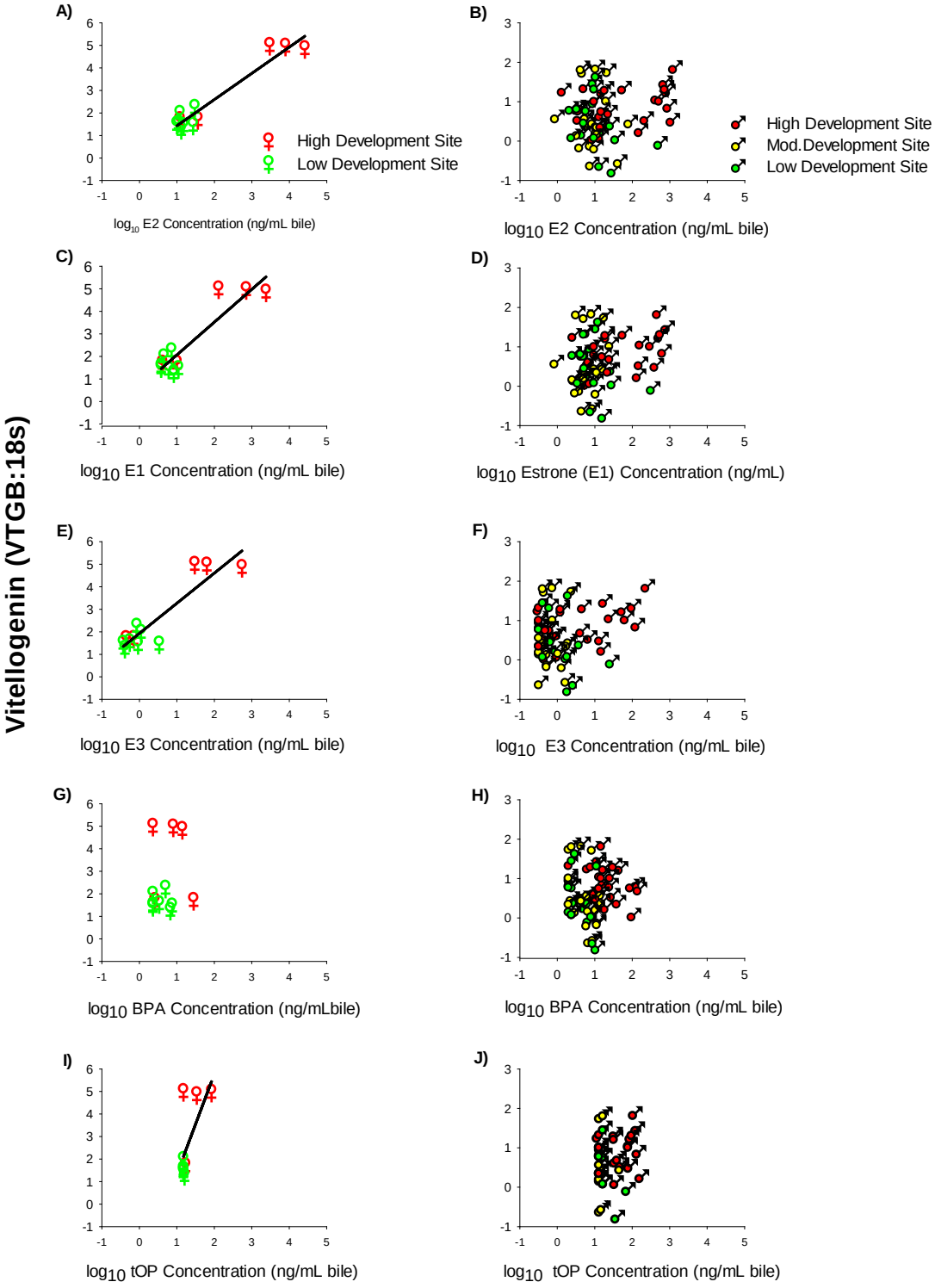

Figure 19. Comparison of liver VTG mRNA levels in male and female English sole with three natural estrogens (E1, E2, E3) and two non-steroidal synthetic chemicals with weak estrogenic activity (BPA and tOP) .

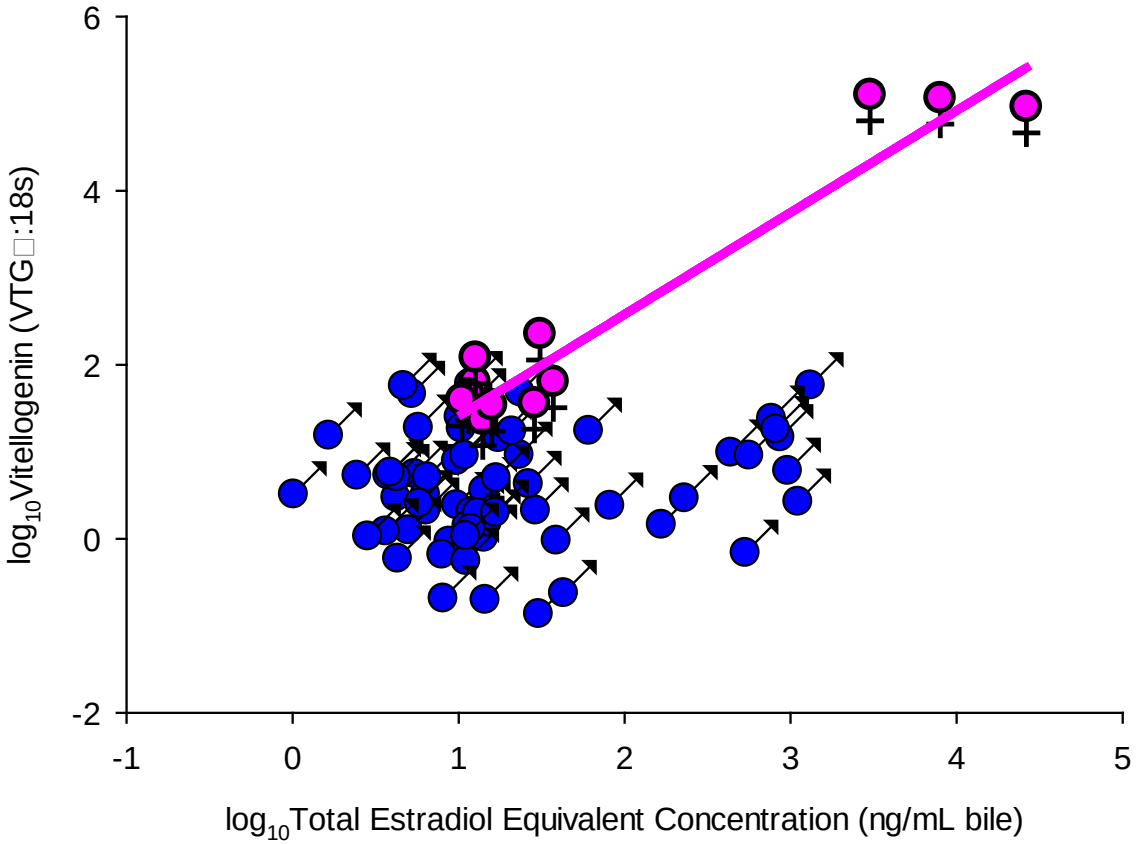

Figure 20. Comparison of liver VTG levels in male and female fish with total estimated estrogenicity of seven estrogenic chemicals measured in their bile, and calculated as estrogen equivalency (Vega-Morales 2013). Male and female are shown as blue and pink symbols, respectively.

had bile extracted as soon as one hour after capture but some fish would have remained in the live tank for up 6 hours, greatly influencing the concentration of EC actually measured in the fish. Normalizing EC biliary concentrations at a site for the degrees of gut fullness (i.e. protein correction) may improve the relationship between EC exposure and VTG induction measured in individual fish. Moreover, given the high with-in site variability in EC and VTG data, the number of paired biliary EC and VTG mRNA samples analyzed was relatively small, with a limited number of fish from areas of high EC exposure. The correlation may improve with additional data. Third, other chemicals commonly found in wastewater discharge, especially those with anti-estrogenic or androgenic properties, may alter or mask effects of ECs.

Baseline levels of naturally–occurring gene expression of VTG in male English sole and the duration of induction when exposed to ECs are unknown, which would affect the relationships we observed herein. The qPCR assay is extremely sensitive, and expression of VTG genes in males is detected even in unexposed fish in laboratory studies (e.g. Ibashi et al. 2016). While VTG mRNA increases more rapidly

than VTG protein in plasma in response to exposure to ECs, the response is also more transient, at least following a single exposure to an EC (Lim et al. 1991; Korte et al. 2002; Bowman et al. 2002; Scholz et al. 2004; Hemmer et al. 2002). In a study with fathead minnow injected with a low dose of E2 (Korte et al. 2002) VTG mRNA reached a peak in 4 hours, then declined markedly and was no longer detectable by day 6. Even with a high dose it was no longer detectable by day 12. Similar time courses for VTG mRNA induction have been observed in tilapia injected with E2 (Lim et al. 1991) and rainbow trout exposed to the xenoestrogen, nonylphenol (Lech et al. 1996). VTG protein concentrations in plasma generally increase less rapidly, but remain elevated for much longer. In fathead minnow, Korte et al. (2002) reports that plasma VTG was detectable 16 hours following treatment and remained elevated for at least 18 days after E2 treatment. The delayed increase and longer persistence of VTG from plasma is also reported in male tilapia (Lim et al. 1991), medaka (Scholz et al. 2004), largemouth bass (Bowman et al. 2002), and sheepshead minnow (Hemmer et al. 2002, Bowman et al. 2002). Thus the effects of a transient exposure to ECs might be detectable for a longer period as VTG in plasma than as VTG gene expression. On the other hand, VTG gene expression may increase at EC exposure levels where plasma VTG is unaffected. For example, in a laboratory exposure to estradiol-17β, Emmersen et al. 1979 found that liver VTG mRNA increased with doses as low as 5 ug, while at this dose plasma VTG did not change. Arukwe et al. 2001 reported similar results in juvenile rainbow trout exposed to nonylphenol (NP); increased VTG mRNA was observed at a low dose of NP at which there was no change in VTG protein. Similarly, in sheepshead minnow, Folmar et al. 2000 reported increased VTG mRNA expression at E2 exposure concentrations of 100 ng/L and above, but increased plasma VTG only at concentrations of 200 ng/L and above. Additional laboratory exposure studies in English sole are needed to determine baseline VTG levels in male English sole and the exposure concentration of ECs in bile that result in a consistent VTG induction.

Given the high variability in ECs concentration and VTG levels in individual English sole within a site, we may only be able to measure a positive correlation between EC exposure and VTG induction in male fish when the EC exposure is exceptionally elevated and/or consistent. Based on the levels of ECs measured in bile of male English sole from 2005 to 2011-2013, the levels of E2 had increased (Figure 11); however, it is not known if the differences in biliary EC concentration measured between the 2005 and 2011 -2013 represent biologically meaningful changes for the fish. Moreover, although E2 concentrations measured in male English sole from Seattle Waterfront in 2011 – 2013 were higher than those measured in male sole collected from 2005, recent loadings of ECs to Seattle Waterfront are likely considerably lower than historic inputs and possibly lower than the those measured in from 1997- 2001 when Johnson et al. (2008) observed high proportion of males with elevated VTG in their plasma, and a higher proportion of females with an altered spawning time.

As early as 1895, two sewage outfalls discharged raw sewage into Seattle Waterfront in the vicinity of Denny Way (Striplin Environmental Associates 2000). Both outfalls operated until 1969 when the Seattle Waterfront interceptor line was installed along the Seattle Waterfront and the Denny Way outfall was converted to a Combined Sewer Overflow (CSO). The Denny Way outfall was the largest CSO in King County and discharged large volumes of combined stormwater runoff and sewer overflow into the inter-tidal waters of Elliott Bay until 2002. In August of 2002, a replacement pipe for the Denny Way

outfall moved the discharge of untreated CSO effluent offshore approximately 120 feet, at a depth of 20 feet below mean lower low water (MLLW).

Additionally, from mid-to late 2005 onward, less untreated CSO effluent was discharged into Elliott Bay as a result of the construction and operation of the Mercer Storage and Treatment Tunnel and the Elliott West treatment facility and CSO outfall. Starting in late May of 2005, during storm events wastewater flows are now diverted into a 6,200-foot-long tunnel under Mercer Street. During most storms, the flows are stored in the tunnel until the storm subsided and the flow is transported to the West Point Treatment Plant for secondary treatment. During large storms, when the Mercer tunnel storage exceeds its capacity, the excess flow undergoes CSO treatment at the Elliott West treatment facility and the treated effluent is discharged through a newly constructed out fall, 490 feet from shore at 60 feet below MLLW. In storms when the amount of CSO water exceeds the capacity of the Elliott West facility, the excess flows is discharged untreated to the aforementioned newly extended Denny Way CSO, 120 feet from shore, 20 feet below MLLW.

The construction of the Mercer Storage and Treatment Tunnel and the Elliott West treatment facility greatly reduced the amount of raw sewage discharged to the Seattle Waterfront area of Elliott Bay during storm events. In the year immediately following the remedial action, the total number of untreated CSO events deceased from an average of approximately 20 events per year to nine events and thereafter ranged from one to four events per year (Figure 21A). Along with a reduction in the number of events per year, the total volume of untreated CSO effluent decreased. Prior to May 2005, when the Elliott West facility began treating CSO effluent, more than 300 million gallons (MG) of untreated CSO effluent discharged to the Seattle Waterfront annually, but after May 2005, the maximum annual volume was reduced to less than 60 MG annually (Figure 21B).

Although less untreated CSO effluent was released into Elliott Bay during the period from 2005 – 2014 compared to earlier years, the amount of treated CSO effluent discharged there increased. In the first full year of operation of the Elliott West treatment facility (June 2005 – May 2006), approximately 315 MG of treated CSO effluent was released from Elliott West facility, 490 feet from shore at a depth of -60 feet MLLW. In the following years, the annual treated volumes were below 200 MG except for water year 2006-2007 and 2010/11 when the annual volumes released were approximately 490 and 340 MG. Indeed, with the exception of 2005/2006, 2006/2007 and 2010/2011, the total volume of treated and untreated CSO effluent was less than 220 MG annually.

Alteration in the number and total volume of these treated and untreated CSO events along the Seattle Waterfront possibly affected the amount of ECs discharged near our Seattle Waterfront site; however the specific temporal trends and the extent to which English sole were exposed to these ECs is uncertain. First, it is not known whether moving the discharge of untreated CSO effluent further offshore in August of 2002 by extending the pipe of Denny Way CSO outfall by 120 feet, resulted in more or less direct exposure of English sole to the untreated effluent. The mean depth of our Seattle Waterfront trawl track is approximately 120 ft (Table 1). Second, we do not know to what extent the concentrations of ECs differ between untreated and treated CSO effluent.

As discussed previously, during the 1997 – 2001 survey, when male sole from Seattle Waterfront were documented to have high plasma levels of VTG, an average of 64% of the female sole from the Seattle Waterfront were in an advanced state of gonadal development or were actively spawning (i.e. ripe), unlike sole from other sites in Puget Sound (Figure 3). However, since 2005, the proportion of ripe female sole sampled from Seattle Waterfront in April and May have decreased slightly to an average of 50% (Figure 21A), possibly due to changes in wastewater effluent inputs to the Seattle Waterfront. Reductions in CSO events but an increase of in treated effluent volume could have altered the amount of EC input to the Seattle Waterfront. However, data on the levels of ECs in CSO effluent and the treated effluent are not available to test this hypothesis. Further, the concentration of ECs needed to induce and a gene expression VTG response in female and male sole, the nature of the dose response relationship, and the duration of the VTG response are unknown. Additional laboratory controlled exposure studies demonstrating the kinetics of VTG mRNA induction in relation to exposure to ECs and other contaminants will improve our ability to interpret these results and better understand their significance regarding fish health.

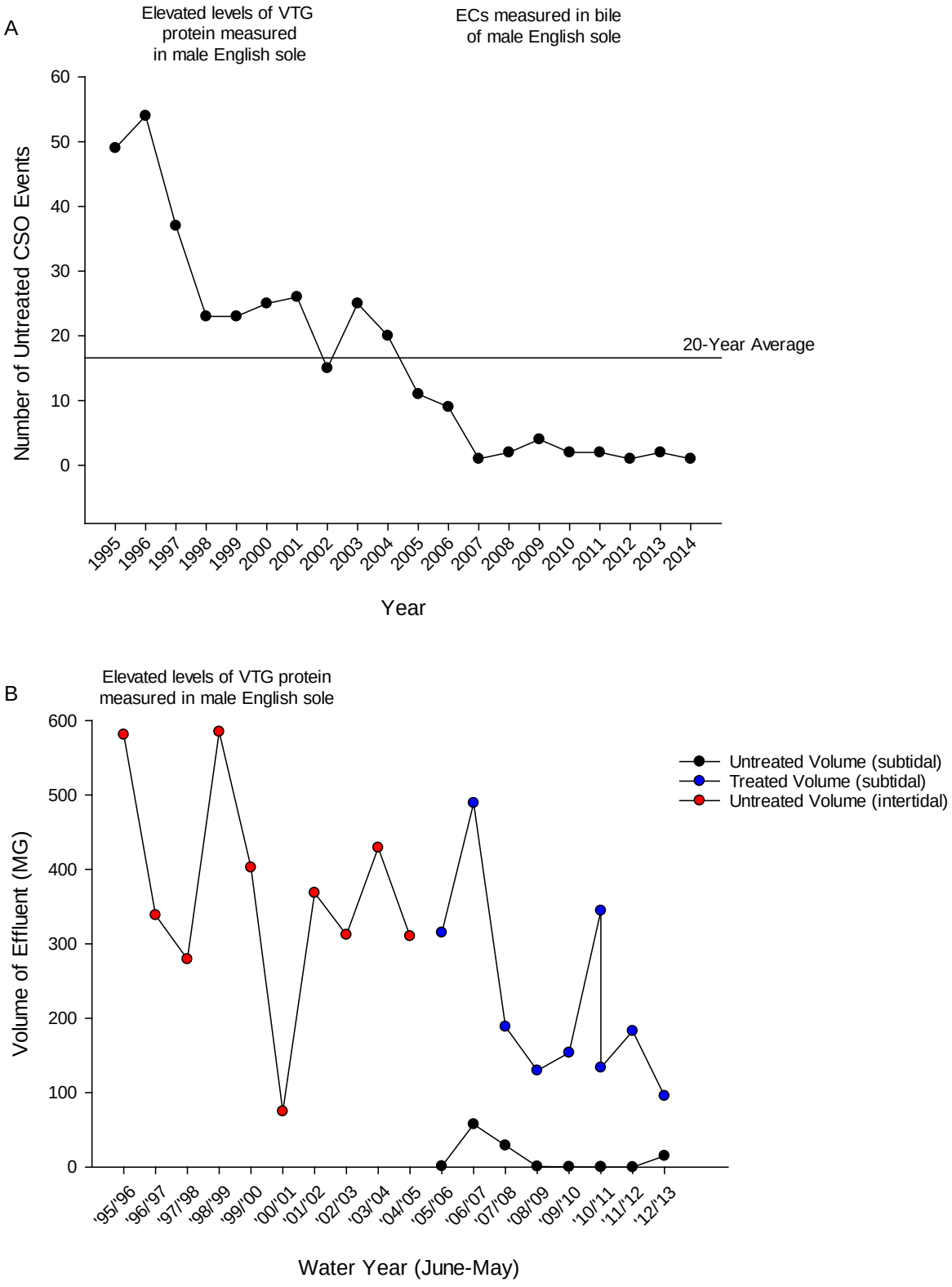

Figure 21. Temporal comparison of the annual number of untreated CSO events (A) and the total volume of treated and untreated CSO effluent released into Seattle Waterfront (B). Data for volume of effluent released are summarized by water year, June through May. Grey bars indicate years in which English sole were sampled in April and May for VTG in plasma. Blue bars indicate years in which English sole were sampled in April and May for liver VTG mRNA.

CONCLUSIONS

This study successfully achieved its goals of developing new tools for exposure metrics for ECs in fish bile, a bioeffects metric for exposure to ECs, and measuring SSRIs in English sole. These tools may be applied in upcoming English sole surveys, and will be further refined as we incorporate them into WDFW/PSEMP's biennial English sole contaminant survey. We also combined these new methods with existing methods on English sole tissue samples collected in 2011 and 2013 to identify:

- continued altered reproductive timing in female fish from Seattle Waterfront in Elliott Bay, likely from exposure to ECs,
- relatively high concentrations of EC in sole from highly-developed urbanized habitats, especially Seattle Waterfront and Sinclair Inlet,
- widespread vitellogenin induction in male sole, with highest values primarily observed in highly developed urbanized habitats, especially Tacoma Waterway and Seattle Waterfront,
- little or no recent exposure of English sole to SSRIs, likely because sole did not occur near enough to, or forage long enough near, putative SSRI sources (such as wastewater treatment plants).

Although ECs appeared concentrated in highly developed areas and we observed the greatest EC effects in urban areas, the correlation between EC exposure-and-effects was less clear on an individual fish basis. The reasons for this could be:

  - a mismatch in timing of our sampling between exposure and effects,
  - differences in kinetics and metabolism of various ECs and VTG mRNA synthesis
  - insufficient sample sizes to achieve enough power to detect relationships, if they existed, because of the highly variable EC and VTG values measured in individual fish within a station
  - the presence of androgenic or anti-estrogenic chemicals that may mask or alter the effects of ECs.

These results highlight the need for continued work to refine these tools for monitoring indicator species such as English sole. Although effects from exposure to ECs seemed clear in some cases, high variability in response metrics precluded unambiguous conclusions in some cases. Power analysis of existing samples will help to define the sample sizes needed to identify spatial and temporal differences for upcoming monitoring efforts. Evaluation of multiple metrics (histological examination of gonads, hepatic VTG induction, and measurement of biliary ECs) is still appropriate and necessary to understand and track the health effects of these chemicals. Controlled dosing studies in the lab may help to elucidate health effects thresholds, which are needed to evaluate the measurements made in wild English sole, and to elucidate the level of normal vitellogenesis in natural, unexposed male English sole, if one exists. Finally, continued evaluation of additional exposure and health metrics, including assessment of VTG proteins in plasma, is warranted as they become available, and more cost effective.

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APPENDIX A: Data Quality Control Check

Estrogenic Chemicals

Below are the data quality control checks for chemical analyses of bile of English sole for chemicals with estrogenic properties (17β-estradiol, estrone, estriol, bisphenol A, nonylphenol, octylphenol, and 17α-ethynylestradiol; referred to collectively as Estrogenic Chemicals, (ECs) determined by liquid chromatography with tandem mass spectrometry (LC-MS/MS). The EC data quality met the criteria outlined in the QAPP for this project (O'Neill et al. 2014) except for minor deviations (discussed below) that did not compromise the usability of the results.

Calibrations

Continuing calibration verification standards were analyzed at the start, middle and end of the LC-MS/MS analytical sequence for each sample set and the results met our continuing calibration criteria [the relative standard deviation (RSD) of each of the analyte responses relative to the surrogate standard was ≤20% for each sample set].

Method Blank Analysis

A method blank was analyzed for ECs with each sample set. Laboratory criteria for method blanks were met for each sample set [no more than one analyte in a method blank is to exceed 2 × the lower limit of quantitation (LOQ)].

Internal Standards and Surrogate Recoveries

Recoveries of internal standards and surrogates for the English sole bile samples and all quality assurance samples [method blank, spiked blank and matrix spike] associated with the analyses of these samples were within our laboratory criteria (recoveries are to be between 60-130%) except for the following: field sample 139304 Nisqually (recoveries of d4-E1 and d4-E2 were 132% and 138%, respectively); the method blank in sample set ED2913 (recovery of d5-NP was 54%) and the method blank in sample set ED2982 (recovery of d16-BPA was 156%, recovery of d4-E1 was 146%, recovery of d4-E2 was 144% and the recovery of d4-EE2 was 145%). In addition, the recovery of d4-E2 for field sample 115482 ELLTBAY (Seattle Waterfront) was not reported due to strong matrix suppression, even after several dilutions.

Sample Replicates

Bile samples from eleven English sole were analyzed in duplicate in the associated sample sets. Laboratory criteria for sample replicates [RSDs are to be ≤ 25% (equivalent to relative percent difference ≤50% for duplicates) for ≥ 80% of the analytes detected] were met for these replicated samples except for sample a DUWAMISH sample (the relative percent difference for BPA was 63%, with only two analytes detected in both replicates). The concentrations of analytes, in some instances, were detected in one sample but were not detected in the other sample but the levels measured were near the lower limit of quantitation. In these cases, the RSD may be >50%, but this is an artifact of the non-detects.

Spiked Solvent Blanks and Matrix Spike Blanks

A spiked solvent blank and a coho salmon bile matrix spike were analyzed with each sample set, and the data were reported on the basis of percent recovery of the analytes spiked. In each of these samples, four individual conjugated ECs [17β-estriol-16-glucuronide (E3-16G), estrone-3-glucuronide (E1-3G),

17β-estradiol-3-glucuronide (E2-3G), 17α-ethynylestradiol-3-sulfate (EE2-3S)] were spiked at a level of 13.4 ng each and three individual unconjugated xenoestrogens (bisphenol A, n-octylphenol, n-nonylphenol) were spiked at a level of 7.52 ng each. The percent recoveries of the ECs and xenoestrogens in the coho bile matrix spike ranged from 36 – 129%, with lower recoveries of E3 (ranging from 36 – 64%) in the matrix spike compared to the other analytes. The percent recoveries ranged from 51 – 132% in the solvent spiked blanks, with lower recoveries of E3 (ranging from 51 – 90%) compared to the other analytes.

SSRIs

Following are the data quality control checks for chemical analyses of English sole liver tissue for fluoxetine, sertraline, and citalopram as determined by gas chromatography – mass spectrometry (GC-MS). The SSRI data quality met the criteria outlined in the QAPP for this project (O'Neill et al. 2014) except for minor deviations (discussed below) that did not compromise the usability of the results.

English sole livers from the following sampling sites were analyzed: Strait of Georgia, Duwamish, Elliot Bay, Port Gardner, Hood Canal, Sinclair Inlet, Vendovi, Nisqually, Tacoma- City Waterway, and Eagle Harbor.

Calibration and Matrix Spiked Blanks

Initial calibration of the instrument for retention time was made using verified fluoxetine, sertraline, and citalopram standards, which were derivatized exactly as was done for sample extracts. This matrix free calibration provided a mass spectrum for each derivatized analyte that was compared to previously published studies to confirm the correct analyte is being measured. Subsequent calibration of the instrument during sample analysis was done using actual English sole liver homogenate that was spiked with different amounts of the analytes. Thus, for this effort no distinction is made between "calibration standards" and "matrix spike standards" because they are the same. This approach to calibration was used because no suitable surrogate matrix for English sole liver tissue could be found. During the method development phase, we investigated using Atlantic halibut liver tissue (collected from lab reared fish & free of prior SSRI exposure) as a calibration matrix. But this was judged to not be representative of English sole liver due to higher lipid content. We decided to use the Atlantic halibut liver tissue for the method blank as it was the only marine flatfish tissue available that was known to be free of prior SSRI exposure. This use of a surrogate tissue matrix for a method blank as opposed to matrix free blank (solvent blank) permits assessment of background interference from endogenous biological molecules that are co-derivatized.

For the calibration standards, we prepared a homogenate of English sole liver tissue removed from fish 135G-ESL-3109, collected from the Strait of Georgia site. This fish was selected as the amount of liver tissue was large (>10g) permitting a large volume of homogenate to be prepared. We initially assayed this sample without any SSRI addition to verify no detectable levels were present. We then subsequently used aliquots of this homogenate to prepare calibration standards. These calibration standards were run once for every two batches of samples. Analyte concentrations were calculated using point-to-point calibration with at least four concentration levels of calibration standards.

Method Blank

At least one method blank, prepared in laboratory reared, Atlantic halibut (*H. hippoglossus*) liver tissue was analyzed for fluoxetine, sertraline and citalopram for every two batches of samples. All analytes met acceptance criteria for blanks: to not exceed 2 times the lower limit of quantitation (LOQ).

Internal Standard and Surrogate Recoveries

Recoveries of the internal standard (hexa-deuterated paroxetine; d6-paroxetine) for the English sole liver samples were generally within laboratory criteria (recoveries between 50-150%). This was based on comparison of integrator area counts observed in calibration standards. The same quantity of d6-paroxetine was added to all samples, calibrators / matrix spiked blanks. This approach to determining recovery was used because pure standards of the derivatized-SSRIs including the internal standard are not available. The d6-paroxetine recovery was calculated by dividing the observed area counts in the sample (obtained after the complete extraction and derivatization procedure) by the average d6-paroxetine area counts observed in the matrix spike calibrators, which were run for every two batches of samples. It is worth noting that the average d6-paroxetine area counts were based on calibrators whose extracts were reconstituted in 100 µl of solvent prior to analysis. Most samples were also reconstituted in 100 µl of solvent, permitting direct comparison. However, some samples (such as those from the Duwamish site and select samples from other sites) were intentionally reconstituted in a smaller volume (25-50 µl) to improve sensitivity to the analytes. Thus, for these samples, recoveries exceeded 150%. Samples reconstituted in a smaller volume were noted on the spreadsheet. In a few isolated samples (13 or 7% of total analyzed samples), a white precipitate was observed after the derivatization step. This was indicative of excessive moisture present in the vial. This normally results in total failure of the derivatization step (causing "0" % recovery) or partial derivatization (recoveries between 0-46%). These were noted on the spreadsheet as "AF" for analytical failure. In other cases, (8 or 4 % of the total), recoveries were between 26 – 49%, which was unrelated to derivatization (no precipitate was observed). These samples were noted on the spreadsheet as "Low recovery". These samples were not reanalyzed due to the sufficient number of samples analyzed at the site and overall lack of detection of the analytes.

Sample Replicates

Sample replicates were not performed due to limited sample size for nearly all samples and the complete lack of detection of the analytes in the samples assayed.

Vitellogenin Gene Expression Analysis (qPCR)

Below are the data quality control checks for analysis of English sole liver vitellogenin gene expression by quantitative real-time polymerase chain reaction (qPCR). The gene expression data quality met the criteria outlined in the QAPP for this project (O'Neill et al. 2014) except for minor deviations (discussed below) that did not compromise the usability of the results.

RNA isolation, DNase treatment and cDNA synthesis methods

Methods for RNA isolation, DNase treatment of RNA and cDNA synthesis were conducted according to Guzmán et al. 2013. Guzmán et al. 2013.

RNA quality

Our laboratory criterion for RNA quality (both after RNA isolation and DNase treatment) is that all RNA samples must fall within an absorbance ratio (260/280 nm) of 1.8 to 2.2. This criterion was met for all liver RNA samples.

Genomic DNA contamination and PCR carryover contamination

DNase-treated, but not reversed transcribed RNA samples (non-amplification controls, NACs, 14 randomly-selected samples total: RNA from fish #133498, 133241, 133886, 116004, 133617, 139443, 133487, 133259, 133737, 133849, 115969, 133398, 133164, and 115054) were analyzed by qPCR to assure that genomic DNA contamination was effectively eliminated by DNase treatment. In sample #133241 for vtgb, and samples # 133498, 11604, 139443, 133259, 133126, 133398, 133164, 115054 for 18s, one of the duplicate samples showed minor signal (cycle threshold (Ct) values ranging from 35-37) and melting curve peaks not specific for the target genes, indicating false positives. As detailed in the QAPP, NAC Ct-values in this range are acceptable. In addition, four no template control (NTC) samples, consisting of water instead of RNA or cDNA samples, were also analyzed to detect possible carryover contamination of qPCR reagents with gene target PCR products. One duplicate for one of the NTC samples showed a minor signal (Ct value of 36) for 18s and a non-target specific melting curve, indicating a false positive. Based again on the QAPP, an NTC Ct-value in this range is acceptable.

qPCR methods and performance

Development of qPCRs for *vtgb* and *18s* followed protocols for other genes developed in our lab Luckenbach et al. (2011), while standards generated from cDNA to quantify copy number were developed as described in Guzmán et al. 2009.

qPCR Efficiency

Our laboratory criterion for qPCR efficiency is that it should fall in a range of 90-110%, based on the second derivative maximum ($E=10^{-(1/slope)}-1$). The calculated efficiency for the *vtgb* qPCR was 103.8% and for *18s* was 107.5%, both falling within the acceptable range.

qPCR Specificity

Our laboratory criterion for qPCR specificity is that assay melt curves should show a single peak. This criterion was met for all experimental samples, as a single melt curve peak was observed at 80ºC and 83ºC for *vtgb*, and *18s*, respectively.

qPCR Reproducibility

Standards were run in triplicate and samples in duplicate for each assay. Our laboratory criterion for qPCR reproducibility/precision is that technical replicates should not show more than a 0.5 Ct difference. All standards met this criterion; however, 23 experimental samples for *vtgb* and 29 samples for *18s* did not initially meet this standard and had to be rerun as a corrective action. After rerunning the samples, all technical replicates fell within 0.5 cycles of each other and were deemed acceptable.

qPCR Sensitivity

Limit of detection of the assays was calculated as the lowest point of reliable detection by qPCR. For the *vtgb* and *18s* assays it was 13.5 copies/µl of reaction and 43 copies/µl of reaction, respectively. All experimental samples were above this limit of detection for both assays.

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