Quality Assurance Project Plan

Chemicals of Emerging Concern in Puget Sound English sole (*Parophrys vetulus*): exposure to and effects of selected xenoestrogens and pharmaceuticals

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Quality Assurance Project Plan

Chemicals of Emerging Concern in Puget Sound English sole (*Parophrys vetulus*): exposure to and effects of selected estrogenic chemicals and pharmaceuticals.

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1.0 Abstract

This project provides a Sound-wide assessment of the presence and biological impact of selected Chemicals of Emerging Concern (CECs) in English sole (*Parophrys vetulus*), an important indicator species for toxics monitoring in Puget Sound. This project leverages 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). Objectives of the project are twofold: (a) develop analytical methods for, and provide a current evaluation of the extent and magnitude of, CEC contamination in English sole for two major classes of CECs, and (b) develop cost-effective bioeffects endpoints for these CECs. The two major classes of CECs are; estrogenic chemicals (ECs), including three natural estrogens (17β-estradiol, estrone, and estriol) and four xenoestrogenic compounds (17α-ethynylestradiol, bisphenol A, nonylphenol and octylphenol); and three selective serotonin reuptake inhibitors (SSRIs) that may amplify the effects of ECs (fluoxetine, sertraline and citalopram). The ultimate goal of this project is to provide Puget Sound recovery targets based on CEC-related health endpoints in indicator species, as well as CEC tools for monitoring Puget Sound ecosystem health. Study objectives are to (1) provide data on ECs and SSRIs, in organism tissues via a Puget Sound-wide reconnaissance survey of English sole and (2) develop a method for measuring vitellogenin (VTG) induction, a widely accepted biological indicator of EC exposure. Establishing VTG induction as a monitoring tool for English sole will fill a critical 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 perspective for prioritizing contaminant-related recovery efforts in Puget Sound.

2.0 Background

This document details specific procedures and quality assurance guidelines proposed by the Washington Department of Fish and Wildlife *Toxics in Biota* staff to implement the following project*: "*Chemicals of Emerging Concern in Puget Sound English sole (*Parophrys vetulus*): exposure to and effects of selected estrogenic chemicals and pharmaceuticals*".*

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. The *Toxics in Biota* effort is one component of PSEMP, a multi-agency effort designed to monitor the health of the Puget Sound ecosystem. PSEMP tracks a broad range of status indicators, including submerged aquatic vegetation, sediment health, fecal contamination in shellfish, water quality and several others. WDFW's *Toxics in Biota* component of PSEMP (a) monitors the status and trends of chemical contamination in Puget Sound biota, (b) evaluates the effects of contamination on the health of these resources and (c) provides information to public health officials for assessing if Puget Sound seafood is safe to eat.

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. Although some contaminants currently monitored by PSEMP 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 estrogenic chemicals (ECs; estrogens and xenoestrogens), in Puget Sound biota, incorporating clear metrics related to EDC effects on fish health. Pharmaceuticals, such as the widely prescribed Selective Serotonin Reuptake Inhibitors (SSRIs) antidepressants are also of interest because they may amplify the effects of ECs. 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.

2.1 Study Area

The project study area is the foraging habitat of adult English sole in Puget Sound and includes ten index sites from which English sole have been monitored for over 20 years for chemical contamination. They represent a broad range of sites from highly urbanized embayments such as Elliott Bay, Commencement Bay, and Sinclair Inlet, to non-urbanized areas such as the Southern Strait of Georgia and northern Hood Canal.

2.2 Logistical Problems

Collection of tissues from field caught fish is always subject to the availability of the fish. However, in this study, analyses for the presence of ECs and SSRI in VTG expression in English sole will be measured in tissue samples collected and archived from previous surveys conducted in 2011 and 2013. Ability to complete all analyses assumes that sufficient amounts of tissue are available. A limited amount of tissue is available for some archived samples, potentially precluding re-analysis for these samples should it be required.

2.3 History of the Study Area

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 CEC, are a special concern due to its semi-enclosed and increasingly urbanized hydrological system, which receives about 2 billion liters (2 million m^3) of treated 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 overflow units (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 Elliott Bay, Duwamish Waterway, Sinclair Inlet, 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--- near Vendovi lsland) and the South Puget Sound (e.g. Nisqually River reach).

We will provide a current evaluation ECs and SSRIs in adult English sole collected from their foraging habitats at ten sampling locations that are monitored by WDFW's Toxics in Biota effort. Collectively, these locations encompass a range of sources, from relatively rural undeveloped areas to heavily urbanized and industrialized areas. Section 6.0 of this document provides a more detailed description of the individual index sites and a map of the sampling locations (Figure 2).

2.4 Contaminants of Concern

Priority estrogenic chemicals emerging internationally as threats to ecological and human health include natural estrogens that come from exogenous sources, 17β-estradiol (E2), estrone (E1) and estriol (E3) and xenoestrogenic compounds such as bisphenol A (BPA), nonylphenol (NP), octylphenol (OP) and the synthetic hormone 17α-ethynylestradiol (EE2) (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 at low concentrations. Data on exposure concentrations and toxic effects of BPA, NP, OP, EE2 and natural estrogen are limited for marine ecosystems, including Puget Sound, but are necessary to assess the risk that they pose for the health of fish and other biota in the ecosystem.

Of the many pharmaceuticals released into the environment, anti-depressants such as 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). They also have been demonstrated to occur at readily detectable levels in WWTP effluent throughout the Puget Sound region (Table 1). Samples will be analyzed for SSRIs commonly detected in WWTPs: Fluoxetine, Sertraline, and Citalopram.

2.5 Results of Previous Studies

PSEMP staff observed effects from estrogenic EDCs in English sole (*Parophrys vetulus*), approximately 15 years ago, which were manifest as unusual reproductive condition in fish from Elliott Bay. Subsequent focused studies by National Oceanic and Atmospheric Administration (NOAA) and WDFW researchers

quantified these effects as altered reproductive timing in females, and vitellogenin (VTG) induction in males from several areas in Puget Sound (Johnson et al., 2008). In sexually maturing oviparous fish, VTG is a female-specific egg yolk precursor protein synthesized in the liver after E2 interacts with nuclear estrogen receptors and activates VTG gene transcription (Yamaguchi et al., 2009). Male fish normally have undetectable or very low blood levels E2 and VTG, but once exposed to exogenous estrogenic chemicals (ECs) the liver synthesizes and releases VTG. Thus, the presence of VTG in males is a specific biomarker of EC exposure (Islinger et al., 2003; Kime et al., 1999). The presence of VTG (measured as mRNA or protein) in wild male or juvenile fish is now commonly used as an index of exposure to estrogenic chemicals in aquatic ecosystems (Kime et al., 1999; Solé et al., 2003).

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 (*Oncorhynchus tshawytscha*) from Elliott Bay and other Puget Sound sites, 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.

Pharmaceuticals, including some drugs that act as endocrine disruptors, represent another class of CECs for the Puget Sound region and elsewhere. There is ample evidence that active pharmaceutical ingredients (APIs) are detectable in wastewater and stormwater, and may adversely affect aquatic organisms (Kositch and Lazorchak, 2008; Kositch et al., 2010; Lubliner et al., 2010; Morace 2012). Prioritizing which pharmaceuticals pose the highest environmental concern is challenging because toxicity data are lacking for most APIs. However, SSRIs, the class of anti-depressants that includes fluoxetine (Prozac) consistently emerge as pharmaceuticals of concern. As with ECs we propose a biota-risk-based approach (after Roos et al., 2012), involving estimation of both true exposure (i.e. internal dosimetry; see Valenti et al., 2012) and effects.

Recent research studies on APIs originating from wastewater treatment plants (WWTPs) provide guidance regarding risk. Lubliner et al. (2010) concluded that advanced nutrient removal systems in Puget Sound's WWTPs failed to remove SSRIs such as fluoxetine. In a 2012 EPA-funded study, Battelle detected several SSRIs at moderately high concentrations (from >200 to 1000 ng/L -- see Table 1) in effluent from eight Puget Sound WWTPs. Other researchers have reported readily detectable SSRIs in fish bile after field exposures to WWTP effluents in concentrations ranging from the LOQ (approximately 0.2 ng/mL bile) to 2.8 ng/mL bile (Togunde et al., 2012). Levels of SSRIs detected in Puget Sound WWTP effluent are within the range of concentrations associated with alterations to reproduction, growth, and development (reviewed in 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 trends monitoring.

2.6 Regulatory Criteria

There are no criteria regulating the exposure of English sole to the contaminants of concern in this study.

3.0 Project Description

This project is designed to (1) provide a Puget Sound-wide assessment of the presence and impact of ECs and pharmaceuticals in English sole, a key indicator species for toxic contaminants in Puget Sound, and (2) measure health effects in English sole related to exposure to these chemicals. This project augments an existing toxics monitoring program of biological indicators in Puget Sound, by adding bio-effects metrics for and tissue residue measurements of CECs for a key contaminant indicator species. We will evaluate the geographic extent to which English sole are exposed to and harmed by ECs and the magnitude of exposure and effects when they occur. This extent will cover a broad range of sites, from highly urbanized embayments such as Elliott Bay, Commencement Bay, and Sinclair Inlet, to relatively pristine areas such as the Southern Strait of Georgia and Hood Canal. This project will focus primarily on (a) seven ECs (BPA, NP, OP, EE2, E1, E2 and E3) which can cause feminization of male English sole, and alter reproductive activities of females, and secondarily on (b) three SSRIs including fluoxetine, sertraline and citalopram. This is a class of environmentally persistent pharmaceuticals (commonly prescribed as anti-depressants), which recent studies suggest can amplify effects of estrogenic chemicals. Additionally we will measure VTG production in adult English sole by specific quantification of VTG mRNA transcripts in liver by reverse transcriptase polymerase chain reaction (RT-PCR), a common approach due to its high sensitivity and shorter response time compared to the appearance of the protein in blood (García-Reyero et al., 2004; Hemmer et al., 2002; Yamaguchi et al., 2009).

The reconnaissance survey of CECs for this project will use tissue, bile and other samples generated from English sole sampled by PSEMP in 2011 and 2013, for ten index sites across Puget Sound. These 2011/13 efforts present a unique opportunity to observe two important classes of CECs that can be used as a starting point for long-term monitoring of these chemicals and conditions. Moreover the effects metrics proposed for ECs and SSRIs will be assessed in combination with other metrics currently monitored by PSEMP, including gonadal condition, lesions, and lipid content, as well as tissue residues of persistent bioaccumulative and toxic chemicals. This comprehensive approach will document exposure and effects of multiple CECs across a wide range of potential environmental contaminant conditions. The biological indicators of EDC exposure and effects proposed herein 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 Puget Sound recovery.

3.1 Project Goal

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

3.2 Project Objectives

Our study objectives are to: (1) develop and apply a rigorous detection and quantitation method to provide data on two classes of currently used CECs with EDC properties: ECs and SSRIs, in English sole (as tissue residues) via a Puget Sound-wide reconnaissance survey, and (2) develop and apply a rigorous detection and quantitation method for VTG induction (a widely accepted biological indicators of EDC-effects) in male English sole, a species previously documented to have exhibited exposure to environmental source of ECs. This latter metric will be developed specifically for use in long-term monitoring of Puget Sound biota.

3.3 Information Needed and Sources

All sampling information (e.g., dates and locations) and biological information (e.g., length, weight and sex) for English sole are available from WDFW's field survey reports (Quinnell 2011, Niewolny 2013) and WDFW/PSEMPs database (Table 7). Sample numbers available for the various analyses are summarized in Section 6.3 (Table 8). The information needed to develop and apply methods for analyzing xenoestrogenic compounds and SSRIs in tissues and for quantitative Polymerase Chain Reaction (qPCR) for expression of vitellogenesis are detailed in sections 8.1 (Chemical Analyses) and 8.2 (Gene Expression Analysis).

3.4 Target Population

The target population for this study is English sole from Puget Sound marine habitats. English sole (*Parophrys vetulus*) have been used for over 25 years as benthic sentinel species for studies on effects of environmental chemical contaminants. This species is broadly distributed in benthic habitats along the Pacific Coast of the United States, where both juvenile and adult phases of its life history are in contact with benthic sediments. English sole can take up chemical contaminants directly from sediments or indirectly through their benthic diet.

Field studies of English sole from Puget Sound, Washington and other embayments on the Pacific Coast of the United States have provided us with a strong epizootiological database that implicates xenobiotic contaminants (especially PAHs, and PCBs) as causative etiological agents of neoplasms and related toxicopathic hepatic lesions detected in wild fish (reviewed in Myers et al., 1999; 2003), as well as other adverse health effects such as reproductive and growth impairments (Johnson et al., 2002; 2008).

English sole is one of the species currently monitored by WDFW's Toxic in Biota Unit, a component of PSEMP. It is an ideal candidate for toxicological work because it occurs in a variety of areas throughout Puget Sound and is relatively sedentary during spring-early fall foraging periods (Day 1976), but does migrate to deeper waters to spawn in the winter months (Moser et al., 2013). During these foraging periods English sole are found in shallow Puget Sound embayments where the greatest contamination of sediments by industrial or urban pollutants typically occurs (Long et al., 2005). We will be using archived bile and liver samples collected as part of the PSEMP survey conducted in 2011 and 2013 to complete the analyses of EDCs and SSRIs.

3.5 Study Boundaries

This boundary area for this study is the marine foraging habitats of adult English sole within Puget Sound. English sole collected from ten index sites that collectively encompass the full range of summer feeding marine habitats used by English sole, including urban, near-urban, and non-urban embayment of Puget Sound. Section 6.0 of this document provides a more detailed description of the individual index sites and a map of the sampling locations (Figure 2).

3.6 Tasks Required

Tasks involved in this study include:

- Task 1: Project Administration & Management
- Task 2: Developing a Quality Assurance Project Plan
- Task 3: Chemical Analyses to measure exposure to estrogenic chemicals
- Task 4: Chemical Analyses to measure exposure to SSRI antidepressants
- Task 5: Measuring the effects of estrogenic chemicals on indicators of fish reproductive health

- Task 6: Data Analyses and Reporting including , formatting data for relational database, analysis of data for DFW final report, and transfer of data to Ecology's Environmental Information Management or EIM database
- QA/QC review

3.7 Practical Constraints

Assessment of presence of xenoestrogen and SSRI in English sole tissues is challenging because standard methods have not been established for measuring these analytes in fish tissue.

Recently, NOAA/NWFSC researchers developed a method to measure estrogens and xenoestrogens in fish bile. For this study, we use the methods developed by da Silva et al. (2013), with minor modifications to improve sensitivity and accuracy of the method, as well as to expand the list of target analytes. NOAA does not anticipate difficulties for modifying the method of analysis for ECs. A bile Standard Reference Material (SRM) is currently not available for the ECs identified in this project.

In the present project we aim to develop specific real-time quantitative PCRs (qPCRs) to determine liver transcript levels of English sole VTGA and VTGB, and evaluate their relative sensitivity to ECs. We do not anticipate difficulties with the development of this assay since VTG sequences from numerous teleosts are available in GenBank, a public database of genomic and proteomic information including several flatfish species, and we have 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). Based on previous experience with adapting this method to new species, we fully expect the lab staff time and equipment identified for this study will be sufficient to develop this assay.

4.0 Organization and Schedule

4.1 Key Individuals and Their Responsibilities

Table 1. Organization of project staff and responsibilities.

Name	Title	Phone #	Email	Responsibilities
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Edward Hayman*	WDFW Scientific Technician	206-860-3470	edward.hayman@dfw.wa.gov	Laboratory technical support for qPCR analyses
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Dr. José Guzmán*	NOAA NWFSC – Post Doc	206-302-2498	jose.guzman-jimenez@noaa.gov	Researcher, lead for VTG qPCR method development & analyses
Dr. Adam Luckenbach*	NOAA NWFSC Research Biologist	206-860-3463	adam.luckenbach@noaa.gov	Researcher, provides advice on VTG qPCR method development
Gina M. Ylitalo*	NOAA NWFSC Program Manager	206-860-3325	gina.ylitalo@noaa.gov	Co-investigator, oversight of method development & analyses of estrogenic chemicals
Dr. Denis da Silva*	NOAA-NWFSC Research Chemist	206-860-3330	denis.dasilva@noaa.gov	Researcher, lead for method development & analyses of estrogenic chemicals
Lyndal Johnson*	NOAA NWFSC – Research Zoologist	206.860.3345	lyndal.l.johnson@noaa.gov	Co-investigator, lead for reproductive fish health assessment
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William Kammin	Ecology QA Officer	360.407.6964	wkam461@ecy.wa.gov	approves QAPP

*Detailed experience and qualifications are presented in Appendix B.

4.2 Project Schedule

Table 2. Proposed schedule for major project tasks detailing deliverable dates for laboratory work, data analyses, and report writing.

Task	Item	Due Date	Lead Staff
Task 1: Project Administration & Management	Progress Report with Invoice	quarterly	Sandra O'Neill
	Project Accomplishments and Outcome	31-Jul-15	
Task 2: Quality Assurance Project Plan	Draft to Ecology for review	23-Jun-14	Sandra O'Neill
	Final to Ecology	31-Jul-14	
Task 3: Chemical Analyses - Measures of Exposure to Estrogenic Chemicals	Laboratory Summary and QA/QC Report	31-Dec-14	Denis da Silva
Task 4 - Chemical Analyses - Measures of Exposure to SSRI Anti-depressants	Laboratory Summary and QA/QC Report	31-Dec-14	Irvin Schultz
Task 5 - Effects of Estrogenic Chemicals on Fish Reproductive Health	Laboratory Data Report	31-Dec-14	José Guzmán
Task 6 - Data Analyses and Reporting	Draft due to peer reviewers and NEP staff	1-Jun-15	Sandra O'Neill
	Final report due	31-Jul-15	
	Data Transfer to EIM	31-Jul-15	Laurie Niewolny

4.3 Budget and Funding

This project is supported by an interagency agreement with Ecology with funding from Toxics and Nutrients Prevention, Reduction, and Control. This overall effort is funded by EPA's National Estuary Program (NEP). Match for this study is provided by WDFW in the form of staff time, sample collection, and laboratory supplies.

Table 3. Proposed WDFW budget for study of CECs in English sole.

Task	**Budget**
Task 1: Project Administration	$10,225
Task 2: Quality Assurance Project Plan	$ 5,113
Task 3: Chemical Analyses - Measures of Exposure to Estrogenic Chemicals	$ 6,452
Task 4: Chemical Analyses – Measures of Exposure to SSRI Anti-depressants	$50,971
Task 5: Effects of Estrogenic Chemicals on Fish Reproductive Health	$58,389
Task6: Data Analysis and Report Writing	$88,850

5.0 Quality Objectives

The quality objectives of this study are designed to ensure that we accurately measure targeted ECs, SSRIs, and VTG induction in a repeatable manner, with an appropriate level of sensitivity, in adult English sole from Puget Sound. Specific methods in the form of standard operating procedures for the three target metrics in this study, (1) ECs in bile, (2) SSRIs in liver, and (3) gene expression of vitellogenin do not currently exist –a primary objective of the study is to develop these methods by modifying existing analytical methods and tools, and applying them as a demonstration using existing English sole samples. Sample sizes and MQOs described herein should be sufficient to identify and measure differences between populations if differences exist, balancing analytical costs with available funds, while achieving adequate geographic coverage. In addition, corrective actions to be taken when quality assurance criteria are not met are identified.

In most cases foundational operating procedures are available in the form of published, peer reviewed methods. However, basic MQOs are detailed and will be followed, as described below, for each of the three target metrics.

5.1 Measurement Quality Objectives (MQOs) for ECs in Fish Bile

The primary objective for development of the LC-MS/MS standard operating procedure to analyze ECs in English sole bile is to define cost-effective methods sufficient to evaluate the target analytes within acceptable limits for precision, accuracy, sensitivity, comparability, representativeness, and completeness. MQOs for quality controls are described herein and summarized in Table 4.

5.1.1 Precision

Standard Reference Materials (SRMs) or Certified Reference Materials (CRMs) are currently not available for ECs in fish bile. We will evaluate precision by analyzing (a) bile free of the target analytes and (b) water free of the target analytes, both spiked with a known amount of all ECs (*spiked-matrix* and *spiked blank*, respectively). One spiked-matrix and one spiked blank will be included in each analytical batch. Precision is expressed as the relative standard deviation (RSD) for repeated measurements of the internal standards, which must be ≤ 15% to be acceptable. Field replicates may be processed if sample volume is sufficient, however we do not anticipate this to contribute significantly to estimating precision.

5.1.2 Bias

Standard Reference Materials (SRMs) or Certified Reference Materials (CRMs) are currently not available for ECs in fish bile. Accuracy of sample measurements will be assessed by including spiked-matrix and spiked-blank samples (one each) in every batch. The recovery of all analytes must be between 60 – 130% to meet accuracy standard (Table 4).

5.1.3 Sensitivity

The Lower Limit of Quantitation (LOQ) for the ECs in this study is the concentration that would be calculated if that analyte had a LC-MS/MS area ratio equal to its area ratio in the lowest level calibration standard used in that calibration (where: area ratio = response area of analyte/response area of surrogate standard). When an analyte is not detected in a sample or it has a response area that is smaller than its area in the lowest level calibration standard used, the concentration of the analyte in that sample is reported to be less than the value of its lower LOQ (Sloan et al., 2006). In this study, individual sample EC LOQs are given as a range because sample LOQs are affected by the field sample volume, as well as the sensitivity of each analyte in the instrument. The LOQ is the lowest concentration at which a result will be reported.

LOQs of seven ECs from unpublished pilot studies evaluating the sensitivity of the LC-MS/MS method have been between 0.75-1.5 ng/mL bile in English sole bile; concentrations of ECs are expected to range from the LOQ to 30,000 ng/mL bile (Table 9), based on da Silva et al. (2013) and subsequent unpublished work. Sensitivity of this method should be sufficient to evaluate geographic differences and time trends in biliary EC concentrations. There are no reported effects levels for ECs in English sole, so it is impossible to evaluate whether the method is sensitive enough to measure effects concentrations.

5.1.4 Comparability

This is a one-time study to establish the use of the LC-MS/MS method as a tool to assess presence and effects of ECs. To date, limited EC data are available (da Silva et al., 2013). In this study, we propose to use da Silva et al. (2013) methods with slight modifications (detailed in section 8.1.4.1) so the results from this study should be comparable to previously reported data.

5.1.5 Representativeness

This study aims to develop a method that can be used to monitor geographic and temporal trends in EC exposure of English sole for toxics monitoring in Puget Sound, and to demonstrate the applicability of the method using previously collected English sole samples. The geographic coverage represents urban, near-urban, and non-urban locations where English sole may be exposed to a wide range in magnitude of ECs. Without knowing the distribution of ECs across these land-use types, or across sexes and fish sizes, it is impossible to predict how well the samples taken for this study will identify geographic differences in EC exposure. Moreover, there is no *a priori* reason to suspect that size of the animal would control EC exposure. Our pilot studies on biliary ECs in English sole suggest existing sample sizes will be large enough to represent geographic patterns, and to begin to address potential differences attributable to sex of the animals.

5.1.6 Completeness

This study will be considered complete when the minimum number of samples for EC and SSRI exposure and VTG induction listed in Table 8 (Section 6.3) are analyzed.

Table 4. QC samples, MQOs, and corrective actions for the analysis of English sole bile for estrogenic compounds (ECs) by liquid chromatography-tandem mass spectrometry (LC-MS/MS)

Quality Controls	Minimum Frequency	Control Limit	Corrective Action
Initial Calibration (minimum 5 different concentrations per analyte)	One per analytical sequence	The % accuracy of each analyte in each level is to be 70 – 130%.	If the acceptance criterion is not achieved, corrective action must be taken. Any samples associated with a failed initial calibration must be reanalyzed.
Method Blank (MB)	One in every batch of 20 or fewer field samples	Each analyte is to be < 2 x Lower Limit of Quantitation (LOQ).	If target analytes in the blank exceed the acceptance limits, the source of the contamination must be investigated. All samples associated with an unacceptable method blank must be re-prepared and reanalyzed. If the analyte was <u>not</u> detected in the samples, then the data may be reported with censoring qualifiers and the issue must be addressed in the project narrative.
Spiked matrix	One in every batch of 20 or fewer field samples	The recovery of each analyte is to be 60-130%.	If recoveries of the analytes are outside the established control limits, corrective action must occur. If recoveries are made outside the control limit and the analyte(s) of interest are not detected in samples, the data may be reported with censoring qualifiers and the issue addressed in the project narrative. In other circumstances, the entire batch must be re-extracted.
Spiked blank	One in every batch of 20 or fewer field samples	Recovery of each analyte is to be 60-130%.	If recoveries of the analytes are outside the established control limits, corrective action must occur. If recoveries are made outside the control limit and the analyte(s) of interest are not detected in samples, the data may be reported with censoring qualifiers and the issue addressed in the project narrative. In other circumstances, the entire batch must be re-extracted.
Continuing Calibration Check (LC-QC)	One in 15 or fewer LC injections	The RSD of each analyte is to be ≤15%.	If the RSD of the continuing calibration check (LC-QC) standards injected throughout the sequence is above the control limit, the entire batch should be re-run on the LC-MS/MS. If the batch is not re-run, the reasons for accepting the batch must be clearly presented in the project records and the report.
Surrogate standards	Added to every sample	The recovery of each surrogate is to be 60-130%	• Check all calculations for error; recalculate the data if an error is found. • Ensure that instrument performance is acceptable; reanalyze the sample if a problem is found. • If neither of the above resolves the problem, re-extract and reanalyze the sample or flag the data as an "Estimated Concentration".

5.2 Measurement Quality Objectives (MQOs) for SSRIs in Fish Liver

The primary objective for development of the GC/MS standard operating procedure to analyze SSRIs in English sole liver is to define cost-effective methods sufficient to evaluate the target analytes within acceptable limits for precision, accuracy, sensitivity, comparability, representativeness, and completeness. MQOs for quality controls are described herein and summarized in Table 5

5.2.1 Precision

Standard Reference Materials (SRMs) or Certified Reference Materials (CRMs) are currently not available for SSRIs in fish liver. We will measure precision across batches by analyzing (a) liver free of the target analytes (a *matrix-spiked blank*), and (b) internal standards run with every sample. Precision is expressed as the relative standard deviation (RSD) for repeated measurements of the internal standards, which must be ≤ 15% to be acceptable. In addition, field replicates may be processed if sample volume is sufficient. When samples are replicated, RSDs are to be <= 20% for analytes that have concentrations >= 2 ng/g. Based on existing samples, we expect to be able to run one duplicate for approximately every 40 samples.

5.2.2 Bias

Standard Reference Materials (SRMs) or Certified Reference Materials (CRMs) are currently not available for SSRIs in fish liver. Accurate identification of target SSRI analytes in English Sole liver tissue is based on monitoring retention times (established from pure standard material) and the presence of corroborating / qualifier ions (m/z 344 for fluoxetine, 501 for sertraline; 324 for citalopram). A composite of English sole liver homogenate, prepared from tissue collected from a non-contaminated site will be used to prepare calibrating standards.

5.2.3 Sensitivity

The Lower Limit of Quantitation (LOQ) for SSRIs in this study is the concentration that would be calculated if that analyte had a GC/MS (for SSRIs) area ratio equal to its area ratio in the lowest level calibration standard used in that calibration (where: area ratio = response area of analyte/response area of surrogate standard). When an analyte is not detected in a sample or it has a response area that is smaller than its area in the lowest level calibration standard used, the concentration of the analyte in that sample is reported to be less than the value of its lower LOQ (Sloan et al., 2006). In this study, individual sample SSRI LOQs are given as a range because sample LOQs are affected by the field sample volume, as well as the sensitivity of each analyte in the instrument. The LOQ is the lowest concentration at which a result will be reported. Overall, however, we expect that if significant geographic trends in SSRI concentrations exist in English sole across our study sites, they will be substantially higher than LOQs. Moreover, there are no reported effects levels for SSRIs in English sole, so it is impossible to evaluate whether the method is sensitive enough to measure effects concentrations. Based on unpublished lab studies we expect the following LOQs for the three SSRIs: 0.5 – 1.0 ng/g for fluoxetine, 1.0 – 2.5 ng/g for sertraline, and 3.0 – 5.0 ng/g for citalopram (Table 9).

5.2.4 Comparability

This is a one-time study to establish the use of the GC-MS method as a tool to assess presence and effects of SSRIs. To date, only limited unpublished lab data are available for comparison, especially for free-roaming field-sampled marine fish such as English sole.

5.2.5 Representativeness

This study aims to develop a method that can be used to monitor geographic and temporal trends in SSRI exposure of English sole for toxics monitoring in Puget Sound, and to demonstrate the applicability of the method using previously collected English sole samples. The geographic coverage represents

urban, near-urban, and non-urban locations that may expose English sole to a wide range in magnitude of SSRIs. Without knowing the distribution of SSRIs across these land-use types, or across sexes and fish sizes, it is impossible to predict how well the samples taken for this study will identify and represent geographic differences in SSRI exposure. Unpublished pilot studies analyzing SSRIs in English sole suggest our sample sizes will be large enough to represent geographic patterns, and to begin to address differences attributable to size or sex of the animals. However, there is no *a priori* reason to suspect that size or sex of the animal would control SSRI exposure for the range of fish sizes in the study.

5.2.6 Completeness

This study will be considered complete when the minimum number of samples for SSRI exposure and VTG induction listed in Table 8 (Section 6.3) are analyzed.

Table 5. QC samples, MQOs, and corrective actions for the analysis of English sole liver tissue using Gas Chromatography/Mass Spectrometry.

Quality Controls	Minimum frequency	Control Limit	Corrective Action
Instrument calibration (using matrix spiked with at least 4 different concentrations)	Once every 40 samples.	Analyte concentrations are to be calculated using point-to-point calibration with at least four concentration levels of calibration standards at 60-130% recovery.	If fewer than 4 calibration concentrations fall outside control limit, stop analyses; rerun standard calibration curve and rerun sample
Spiked matrix	Once every 40 samples.	60-130% of recovery	If recoveries of the analytes are outside the established control limits, corrective action must occur. If recoveries are made outside the control limit and the analyte(s) of interest are not detected in samples, the data may be reported with censoring qualifiers and the issue addressed in the project narrative. In other circumstances, the entire batch must be re-extracted.
Method blank (blank matrix)	Once every 40 samples.	No analyte may be more than 2 x LOQ.	If target analytes in the blank exceed the acceptance limits, the source of the contamination must be investigated. All samples associated with an unacceptable method blank must be re-prepared and reanalyzed. If the analyte was not detected in the samples, then the data may be reported with censoring qualifiers and the issue must be addressed in the project narrative.
Sample replicates (e.g. duplicates of specific samples), when sample mass is available.	Not regularly performed because sample mass is limited. Approximately 5% will be have enough tissue to perform replicate analyses.[a]	When samples are replicated, RSDs are to be <= 20% for analytes that have concentrations >= 2 ng/g	Limited sample mass will probably preclude a rigorous evaluation of intra- and inter-batch repeatability.
Internal standard	Hexa-deuterated paroxetine (d6-paroxetine) is added to every sample.	Integrator area counts are to be 50 – 150% of values in calibration standards.	If >10% of samples in a batch exceed these values, failed samples will be re-injected and reanalyzed. If fewer than 10% exceed, no rerun, and failed samples are censored with a qualifier.

[a]Although mass of individual liver samples may limit availability of analytical replication, the high number of individual samples (approximately 50 per site) can be used to evaluate repeatability.

5.3 Measurement Quality Objectives for VTG Expression

The objective for development of a standard operating procedure for measuring VTG gene expression is to define cost-effective methods sufficient to evaluate VTG within acceptable limits for precision, accuracy, sensitivity, comparability, representativeness, and completeness. MQOs for each of these quality controls are described herein, and summarized in Table 6.

Teleost fish have multiple VTG genes due to genome duplication events (Finn and Kristoffersen, 2007). At least two distinct VTG genes –named 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 reported differences in the regulation of distinct types of hepatic VTG gene transcription 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). Although the opposite scenario was recently reported for *Channa punctatus*, where the VTGB gene had a higher sensitivity to ECs (Rawat et al., 2013). 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. Good

In the present project we aim to develop specific real-time quantitative PCRs (qPCRs) to determine liver transcript levels of English sole VTGA and VTGB, and evaluate their relative sensitivity to ECs. We do not anticipate difficulties with the development of this assay since VTG sequences from numerous teleosts are available in Genbank, a public database of genomic and proteomic information including several flatfish species, and we have 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 real-time PCR 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, will 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 will be 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).

5.3.1 Precision

RNA Quality: all RNA samples must fall within an absorbance ratio (260/280 nm) of 1.8 to 2.2. Failing this, the sample is probably contaminated (i.e. protein, phenol) and will not be included in downstream analyses. If additional liver is available, RNA isolation would be repeated and quality assessed.

qPCR performance: Precision of qPCR assays is indicated by the reproducibility of technical replicates (duplicates or triplicate cDNA samples loaded into different wells), which must not have more than 0.5 Ct (cycle) difference. If this criterion is exceeded, wells will we reloaded with cDNA and rerun.

5.3.2 Bias

RNA Quality: during this step, contamination by genomic DNA from the same tissue for a sample from which RNA is being isolated is the primary concern. A bias among RNA samples would be contamination of genomic DNA in any RNA preparations. To eliminate this bias, we conduct DNase treatment of RNA samples (see details in Section 8.2.5.1). If genomic DNA has been successfully eliminated, negative controls that consist of RNA that was not reversed transcribed (no amplification control, NAC) will show no detection over 40 cycles of PCR. If bias across samples persists, DNase treatment will be repeated on problematic samples.

qPCR performance: The slope of the standard curve defines the efficiency of each assay. If the slope of the standard curve is -3.32 then the qPCR is 100% efficient (each cycle the amount of template is doubled). Efficiencies between 90 and 110% will be considered acceptable. If this criterion is not met, the assay will be rerun, and if the problem persists, the assay will be redesigned, including redesigning a new pair of primers for qPCR.

The specificity of the qPCR assay (i.e. the target gene is correct) is determined by analysis of the melting curve. A gene-specific qPCR should have a single peak in the melt curve, corresponding to a single amplicon. In addition, a qPCR product from each English sole qPCR assay will be sequenced to verify that the intended target was amplified. If the criteria of single peak/single amplicon are not met, the assay will be redesigned for a new pair of primers for qPCR.

5.3.3 Sensitivity

Establishing limit of detection for VTG assay: qPCR is one of the most sensitive biological assay in existence; it is generally considered to be sensitive enough to detect a single or only a few copies of a given gene transcript. Liver VTG copy number is the measure of sensitivity and a primary goal of this study. We will empirically determine sensitivity (i.e., minimum copy number) of the assays developed. This will be achieved via dilution of cDNA standards (for VTG and potential housekeeping genes).

Distinguishing gene-specific signal from background: VTG mRNA may be completely absent or below the limit of detection in some sole liver samples. Samples with no VTG or undetectable VTG will be defined as samples that either do not amplify by 40 cycles of qPCR or that show some minor background signal (cycle threshold (Ct) values ranging from 35-39) that do not exhibit a melt curve peak for VTG. Thus, for samples showing very low signal, we will use a 2-step process of first checking the Ct and then the melt curve to determine if it is truly detecting VTG.

5.3.4 Comparability

In this project, transcripts for VTG will be quantified using quantitative real time polymerase chain reaction (qPCR). Johnson et al. (2008) previously measured VTG (protein) in English sole using a semi-quantitative enzyme linked immunosorbent assays (ELISAs). However, VTG gene expression has generally replaced ELISAs because it provides more consistent and cost-effective results and is likely sensitive enough for detecting response to exposure to ECs (Flick et al. 2014).

These analyses may become standard metrics in monitoring the health of the Puget Sound. Data generated in this study will serve as a baseline for future time trend analyses and other evaluations.

5.3.5 Representativeness

This study aims to develop a method that can be used to monitor geographic and temporal trends in VTG exposure of English sole for toxics monitoring in Puget Sound, and to demonstrate the applicability of the method using previously collected English sole samples. The geographic coverage represents urban, near-urban, and non-urban locations that may expose English sole to a wide range in magnitude of ECs. Without knowing the distribution of VTG induction across these land-use types, or fish sizes, it is impossible to predict whether the samples taken for this study will be enough to identify geographic differences in VTG induction. Moreover, there is no *a priori* reason to suspect that size of the animal would affect VTG induction, because all male fish taken in the study were sexually mature. Our pilot studies on VTG induction in English sole suggest existing sample sizes will be large enough to represent geographic patterns.

5.3.6 Completeness

This study will be considered complete when the minimum number of samples for VTG induction listed in Table 8 (Section 6.3) is analyzed.

Table 6. QC samples, MQOs, and corrective actions for the analysis of vitellogenin gene expression by quantitative PCR.

Quality Controls	Minimum Frequency	Control Limit	Corrective Action
Absorbance Ratio for RNA Isolation	Every sample	Ratio of 1.8-2.2 at 260/280nm	Deviations from this range of ratios indicate unacceptable contamination. The sample must be reprocessed for RNA isolation.
qPCR Efficiency: Slope of the best-fit line of the standard curve (estimate of bias)	Every assay	Range of 90-110% efficiency is acceptable	If this criterion is not met, the assay will be rerun, and if the problem persists, the assay will be redesigned, including redesigning a new pair of primers for qPCR.
Gene Specificity (qPCR Assay): analysis of melting curve to determine whether the target gene is correct.	Every assay	Only one peak (amplicon) should appear	If this criterion is not met, the assay is non-specific. Primers pair needs to be redesigned.
Gene Specificity (qPCR Assay): Sequencing of qPCR product	Every assay	The sequence of the qPCR product should coincide with the target gene sequence	If this criterion is not met, the assay is non-specific. Primers pair needs to be redesigned.
False Positive Control (no cDNA template control, or NTC) or RNA that was not reverse-transcribed (no amplification control, or NAC)	Every assay	Both NTCs and NACs should show either no detectable amplification over 40 qPCR cycles, or minor signal between 35-39 cycles with a melt curve peak not specific for the gene of interest	If NTC's show a major sign or a gene-specific melt peak, the qPCR reagents (e.g. water, primers, master mix) are likely contaminated; assay should be repeated using new reagents. If NAC's show a major sign or gene-specific melt peak, the cDNA sample is likely contaminated with genomic DNA; DNase treatment should be repeated on RNA and downstream steps repeated.
Reproducibility (precision) of technical replicates. Standards will be run in triplicate and samples in duplicate.	Every assay/every sample	Amplification of replicates should not show more than 0.5 Ct (cycle) of difference	If the standards at a certain concentration do not meet this criterion, will not be considered for slope/efficiency calculation. If more than one standard do not meet this criterion the assay will be repeated. If a sample does not meet this criterion, it will be rerun.

6.0 Overall Study Design

The study design includes:

- Chemical Analyses to measure exposure to ECs
- Chemical Analyses to measure exposure to SSRIs
- Measuring effects of estrogenic chemicals on indicators of fish reproductive health
- QA/QC review

Tissue concentrations of selected ECs and SSRIs in English sole fill important data gaps for two major classes of CECs currently released into Puget Sound. We will conduct a Sound-wide reconnaissance survey of ECs and SSRI anti-depressants and VTG induction in adult English sole collected from ten urban, near-urban and non-urban foraging habitats throughout the Puget Sound during 2011 and 2013. The proposed project will support a more comprehensive effects-based monitoring program for Puget Sound by providing baseline exposure level of these CECs in the marine biota of Puget Sound.

In this project we aim to measure VTG induction in English sole related to their exposure to estrogenic chemicals, using hepatic VTG gene expression (e.g., Hiramatsu et al., 2005; Lange et al., 2012). Transcripts for VTG will be quantified using quantitative real time polymerase chain reaction (qPCR). We have already established a qPCR assay for salmon hepatic VTG mRNA, which is positively correlated with exposure to EE2 -- Figure 1). A similar qPCR assay for English sole VTG will be developed after cloning and sequencing English sole VTG. Although a controlled exposure-response experiment is beyond the scope of this study, we will correlate VTG activity with biliary and hepatic concentration of ECs and SSRIs.

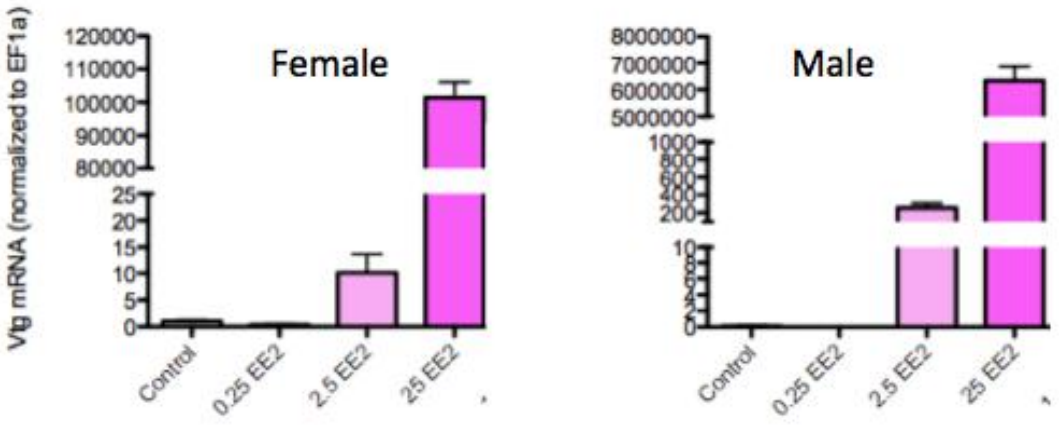

Figure 1. Relative levels of liver VTG mRNA (mean+/- SEM) in juvenile coho salmon smolts exposed to 17α-ethynylestradiol (0.25-25 ng/L) via tank water for two weeks. Levels of VTG were measured by quantitative real time RT-PCR. N=12 fish per sex. Swanson et al., unpublished.

Gene expression of VTG combined with EDC tissue residues supports the development of biological indicators that may predict EDC-related health impairments. EDC exposure and effects have been identified as a key component of the Puget Sound Partnership's Toxics in Fish Vital Sign but currently are not funded. Information generated for this project will fill this gap the Toxics in Fish Vital Sign.

6.1 Sampling location and frequency

Samples were collected at ten locations in the spring of 2011 and 2013 during the PSEMP English sole surveys. Collectively, the ten sampling locations encompass a range of sources from relatively rural, undeveloped areas such as the Hood Canal, Strait of Georgia, and the Gulf of Bellingham (near Vendovi Island) and the Nisqually Reach, the moderately urbanized Port Gardner Bay, and more heavily urbanized and industrialized areas such as Elliott Bay, Duwamish Waterway, Sinclair Inlet, Commencement Bay, and Eagle Harbor locations (Table 7). Section 6.2 provides a map of the ten sampling locations (Figure 2).

Table 7. English Sole PSEMP Survey Station Descriptions and Latitude/Longitude.

Station Name	Description (Puget Sound Basin)	Latitude	Longitude
STRTGEOR	Strait of Georgia (North Sound)	48.87138	-122.94048
VENDOVI	Gulf of Bellingham -northwest of Vendovi Island (North Sound)	48.64247	-122.63780
PTGARDNR	Port Gardner (Main)	47.98556	-122.24409
HDCANAL	Northern Hood Canal (Hood Canal)	47.83458	-122.64128
EGLHARBR	Eagle Harbor (Main)	47.62017	-122.51010
ELLTBAY	Elliott Bay - Seattle Waterfront (Main)	47.60654	-122.34747
DUWAMISH	Duwamish Waterway (Main)	47.56380	-122.34771
SCLINLET	Sinclair Inlet (Main)	47.54797	-122.64925
COMMBAY	Commencement Bay -Thea Foss Waterway (Main)	47.25946	-122.43618
NISQUALY	Nisqually Reach (South Sound)	47.15864	-122.66799

6.2 Map of study area

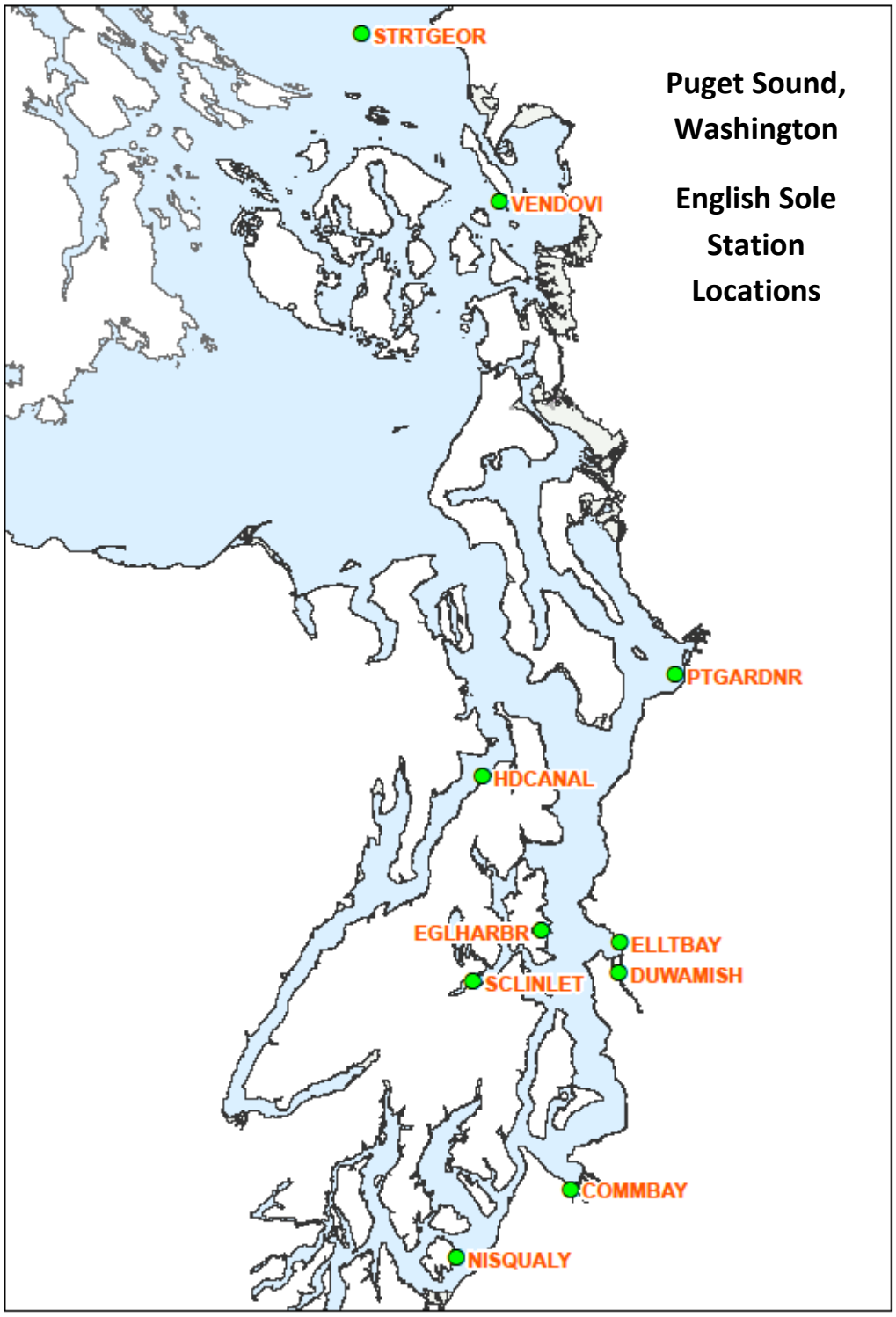

Figure 2. Location of ten long-term monitoring stations for the PSEMP English sole surveys. Station names are defined in Table 7.

6.3 Samples collected for analyses

This study will use samples collected from the 2011 and the 2013 English sole PSEMP Surveys. Table 8 depicts the target number of samples for each analysis. Generally, sample sizes and locations were selected to maximize power for representing potential low to high exposure scenarios.

Table 8. Target number of adult English sole samples to be analyzed for ECs, the presence of VTG, and SSRIs.

Station Name	Station Description	ECs in Bile		VTG Expression in Liver		SSRIs in Liver	
		M	F	M	F	M	F
STRTGEOR	Strait of Georgia (North Sound)	7	10*	7	0	0	19
VENDOVI	Gulf of Bellingham - northwest of Vendovi Island (North Sound)	8	7*	10	0	0	20
PTGARDNR	Port Gardner (Main)	6	7*	10	0	0	20
HDCANAL	Northern Hood Canal (Hood Canal)	10	7*	10	10	11	9
EGLHARBR	Eagle Harbor (Main)	6	7*	10	0	7	13
ELLTBAY	Elliott Bay - Seattle Waterfront (Main)	10	5	10	7	14	6
DUWAMISH	Duwamish Waterway (Main)	6*	7*	10	0	8	12
SCLINLET	Sinclair Inlet (Main)	6	12	10	0	10	10
COMMBAY	Commencement Bay -Thea Foss Waterway (Main)	2	5	10	0	10	10
NISQUALY	Nisqually Reach (South Sound)	4	7*	10	0	0	20
Sample Grand Total		***139***		***114***		***200***	

*One sample combined from two individual fish as a composite

Gene Expression: For gene expression analyses, 10 samples from male English sole will analyzed for VTG expression per station, except for the Strait of Georgia Station were only 7 males fish were collected. Male fish normally have undetectable levels VTG, but once exposed to exogenous estrogenic chemicals (ECs) the liver synthesizes and releases VTG. Additionally, 7- 10 samples from female fish from one non-urban station (Hood Canal) and one urban station (Elliott Bay) will be analyzed for VTG expression. Female English sole naturally produce VTG, however, differences in VTG gene expression in females from an urban and a non-urban station at the same time of the year indicate altered reproductive timing and potential reproductive impairment.

Presence of ECs: All of the male fish that will be analyzed for VTG gene expression, are also targeted to be analyzed for the presence of ECs, however, the amount of bile was limited for many samples. Accordingly, as detailed in Table 8, the presence of ECs in male English sole will be measured in two – ten fish per station. Paired measures of ECs levels and VTG gene expression from individual fish will be used to assess the quantitative relationship between EC exposure and VTG induction in male English sole. Additionally, five -twelve bile samples from female fish will be analyzed for the presence of ECs as (Table 8). The number of samples is determined by whether sufficient volumes of bile were collected for analysis.

Presence of SSRIs: Twenty liver samples per station (male and females) will be measured for the presence of SSRIs in English sole. All of these samples will come from 2013 WDFW English sole survey as liver for chemical analyses were not collected 2011. Also, due to limited amount of liver tissue available, the presence of SSRI and VTG gene expression will not usually be measured in the same fish.

6.4 Parameters to be determined

This study will report the following information:

- Field Sampling information
  - Location
  - Date/time
  - Sampling method
- Biological metrics of English sole
  - Fish length (mm)
  - Total body mass (g)
  - Fish sex
- Tissue chemistry
  - Estrogen and Xenoestrogens in bile of English sole
  - SSRIs in liver tissue of English sole
- VTG mRNA Induction in male English sole liver

6.5 Field measurements

Field measurements made in 2011 and 2013 related to capturing English sole include date, time, location (latitude/longitude sampling device). Coordinates were recorded to the nearest 0.00001 decimal degrees (1.11 m/3.64 ft).

6.6 Assumptions underlying design

- Tissue residues of ECs and SSRI are correlated with exposure to these contaminants, so that tissue residues are a reasonable proxy for contaminant exposure in the environment.
- VTG induction in male English sole is a suitable proxy for evaluating health risks from exposure to estrogenic EDCs.
- holding times for frozen bile and preserved livers do not negatively affect accurate measurement of chemicals and gene expression.
- The sample size and for all metrics is large enough to detect significant location differences if they exist.
- The sample locations selected for this study represent a wide range of land-use characteristics across the Puget Sound basin.

6.7 Relation to objectives and site characteristics

The study design was aimed at representing a variety of exposures to ECs and SSRIs in order to elicit a dose response for VTG expression. Generally, sample sizes and locations were selected to maximize power for representing potential low to high exposure scenarios. Collectively, the ten sampling locations encompass a range of sources from relatively rural, undeveloped areas such as the Nisqually reach, Hood Canal, Strait of Georgia, and Vendovi locations to more heavily urbanized and industrialized areas such as Elliott Bay, Duwamish, Sinclair Inlet, Commencement Bay, and Eagle Harbor locations.

6.8 Characteristics of existing data

PSEMP staff observed effects from estrogenic compounds in English sole (*Parophrys vetulus*), approximately 15 years ago, manifest as unusual reproductive condition in fish from Elliott Bay. Subsequent focused studies with by NOAA and WDFW/PSEMP researchers quantified these effects as altered reproductive timing in females, and VTG induction in males from several areas in Puget Sound (Johnson et al., 2008). For 14 of 17 sites in Puget Sound, most females (roughly 60 to 100%) were in a spent or post-spawning condition, which was considered the normal condition for the time period when they were sampled. More than 50% of the female English sole from three urban (Elliott Bay) sites were in spawning condition during this period. Prevalence of VTG, measured in blood plasma using enzyme-linked immunosorbent assay, ranged from 5.9 to 47% in urban fish, and 0-6.8% in non-urban fish. The likely cause of these effects is environmental xenoestrogens -- chemicals which were subsequently detected in bile of this species in a follow-up study (da Silva et al., 2013).

7.0 Field Sampling Procedures

7.1 Field Measurements and Field Sampling Standard Operating Procedures

English sole were collected in 2011 and 2013 at ten baseline assessment stations (Figure 2). The collection of English sole specimens and the creation of samples for chemical and VTG analyses are detailed below.

7.1.1 Collecting English Sole

The trawl surveys to collect English sole in 2011 and 2013 followed the PSEMP protocol detailed in WDFW-PSEMP (2013).

7.1.2 Field log

The lead scientist for each field survey maintained a bound Rite-in-the-Rain field log with detailed notes for each day's activities. Entries were made in the daily log either in permanent ink or pencil. Minimum information recorded was:

- Name and location of project
- Field personnel
- Sequence of events
- Gear used and description of fishing activity
- Any changes to plan
- Weather conditions
- Date, time, location name and/or coordinates,
- ID and description of each sample
- Unusual circumstances that may affect interpretation of results

7.1.3 Sample Identification

English sole of greater than 230mm in length were targeted. Bile and liver samples were removed from recently euthanized fish aboard the fishing vessel. Up to 60 fish were sampled in this manner. Up to 60 more fish were collected to be transported back to the WDFW Marine Resources Lab where further liver samples were collected.

7.2 Lab Measurements and Standard Operating Procedures

7.2.1 Equipment, reagents and supplies for analytical chemistry

The following inventory was confirmed prior to all fish-processing activities:

- Terg-A-Zyme® for cleaning lab surfaces and instruments
- Isopropyl Alcohol - B&J Brand® Multipurpose ACS, HPLC
- Tap water
- Teflon Squeeze bottles
- Heavy duty aluminum foil – Reynolds 627 (60.96 cm wide x 0.94 mm thick)
- Scissors - stainless steel
- Forceps - stainless steel
- Spatula – stainless steel, flat blade/round blade
- Measuring tape – cloth
- Stainless Steel mixing bowl
- 28G1/2 1CC Tuberculin syringes
- 1 oz jars, I-CHEM Certified 200-0 series, Type III glass with Teflon-lined polypropylene lid
- 2mL Cryovials
- 1.5ml amber Max Recovery LC/MS certified clean
- Bench scales– such as A&D EK-6000H (6,000 x 0.1 grams)
- Labels – cryogenic, laser printer ready, Diversified Biotech LCRY-2380 0.94in. x 0.50in and LCRY-1258 2.625in x 1.0in.
- Lab coat/apron
- Nitrile exam gloves – talc-free
- Eye protection
- Freezers – walk-in freezer at -20°C, chest freezer at -15°C

7.2.2 Lab setup and preparation for tissue chemistry

7.2.2.1 Preparation of Lab Record forms

Specimen forms were created for this study that identified samples using nomenclature described below. A daily log of operations was kept in the lab. A series of codes were assigned and printed on all lab forms; identification code for the survey (SurveyID), station StationID, specimen (FishID) and sample (SampleID).

7.2.2.2 Use and creation of sampling codes

SurveyID: Each survey carried out by the PSEMP unit was assigned a SurveyID to differentiate it from surveys of the past and future. The PSEMP database manager created a unique alpha numeric code that identified the survey type and the year.

StationID: Each station sampled by PSEMP was assigned a StationID code to help differentiate it from other locations sampled in the past, present and future. The database manager compared the

latitude/longitude information for the sampling location in question against those of StationIDs listed in the database to determine if the location had been sampled in the past. A new location was assigned a descriptive name that was unique from all other StationIDs (using all capital letters for the text in the code) and a location which had been sampled in the past was assigned the same SampleID as the past sampling effort(s).

For specimens acquired from a source outside PSEMP (e.g. WDFW test fishery, WDFW survey, Tribal test fishery), if derived from a fixed[1] site, PSEMP uses the sources assigned name as the StationID; however, if the fixed site corresponds to an establish PSEMP station, the PSEMP StationID is used.

SampleID: All samples created by PSEMP were assigned a unique SampleID code that differentiated each sample from similar samples collected in the past, present or future. A SampleID was a unique alpha-numeric code assigned to an analytical sample; either a sample taken from an individual or a composite of individual tissues. Each Id consisted of six parts, a two-character year code, a two or more character site code, a dash, a two-character species code, a one or two-character matrix code and either a two-digit (composite sample) or 4-digit (individual FishID) sample number.

Unique SampleIDs were assigned by concatenating numbers of label acronyms as follows:

- Two digit year,
- Two or three (typically) digit station identifier
- A dash "-"
- Two digit species
- Single digit matrix
- A sequential number

For example : **13DU-ESB3521**, from 20**13**, **Duwamish Waterway**, **E**nglish sole, #3521.

7.2.2.3 Use and creation of forms

Once the database manager determined the sampling codes, he/she then prepared a Specimen Form for use in the lab. The forms were printed on waterproof paper to facilitate use in the lab environment. The following information was captured on a Specimen Form:

1. Station Information
    a. SurveyID – database manager provides, preprinted on form
    b. StationID – database manager provides, preprinted on form
    c. Collection Date – preprinted on form and Time?
2. Specimen Information
    a. Species – preprinted on form
    b. Effort – Enter the EffortID if one has been assigned or a general description of the effort (e.g. Tow-1, Tow-2, Set-1, Set-2, etc.)
    c. FishID code
    d. SampleID – database manager provides, preprinted on the form.
3. Observations

[1] fixed site – a specific location that is returned to repeatedly over time.

7.2.2.4 Labeling sample jars

To facilitate identification of composite samples compiled in glass jars, corresponding labels were attached to both the lid and the jar. Both labels were printed on cryogenic, laser printer ready labels produced by Diversified Biotech. The lid label had the SampleID printed on it and the jar label had the Year, Station, Species, Matrix, SampleID, Date (capture), jar Weight (empty weight with lid on) and tissue weight.

7.2.2.5 Chain of Custody

A Chain of Custody/Task Order form was initiated when sample jars were created, to track location, disposition, and entity responsible for each jar. COC forms were signed and dated each time sample jars changed hands, most importantly when they were delivered from WDFW to the analytical laboratory.

7.2.2.6 Equipment cleaning procedure

When processing specimens for contaminant analysis, anything (work-surfaces, instruments, etc.) that contacted portions of a specimen subject to contaminant analysis were cleaned before use.

A “clean” work-surface, meant a surface (lab counter, cutting board, sorting tray, etc.) covered by aluminum foil fresh off the roll. The work surface was covered with at least one layer of aluminum foil and the foil was changed between composites.

"Clean" instruments meant stainless steel dissection tools and grinding apparatus (hand grinder and cutting blades) that had been washed in warm soapy water (Terg-A-Zyme®), thoroughly rinsed three times under warm running tap water, followed by a rinse with deionized water (held in teflon squeeze bottle), solvent rinsed using isopropyl alcohol (held in a teflon squeeze bottle) and then placed on aluminum foil for air drying.

The same clean instruments/surface could be used repeatedly, without re-cleaning, on specimens contributing to the same composite. They were subjected to the complete cleaning procedure between composites. Lab personnel changed nitrile gloves between composites.

7.2.3 Sample Creation

Tissue resections generally followed Washington Department of Ecology’s Standard Operating Procedure for whole bodies and body parts (Ecology, 2010). Bile and liver samples were removed from recently euthanized fish aboard the fishing vessel at the time of sampling. Up to 60 fish were sampled in this manner. Up to 60 more fish were collected to be transported back to the WDFW Marine Resources Lab where further liver samples were collected.

7.2.3.1 Bile for EC Analysis

Bile was extracted with 28G1/2 1CC Tuberculin syringes immediately after the English sole was euthanized. Bile was dispensed into amber LC/MS certified clean 1.5mL amber Max Recovery vials and stored on ice until all samples were taken. Then the bile samples were returned to the lab (MRL) and frozen to -20°C until analysis.

7.2.3.2 Liver for VTG Expression Analysis

A liver snip was taken in the field and placed in RNALater® in a 2mL cryovial. The liver snip was to be no more than the tip of a pencil eraser to ensure proper preservation. After the trawl surveys, the liver samples were returned to the MRL and frozen to -20°C, awaiting analysis for VTG gene expression (Section 8.2).

7.2.3.3 Liver for SSRI Analysis

Livers for the SSRI analysis were removed from the English sole in the field, transported to the MRL, and stored at -20°C until resectioning. Some livers were removed from slightly thawed English sole at the MRL and placed in 1 ounce pre-cleaned, pre-labeled I-Chem Series 200 jars. Samples were labeled and frozen to -20°C until transfer to the analytical lab.

8.0 Measurement Methods

Two types of analyses will be performed for the measurement of contaminants of emerging concerns: 1) direct chemical analysis of two groups of chemicals; ECs and SSRIs, and 2) gene expression analyses for VTG.

8.1 Chemical Analyses

8.1.1 Analytes

Target ECs include 4 xenoestrogens (EE2, BPA, NP, and OP) and 3 natural estrogens (E1, E2, and E3). Three SSRIs will be analyzed; fluoxetine (common brand is Prozac), sertraline (common brand is Zoloft), and citalopram (common brand is Celexa).

Table 9. Chemicals of emerging concern to be measured in this study.

Chemical of Emerging Concern:	No. Analytes	Method	Limit of Quantitation - LOQ (wet weight)	Expected Range (wet weight)
Estrogens and Xenoestrogens (E1, E2, EE2, E3, BPA, OP, and NP)	7	Da Silva et al., 2013	0.75-1.5 ng/mL	LOQ to 30,000 ng/mL
SSRIs (fluoxetine, sertraline, and citalopram)	3	Eap et al., 1996; Wille et al., 2007; and Wille 2008	0.5 – 1.0 ng/g for fluoxetine, 1.0 – 2.5 ng/g for sertraline 3.0 – 5.0 ng/g for citalopram.	Unknown

8.1.2 Matrix

Two matrices from adult English sole are targeted in this study (1) bile for analysis of ECs and (2) liver tissue for analysis of SSRIs. Although the individual bile and liver samples were not always collected from the same fish, the sample size of paired samples is large, and across a wide range of potential exposures, which should provide have sufficient pairings to model the relationship between exposure and effects.

8.1.3 Number of samples

For estrogens and xenoestrogens, the minimum number of samples to be submitted for chemical analysis in this study is expected to be 139, comprising bile from individual English sole collected in 2011 and 2013.

For SSRIs, the anticipated number of samples is 20 samples collected from ten different sites, for a total of 200.

8.1.4 Analytical methods

All analyses biliary estrogenic compounds will be conducted by the Environmental Chemistry Program at NOAA Fisheries, Northwest Fisheries Science Center in Seattle, Washington. Methods proposed for EC analyses are detailed below in section 8.1.4.1. All SSRI analyses of English sole liver will be conducted by Battelle's Pacific Northwest National Laboratories at the Marine Sciences Laboratory in Sequim, Washington. Analytical methods proposed for SSRI analyses are detailed below in section 8.1.4.2.

8.1.4.1 Estrogenic Compounds

Bile of adult English sole will be analyzed for estrogen and xenoestrogens using a liquid chromatography-tandem mass spectrometry (LC-MS/MS) method developed herein specifically for this purpose (de Silva et al., 2013) as detailed below. The natural estrogenic compounds E1, E2, E3, and xenoestrogenic compounds EE2, BPA, NPs, and OPs will be analyzed.

This method comprises three steps: (a) protein precipitation and enzymatic hydrolysis, (b) solid-phase extraction (SPE) and (c) quantitative analysis by liquid-chromatography/tandem mass spectrometer (LC-MS/MS). Approximately 100 μL of bile will be diluted in LC-MS grade water, followed by the addition of a mixture containing surrogate standards (S-std) containing deuterated compounds BPA-d16, E1-d4, E2-d4, EE2-d4 and NP-d5 used for quantitation of the corresponding ECs. Acetone (2mL) will be added and the samples held at -20°C for 45 min for partial protein precipitation. The samples will be then centrifuged at 4000 rpm for 10 minutes, and the supernatant transferred and evaporated under N2 flow at 35°C. A 1M sodium acetate trihydrated/acetic acid pH5 buffer containing 2000 units of β-Glucuronidase/sulfatase will be used for enzymatic hydrolysis (2h at 40°C) of the glucuronide and sulfate conjugated metabolites of the ECs. This procedure will allow us to measure the total ECs in fish (free non-conjugated form plus conjugated metabolites)

The final hydrolyzed bile mixture will be extracted /cleaned up using solid phase extraction (SPE) technique. An SPE cartridge packed with 60mg of polymeric reversed-phase sorbent will be used. The cartridge will first be conditioned with 2mL of methanol and 2mL of water. After loading the hydrolyzed sample, the cartridge will be washed with 1.5 mL of water, then 1.5 mL of methanol/water (60/40, v/v), followed by drying under vacuum for 30min. The target ECs will be eluted with 1.5mL of methanol into vial containing BPA-d4, used as recovery standard (Rec-std). Finally, an aliquot of the final methanolic extract will be diluted 10 times in methanol and injected into the LC-MS/MS.

Final bile extracts will be analyzed by liquid chromatography (LC, Acquity system, Waters Co., Milford, MA, USA) coupled with a triple quadrupole tandem mass spectrometer (MS/MS, QTRAP 5500, AB Sciex, Framingham, MA, USA). For each sample, 10 μL of diluted extract will be injected into the LC-MS/MS. The LC will be equipped with a 0.2-μm pre-filter followed by a 2.1 x 5.0 mm (1.7 μm particle size) C18 guard column and a 2.1 x 150 mm (1.7 μm particle size) reversed-phase column. Water (solvent A) and methanol (solvent B) will be used as the mobile-phase. The total analysis time is expected to be 26 minutes using a linear gradient, as follows (solvent A/solvent B): initial gradient 60/40 at 0.2 mL/min; 14 min to 20/80 at 0.2 mL/min; 1 min to 100% B at 0.2 mL/min; 0.1min to increase the flow up to 0.35mL/min and held for 4.9 min; 0.1min to reduce flow to 0.30 mL/min; 0.9 min to initial gradient 60/40 at 0.3 mL/min and held for 5 min. The column temperature will be maintained at 45ºC. Electro-spray ionization (ESI) mode will be used for the ionization of all analytes. The MS/MS will be operating in negative ion mode and the analytes will be detected via multiple-reaction monitoring (MRM). The ion source will be kept at 700°C and capillary voltage at -4.5 kV. Declustering potential and entrance potential will be set at -60V and -10V, respectively. Additional details on the MRM parameters are given in the Table 10. The analytes will be quantified by S-std and based on the calibration curve of each analyte. The recovery of each S-std will be calculated by the Rec-std.

Table 10. Individual multiple-reaction monitoring (MRM) parameters of target EDCs and deuterated standards

	ions (m/z)					ions (m/z)			
EDC	Precursor	Product [a]	CE (V)	CXP (V)	Deuterated Standard	Precursor	Product [a]	CE (V)	CXP (V)
E3	287.043	171	-50	-13	BPA-d16	242.058	142.2	-36	-9
		-145.1	-52	-9			-224.1	-26	-9
BPA	226.908	212.1	-24	-7	BPA-d4	231.038	216	-24	-9
		-133.1	-34	-7			-135	-40	-15
E1	269.155	144.9	-50	-5	E1-d4	273.042	147.1	-52	-7
		-142.8	-64	-11			-145.1	-68	-7
E2	271.055	145.1	-50	-7	E2-d4	274.898	147	-54	-7
		-182.9	-54	-7			-187.1	-56	-7
EE2	295.084	144.9	-54	-7	EE2-d4	299.084	147.1	-48	-7
		-143	-62	-11			-145.1	-72	-7
OP	205.052	133	-26	-17	NP-d5	224.099	110.6	-28	-9
		-177	-26	-13			-110	-28	-5
NP	219.1	133	-28	-3					
		-82.8	-22	-13					

[a] Product ions in parenthesis will be used to help identify the analytes only. CE = collision energy; CXP = collision cell exit potential.

8.1.4.2 SSRI Analyses

SSRIs will be analyzed using Gas Chromatography (GC) Mass Spectrometry (MS) using the method described by Wille (2008) and Wille et al. (2008). This method comprises 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 (SIM). As an internal standard, hexadeuterated paroxetine (d6-paroxetine) will be added during the extraction step. The GCMS will be operated in electron ionization (EI) mode with monitored ions (m/z): 117, 344 (fluoxetine), 274, 501 (sertraline), 58 (citalopram) and 531, 138 (d6-paroxetine). The retention times and spectra of all analytes will be determined or confirmed from authentic standard solutions made at PNNL.

Samples will first be homogenized in 2-volumes of deionized water using either a dispersive type tissue grinder (samples >0.5 g) or a ground glass hand grinder for smaller samples. Next, homogenates will be mixed with 1 M carbonate buffer to raise the pH to above 9.5. Homogenates will then be extracted with 6 ml (per g tissue) of Hexane:methyl-tert-butyl-ether (MTBE; 50:50 v/v) and vortexed for 30 sec. After centrifugation (for 1 min at 2000xg) to separate layers, the upper organic layer will be transferred to a

clean glass tube and mixed with 1.2 ml (per g tissue) of 0.1 M HCL. Next, the bottom aqueous layer will be transferred to a new tube and mixed with excess 1M carbonate buffer. The sample will then be extracted with toluene:iso-amyl-alcohol (85:15 v/v). After vortexing and centrifugation (for 1 min at 2000xg), the toluene layer will be transferred to a 2-ml glass GC autosampler vial, evaporated to dryness at 40°C under N2, and then mixed with 40 µL of freshly thawed HFBI reagent. The mixture will then be incubated for 20 min at 85 °C. Afterwards, the vials will be cooled, mixed first with 400 µL to terminate the reaction, then mixed with 600 µm toluene, vortexed and centrifuged for 1 min at 2000xg. The toluene layer (containing the HFBI derivatives) volume is reduced to 125 µm (per g of original tissue weight) by evaporation under N2 gas, and then injected onto the GCMS. To facilitate evaporation, the vials are held in a rack placed on top of dry bath set to 60 deg. C. The time needed for volume reduction can be up to 30 minutes. The final, residual volume in the vial is estimated by eye.

8.2 Gene Expression Analysis

8.2.1 Analyte

Vitellogenin gene expression will be measured as a marker of exposure to ECs.

8.2.2 Matrix

Transcript levels for VTG will be quantified in liver using quantitative real time polymerase chain reaction (qPCR).

8.2.3 Number of Samples

For VTG gene expression analyses, liver samples from a total of 114 individual fish will be analyzed. Transcripts for VTG will be measured in liver samples from ten male fish from each of the ten sites except for the Strait of Georgia where only 7 male fish were collected and in 10 female fish from Elliott Bay and 7 female fish in Hood Canal.

8.2.4 Expected Range of Results

There is no information on VTG gene expression in English sole; therefore, it is not possible to make any estimation of the hepatic abundance of these in transcripts in fish from this study.

8.2.5 Analytical Methods

All analyses of VTG gene expression will be conducted by NOAA Fisheries' Fishery Resource Analysis and Monitoring Program. Details of proposed methods follow:

Because there is not an assay available to detect/quantity VTG gene expression in English sole, we will develop for the first time a specific qPCR for this purpose. This process comprises four steps: 1) liver total RNA isolation and cDNA synthesis; 2) cloning of partial cDNA encoding VTGs and candidate housekeeping genes; 3) selection of VTG and housekeeping gene; 4) development of qPCRs (Figure 3).

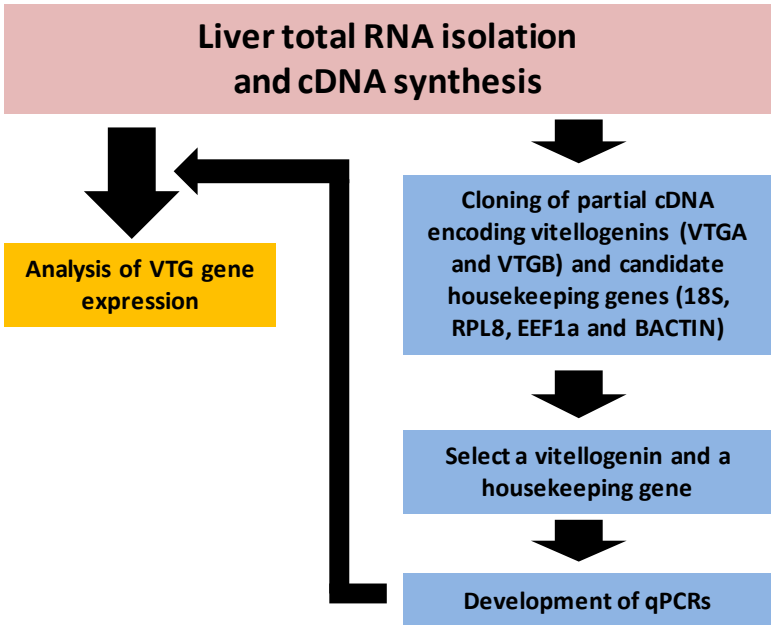

Figure 3. Schematic representation of the major steps toward VTG gene expression assay development in English sole.

8.2.5.1 Liver total RNA isolation and cDNA synthesis

Methods for RNA isolation and cDNA synthesis will be conducted according to Guzmán et al. (2013). Total RNA from English sole liver tissue (pieces of approximately 20-50 mg wet weight) will be isolated with Tri-Reagent (Molecular Research Center, Cincinnati, OH) using a TissueLyserII homogenizer (Qiagen, Valencia, CA). Total RNA will be diluted to ~250 ng RNA/µl in nuclease-free water and DNase treated using the DNA Free kit's ''rigorous'' protocol (Ambion, Life Technologies, Grand Island, NY). For cDNA synthesis, 1 µg of total RNA of each sample will be reverse transcribed in a 20-µl reaction with the Superscript II kit (Invitrogen, Life Technologies). Other necessary components for reverse transcription (RT), such as random primers and RNase inhibitor, will be purchased from Promega (Madison, WI). Negative control reactions will be performed without the addition of the RT enzyme for a subset of the RNA samples.

8.2.5.2 Cloning of partial cDNA encoding English sole VTGs and candidate housekeeping genes

Protocols described in Guzmán et al. (2013) and Smith et al. (2013) will be followed for partial cloning of VTG s and four housekeeping genes using gene specific primers designed as part of developing methods for English sole VTG qPCR. For partial cloning of English sole VTG (VTGA and VTGB) and potential housekeeping genes (18S, RPL8, EEF1a and BACTIN, see Filby and Tyler, 2007), specific sequences will be amplified by RT-PCR using English sole liver cDNA. For a specific gene, primer pairs will be designed in conserved coding regions (exons) of orthologous gene sequences from other marine species and using the software Primer3.

Briefly, PCRs will be 25 µl in volume and include Platinum Taq DNA Polymerase High Fidelity (Invitrogen) using an initial denaturation at 94 ºC for 2 min followed by 35 cycles of 94 ºC for 30 sec, 59 ºC for 30 sec (annealing) and 72 ºC for 1 min (elongation), and a final elongation of 72 ºC for 5 min. The PCR products will be separated on a 2% agarose E-Gel (Invitrogen, Life Technologies) and DNA bands of the desired

size excised and purified using the QIAquick PCR purification kit (Qiagen). cDNAs will be ligated into a pGEM-T Easy vector plasmid (Promega) and transformed into high-efficiency *E. coli* competent cells (Novagen, Merck KGaA, Darmstadt, Germany). Sequencing will be performed using SP6/T7 universal primers on an ABI 3730XL sequencer (Applied Biosystem, Life Technologies). The identity and correctness of partial cDNA nucleotide sequences of English sole VTG and potential housekeeping genes will be further confirmed by BLASTN (National Center for Biotechnology Information, NCBI, GenBank). BLASTN is a free web-based service that analyzes the homology of the queried sequence(s) to all cDNA sequences deposited into the NCBI database. Results are displayed by level of homology and confidence scores (e-values) are provided to aid the user in determining the identity of submitted sequences.

8.2.5.3 Selection of VTG and housekeeping gene

To determine whether one type of VTG gene (A or B) is more sensitive than the other to ECs in English sole, expression of both genes will be compared in a random subset of liver samples representing samples from all collection sites (i.e. urban, near-urban, and non-urban locations that may expose English sole to a wide range in magnitude of ECs), including Elliott Bay where there is published data on presence of plasma VTG in male soles (Johnson et al., 2008). The selection of the housekeeping gene will be based on its expression stability among samples from the collection sites (Filby and Tyler, 2007).

For the selection of VTG and housekeeping gene, qPCRs will be developed as described in Luckenbach et al., 2011. For this, a serial dilution of cDNA (from pooled liver RNA) ranging from 0.1 to 10 ng cDNA (based on the amount of RNA added to the RT reactions) will be used as standard. Specific qPCR primers will be designed (Primer3) based on the partial cDNA sequences (see Figure 3).

8.2.5.4 Development of qPCRs

Development of qPCRs will follow protocols for other genes developed in our lab (Luckenbach et al., 2011), except standards generated from cDNA of the target genes will be used to quantify copy number as described in Guzmán et al., 2009. Briefly, plasmids containing the target cDNA will be linearized and used as templates for gene-specific RNA standard syntheses using an SP6/T7 transcription kit (Roche, Indianapolis, IN). RNA standards will be purified through a size exclusion column (Chroma Spin-200; BD Biosciences, Palo Alto, CA), and the amount of each RNA standard will be determined using a RiboGreen RNA quantification kit (Molecular Probes, Eugene, OR).

Quantitative PCR assays will be run on an ABI 7700 Sequence Detector in 384-well plates using standard cycling conditions: 50 ºC for 2 min, 95 ºC for 10 min, followed by 40 cycles of 95 ºC for 15 s and 60 ºC for 1 min. Reactions will be 12.5 µl each and consist of 1X Power SYBR Green master mix (Applied Biosystem), 150 nM of the forward and reverse primer (based on the partial sequences, see above), and cDNA template, which will be determined empirically based on initial dilution series tests. Standard curve samples generated from a serial dilution of cDNA will be included in each plate. Linearity of the standard curve will be confirmed for the assays by regression analysis and results analyzed using the relative standard curve method. The lowest standard for each gene will be determined empirically by serially diluting the standard and finding the lowest point of reliable detectability by qPCR. We anticipate that this will be 32-35 PCR cycles based on previous experience. The lowest standard should also bracket the lowest measurable biological samples (i.e., samples showing measurable VTG expression). Each standard curve cDNA (generated from cDNA standards) dilution will be run in triplicate, while each sample in duplicate. For each gene, all samples will be assayed in the same plate to avoid across plate variation.

9.0 Quality Control Procedures

Quality control of all laboratories activities will be coordinated by the Principal Investigator, and individual collaborating PIs will be responsible for their specific analytical methodologies. All personnel will have available to them copies of the QAPP and all pertinent supporting research papers and SOPs.

Anticipation and prevention of problems during the setup, sample processing, and analytical phases of the study are key to success of the project. As described in the Study Design section, all field samples for this study have already been collected, according to established collection procedures (WDFW-PSEMP 2013). Sample mass or volume is limited to the samples on hand for the method development portion of this project, and tissue mass and bile volume requirements identified in this study will help predict mass/volume needed from future collections for future monitoring efforts.

Quality Control procedures, quality assurance criteria and corrective actions for analytical chemistry and vitellogenin gene assays are detailed in Section 5.1 and will not be repeated here. These QC procedures are designed to identify unusual results as early in processing as possible, to allow re-processing if needed. Tables 4, 5, and 6 identify corrective actions planned for sample results that fail to meet Quality Assurance control limits regarding bias, precision, and sensitivity. Because field samples for this study have already been taken, corrective actions to overcome problems associated with sample size are not possible. However, it is likely that sufficient samples exist to estimate the statistical power of each metric for assessing geographic and temporal trends, and predict optimal sample sizes for future monitoring.

10.0 Data Management Procedures

10.1 Data recording/reporting requirements

Data for both field samples and QC samples will be received from analytical laboratories in Excel spreadsheets in various formats. PSEMP staff will format these data into a structure compatible with the Toxics in Biota (TIB) database. The TIB database is a relational format created in Access, with separate tables for (1) field effort data, (2) biological characteristics of individuals used to create samples, (3) many-to-many cross reference for individuals-to-composites, (4) sample tracking, condition and summary statistics, and (5) chemical analyses. Data will be examined visually using Excel filters and sorting procedures to identify formatting or transcription errors. Raw analyte concentrations will be compared with expected ranges to identify potential outliers. In addition, preliminary summary statistics tables, scatter plots, and time trend plots will be created to examine the new data.

10.2 EIM data upload procedures

All data generated by this project will be submitted to Ecology's EIM for later export to EPA's STORET database, as appropriate.

11.0 Audits and Reports

11.1 Frequency of Audits

The NWFSC analytical lab participates in annual NIST or IAEA interlab comparison studies.

11.2 Responsibility for reports

WDFW, NOAA and Battelle staff will submit a draft report to Ecology, who will select peer reviewers for comment. The report will include summary statistics of all analytes, a statistical comparison of each analyte (or group total) by study location and site type, with inclusion of covariates if needed. Pattern analysis for selected analytes may be included. Tissue concentrations will be compared with other studies available from the literature.

The final report will address comments received as deemed appropriate. Data packages will be prepared for submittal to EIM and later export to EPA's STORET database, as appropriate, and as detailed in the Scope of Work. Sandra. M. O'Neill is responsible for these products.

12.0 Data Verification and Validation

12.1 Field data verification, requirements, and responsibilities

All sample location data for this study were verified by comparing GIS-plotted latitude and longitude data with field notes to confirm locations plotted correctly. GPS locations plotted incorrectly were replotted using narrative documentation of locations from archived field notes from the 2011 and 2013 PSEMP English sole surveys.

12.2 Lab data verification and validation

Data generated by the analytical lab will be reviewed for out-of-bounds values, transcription errors, and other problems by at least two chemists. Final review will be conducted by a lab manager who will approve data before they are released. Prior to database entry, WDFW staff will compare results with MQOs identified in Section 5.0. Individual results, means, and standard deviations will be plotted and putative outliers evaluated for validity. Evaluation of the validity of putative outliers will include reviewing all collection, biological, and analytical data for potential transcription errors, communication with analytical labs to verify reported values are correct, and evaluation of biological covariates that might explain otherwise unanticipated values.

13.0 Data Quality (Usability) Assessment

13.1 Process for determining whether project objectives have been met

The success of meeting data quality objectives will be evaluated based on the outcome of quality control procedures during analytical procedures. Typically if QC criteria are not met the problem will be identified by staff from the analytical lab, corrected, and sample (or extract) re-run. In cases where QC criteria have not been met and there will not enough tissue to be reanalyzed, the data will be censored with appropriate qualifiers to allow an objective evaluation of the usability of the final record. Rejected data will be censored with an "R" or equivalent qualifier. The project manager will review all results for the project overall, and determine if there are any additional reasons to censor results.

13.2 Data analysis and presentation methods

Estrogenic chemicals (ECs) and SSRI data and VTG expression data collected for this study are part of a long-running tissue residue monitoring program. This program has a long history of data analysis and presentation, which will be continued in the present study. Analysis and presentation of contaminant and covariate data will be conducted using programs commonly employed by PSEMP to compare spatial distribution of contaminants. This will include a General Linear Model that compares contaminant concentrations across geographic locations while adjusting for potentially confounding covariates such as animal size. Analyte results may be log-normalized to achieve normality and homoscedasticity. A Tukey's *post hoc* multiple range test will be used to discriminate the significance of observed differences by sample locations. If normality and homoscedasticity are not achievable with data transformation, non-parametric analogs of ANOVA may be used. Similarity matrices of various combinations of individual analytes may be created to perform Multivariate Dimensional Scaling comparisons among sample types, and used to compare contaminant patterns.

13.3 Treatment of non-detects

Non-detected analytes are censored with a "less than limit of quantitation" (<LOQ) or "U" qualifier for estrogenic chemicals and SSRIs. The value reported for non-detected analytes will be the LOQ. It is the responsibility of users to decide how to use such censored data.

In some samples there may be no VTG induction in male English sole (true zeroes) or undetectable VTG mRNAs. Undetectable VTG mRNA is defined in section 5.3.3, and is indistinguishable from true zeroes.

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15.0 Appendix A. Glossary, Acronyms, and Abbreviations

15.1 Glossary – General Terms

Bisphenol A – Bisphenol A (BPA) is a high production volume (HPV) chemical widely used in manufacturing polycarbonate plastics and epoxy resins used in many industries. Because BPA is a reproductive, developmental, and systemic toxicant in animal studies and is weakly estrogenic, there are questions about its potential impact particularly on children's health and the environment. (USEPA, Bisphenol A Action Plan)

Endocrine disruption – The inhibition or blockage of normal endocrine system function in humans and wildlife caused by synthetic chemicals that block or mimic **hormones**. Known endocrine disruptors include PCBs, dioxins, DDT and some other pesticides.

Hormone – A cellular signaling chemical produced in biological systems by a gland and secreted into the bloodstream to a target tissue.

Housekeeping gene – A gene that is typically required for the maintenance of basic cellular function, and is thus expressed in all cells of an organism under normal and pathos-physiological conditions. These genes are believed to be expressed at a constant, steady level, making them ideally suited for the purposes of normalizing the expression of other genes. Examples include β-actin, GAPDH, and 18S ribosomal subunit.

Metabolic function – Refers to the wide array of biochemical reactions that occur within a cell that release or store energy at the molecular level in order to maintain normal functions.

Microarray – A high-throughput screening method used to sequence large amounts of genetic material or entire genomes. In the case of this study, a DNA microarray will be employed, which hybridizes with mRNA harvested from tissue samples that has been converted into cDNA.

Molecular pathways – Refers to molecular signaling responses and events that maintain and drive normal functioning at the cellular level. Environmental agents that perturb these signals cause adverse health effects and toxicity.

Pharmaceuticals and personal care products (PPCP) – Any product used by individuals for personal health or cosmetic reasons or used by agribusiness to enhance growth or health of livestock. PPCPs comprise a diverse collection of thousands of chemical substances, including prescription and over-the-counter therapeutic drugs, veterinary drugs, fragrances, and cosmetics. (USEPA, Feb 29, 2012)

Relative percent difference (%RPD) - Percent difference or relative percent difference (RPD) between two numbers is the difference between them as a percent of one of them. It is often used as a quantitative indicator of quality assurance and quality control for repeated measurements where the outcome is expected to be the same. (Wikipedia)

Relative standard deviation (%RSD) - The relative standard deviation is widely used in analytical chemistry to express the precision and repeatability of an assay. 100 × [(standard deviation of array X)/ (average of array X)] = relative standard deviation, expressed as a percentage. A lower percentage

indicates a lower variability in the data set. Equally, a higher percentage indicates the data set is more varied. (Wikipedia)

Replicate – Duplicate analysis of an individual sample. Replicate analyses are used for quality control. (USEPA, Waste and Cleanup Risk Assessment).

Experimental replicates – Duplicate samples from identical treatment conditions of a controlled laboratory experiment. These replicates are used to assess differences between treatment conditions (i.e. treated vs. control, untreated).

Field replicates – Two or more portions of environmental media collected at the same point in time and space so as to be considered identical. These replicates are used to estimate sampling and laboratory analysis precision. (USEPA)

Laboratory replicates - A laboratory replicate is a sample that is split into subsamples at the lab. Each subsample is then analyzed and the results compared. These replicates are used to test the precision of the laboratory measurements. (USEPA)

Technical replicates – In a bioassay, these are duplicate measurements of a single sample. These replicates are useful for assessing reproducibility of results.

15.2 Acronyms and Abbreviations

Following are acronyms and abbreviations that may have been used in this report.

BPA	bisphenol A
cDNA	complementary DNA
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 compound
EDC	endocrine disrupting compound
EIM	Environmental Information Management database
EPA	U.S. Environmental Protection Agency
et al.	And others
GC/MS	gas chromatography/mass spectrometry
GPS	Global Positioning System
i.e.	In other words
LC-MS/MS	liquid chromatography-tandem mass spectrometry
MQO	Measurement quality objective
mRNA	messenger ribonucleic acid
NP	nonylphenol
OP	octylphenol
PBDE	polybrominated diphenyl ethers
PCB	polychlorinated biphenyls
PCR	polymerase chain reaction

PI	Principal Investigator
PSEMP	Puget Sound Ecosystem Monitoring Program
QAPP	Quality Assurance Project Plan
qPCR	quantitative polymerase chain reaction
RPD	Relative percent difference
SOP	Standard operating procedures
SSRI	selective serotonin reuptake inhibitors
VTG	vitellogenin

Units of Measurement

°C	degrees centigrade
dw	dry weight
ft	feet
g	gram, a unit of mass
kg	kilograms, a unit of mass equal to 1,000 grams.
km	kilometer, a unit of length equal to 1,000 meters.
LOQ	Limit of Quantitation
l/s	liters per second (0.03531 cubic foot per second)
m	meter
mg	milligram
mg/Kg	milligrams per kilogram (parts per million)
mg/L	milligrams per liter (parts per million)
mg/L/hr	milligrams per liter per hour
mL	milliliters
mm	millimeter
mmol	millimole or one-thousandth of a mole. A mole is an S1 unit of matter.
ng/g	nanograms per gram (parts per billion)
ng/Kg	nanograms per kilogram (parts per trillion)
ng/L	nanograms per liter (parts per trillion)
pg/g	picograms per gram (parts per trillion)
pg/L	picograms per liter (parts per quadrillion)
s.u.	standard units
µg/g	micrograms per gram (parts per million)
µg/Kg	micrograms per kilogram (parts per billion)
µg/L	micrograms per liter (parts per billion)
µm	micrometer
µM	micromolar (a chemistry unit)
ww	wet weight

17.0 Appendix B. Detailed Experience and Competencies for Project Participants

Sandra M. O'Neill: has over 20 years of experience conducting environmental monitoring and assessment, and evaluating factors affecting contaminant exposure and accumulation in biota.

James E. West: leads WDFW's Toxics in Biota Unit, and has 20 years of experience evaluating factors affecting contaminant exposure and accumulation in biota.

Laurie A. Niewolny: Laurie Niewolny has 15 years of experience working as both an environmental analytical chemist and freshwater/marine toxicologist. She currently is WDFW-PSEMP's Field and Operations Biologist coordinating and facilitating field and lab work and data and sample management.

Dr. Penny Swanson: Dr. Penny Swanson has 32 years of experience working in the field of fish physiology, specifically in the area of fish endocrinology and reproduction. She has 130 peer-reviewed publications in these areas, 10 of which deal specifically with endocrine disruption in fishes. Her laboratory has 30 years of experience in immunoassay development and 20 years of experience in molecular biology techniques. In the past 10 years her lab has developed quantitative PCR assays for over 100 genes.

Dr. José Guzmán: Dr. José M. Guzmán has 13 year of experience in the field of fish endocrinology, with special emphasis on reproductive physiology. He has 21 peer-reviewed publications and has participated in more than 30 national and international conferences. He focused on the reproductive physiology of the flatfish Senegalese sole during his PhD, monitoring levels of vitellogenin to characterize its reproductive cycle. Jose also has wide experience molecular biology techniques, including gene cloning and development and validation of quantitative PCR assays.

Dr. Adam Luckenbach: Dr. Adam Luckenbach has over 15 years of experience in the area of fish reproductive physiology and endocrinology. He has 28 peer-reviewed publications and has given numerous national and international presentations. Adam specializes in molecular analyses of fish reproductive development and has been involved in previous projects focusing on environmental stressors and endocrine disrupting compounds. Adam has cloned over a thousand genes and developed and validated over 100 quantitative PCR assays for fish species.

Gina M. Ylitalo: Ms. Ylitalo has worked for the NWFSC since 1989 and is currently the Program Manager of the Environmental Chemistry Program at the NWFSC. She has helped develop analytical methods to measure environmental contaminants in marine organisms and sediments, including chemicals of emerging concern. She is a coauthor on a manuscript recently published in *Chemosphere* that reported on the concentrations of bisphenol A, ethynyl estradiol and estradiol metabolites in bile of English sole from Puget Sound. Her work addresses management concerns such as the effects or injuries to natural resources resulting from releases of hazardous chemicals.

Dr. Denis da Silva: has developed new methods by LC-MS/MS for analysis of CEC, especially xenoestrogens, over the past 7 years.

Lyndal Johnson: (NWFSC) has over 30 years of experience evaluating endocrine and reproductive toxicology in marine and anadromous fishes and environmental monitoring and assessment.

Dr. Irvin Schultz: Dr. Schultz has been involved in toxicological research since 1986 with broad expertise linked to both ecological and human health problems. Specific areas of expertise include bioaccumulation, toxicokinetics & computational biological modeling, biotransformation, analytical chemistry and environmental and human toxicological issues such as endocrine disruption and carcinogenesis. Dr. Schultz has worked for the Battelle Pacific NW National Laboratory (PNNL) since 1996. Dr. Schultz's lab was originally based at the Richland WA site and then subsequently based at the Marine Science Lab (MSL) in Sequim since 2001. Dr Schultz is currently a scientist V in the Ecotoxicology group at MSL. Dr. Schultz also holds an adjunct assistant professorship position with the University of Idaho. Dr Schultz has authored over 72 peer-reviewed publications, prepared 2 book chapters, 10 technical reports and over 95 lectures or invited talks.

Edward Hayman: Edward Hayman is a recent graduate of University of Washington (Biochemistry major) and laboratory technician working in the area of fish molecular biology. He has ~1 year of experience in development and validation of quantitative PCR assays. He also has experience in RNA isolation and QC for downstream quantitative PCR.