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Contaminants of Emerging Concern in Puget Sound: Screening, Prioritization, and Estrogenic Mixture Response Assessment

Maya Faber
Western Washington University, faberm@wwu.edu

Contaminants of Emerging Concern in Puget Sound: Screening, Prioritization, and Estrogenic Mixture Response Assessment

By

Maya Faber

Accepted in Partial Completion
of the Requirements for the Degree
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ADVISORY COMMITTEE

Dr. Ruth Sofield, Chair

Dr. C. Andrew James

Dr. Louisa Harding

Dr. Kathryn Sobocinski

GRADUATE SCHOOL

David L. Patrick, Dean

Master's Thesis

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Maya Faber

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A Thesis
Presented to
The Faculty of
Western Washington University

In Partial Fulfillment
Of the Requirements for the Degree
Master of Science

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Abstract

Contaminants of emerging concern (CECs) are chemicals used in daily life, such as pharmaceuticals, personal hygiene products, steroids, pesticides, and flame retardants. The environmental occurrence and toxicology of CECs are poorly characterized, and they are generally unregulated. Traditional toxicological approaches rely on *in vivo* methods to test whole organisms for apical endpoints, including survival, reproduction, and growth. This is time-consuming and costly, both financially and in terms of laboratory animal well-being, limiting ecotoxicological data for CECs. To overcome this challenge, we are utilizing alternative approaches, including New Approach Methodologies (NAMs), to perform a screening-level evaluation of CECs present in Puget Sound to prioritize those most likely to elicit a biological response.

In the initial phase, regional monitoring data from 17 studies were compiled, including sampling data from water, wastewater treatment plant (WWTP) effluent, and biota. A total of 380 chemicals were analyzed (215 detected), and screening was performed with four different toxicological response measures to provide diverse lines of evidence and enhance confidence in prioritization. To address the lack of traditional ecotoxicity data for CECs, measured environmental concentrations were compared to responses reported *in vitro*, using high-throughput screening (HTS) data, and existing biological effects concentrations reported *in vivo*. *In vitro* HTS data, such as that obtained from the ToxCast/Tox21 programs, is a NAM that was introduced as a practical solution to rapidly evaluate chemicals with limited or unknown toxicity. This prioritization phase identified 56 chemicals as *High Priority* (likely to cause biological effects), and 84 as *Watch List* (potential to cause biological effects). CEC screening results are

intended to focus further monitoring and research efforts and inform management actions to mitigate the potential impacts of *High Priority* CECs.

In the subsequent phase of this work, mixture response was estimated by identifying chemicals with a common mode of action using *in vitro* HTS data. Specifically, the focus was on the estrogen receptor (ER) agonists, as the ER is the assay target that has been the most extensively tested in ToxCast. This information was used to identify CECs that act as estrogenic endocrine disrupting compounds (e-EDCs). To assess the presence of e-EDCs, bile samples from English sole (n = 500) were included in this phase of the analysis as bile was the only biological matrix that was analyzed for steroidal estrogens. Bile is also a primary excretory pathway for such compounds, so they are more likely to be at higher concentrations in bile compared to other tissue types. Samples were evaluated against estrogenic mixture screening values, derived for this work for all matrices, to identify mixtures and bile sampling sites with high, medium, and low potential for estrogenic effects. The absence of pre-existing values makes the derivation of screening values for fish bile, unique to this work. Priority chemicals were subsequently identified within the mixtures to categorize those that would drive estrogenic mixture response or exert influence, as either major or minor contributors. Results indicate that natural exogenous estrogens (i.e., estrone, 17β-estradiol, and estriol) were the primary drivers of ER agonism, with contributions from bisphenol A (BPA), all of which were classified as *High Priority* chemicals, likely to drive or significantly contribute to estrogenic effects in mixtures. The application of *in vitro* HTS data in this approach for mixtures can be expanded to evaluate mixtures in other regions or watersheds, and to investigate other biological targets, thereby enhancing its utility.

Acknowledgments

I would like to thank my graduate adviser, Dr. Ruth Sofield, for her guidance, support, and mentorship throughout my academic journey. I would like to extend my appreciation to the esteemed members of my thesis committee, Dr. C. Andrew James, Dr. Louisa Harding, and Dr. Kathryn Sobocinski for their dedicated time and patience. The insightful contributions from my entire committee were instrumental in shaping a novel methodology for my research. I would like to thank the Puget Sound Ecosystem Monitoring Program (PSEMP) Toxics Workgroup members, who provided feedback and guidance on this work. They include Francis Bothfeld (Washington State Department of Ecology), Jenee Colton (King County), Will Hobbs (Washington State Department of Ecology), Mark Jankowski (US Environmental Protection Agency, Region 10), Jenna Judge (Puget Sound Partnership), James Meador (NOAA retired), Jennifer Morace (United States Geological Survey Oregon Water Science Center), Sandra O'Neill (Washington Department of Fish and Wildlife), Katrina Radach (Puget Sound Partnership), Irv Schultz (National Oceanic and Atmospheric Administration), Marissa Smith (Washington State Department of Ecology), John Stark (Washington State University), and James West (Washington Department of Fish and Wildlife). Finally, I would like to thank the Puget Sound Partnership for providing the funding needed for this project and the project partners, Western Washington University, University of Washington-Tacoma, the Puyallup Tribe of Indians, and the Washington Department of Fish and Wildlife.

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Preface

This thesis includes portions that are organized for publication in a peer-reviewed scientific journal. Therefore, there is repetition between the content presented in each chapter. Chapter 1 is a general introduction and literature review, including project objectives. Chapter 2 includes a link to the open access manuscript that was published as part of this project in a peer-reviewed journal. Chapter 3 is organized as a manuscript intended for publication in a peer-reviewed scientific journal. Supplementary materials related to Chapter 3 have been included in the Appendices section at the end of this thesis. Chapter 4 is an overall conclusion with recommendations for future research. References cited were included at the end of each chapter.

The work on this project, through collaboration, conversations, and outreach, successfully expanded the interested Community of Practice around the science and management of anthropogenic contaminants in the environment. This included strong core collaborations within the research, monitoring, and management community which helped focus the research but also to clarify data gaps and proposed solutions. This engagement has been within the region (e.g., PSEMP, Ecology, EPA) but also included outreach to other systems (e.g., San Francisco Estuary Institute, Columbia River Toxics Reduction Program, Environment and Climate Change Canada). As a result of this collaboration, this work benefitted from a variety of contributions from a number of people, with substantial contributions from the core project team, Dr. Ruth Sofield, Dr. C. Andrew James, Dr. Louisa Harding, and me. This is why I chose to use “we” to reflect the collaborative nature of the work. However, I was the primary contributor to much of the work. Detailed information regarding author contributions for Chapter 2 can be found in that specific chapter. As for my contributions to Chapters 1, 3, and 4, they include conceptualization,

data curation, formal analysis, methodology, validation, visualization, as well as writing both the original draft and reviewing and editing the final version of the thesis.

1.0 Introduction

1.1 Contaminants of Emerging Concern

A global inventory of registered substances conducted in 2020 identified over 350,000 compounds in commerce (Wang et al., 2020), though only a fraction of them have undergone any environmental assessment to ensure their safety (Sipes et al., 2013). For example, to regulate the discharge of chemicals into waterways, the U.S. Clean Water Act (CWA) provides a regulatory framework based on information in available scientific literature or government laboratory reports. Currently, the CWA references a Priority Pollutant List comprising 126 entries (40 CFR Part 423, Appendix A). Contaminants of emerging concern (CECs) include a subset of predominantly anthropogenic chemicals that are generally unregulated and have poorly characterized occurrence and toxicity (Meador et al., 2017). CECs are widely used in common household and consumer products and include pharmaceuticals and personal hygiene products (PPCPs), steroids, plasticizers, flame retardants, and current-use pesticides, among others (Tang et al., 2020). While some CECs are newly developed, the majority of chemicals were introduced to the environment years ago and are only recently being investigated both in terms of their occurrence in the environment and their toxicological impact (Dulio et al., 2018).

1.1.1 Sources of CECs and Occurrence in the Environment

Many CECs are used regularly by humans and continue to be manufactured in large quantities every year (Gefell, et al., 2019). CECs enter the environment through a number of pathways and are found in surface waters (Blackwell et al., 2017a; Corsi et al., 2019; Meador et al., 2016; Tian et al., 2020). Wastewater treatment plant (WWTP) effluent, combined sewer overflows, urban stormwater runoff, and agricultural runoff are among the various pathways through which CECs are routinely released into the aquatic enviornment (Diamond et al., 2015).

For example, pharmaceuticals that are ingested by humans, like synthetic estrogens and therapeutic chemicals, are metabolized and excreted as the parent compound or metabolite(s) and transported to WWTPs (Meador et al., 2017). The WWTP infrastructure is generally not designed to remove these substances, and they pass on to receiving waters (Diamond et al., 2015; Malev et al., 2020; Meador et al., 2016; Tang et al., 2020).

Additionally, different land use activities from agriculture, to urbanization, to industry can result in different chemical profiles (e.g., fertilizers and pesticides in agricultural areas) (Diamond et al., 2015). Compared to rivers draining from primarily undeveloped areas, rivers in urbanized or agricultural watersheds are associated with more frequent detections and higher concentrations of CECs (Baldwin et al., 2016). While CECs have been detected across different land uses, certain classes of CECs reflect the nature of land use (Tian et al., 2021). For example, the occurrence of herbicides, such as atrazine and metolachlor, is more common near agricultural sites (Corsi et al., 2019). Alternatively, CECs such as solvents, detergents, fragrances, and pharmaceuticals, have been associated with urban land use (Alvarez et al., 2021; Baldwin et al., 2016). Agricultural runoff, urban stormwater runoff, and on-site sewage systems are examples of non-point sources of CECs to the environment. In addition to non-point sources, point sources, such as WWTP effluent, have been associated with the presence of CECs. Some common CEC classes associated with WWTP effluent include PPCPs and surfactants (Baldwin et al., 2016). Both distance from point sources and land use have been identified as critical parameters for predicting CEC occurrence in aquatic systems (Kiesling et al., 2019).

While land use may be a highly influential factor in the occurrence of CECs in surface waters, there are other noteworthy factors. For example, different streamflow conditions can affect CEC transport and occurrence (Baldwin et al., 2016). Rain events can mobilize specific

suites of CECs, such as those associated with automobile use and tire wear particles (Tian et al., 2020). Alternatively, discharge from point sources may be diluted during high flow conditions and CEC concentrations may also decrease (Baldwin et al., 2016). Seasonal patterns can also impact the occurrence of CECs. For herbicides primarily used seasonally, higher surface water concentrations were found during spring rain events, following application (Gilliom et al., 2007).

1.1.2 CEC Occurrence in Puget Sound

CECs have been found in various marine habitats in Puget Sound including benthic, nearshore, and pelagic habitats (James et al., 2020; Meador et al., 2016; O'Neill et al., 2020). In the nearshore environment of Puget Sound, one study identified 87 unique CECs in estuarine waters (Tian et al., 2020). Many of these CECs are present in the environment due to WWTP discharge, and one study in Puget Sound detected 81 analytes in WWTP effluent (Meador et al., 2016), including hormones (i.e., androstenedione, estrone, and testosterone), which are some of the most potent endocrine disrupting compounds (Diamanti-Kandarakis et al., 2009; Vandenberg et al., 2012). In addition to water sampling, researchers have used indicator species as biomonitors, and have found CECs at potentially hazardous levels in Puget Sound biota (James et al., 2020; Meador et al., 2016). In 2013, bay mussel (*Mytilus trossulus*) tissue samples were collected through a monitoring program from 18 sites in Puget Sound, from which 30 detections of unique CECs were found (James et al., 2020). Among these detections, some chemicals were present at concentrations of biological concern, including the highly toxic chemotherapy drug, melphalan, which is associated with DNA damage in freshwater mussels (Buschini et al., 2003; James et al., 2020). CECs have also been detected in the tissue of juvenile Chinook salmon (*Oncorhynchus tshawytscha)* in estuarine waters (Meador et al., 2016). Meador et al. (2016) found detections of 42 CECs, including pharmaceuticals (i.e., antidepressant and metabolic

regulators) and industrial chemicals (i.e., 4-nonylphenol (NP), formed through degradation of industrial detergents; and bisphenol A (BPA), used as a precursor in polymer production and plastic additive) (Goeury et al., 2022). For example, fluoxetine (an antidepressant) was detected in juvenile Chinook salmon tissue (Meador et al., 2016), which has been found to cause increased mortality and increased vitellogenin induction in male fathead minnows (*Pimephales promelas)* at low ambient water concentrations (28 ng/L) (Schultz et al., 2011).

Using multiple environmental matrices in a sampling program can provide a more comprehensive understanding of CEC occurrence and distribution (James et al., 2023). For example, WWTP effluent contains many CECs and their metabolites that are too low of levels to detect after they are diluted in the receiving waters (Meador et al., 2017). Tissue matrices can also provide exposure information that is not available through water sampling. For example, lipophilic compounds that accumulate in the fatty tissue of organisms reach higher concentrations in tissues compared to the surrounding water, thereby improving the likelihood of detection (Meador et al., 2017). Additionally, certain chemicals exhibit a higher bioaccumulation potential in specific tissue types, such as fish bile over fish muscle (Lv et al., 2019), emphasizing the need to consider a range of tissue types in monitoring efforts. Furthermore, some metabolic processes can strongly differ among species, which supports the consideration of a range of species when biota are monitored (Van Den Berg et al., 2003). Finally, sampling of species from distinct habitats, such as benthic or pelagic, or from areas with different primary land use types, can provide more information on the sources/pathways of CECs because of their different exposure routes (Choy et al., 2010).

1.1.3 CEC Detection: Targeted and Nontargeted Analysis

The detection and quantification of CECs in environmental matrices, such as surface water, WWTP effluent, and biota, is key to understanding the presence of CECs in the environment and exposure to organisms. However, this can be challenging due to the large number of compounds and low concentrations found in environmental samples. Thanks to technological developments, analytical instrumentation can increasingly detect and measure CECs at environmentally relevant concentrations (Tang et al., 2020). Advancements in high resolution mass spectrometry (HRMS) instrumentation and developments of non-target and suspect screening workflows have greatly increased our ability to screen for CECs in the environment.

Traditional analytical methods utilize targeted approaches. In a targeted approach, the laboratory pre-selects the analytes that are included in validated analytical methods, and which have available analytical standards (Tang et al., 2020). This approach is highly sensitive and can detect concentrations as low as part-per-trillion levels (Blackwell et al., 2017). Targeted analysis, however, does not provide information on chemicals that are not included in the analytical schedule. Alternatively, non-targeted analysis is a highly sensitive emerging technique that relies on HRMS to detect and identify compounds without predefined chemical standards (Blackwell et al., 2017; McCord et al., 2022). Many analytical frameworks include both the use of targeted and nontargeted analysis to detect CECs effectively and accurately at low concentrations in the environment (Tang et al., 2020). While these technological advances have allowed for increased

CEC detection, there remains a lack of information regarding the biological response[1] of exposures to specific CECs.

1.2 Biological Response Associated with CEC Exposures

Information on the occurrence and concentrations of CECs in aquatic systems provides insight into the exposure profiles experienced by aquatic biota. Another important aspect of understanding the potential risk of a chemical in the environment is the potency and potential for biological response associated with exposures. For example, artificial sweeteners, such as sucralose, are widely detected in the environment and have been found at 10 μg/L in the environment, but they are not expected to cause biological impacts to growth, reproduction, or survival even at exposures that far exceed environmental concentrations (e.g. concentrations >9,000 times higher than environmental concentrations) (Tollefsen et al., 2012). On the other hand, steroidal hormones, such as 17α-ethynylestradiol (EE2), a synthetic estrogen used in birth control, can cause population-level consequences at low environmental concentrations (e.g., 5-6 ng/L range) (Kidd et al., 2007). As these examples demonstrate, consideration of potency and potential biological responses associated with exposure to a chemical is integral for determining the associated risk.

Traditional toxicological methods are based on whole organism responses to exposure through *in vivo* toxicity tests, which typically expose whole organisms to an individual chemical at multiple concentrations to measure effects. Effects of interest include lethality, changes in reproductive fitness, and changes in growth; these are known as apical endpoints (Villeneuve and Garcia-Reyero, 2011). Evaluation of whole organisms for apical effects is time-consuming and

[1] Biological response is used here to include both the disturbance of a biological process at lower levels of biological organization (such as in vitro sub-cellular responses) and those that are traditionally considered conventional measures of toxicity, such as apical effects.

costly, both financially and in terms of laboratory animal well-being (Spromberg and Meador, 2005). For these reasons, traditional methods can be a barrier to the assessment of existing chemicals with little or no toxicity data available (Judson et al., 2009; Krewski et al., 2010), which is particularly problematic for CECs. To overcome the limited availability of ecotoxicological information, alternative data sources and data evaluation methods are being developed. These are collectively referred to as New Approach Methodologies (NAMs), and include a variety of *in silico, in vitro,* and alternative approaches that do not use whole organisms (*in vivo).* NAMs can provide large amounts of data to fill gaps in ecotoxicological information. (Hsieh et al., 2021; Kavlock et al., 2012; McCord et al., 2022).

1.2.1 Response Measures Developed based on in vivo Data

Traditionally, acute and chronic toxicity experiments are conducted *in vivo,* on whole organisms, to determine the chemical concentration that results in organismal-level consequences for survival, growth, or reproduction (Braund et al., 2015). Empirical data from these experiments are used to develop response measures, such as the Predicted No Effects Concentration (PNEC), which predicts the concentration below which no adverse effect is expected to occur (Moermond et al., 2016; Tian et al., 2020). PNEC derivation has the highest certainty when the chemical concentration which causes an effect on 50% of the population (L(E)C50), is available from three trophic levels: fish, invertebrates, and algae (Moermond et al., 2016; Peter Carsten von der Ohe & Dulio, 2013). PNECs have been used as a tool to evaluate the potential adverse effects of a contaminant on the environment. However, determining PNECs can be a time consuming and expensive process. To address this challenge, the NORMAN network was developed following a call by the European Union to create a reference for CECs and support the exchange of information regarding CECs (Dulio et al., 2014). The NORMAN

network database, ECOTOX, is a source of PNECs that are derived using expert judgment from experimental toxicity data or predicted from quantitative structure-activity relationship (QSAR) models (Dulio et al., 2018). QSARs are read-across models applied by NORMAN to systematically predict acute toxicity for chemicals with no experimental data, based on their chemical structure and properties (von der Ohe and Dulio, 2013). Currently, there are over 1,000 chemicals with PNECs based on ecotoxicology data that have been verified by NORMAN ecotoxicology experts (NORMAN Network, 2022). Over 94,000 chemicals have predicted PNECs based on QSAR models, greatly expanding the list of chemicals with available data (NORMAN Network, 2022).

Another response measure that integrates data from chronic and acute toxicity tests is the fifth percentile hazard concentration (HC_5) from sensitive species distributions (SSDs) (Posthuma et al., 2019). SSDs have been derived for both acute and chronic water exposures. Posthuma et al. (2019) derived > 12,000 SSDs. These SSDs were modeled based on chronic no-effect or negligible effects data for similar chemicals to capture chronic-exposures rather than QSARs.

A final response measure, known as screening values (SVs) (Gefell et al. 2019), did not incorporate any modelled effects and instead relied exclusively on available *in vivo* exposure data found in the literature. SVs are currently available for 14 CECs from the US Fish and Wildlife Service Ecological hazard assessment, with two additional SVs currently in review (Gefell, et al., 2019). The derivation methods and information outputs, including the specific CECs that are evaluated, vary across these response measures. The advantage of these response measures is that they are readily available for some chemicals and the concentrations of those

chemicals in an environmental sample can be compared to the response measures for prioritization purposes (Villeneuve et al., 2019).

1.2.3 In vitro Biological Response Data

In 2007, the National Research Council (NRC) published a report titled *Toxicity Testing in the 21st Century: A Vision and a Strategy* which emphasizes the use of *in vitro* high throughput screening (HTS) methods as an alternative to *in vivo* animal testing (National Research Council, 2007). HTS, considered a NAM, is an efficient, cost-effective alternative to traditional methods that does not include the use of whole organisms (Kavlock et al., 2012). High throughput *in vitro* tests, or "assays", are automated, rapid experiments which are conducted using cell or cell components rather than intact whole organisms (Villeneuve et al., 2019). In an assay, a whole cell or a cell component, such as a protein or receptor, is exposed to a chemical at a series of concentrations, and disturbances of a particular biological process of interest are monitored (National Research Council, 2007; Villeneuve et al., 2019). The measured *in vitro* responses cover a wide variety of biological endpoints, which do not translate directly to apical endpoints though they identify preceding events that may ultimately lead to adverse health outcomes in a whole organism (Villeneuve et al., 2019). Understanding the underlying mechanisms by which chemicals interact with cells and cell components can support the prediction of adverse effects at the organism level (Fay et al., 2018; Judson et al., 2016; Krewski et al., 2010).

In response to the NRC report, the Environmental Protection Agency (EPA) created an HTS program known as the ForeCaster program, which has employed a battery of more than 700 different bioassays for approximately 2,000 chemicals (Schroeder et al., 2016). The EPA has also pooled resources in collaboration with the National Institute for Environmental Health

Sciences, the Food and Drug Administration, and the National Institutes of Health to form the Tox21 program which has generated data for approximately 50 assays covering approximately 10,000 chemicals. Both programs are now collectively called ToxCast. Data in ToxCast is produced by EPA labs and EPA-contracted companies that perform the bioassays (Williams et al., 2017). ToxCast bioassays typically target mammalian cells or cell components, though chemical-response data generated through these programs has been increasingly applied to nonmammalian species (Alvarez et al., 2021; Blackwell et al., 2017; Corsi et al., 2019; Elliott et al., 2019).

1.2.3.1 Identifying a Biological Response in in vitro HTS Data

ToxCast uses more than 700 assays to test a range of biological responses known as endpoints. There are hundreds of endpoints such as activation of the androgen receptor, antagonism of the estrogen receptor, and inhibition of the CYP19A1 enzyme (Alvarez et al., 2021). These examples are key events from Adverse Outcome Pathways (AOPs) that are all related to endocrine disruption and may ultimately lead to behavioral, reproductive, and/or developmental alterations in vertebrate species (Alvarez et al., 2021). Chemicals analyzed though ToxCast follow standardized testing methods and uniform analysis (Filer et al., 2016). This ensures that data can be reliably compared across assay platforms.

As part of the standardized data analysis pipeline in ToxCast, a dose-response curve is modeled for each chemical-assay pair by comparing a measure of response with a chemical concentration (Fig. 1-I). The dose-response relationship is used to identify the maximum response (efficacy) and a point of departure (POD) concentration. Each chemical tested in a given assay will likely have a different efficacy (Villeneuve et al., 2000). An active chemical will have a response that exceeds the activity cutoff threshold (Fig. 1-I). The activity cutoff threshold

is specific to each assay and is defined as the response level that is significantly different from the background response (Blackwell et al., 2017). An individual chemical is likely to be tested in several assays and can have any number of active assays (Tice et al., 2013).

Once the dose-response model for a chemical has been fit to the data, POD estimates can be derived. A POD is a chemical concentration at which a measurable adverse effect is first observed (U.S. EPA, 2012). A common POD estimate applied in pharmacological research and in applications of ToxCast data is the concentration where half-maximal activity occurs (AC_{50}) (Fay et al., 2018; Shockley, 2016). In recent studies, an alternative POD known as the activity concentration at cutoff (ACC) has been used for CEC screening and prioritization (Alvarez et al., 2021; Blackwell et al., 2017; Corsi et al., 2019; Elliott et al., 2019; Malev et al., 2020; Rose et al., 2019). The ACC is the concentration where the dose-response curve meets the activity cutoff threshold. (Fig. 1-I). The ACC may be preferred over the AC_{50} because it is independent of variable chemical efficacies since it is based on the activity cutoff threshold which is constant across all chemicals in an assay and thus provides a better relative measure of response (Filer et al., 2016).

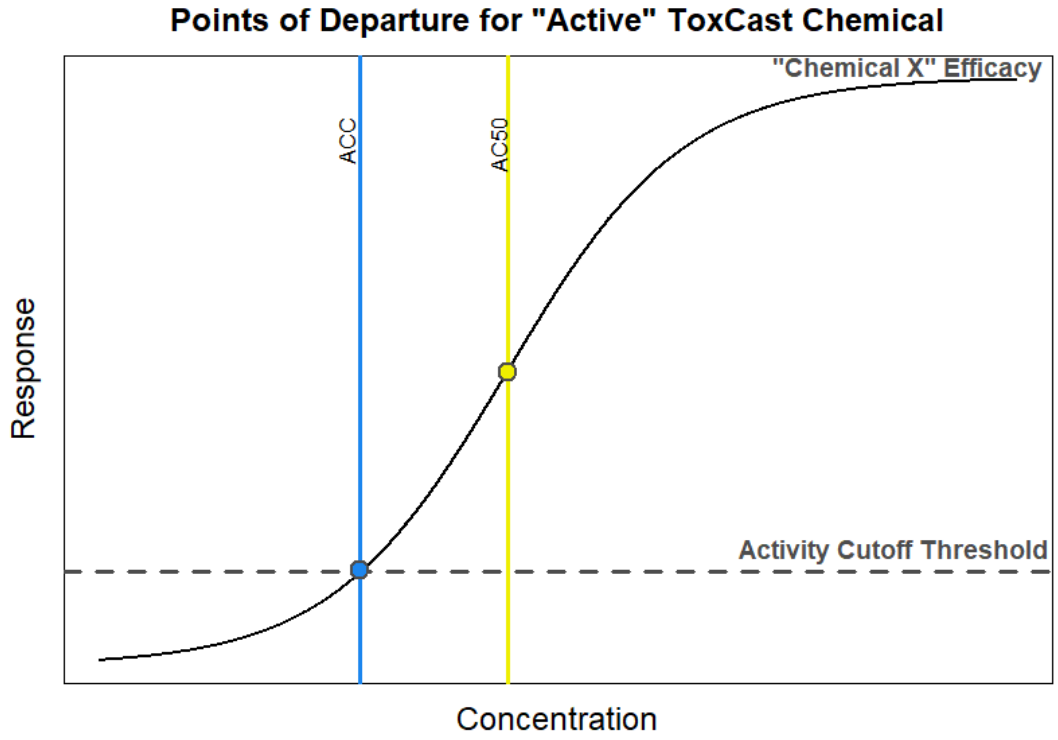

Fig. 1-I. Dose-response curve of an example chemical, "Chemical X". Chemical X is active in this assay because the maximum achievable response, known as the efficacy, exceeds the activity cutoff threshold. The activity cutoff threshold is defined as the response level that is significantly different from the background response. The activity cutoff threshold is generally consistent across all chemicals in an assay. The AC_{50} is identified as the concentration where a half-maximal response occurs compared to the control. The ACC is identified where the curve meets the activity cutoff threshold. ACC is more commonly used in recent studies using ToxCast data. Figure made using R and the tcpl *package (Filer, 2022).*

1.4 CEC Screening and Prioritization

One common objective of CEC investigations is to identify those chemicals that adversely impact aquatic organisms through CEC screening and prioritization (Corsi et al., 2019). One approach is to compare a measured environmental concentration to a measure of biological response. Biological response measures can be derived from *in vivo* toxicity tests (i.e. PNECs, HC5, and SVs) and *in vitro* HTS data (i.e., ACC). A toxicity quotient (TQ) is calculated by comparing the detected concentrations in the environment to the biological response measure determined *in vivo* (Peter Carsten von der Ohe and Dulio, 2013). A ratio equal to or greater than

1 indicates that the exposure may elicit a biological response (Braund et al., 2015), and so a threshold of 1 is commonly used to screen for chemicals that may have a harmful effect (Sardiña et al., 2019; Tian et al., 2020; von der Ohe et al., 2011; von der Ohe and Dulio, 2013). Other studies have used a more conservative approach, screening for chemicals at a TQ $\geq$ 0.1 (Alvarez et al., 2021). TQs below this threshold likely pose an insignificant environmental risk (Wennmalm and Gunnarsson, 2009). The framework detailed by von der Ohe et al. (2011), recommends that CEC prioritization consider the extent of the exceedance (magnitude by which the environmental concentration exceeds the biological response measure), and the frequency of the exceedance (number of sites with observations of a compound above the biological response measure). This approach allows consideration of both distribution and exposure levels (Slobodnik et al., 2012).

In vitro HTS data can also be used to screen CECs (Villeneuve et al., 2019). Similar to a TQ, Blackwell et al. (2017) introduced the exposure activity ratio (EAR), which is the ratio of the observed chemical concentration and the associated ACC. Corsi et al (2019) introduced EAR_{chem}, a summation of EARs calculated across all active assays for individual chemicals. EAR_{chem} considers both the number of active assays and magnitude of individual chemical-assay EAR values to provide a means to prioritize chemicals based on their overall potential to elicit a biological response (Corsi et al., 2019). Chemicals with higher EAR_{chem} values are more likely to result in a biological response, allowing for the prioritization of chemicals based on their likelihood to cause biological effects (Corsi et al., 2019; Elliott et al., 2019; Malev et al., 2020; Rose et al., 2019). The TQ and EAR are both quotients comparing measured concentrations to a biological response; they are referred to as Biological Response Ratios (BRR) throughout this work when no distinction between the two is needed.

EAR thresholds have been identified to support prioritization of CECs measured in the environment. Corsi et al. (2019) identified an EAR threshold of 10^{-3}, which was based on the comparison of EARs to thresholds from water quality benchmarks, which are meant to be protective of most aquatic organisms. The EAR threshold of 10^{-3} is specific to water and has been used for prioritization (Alvarez et al., 2021; Corsi et al., 2019; Rose et al., 2019). EAR thresholds have also been developed for chemical concentrations in plasma (Elliott et al., 2019; Malev et al., 2020). Elliott et al. (2019) predicts the effects of CECs based on blood plasma concentrations found in upper midwestern U.S. bald eagles and response data from ToxCast, and the authors applied an EAR threshold of 1 (Schroeder et al., 2016). EAR thresholds can be adjusted to the specific needs of the study as long as the reasoning for threshold adjustment is provided and there is scientific evidence supporting their use (Blackwell et al., 2017)

1.5 CEC Mixtures

Industrialization, urbanization, and certain agricultural practices have resulted in complex chemical mixtures being released into the aquatic environment (Diamond et al., 2015), and multiple studies have demonstrated the heterogeneity of CEC mixtures found in surface water samples (Alvarez et al., 2021; Baker et al., 2021; Baldwin et al., 2022; Blackwell et al., 2019; Corsi et al., 2019). Industrial discharge, WWTP effluent, combined sewer overflows, and urban stormwater runoff contribute to mixture complexity in urbanized areas (Baker et al., 2021; Baldwin et al., 2022). Improved analytical technologies have resulted in better characterization of chemical mixtures, however, there are significant gaps in understanding the biological responses from mixture exposures. This is in part because traditional toxicological methods, used to develop water quality benchmarks, are based on single chemical evaluations (Alvarez et al., 2021). Additionally, some CECs that target the same biological pathway or mechanism, that

have similar modes of action, have the potential for cumulative effects (Nilsen et al. 2019). For example, in zebrafish (*Danio rerio),* combined exposures to steroidal hormones (17β-estradiol (E2), EE2, and Diethylstilbestrol (DES)) and xenoestrogens (4-tert-octylphenol, NP, and BPA) exerted stronger reproductive toxicity, by decreasing sperm counts, than exposure to EE2 alone (Wang et al., 2019). Mixture effects can also arise from chemicals with different modes of action. For example, co-exposures of fluoxetine (selective serotonin reuptake inhibitor, SSRI) and roxithromycin (antibiotic) induced stronger antioxidant responses than single pharmaceutical exposures in crucian carp (*Carassius auratus*) (Ding et al., 2016). Therefore, considering the toxicity of an individual chemical may not accurately represent the effects of the mixture on a given biological endpoint (Dyer et al., 2011).

NAMs are particularly useful for evaluating the effects of chemical mixtures in the environment (Hsieh et al., 2021). *In vitro* HTS data, such as ToxCast data, is a particularly useful NAM in the screening and prioritization of complex mixtures of CECs (Corsi et al., 2019). As stated above, each assay in ToxCast focuses on specific biological targets or pathways, and multiple assays can provide information on the same biological target, such as the estrogen receptor (ER), and/or biological pathway, such as ER signaling, that may be affected by multiple chemicals in a mixture (Corsi et al., 2019). The cumulative response is often assessed based on the assumption of additivity, by adding the responses of all individual chemicals acting on the same target or biological pathway (Alvarez et al., 2021). One approach for this is to calculate an $EAR_{Mixture}$, which is the summation of EARs for all chemicals found in the mixture, with bioactivity in a common assay (Alvarez et al., 2021; Blackwell et al., 2017b; Corsi et al., 2019). This is consistent with the toxic unit approach where toxic units, defined as the ratios of the environmental concentration of a chemical over the biological response threshold, are summed

for chemicals with similar modes of action (Alvarez et al., 2021; Nirmalakhandan et al., 1994). Similarly, EARs have been summed for chemicals acting on a set of assays associated with the same adverse outcome pathway (EAR_{AOP}) (Corsi et al., 2019). To describe the contribution of individual chemicals to overall mixture response, CECs are considered noteworthy when their contribution ranges from a minimum of 20% (Maloney et al., 2023), down to at least 1% (Corsi et al., 2019) towards a mixture response.

By leveraging the capabilities of *in vitro* HTS data, researchers can achieve a more efficient and cost-effective evaluation of mixture toxicity, which reduces the reliance on expensive or less accurate alternatives (Stossi et al., 2022). For example, an ER agonist model was developed, integrating data from 16 ToxCast assays that measured various responses along the ER pathway (Judson et al., 2015). This model captures a broader range of responses associated with ER agonists than effects-based assays. Three types of effects-based assays that have been used to measure estrogenic activity of mixtures include the ER-binding, yeast estrogen screen (YES), and ER-mediated chemically activated luciferase gene expression (ER-CALUX) (Murk et al., 2001). Each measures a different event along the ER pathway leading to estrogenic effects. For example, ER-binding occurs before ER-induced cell proliferation, measured by the YES assay (Drier et al., 2017). Results from these assays are used to calculate estradiol equivalency quotients (EEQ) and results have indicated that ER-binding assays result in higher EEQs (greater predicted estrogenicity) than both the YES and ER-CALUX assays for a given sample (Murk et al., 2001). The ER agonist model of Judson et al. (2015) allows for the estimation of the overall estrogenic potential of individual chemicals while considering multiple events, expanding the scope to identify estrogenic chemicals that had not been previously investigated (Judson et al., 2017). Building upon the well-established principle of additivity and

combining aspects of Judson et al. (2015) and Corsi et al. (2019), characterizing the combined mixture responses arising from chemicals in a mixture acting on a common biological pathway is possible.

1.6 Project Objectives

The primary focus of previous CEC screening and prioritization has been on freshwater systems, specifically centered around water monitoring data, and there has been limited investigation into the effects of CECs in marine environments utilizing biota sampling data. Therefore, the objectives of my research were to: *a)* compile regional CEC monitoring data from multiple environmental matrices such as surface water, wastewater, mussel tissue, and fish tissue in Puget Sound, *b)* demonstrate how various CEC assessment approaches can be integrated to effectively screen and prioritize individual CECs using regional data, *c)* develop screening values to categorize mixtures according to their estrogenic potential, leveraging *in vitro* HTS assay results, and validated by field measures of exogenous estrogen exposure, and *d)* prioritize chemical constituents in a mixture that drive or contribute toward estrogenic response.

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2.0 The Screening and Prioritization of Contaminants of Emerging Concern in the Marine Environment Based on Multiple Biological Response Measures

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Contributions:

C. Andrew James: Conceptualization; Data curation; Formal analysis; Funding acquisition; Investigation; Methodology; Project administration; Supervision; Validation; Visualization; Writing - original draft; Writing - review & editing.

Ruth Sofield: Conceptualization; Data curation; Formal analysis; Funding acquisition; Investigation; Methodology; Project administration; Supervision; Validation; Visualization; Writing - original draft; Writing - review & editing.

Maya Faber: Data curation; Formal analysis; Methodology; Software; Validation; Visualization; Writing - review & editing.

Dave Wark: Data curation; Visualization; Writing - review & editing.

Amy Simmons: Data curation; Writing - review & editing.

Louisa Harding: Conceptualization; Data curation; Methodology; Writing - review & editing.

Sandie O'Neill: Conceptualization; Data curation; Writing - review & editing.

3.0 Advancing the Evaluation of Estrogenic Mixtures Using High Throughput Screening and Fish Bile Data Integration: A Case Study in Puget Sound

Abstract

A variety of anthropogenic chemicals known as contaminants of emerging concern (CECs), are regularly released into the aquatic environment due to human activities. CECs in the environment are present in contaminant mixtures that may share a common mode of action such as estrogen receptor agonism, which can lead to feminization of male fish. This study aims to demonstrate how *in vitro* high throughput screening (HTS) data can be used to evaluate estrogenic mixtures in a watershed. The evaluation included data compiled from 18 studies, analyzing 386 CECs (not all chemicals were analyzed in each study; 222 detected), within various matrices including water, wastewater treatment plant (WWTP) effluent, fish and mussel tissue, and fish bile. Samples were evaluated against estrogenic mixture screening values, derived for this work for all matrices, to identify mixtures with high, medium, and low potential for estrogenic effects. Priority chemicals were subsequently identified as those that would drive estrogenic mixture response or exert influence as either major or minor contributors. Among fish bile samples with medium or high potential for estrogenic effects, 64% of mixture response was explained by at least one chemical driver rather than a mixture of multiple contributing chemicals. Estrone (E1), 17β-estradiol (E2), and to some extent, estriol (E3) were responsible for most of the estrogenic activity, and bisphenol A (BPA) was consistently a major contributor. Among WWTP effluent samples, four of nine samples were identified as having medium or high potential for estrogenic effects. In addition to prioritizing individual chemicals, this study presents a methodology for classifying field sites using bile from multiple fish samples per site. The absence of pre-existing values makes the derivation of screening values for fish bile unique to this work. Additionally, the utilization of HTS data for mixtures expands the evaluation of estrogenic CECs by incorporating those that lack traditional benchmark concentrations.

3.1 Introduction

Anthropogenic activities related to agriculture, urbanization, and industrialization introduce a diverse array of chemicals into the aquatic environment (Baldwin et al., 2016; Diamond et al., 2015). Among the various contaminant inputs, contaminants of emerging concern (CECs) are increasingly recognized because of their potential to cause adverse effects on aquatic organisms. CECs are inadequately understood in terms of their environmental occurrence and toxicological impact, and typically lack regulation (James et al., 2023). One category of CECs, endocrine disrupting chemicals (EDCs), have been frequently investigated because the endocrine system is conserved across most vertebrate species (Lalone et al., 2018), and disruption can impact development (Colborn et al., 1993; Deich et al., 2020), reproduction (Blazer et al., 2018; Nilsen et al., 2019; Vajda et al., 2008), and behavior (Oshima et al., 2003).

Estrogenic endocrine disrupting chemicals (e-EDCs) are a subclass of EDCs that disrupt estrogen receptor signaling and are of particular concern because of their ability to interfere with the function of the natural reproductive hormone, 17β-estradiol (E2) (Vega-Morales et al., 2013). Exogenous estrogens, both natural (e.g., E2, estrone (E1), estriol (E3)) and synthetic (e.g., 17α-ethynylestradiol (EE2)), are considered e-EDCs, as are anthropogenic chemicals such as industrial phenolic compounds (e.g., bisphenol A (BPA), octylphenol (OP), and 4-nonylphenol (NP)). There is substantial evidence of impacts from e-EDC exposures (Kavlock and Ankley, 1996; Kidd et al., 2007; Vajda et al., 2008; Vega-Morales et al., 2013). Both synthetic and natural estrogen exposures are associated with vitellogenin (Vtg) induction (a common biomarker of exposure) in male fish and juveniles, reduced gonad size, and gonadal intersex (Azizi-Lalabadi & Pirsaheb, 2021; Lange et al., 2012; Vajda et al., 2008). Laboratory studies have demonstrated that chronic exposure to environmentally relevant concentrations of BPA can

lead to reproductive impairments in fish, such as decreased sperm density and mobility, as well as delayed or decreased ovulation (Canesi and Fabbri, 2015; Lahnsteiner et al., 2005). Environmental exposures to EE2 in the ng/L range have been linked to reduced reproductive success and population collapse in fish (Kidd et al., 2007).

In the environment, chemicals occur as complex mixtures. Traditional toxicological methods employed to establish water quality benchmarks focus on single-chemical evaluations. However, studies have shown that effects tend to be higher when co-occurring chemicals are considered, especially those with shared modes of action (Alvarez et al., 2021; Dyer et al., 2011; Nilsen et al., 2019; Schoenfuss et al., 2016). Therefore, even when individual chemical concentrations are below effects thresholds, the combined effects of chemicals in mixtures may drive a biological response (Rodea-Palomares et al., 2023; Thrupp et al., 2018).

Assessing the effects of chemical mixtures in the environment is challenging because of the interactions between co-occurring contaminants and the constantly changing mixture composition (Dyer et al., 2011; Schoenfuss et al., 2016; Thrupp et al., 2018). Common approaches used to determine the estrogenicity of an environmental sample are effect-based methods and include the Yeast Estrogen Screen (YES), estrogen receptor (ER)-binding assays, and ER-CALUX assays (Murk et al., 2002). These methods indicate total activity of an environmental sample, although they do not differentiate which individual chemicals contribute to the overall estrogenicity. Another common approach is, the 17β-estradiol equivalency quotient (EEQs) (Jarošová et al., 2014; Vajda et al., 2008; Vega-Morales et al., 2013) which considers individual chemicals and allows for predictions of estrogenicity based on concentrations of chemicals in a mixture and established organism response to those chemicals. Since estrogenic effects of e-EDCs have demonstrated additivity, the EEQ is calculated as the sum of

concentrations for each individual e-EDC after normalizing by an estradiol equivalency factor (EEF), which are often derived from effects-based assays (Vajda et al., 2008; Vega-Morales et al., 2013). EEFs have only been derived for a small number of chemicals, including steroidal estrogens and some phenolic chemicals that can mimic estrogens (Vajda et al., 2008; Vega-Morales et al., 2013), which is limiting, considering the substantial number and diverse types of CECs that are estrogenic (Judson et al., 2015). Alternatively, there are New Approach Methodologies (NAMs) available, such as *in vitro* high-throughput screening (HTS) assays, an automated technology used for screening chemicals for a specific biological activity (Krewski et al., 2010). These offer a cost effective and efficient way to assess the biological responses of a large number of chemicals to fill data gaps (Villeneuve et al., 2019) and can be used to assess chemicals with shared modes of action which is particularly valuable for mixture evaluation.

Large-scale *in vitro* HTS programs known as Tox21 and ToxCast, hereafter referred to collectively as ToxCast, capture information from *in vitro* HTS bioassays by exposing primarily mammalian cells or isolated proteins to chemicals and measuring changes in biological activity (Alvarez et al., 2021; Lalone et al., 2018). ToxCast includes *in vitro* HTS data for over 9,000 chemicals for more than 300 unique signaling pathways, including data for several chemicals lacking traditional health or environmental effects data (Blackwell et al., 2017). In ToxCast assays, measurements of biological response include the disruption of specific pathways, such as receptor agonism or antagonism, or generalized disruption leading to cytotoxicity (Judson et al., 2016). The assay target that has been the most extensively tested in ToxCast is the ER, and e-EDCs are well represented among the chemicals tested in ToxCast (Blackwell et al., 2017). Recent studies have provided evidence that the use of ER agonist assays in ToxCast can be effective for identifying e-EDCs(Dreier et al., 2017; Judson et al., 2015, 2016, 2017). For

example, Judson et al. (2015) created a model using ToxCast data to predict ER agonist activity for over a thousand chemicals, with a range of structures and chemical use categories, by integrating results from 16 *in vitro* assays targeting the estrogen receptor 1 (ESR1) and estrogen receptor 2 (ESR2) in the activation direction. The model results correctly identified ER agonists known to perturb the ER pathway (e.g., receptor binding, receptor dimerization, DNA binding, RNA transcription, protein production, and ER-induced proliferation) suggesting the model may be used to identify new e-EDCs.

Another application of the ToxCast data is with exposure-activity ratios (EARs) which predict biological response by comparing chemical concentrations in the environment with corresponding chemical-assay response measures from ToxCast (Blackwell et al., 2017, 2019). The preferred chemical-assay response measure is the activity concentration at cutoff (ACC; Alvarez et al., 2021; Blackwell et al., 2017; Corsi et al., 2019; Loken et al., 2023), which is the minimum concentration of a chemical that can produce a measurable effect in an *in vitro* assay (Blackwell et al., 2017; Fay et al., 2018; Filer et al., 2016; Judson et al., 2009). EARs are a valuable tool for risk-based evaluations for individual chemicals, enabling the screening and prioritization of chemicals (Corsi et al., 2019; Elliott et al., 2019; James et al., 2023; Malev et al., 2020; Rose et al., 2019). Additionally, EARs can be used in assessing chemical mixtures that act on a common biological pathway (Alvarez et al., 2021; G. T. Ankley et al., 2021; Corsi et al., 2019; Loken et al., 2023).

While previous work applying the EAR approach has focused on the assessment of data from a single environmental matrix, predominantly water, one exception is the work by James et al. (2023). In that study in Puget Sound, WA, the second largest estuary in the United States, chemical occurrence data from water, wastewater treatment plant (WWTP) effluent, and fish and

mussel tissue were compiled to screen and prioritize individual chemicals. e-EDCs were detected in all matrices, which provides a valuable opportunity to apply the EAR approach to ER-agonist assay responses while considering multiple matrices. Notably, other research has shown that e-EDCs may accumulate preferentially in various fish tissue, so it is important to include multiple tissue types (Lv et al., 2019). For example, e-EDCs accumulate in bile, as biliary excretion serves as the primary excretory pathway for natural endogenous and exogenous estrogens, and some phenolic estrogen mimics (Gibson et al., 2005; Houtman et al., 2004; Lv et al., 2019). As such, bile extracts have been used as a tool for monitoring e-EDC exposure (Allard et al., 2004; Da Silva et al., 2013; Houtman et al., 2004; Legler et al., 2002; Lv et al., 2019; Yang et al., 2014).

The present study aims to develop and evaluate a methodology for understanding the effects of exposures to e-EDC mixtures in Puget Sound using *in vitro* exposure-response information. One key objective is to develop screening values to classify mixtures based on their estrogenic potential, validated by field measures of exogenous estrogen exposure such as Vtg induction in male fish, reproductive maturity of females, and concentrations of CECs and persistent organic pollutants (POPs) in biota. The outcomes of our research enable two important applications: (1) the prioritization of individual chemicals within mixtures that contribute to estrogenic effects, and (2) the classification of sites where exposures to mixtures with estrogenic chemicals pose potential concerns for aquatic organisms.

3.2 Experimental Methods

3.2.1 Environmental Monitoring Data

Regional chemical monitoring data were compiled from 18 individual studies from local, state, and federal sources. Data was from multiple environmental matrices, including water,

WWTP effluent, mussel tissue (whole body), fish tissue (whole body/filet), and fish bile. Seventeen of these studies are described in James et al., (2023); the additional study included contaminant concentrations in English sole bile (Da Silva et al., 2013; Harding et al., 2022). The use of multiple studies maximized the amount of data available for analysis and geographic coverage of contaminant occurrence data. As sampling was not coordinated under a single sampling program, the study designs, analytical methods, and suite of analyzed chemicals varied and were not always focused on e-EDCs. Methods from James et al. (2023) were followed for quality assurance and blank correction for all data (Appendix A1.1).

The compiled dataset included more than 900 samples, which were analyzed for 385 unique compounds. Tissue samples were collected from bay mussels (*Mytilus trossulus*; n=75) and multiple species of fish including Smallmouth bass (*Micropterus dolomieu;* n=9), Pacific staghorn sculpin (*Leptocottus armatus*; n=5), Pacific herring (*Clupea pallasii*; n=20*)*, juvenile and subadult resident Chinook salmon (*Oncorhynchus tshawytscha*; n=74 and 34, respectively), English sole (*Parophrys vetulus*; n=123*)*, Pacific sand lance (*Ammodytes personatus*; n=10), Quillback rockfish (*Sebastes maliger*; (n=1), and Brown rockfish (*Sebastes auriculatus*; n=18). Samples were primarily marine or estuarine, except Smallmouth bass collected from an estuary-adjacent freshwater lake. WWTP effluent samples (n=9) were collected from five facilities and were considered as representative of the highest concentrations as compared to receiving waters, as samples are undiluted effluent. Marine water samples (n=134) were collected from estuarine, nearshore, and pelagic environments.

Bile samples were collected from English sole (n=500) by the Washington Department of Fish and Wildlife (WDFW) from 18 locations across Puget Sound from 2011–2019 (Fig. 1). Fish bile samples were analyzed for alkylphenols (NP, 4-nonylphenol triethoxylate, 4-tert-octylphenol

(tOP), 4-tert-octylphenol triethoxylate, 4-tert-octylphenol diethoxylate, 4-tert Octylphenol monoethoxylate, and 4-tert-octylphenol triethoxylate), bisphenols (BPA, bisphenol AF (BAF), bisphenol F (BPF), bisphenol S (BPS), and tetrabromobisphenol A), and steroidal estrogens (E1, E2, E3, and EE2) at NOAA's Northwest Fisheries Science Center (Da Silva et al., 2013; and unpublished). Samples were deconjugated prior to analysis with β-glucuronidase/sulfatase for enzymatic hydrolysis to obtain the total analyte concentration including the glucuronide and sulfate conjugated metabolites (Da Silva et al., 2013; Harding et al., 2016). To avoid confounding presence of endogenous steroid hormones, female samples were omitted from our current analysis.

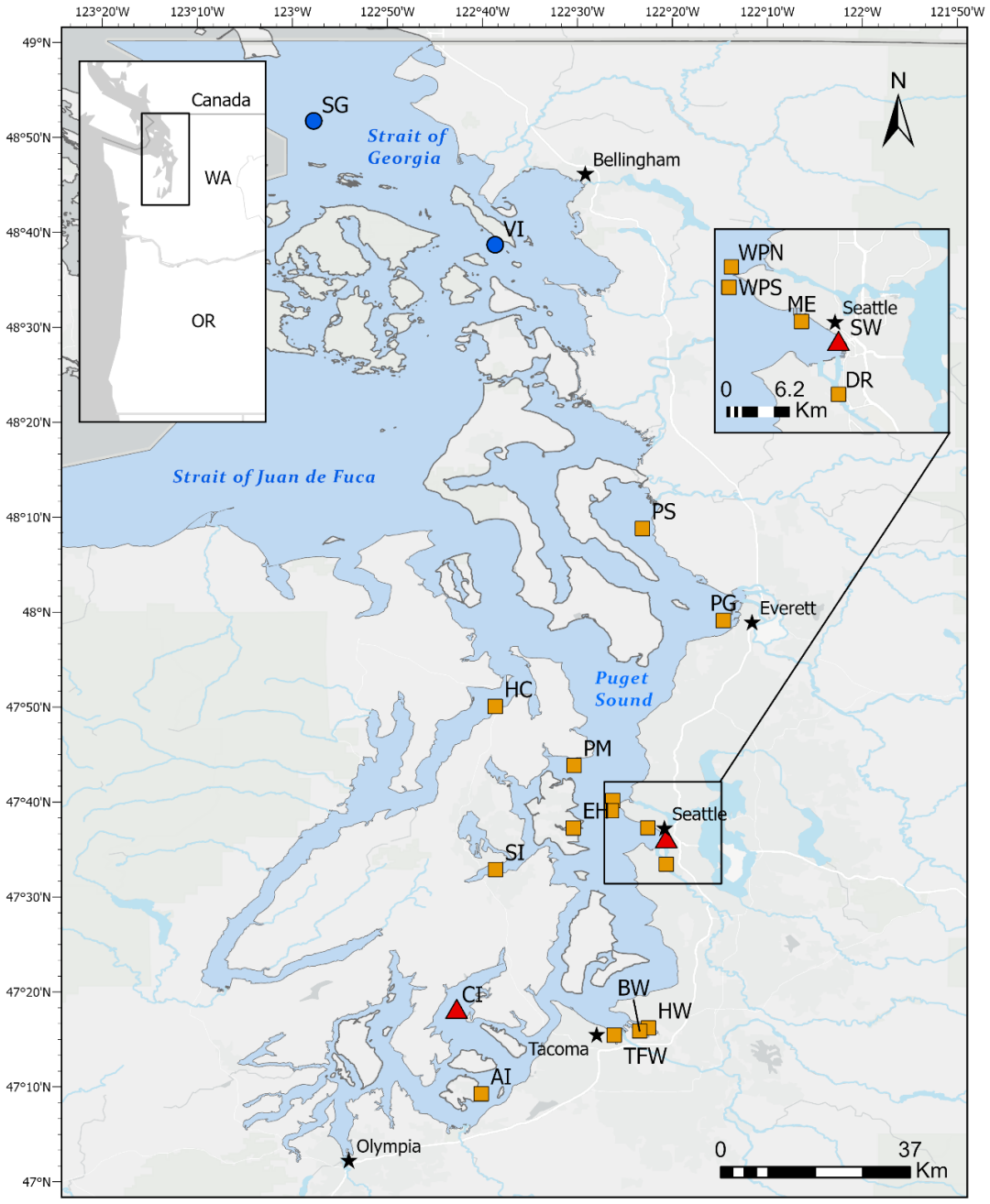

Fig. 1. Sites sampled by Washington Department of Fish and Wildlife for English Sole bile in Puget Sound. SG: Strait of Georgia, VI: Vendovi Island, PS: Port Susan, PG: Port Gardner Bay, HC: Hood Canal, PM: Port Madison, WPN: West Point North, WPS: West Point South, EH: Eagle Harbor, ME: Myrtle Edwards, SW: Seattle Waterfront, DR: Duwamish River, SI: Sinclair Inlet, HW: Commencement Bay Hylebos Waterway, BW: Commencement Bay Blair Waterway, TFW: Commencement Bay Thea Foss Waterway, CI: Carr Inlet, AI: Anderson Island. Red triangles indicate impacted sites, blue circles indicate unimpacted sites (as defined in section 3.2.7), and orange squares are all other sampling sites.

3.2.2 Estrogenic Predictions of Chemical Mixtures using ToxCast in vitro HTS data

3.2.2.1 Selection of ER Agonist Assays for Identifying Estrogenic Chemicals

A total of 18 ER agonist assays were used (Table 1). Sixteen assays were identified by Judson et al. (2015) and two additional assays present in the updated ToxCast database (invitroDBv3.5_database) were identified as ER agonist assays by Maloney et al. (2023) (Table 1). These assays would be classified by Escher et al. (2018) as Category 1 bioassays, which include those that target one highly specific molecular initiating event.

To distinguish between estrogen agonism and false-positive results caused by assay interference (Fay et al., 2018; Judson et al., 2016), we used an EPA ER agonist pathway model to predict the probability that an assay response was associated with the estrogen receptor and not a result of assay interference (Judson et al., 2015). The EPA model uses ToxCast data to generate an area-under-the-curve (AUC) across the range of assay concentrations. The AUCs are scaled from 0-1 relative to EE2, which is assigned an AUC of 1. The EPA defines an AUC score ≥ 0.1 as active, scores between 0.1 and 0.01 as inconclusive, and scores ≤ 0.01 as inactive and unlikely to interact with the ER receptor (Judson et al., 2015). The AUC score of 0.01 was recommended by the Collaborative Estrogen Receptor Activity Prediction Project (CERAPP) as a cutoff to identify active agonists (Mansouri et al., 2016). In our study, chemicals exhibiting activity in ER agonist assays but having AUC scores ≤ 0.01 were considered non-estrogenic and were excluded from further analysis. Twenty chemicals that were detected in Puget Sound and active in ER agonist assays without available AUC scores were manually evaluated based on review of chemical-assay response, and results from the CERAPP agonist consensus model (Mansouri et al., 2016) (Appendix A1.2).

Table 1. Summary of ToxCast assays related to the estrogen receptor agonist signaling pathway. Unless stated otherwise, ER agonists assays were identified by Judson et al. (2015)

Assay Name	Assay Source	Gene Target	Biological Process	Timepoint (hr)	Organism	Tissue	Cell Format	Cell Line Name
NVS_NR_bER	Novascreen	ESR1	Receptor binding	18	Bovine	Uterus	Cell-free	NA
NVS_NR_hER	Novascreen	ESR1	Receptor binding	18	Human	NA	Cell-free	NA
NVS_NR_mERa	Novascreen	ESR1	Receptor binding	18	Mouse	NA	Cell-free	NA
OT_ER_ERaERa_0480	Odyssey Thera	ESR1	Protein stabilization	8	Human	Kidney	Cell line	HEK293T
OT_ER_ERaERa_1440	Odyssey Thera	ESR1	Protein stabilization	24	Human	Kidney	Cell line	HEK293T
OT_ER_ERaERb_0480	Odyssey Thera	ESR1/ESR2	Protein stabilization	8	Human	Kidney	Cell line	HEK293T
OT_ER_ERaERb_1440	Odyssey Thera	ESR1/ESR2	Protein stabilization	24	Human	Kidney	Cell line	HEK293T
OT_ER_ERbERb_0480	Odyssey Thera	ESR2	Protein stabilization	8	Human	Kidney	Cell line	HEK293T
OT_ER_ERbERb_1440	Odyssey Thera	ESR2	Protein stabilization	24	Human	Kidney	Cell line	HEK293T
OT_ERa_EREGFP_0480	Odyssey Thera	ESR1	Regulation of gene expression	8	Human	Cervix	Cell line	HeLa
OT_ERa_EREGFP_0120	Odyssey Thera	ESR1	Regulation of gene expression	2	Human	Cervix	Cell line	HeLa
ATG_ERE_CIS_up	Attagene	ESR1	Regulation of transcription factor activity	24	Human	Liver	Cell line	HepG2
ATG_ERa_TRANS_up	Attagene	ESR1	Regulation of transcription factor activity	24	Human	Liver	Cell line	HepG2
Tox21_ERa_LUC_VM7_Agonist[1]	Tox21	ESR1	Regulation of transcription factor activity	22	Human	Ovary	Cell line	VM7
Tox21_ERa_LUC_VM7_ICI182780[2]	Tox21	ESR1	Regulation of transcription factor activity	22	Human	Ovary	Cell line	VM7
Tox21_ERa_BLA_Agonist_ratio	Tox21	ESR1	Regulation of transcription factor activity	24	Human	Kidney	Cell line	HEK293T
Tox21_ERb_BLA_Agonist_ratio[3]	Tox21	ESR2	Regulation of transcription factor activity	24	Human	Kidney	Cell line	HEK293T
ACEA_ER_80hr[4]	ACEA Biosciences	ESR1	Cell proliferation	80	Human	Breast	Cell line	T47D

[1] Tox21_ERa_LUC_VM7_Agonist was previously named Tox21_ERa_LUC_BG1_Agonist as it was previously annotated as being run in BG1 cells, but was recently shown to be of MCF7 origin (Judson et al., 2017)

[2] This is a secondary assay developed for Tox21_ERa_LUC_VM7_Agonist. Identified as an ER agonist assay in Maloney et al. (2023)

[3] Assay targeting the ESR2 receptor was new in 2020. Identified as an ER agonist assay in Maloney et al. (2023)

[4] Assay renamed from ACEA_T47D_80hr

3.2.2.2 Exposure-Activity Ratio (EAR) Calculations

Exposure-activity ratios (EARs) were calculated for all chemicals detected in the compiled environmental monitoring data, with activity in one or more of the 18 ER agonist assays and without assay interference (Corsi et al., 2019). Since chemicals can be active in multiple ER agonist assays, with some targeting the same response (e.g., receptor binding, protein stabilization, or regulation of gene expression), the 5th percentile of the log ACC (which were transformed to the ACC_5 with the antilogarithm; µM) was estimated for each chemical across all ER agonist assays and converted to µg/L. EARs were calculated per Equation 1.

$$EAR_{ACC5} = \frac{Measured\ Environmental\ Concentration}{ACC_5} \quad (1)$$

In the case of mussel and fish tissue, the measured environmental concentration was converted to an estimated water concentration using BCFs predicted with OPERA (OPEn saR App) models available through CompTox, or with Burkhard (2021) for PFAS compounds, as available (see James et al., 2023).

EAR_{ACC5} values for each chemical in a sample were summed to calculate an EAR_{mix} for each individual sample (Eq. 2), assuming the additivity of effects (Filby et al., 2007; Nirmalakhandan et al., 1994; Thorpe et al., 2003).

$$EAR_{mix} = \sum (EAR_{ACC5})_{[i]} \quad (2)$$

i = the individual chemicals in the mixture, active in the selected assays.

Computation of EAR_{mix} was carried out using the *Toxicity Explorer* (Faber et al., 2022), with additional calculations using *dplyr* (Wickham et al., 2015), and visualizations using *ggplot2* (Wickham, 2009) and the *ToxEval* R package (DeCicco et al., 2022). Data from ToxCast were filtered based on consideration of data quality flags following Corsi et al. (2019) (Appendix A1.2).

3.2.3 EAR_{mix} and 17β-estradiol Equivalency Quotients Comparisons

To validate the application of EAR_{mix} values as an appropriate evaluation of mixture estrogenicity, a comparison was made with EEQs. The EEQs estimate the potency of estrogenic substances relative to E2, by multiplying the environmental concentration of an individual chemical by its chemical-specific EEF (Eq. 3),

$$EEQ = Measured\ Enviornmental\ Concentration_{[i]}\ x\ EEF_{[i]} \quad (3)$$

where i = individual chemical information.

Eight chemicals have both EEFs compiled in Vega-Morales et al. (2013) and ER agonist assay results in ToxCast (Table B1). A Pearson product-moment correlation test was conducted to compare EEQ values, calculated using the median EEF, to EAR_{ACC5} (assuming 100 µg/L concentrations for each). The line of best fit was modelled to the data to evaluate the nature of the relationship between the EEQ and EAR_{ACC5}. The slope of the best-fit line was used as an empirical foundation for identifying screening values (i.e., levels of concern) for EAR_{mix} levels.

3.2.4 Screening Values and Classification of Samples based on Estrogenic Potential in Mixtures

Screening values for EAR_{mix} were derived from the no observed effect concentration (NOEC) and lowest observed effect concentration (LOEC) for Vtg induction from estradiol exposures in male fish. In previous studies, EEQs from water were compared to a NOEC of 0.005 µg/L and LOEC of 0.025 µg/L for Vtg induction in male cyprinid fish and used as benchmarks for endocrine disruption (Baldwin et al., 2016; Jobling et al., 2006). The NOEC and LOEC were used to determine two sets of EAR_{mix}-based NOECs and LOECs. A $NOEC_{EAR}$ and $LOEC_{EAR}$ (Eq 4) were used for comparison with EAR_{mix} based on measured and estimated water concentrations, and WWTP effluent; a $NOEC_{bEAR}$ and $LOEC_{bEAR}$ were used for comparison to bile (Eq 5),

$$NOEC_{EAR} = NOEC * Slope \quad (4)$$

$$NOEC_{bEAR} = NOEC_{EAR} * BCF_{bw} \quad (5)$$

where *Slope* is the slope of the best fit line of EEQ vs EAR_{AAC5} (see Section 3.2.3), and BCF_{bw} is the bioconcentration factor for E2 in bile and water (Larsson et al., 1999). For the $LOEC_{EAR}$ and $LOEC_{bEAR,}$ the LOEC was used in place of the NOEC in the above equations.

Screening values were compared to EAR_{mix} values calculated for each sample to classify samples that have either low potential ($EAR_{mix} < NOEC_{EAR}$ or $NOEC_{bEAR}$), medium potential ($LOEC_{EAR}$ or $LOEC_{bEAR} \geq EAR_{mix} \geq NOEC_{EAR}$ or $NOEC_{bEAR}$), or high potential for estrogenic

effects ($EAR_{mix} \geq LOEC_{EAR}$ or $LOEC_{bEAR}$). Samples identified as medium or high potential for estrogenic effects were further examined to prioritize chemical constituents based on their contribution to the estrogenic response.

3.2.5 Prioritization of Individual Chemical Constituents in Mixtures

For the water, mussel and fish tissue, WWTP effluent, and bile samples identified as medium and high potential for estrogenic effects, chemical constituents contributing at least 1% were retained to identify which chemicals drive (drivers) and or contribute (minor and major contributors) to estrogenic activity in a mixture. A chemical was considered a "driver" of the estrogenic activity if that chemical individually exceeded the screening value, a "major contributor" if it did not individually exceed the screening value but contributed ≥ 1% to the mixture response, or "minor contributor" if it contributed < 1%. A chemical may be both a driver and a contributor depending on matrix, sample, and prioritization category. Chemicals can also be assigned to multiple priority categories because different samples within a matrix or between matrices may result in conflicting assignments. In cases when chemicals were classified in multiple categories, they were assigned to the higher priority category.

A decision tree for the prioritization framework is included (Fig. 2). The prioritization categories for e-EDCs are:

- *High Priority*: Chemicals in mixtures with an $EAR_{mix} \geq LOEC_{EAR}$ or $LOEC_{bEAR}$; and either the $EAR_{ACC5} \geq LOEC_{EAR}$ or $LOEC_{bEAR}$, or the chemical contributes ≥ 1% toward cumulative mixture response.
- *Watch List:* Chemicals in mixtures with an EAR_{mix} between the $NOEC_{EAR}$ and $LOEC_{EAR}$ or $NOEC_{bEAR}$ and $LOEC_{bEAR}$; and either the $EAR_{ACC5} \geq NOEC_{EAR}$ or $NOEC_{bEAR}$; or, the

chemical contributes ≥ 1% toward cumulative mixture response. Although this was not observed in our work, it is possible that a chemical in a mixture with an EAR_{mix} above the $LOEC_{EAR}$ or $LOEC_{bEAR}$ could individually exceed the screening value ($EAR_{ACC5} \geq NOEC_{EAR}$ or $NOEC_{bEAR}$) but not contribute ≥ 1% to the mixture; these chemicals should be considered for prioritization on a case-by-case basis.

- *Low Priority:* Chemicals in mixture that were active in ER agonist assays but $EAR_{mix} < NOEC_{EAR}$ or $NOEC_{bEAR}$ or did not contribute ≥ 1% of mixture response.
- *Insufficient Information:* Insufficient information to assess these e-EDCs. This category includes chemicals that were detected in environmental samples but were not assessed in ToxCast ER agonist assays. Although not evaluated in this work, this also includes chemicals that were analyzed for and not detected, but whose median detection limit was greater than 0.1 x ACC5, indicating that there could be estrogenic effects at concentrations below the limit of detection. Due to the lack of information, it is not possible to assess the potential impact of these chemicals as e-EDCs.

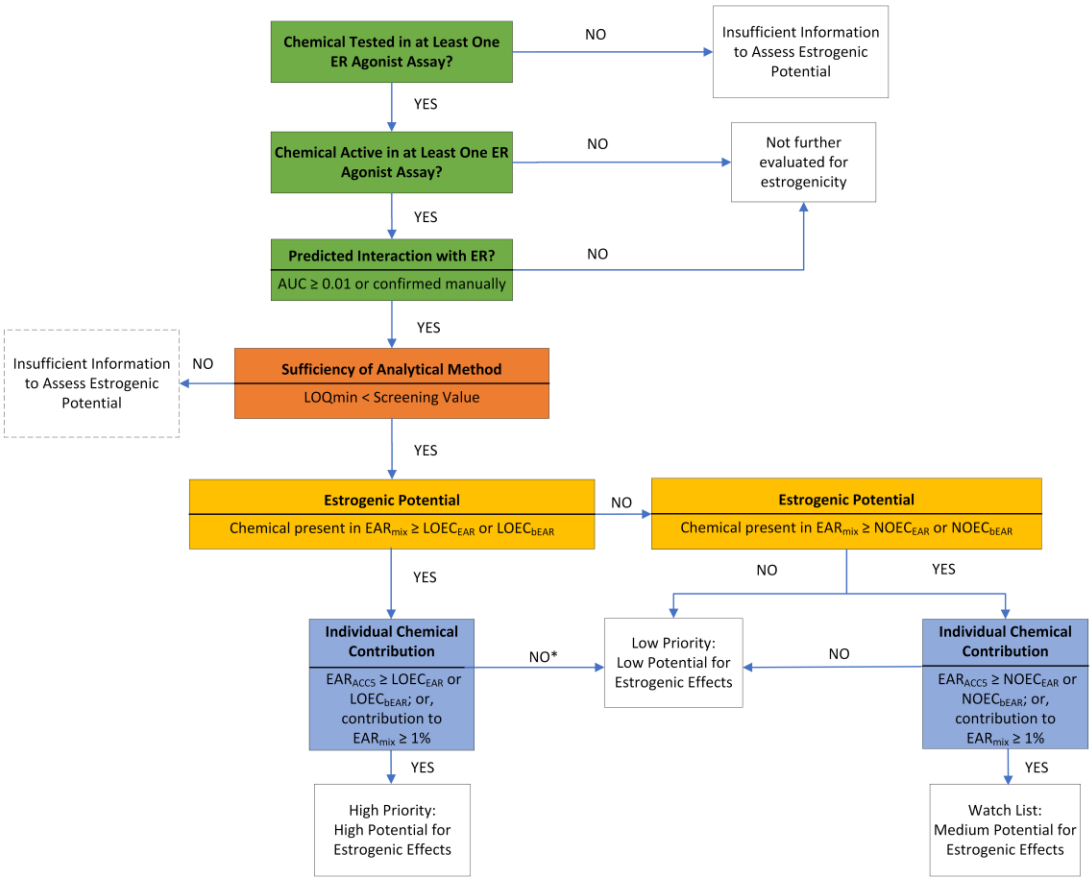

*Fig. 2. Decision flow diagram for e-EDC chemical constituent prioritization framework. Dotted line around box indicates that this evaluation was not completed for this work; an example of this assessment is included in James et al. (2023) using the effects thresholds and matrices from that work. * Chemicals in mixture with $EAR_{mix} > LOEC_{EAR}$ or $LOEC_{bEAR}$ but with $LOEC_{EAR}$ or $LOEC_{bEAR} > EAR_{ACC5} \geq NOEC_{EAR}$ or $NOEC_{bEAR}$ that do not meet 1% response contribution should be evaluated on a case-by-case basis to determine the category assignment.*

3.2.6 Classification of Sampling Sites

Site classification was performed at locations where bile samples were collected, because 1) multiple samples were available from each site, and 2) it was the only biological matrix that was analyzed for steroidal estrogens. The median EAR_{mix} was used to evaluate each of the 18 bile sampling locations (Fig. 1). Median values were compared to the $NOEC_{bEAR}$ and $LOEC_{bEAR}$ to classify sites into three categories based on their estimated potential for impacts from estrogenic effects; low potential site (site median $EAR_{mix} < NOEC_{bEAR}$), medium potential site

(site median EAR_{mix} between $NOEC_{bEAR}$ and $LOEC_{bEAR}$), and high potential site (site median $EAR_{mix} \geq LOEC_{bEAR}$).

3.2.7 Field-Based Screening Value Derivation

To validate the application of the $NOEC_{bEAR}$ and $LOEC_{bEAR}$, these screening values were compared to a second set of field-based screening values derived for "unimpacted" or "impacted" sites based on biological measures of exogenous estrogen exposure.

Field-based monitoring results from unimpacted sites were used to establish a baseline where: 1) no Vtg induction was detected in male English sole plasma samples; and 2) POP and CEC concentrations in fish tissue are low, suggesting minimal exposure to anthropogenic contaminants (Harding et al., 2022; West et al., 2017). Vendovi Island and Strait of Georgia, the two northern-most sites (Fig. 1), were identified as unimpacted sites. The EAR_{mix} values calculated for each bile sample from both sites were aggregated (n=20), log-transformed, and the 95% confidence limits of the mean were determined. The upper confidence limit (UCL) of the EAR_{mix} for unimpacted sites was defined as the field-based screening value below which biological impacts would not be expected and was compared to the $NOEC_{bEAR}$.

Impacted sites were those where there was: 1) a high occurrence of Vtg induction in male English sole, relative to fish from unimpacted sites (>35%); 2) significantly higher mean plasma Vtg concentrations in male English sole relative to fish from unimpacted sites; and 3) altered reproductive timing in female English sole (Harding et al., 2022). The impacted sites were identified as Carr Inlet and Seattle Waterfront (Fig. 1). The EAR_{mix} calculated for each bile sample from both impacted sites were compiled (n = 63) and log-transformed, and the 95% confidence limits of the mean were determined. The lower confidence limit (LCL) of the EAR_{mix}

for impacted sites was defined as the field-based screening values above which biological impacts would be expected and was compared to the $LOEC_{bEAR}$.

3.3 Results/Discussion

3.3.1 Identification of e-EDCs by Integrating ToxCast ER Agonist Assay Results

Of the 387 chemicals analyzed, 222 were detected in at least one sample in any matrix. Of these detected chemicals, 158 were evaluated against ToxCast ER agonist assays. Of these, 68 were active and 90 were inactive in ER agonist assays. The 68 active chemicals were further screened for activity above the threshold and assay interference using AUC scores or through manual evaluation, and 19 chemicals were identified as causing disruption specific to the estrogen signaling pathway (Table B2) and were retained for further evaluation.

3.3.2 Occurrence and Distribution of e-EDCs in Environmental Monitoring Samples

The 19 e-EDCs include hormones (n = 9), bisphenols (n = 4), alkylphenols (n = 3), phthalates (n = 2), and PFAS (n = 1). The analysis of e-EDCs varied across sampling matrices due to the opportunistic nature of including multiple datasets across monitoring projects. For example, the analysis of steroidal estrogens was limited to fish bile and WWTP effluent samples. The detection of e-EDCs also varied across matrices. The steroidal estrogen, E1, was detected in 100% of male English sole bile samples, while it was only detected in 29% of WWTP effluent samples. Phenolic substances, like BPA and NP, used in high volumes by industry, were widely analyzed and detected across multiple matrices at varying detection frequencies. For instance, BPA was detected in 79% of fish bile samples and 56% of WWTP effluent samples, but only 4% of water samples and 5% of mussel and fish tissue samples. A summary of the number of detected e-EDCs in each matrix is presented in Fig. 3.

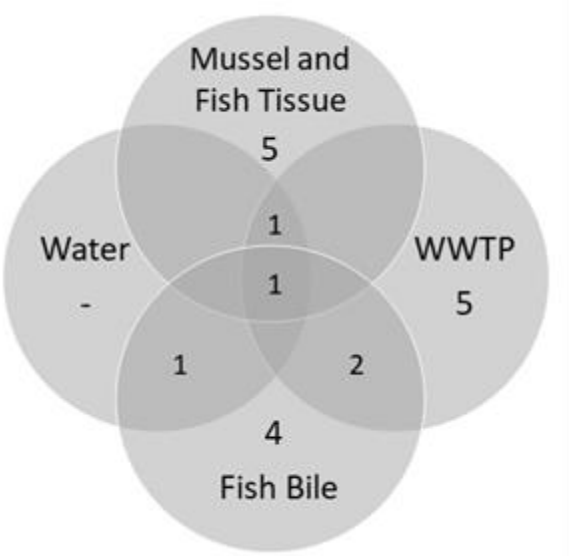

Fig. 3. Number of e-EDCs detected in each matrix. WWTP is wastewater treatment plant effluent.

In addition to different matrices, samples were collected from different aquatic species and tissue types. Biological tissue samples were from nine fish species and one shellfish species, representing different diets, habitats, and thus, varying levels of exposure to contaminants. The variation in chemical occurrence profiles across different matrices (Fig. 3) and across species with different life histories and habitats (Table B3), highlights the importance of conducting a comprehensive and diverse sampling campaign to adequately characterize contaminant exposure. Sampling different tissue types is also critical to understand chemical exposures as various tissues (e.g., plasma, bile, liver, and muscle) in fish accumulate and store contaminants differently (Lv et al., 2019). For example, bile is a useful matrix to monitor exposure to exogenous estrogens and industrial phenolic estrogen mimics, as they tend to accumulate preferentially in fish bile rather than muscle or other tissue types because of the important role that bile plays in the excretion of these chemicals (Lv et al., 2019; Wu et al., 2016). This contributes to the greater detection frequency of BPA in bile when compared to other fish

tissues. Therefore, incorporating bile sampling into monitoring programs can provide valuable information on exposure to estrogens or other chemicals in fish that may accumulate in bile.

The monitoring data demonstrates that complex mixtures of CECs are present in Puget Sound. In a given sample, between 0-55 unique chemicals were detected with 0-6 of those being e-EDCs. The range of e-EDCs detected in mixtures in each matrix were 0-2 in marine water, 0-6 in WWTP effluent, 0-3 in mussel tissue, 0-4 in fish tissue, and 1-6 in fish bile (Table B4). This study focuses on CECs exhibiting estrogen receptor agonistic activity, excluding legacy contaminants, which means that estrogenicity may be underestimated. Additionally, hormone disruption can occur via other mechanisms; for examples, studies have demonstrated the potential for chemicals to inhibit testosterone or interfere with the thyroid hormone system (Ankley et al., 2010; Tietge et al., 2013). Estrogen receptor agonism was selected as it is one of the most well-studied mechanisms and it is known to result in measurable reproductive impact. Finally, samples had as many as 49 chemicals (in a WWTP effluent sample) that were not e-EDCs and so not considered in this work. This suggests that there are likely other mixture-based impacts to aquatic wildlife not accounted for here.

3.3.3 Determination of EAR-based Screening Values

Previous studies have relied on NOEC and LOECs for Vtg induction as benchmarks for endocrine disruption (Baldwin et al., 2016; Jobling et al., 2006). To incorporate these benchmarks into our work, we first compared EARs to EEQs and then used that relationship to convert the NOECs and LOECs to an EAR-based threshold. A consistent relationship between the two measures would support using the HTS data to evaluate EDC mixtures. The EEQ and EAR_{ACC5} were strongly correlated ($r = 0.93$, $p < 0.01$, $n = 8$) (Fig. 4), which supported the conversion of NOECs and LOECs to an EAR-based threshold.

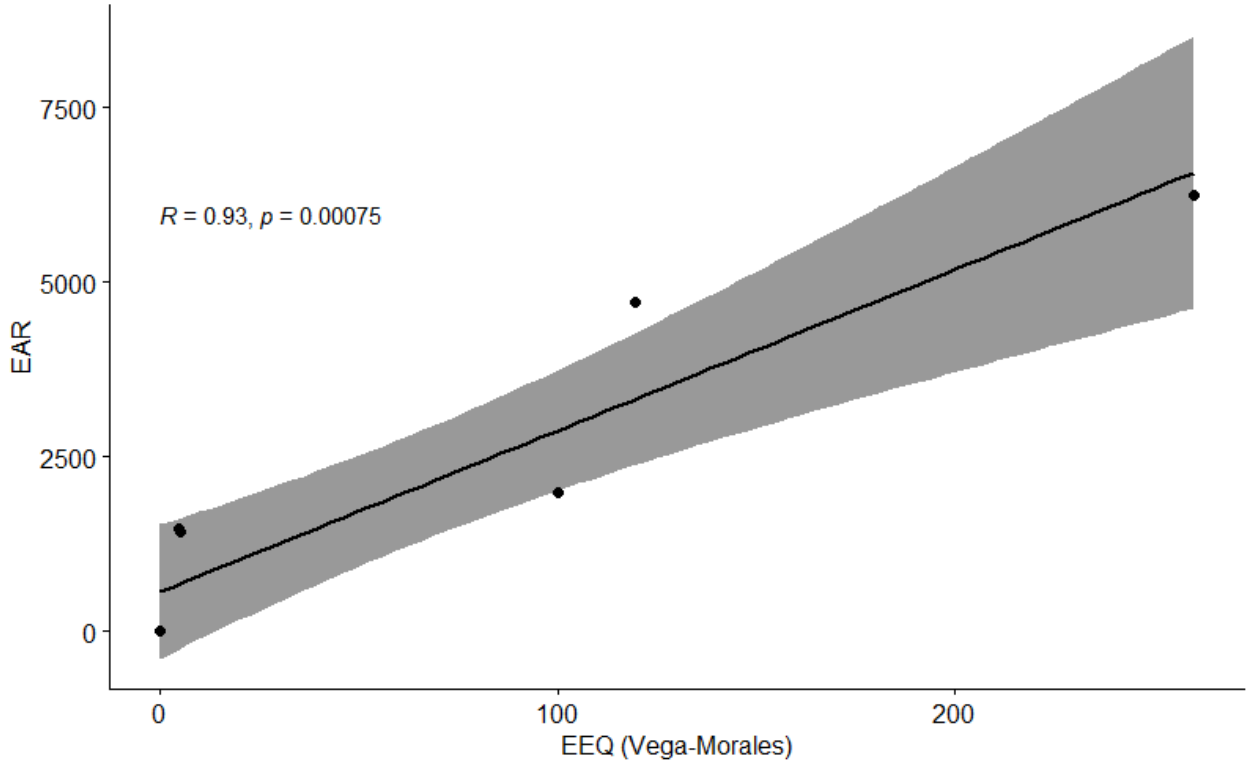

Fig. 4. Relationship between EEQ and EAR_{ACC5} for eight known e-EDCs. The shaded region displays the 95% confidence interval for the best fit line.

A best fit line was then modelled to obtain the slope (SLOPE=23). The NOEC and LOEC values for estradiol were multiplied by the slope of 23, resulting in $NOEC_{EAR}$ and $LOEC_{EAR}$ values of 0.1 and 0.6, respectively, for use as EAR_{mix} screening values for e-EDCs in water. To estimate EAR_{mix} screening values for bile, the $NOEC_{EAR}$ or $LOEC_{EAR}$ was multiplied by the BCF_{bw}, (4000; Larsson et al., 1999), resulting in $NOEC_{bEAR}$ and $LOEC_{bEAR}$ values of 460 and 2300, respectively. This approach doubled the list of chemicals evaluated in mixtures for estrogenic potential from less than 10 using EEQs to 19 chemicals that were active in ER agonist assays with confirmed estrogen receptor interaction.

3.3.4 Prioritization of Chemical Constituents

3.3.4.1 Screening of Water, Biota, and WWTP Effluent Samples and Prioritization of Chemical Constituents

EAR_{mix} for water, WWTP effluent, and mussel and fish tissue samples were used to characterize the degree to which e-EDC mixtures in environmental samples had potential for estrogenic effects. Out of nine WWTP effluent samples, six had detectable e-EDCs. One sample exceeded the $LOEC_{EAR}$ of 0.6 (high potential for estrogenic effects) and three samples exceeded the $NOEC_{EAR}$ of 0.1 (medium potential for estrogenic effects) (Fig. 5). None of the water, mussel tissue, or fish tissue samples exceeded the screening values, suggesting a low potential for estrogenic effects.

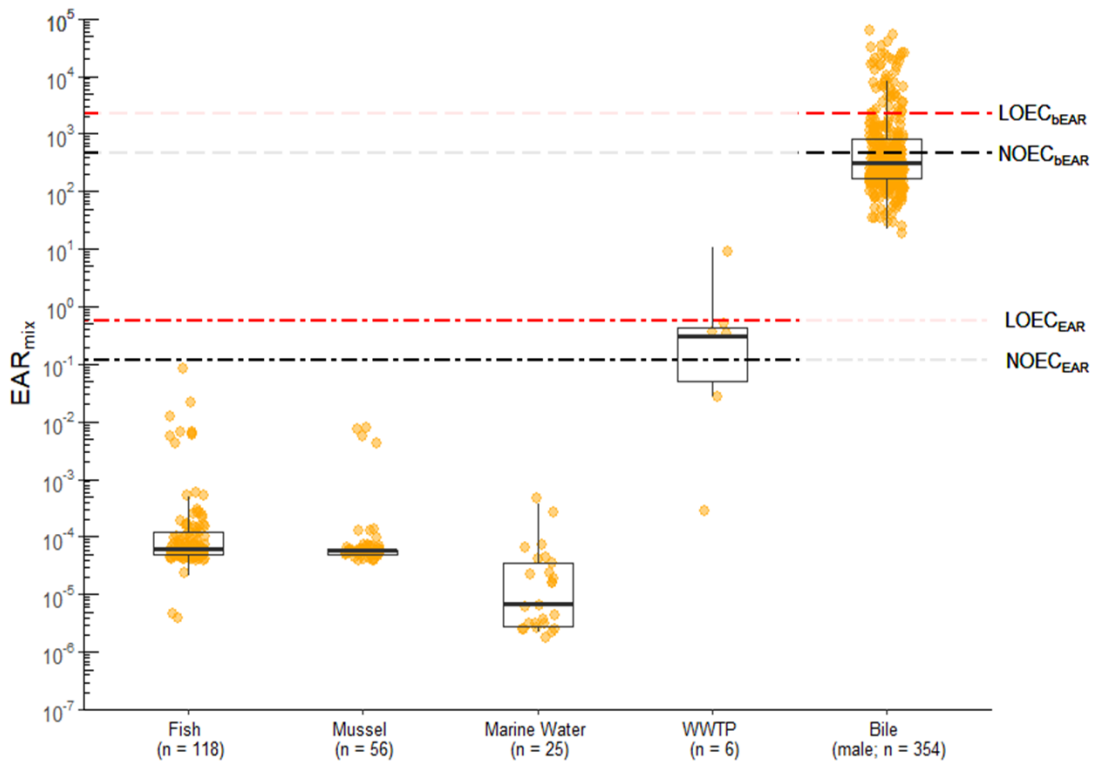

Fig. 5. Distribution of EAR_{mix} values for each matrix. Box and whisker plot shows the median, first/third quartile, and a distance of 1.5 times the interquartile range. The dots represent the EAR_{mix} values for each sample, and n is the number of samples with detected e-EDCs. For fish and mussel samples, concentrations were back calculated to water concentrations (Section 2.2.2). The total number of samples with e-EDC analysis was: fish = 200; mussel = 75; marine water = 134; WWTP = 9, and bile = 500.

The four WWTP effluent samples that exceeded the screening values were then evaluated for which chemical constituents drive or contribute to an estrogenic effect (Fig. 6). In the WWTP effluent sample that exceeded the $LOEC_{EAR}$, E1 drove the estrogenic response and was, therefore, included as a *High Priority* chemical because the E1 EAR_{ACC5} exceeded the $LOEC_{EAR}$. Within that same sample, BPA and E2 were major contributors, with greater than 1% contribution toward estrogenic response. Of the three WWTP effluent samples exceeding the $NOEC_{EAR}$, BPA and E1 either drove or contributed to the estrogenic response. Both chemicals were included as *High Priority*, which is consistent with James et al. (2023).

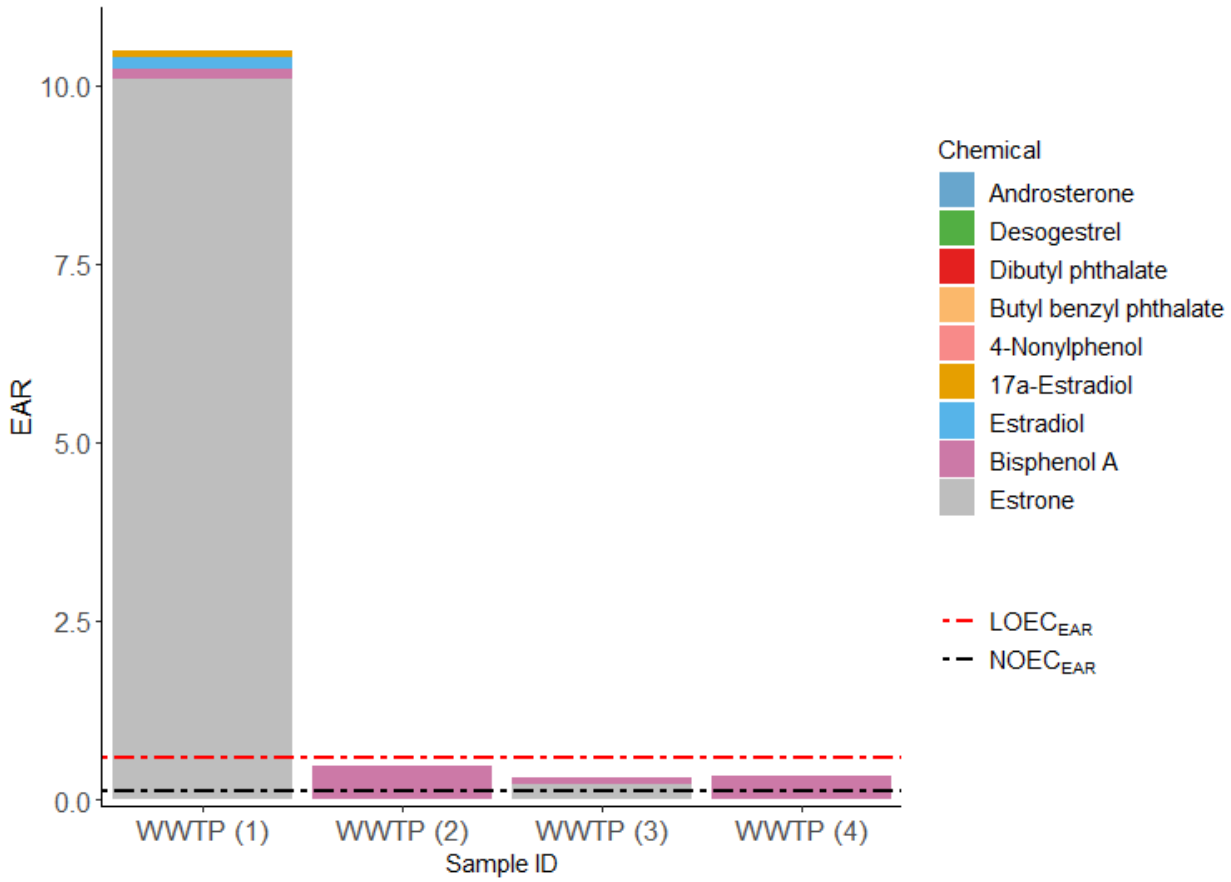

Fig. 6. Stacked bar plot with exposure-activity ratios (EARs) for each WWTP effluent sample exceeding either of the screening values. The EAR_{ACC5} is represented by the EAR for each chemical in a sample and the EAR_{mix} is the sum of the EAR_{ACC5} for a sample. Black dotted and dashed line represents the $NOEC_{EAR}$, and red dotted and dashed line represents the $LOEC_{EAR}$. Samples above the $LOEC_{EAR}$ are classified as having ahigh potential for estrogenic effects, and samples between the $LOEC_{EAR}$ and $NOEC_{EAR}$ are classified as medium potential for estrogenic effects. Not visible here, but EAR_{mix} includes minor contributions (<1%) from androsterone (WWTP (1)), desogestrel (WWTP (1)), butyl benzyl phthalate (WWTP (2)), dibutyl phthalate (WWTP (2)), and NP (WWTP (4)).

It is important to recognize that WWTP effluent is considered a worst-case scenario for chemical exposures to marine organisms, due to the rapid dilution of the effluent upon discharge. Ongoing monitoring of WWTP effluent is useful, however, as it provides information on contaminants discharged into aquatic environments, which can be used to estimate

environmental exposures with dilution models, even if there are no data available in the aquatic environment.

3.3.4.2 Screening of Bile Samples and Prioritization of Chemical Constituents

Of the 354 bile samples from male English sole, 50 had EAR_{mix} values above the $LOEC_{bEAR}$ ($EAR_{mix} \geq 2{,}300$) and were classified as having high potential for estrogenic effects (Fig. 5). From those 50 samples, four chemicals were responsible for the predicted estrogenic effects as either drivers or major contributors (Fig. 7 and Fig. B1). 82% of samples had at least one driver, and in all cases, it was a steroidal estrogen (E1, E2, and E3); of these samples, 29% had one driver, 61% had two drivers, and 10% had three drivers. These results align with several studies that identify steroidal estrogens as drivers of estrogenic response (Deich et al., 2020; Desbrow et al., 1998; Gómez et al., 2021; Legler et al., 2002). This is likely due to potency; results from *in vivo* assays demonstrated that steroidal estrogens have potencies more than a thousand-fold greater in fish than other xenoestrogens (Caldwell et al., 2012; Jarošová et al., 2014; Young et al., 2002). BPA was identified as a major contributor in 98% of the samples but never as a driver. Based on the $LOEC_{bEAR}$ exceedances in bile samples, E1, E2, E3, and BPA were classified as *High Priority* chemicals.

Eighty-three of the 354 bile samples had EAR_{mix} values between the $LOEC_{bEAR}$ and $NOEC_{bEAR}$ ($460 < EAR_{mix} \leq 2{,}300$), indicating they represented a medium potential for estrogenic effects. Of those 83 samples, 44 had at least one chemical (E1 or E2) that was a driver because its EAR_{ACC5} exceeded the $NOEC_{bEAR}$; E3 and BPA were major contributors (Fig. 7 and Fig. B2). The remaining samples did not include any drivers, and only the four previously identified chemicals (E1, E2, E3, and BPA) were identified as major contributors, meaning that for these 39 samples, the potential for estrogenic effects was solely due to the presence of

mixtures, and not a single chemical alone. Overall, the estrogenicity in 64% of bile and WWTP effluent samples was largely explained by a single chemical alone, rather than a mixture. This is consistent with Escher et al. (2018) who summarized that a small number of highly bioactive natural hormones or synthetic drugs often explain mixture effects.

E1, E2, and BPA were previously identified as *High Priority* chemicals through individual chemical prioritization and multiple modes of action with BPA supported by data from multiple matrices (James et al., 2023). E1 and E2 were only analyzed in WWTP effluent samples in that study and flagged as such. With the inclusion of bile data, we strengthen the E1 and E2 categorization with the support from biological samples. Analysis in other matrices should be considered for other chemicals that were only analyzed in WWTP effluent and prioritized as *High Priority* in James et al. (2023); this list of chemicals includes phenol, beta-stigmastanol, carbamazepine, campesterol, ergosterol, cholesterol, cholestanol, beta-sitosterol, stigmasterol, and butyl benzyl phthalate. E3 was also added to the *High Priority* list; this was not previously categorized by James et al. (2023) because it was only analyzed in WWTP effluent and was not detected.

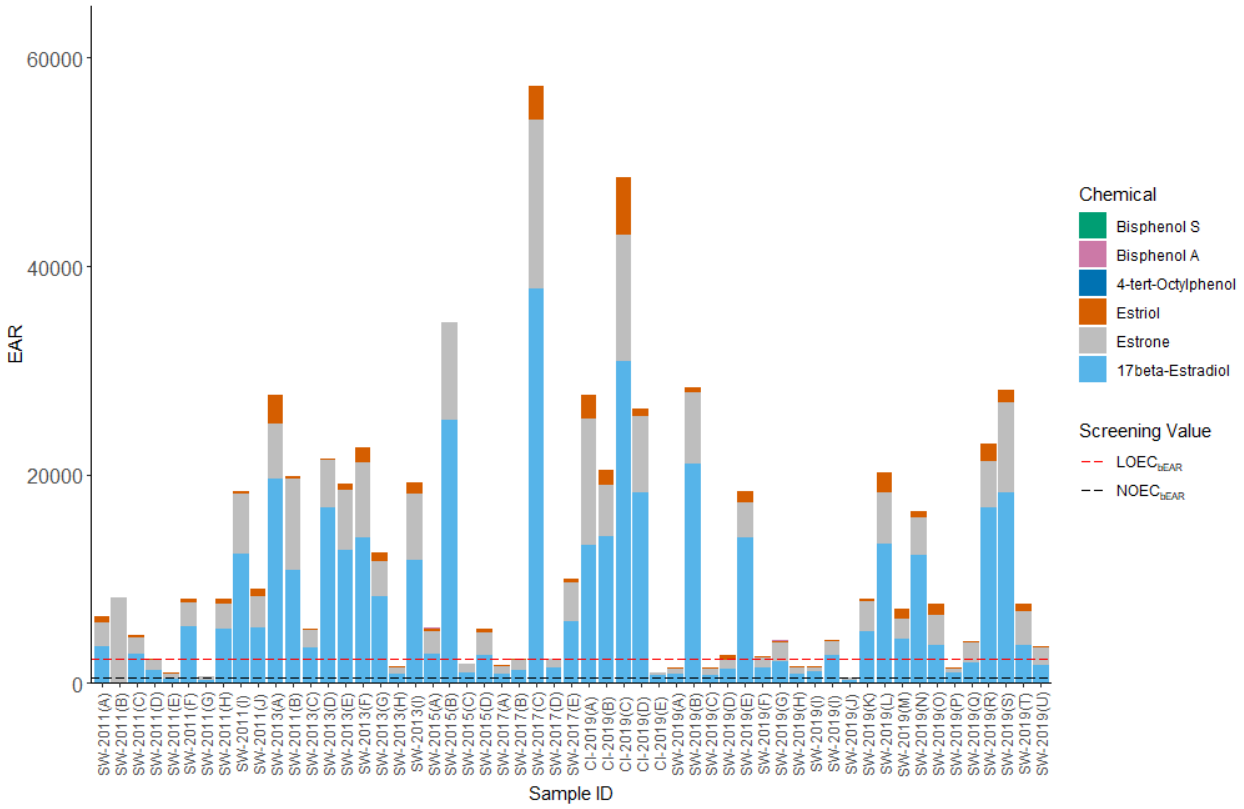

Fig. 7. Stacked barplot with exposure-activity ratios (EARs) for each bile sample collected at the impacted sites, Carr Inlet (CI) and Seattle Waterfront (SW) and exceeding either of the screening values. The sample labels include the collection year and a letter to distinguish samples. The EAR_{mix} is the sum of all EARs in a sample, where each component of the stacked bar is the EAR_{ACC5} for that chemical. Black dotted line represents the $NOEC_{bEAR}$, and red dotted line represents the $LOEC_{bEAR}$. Samples with EAR_{mix} above the $LOEC_{bEAR}$ have high potential for estrogenic effects, and samples between the $LOEC_{bEAR}$ and $NOEC_{bEAR}$ have medium potential for estrogenic effects.

3.3.4.3 Considerations for High Priority and Watch List Compounds

BPA was analyzed in 100% of bile and WWTP effluent samples, as well as 97% of fish tissue, 84% of mussel tissue, and 40% of marine water samples, with detection frequencies of 79%, 56%, 5.1%, 5.5%, and 3.7%, respectively (Table 2). WWTP effluent and fish bile are expected to have higher concentrations of e-EDCs making non-detects less likely if a chemical is present, though as analytical techniques improve, the sensitivity with respect to other matrices may become greater.

Steroidal estrogens were measured in all bile and WWTP effluent samples. In bile, the detection frequencies for steroidal estrogens were 100, 93, and 26%, for E1, E2, and E3, respectively, showing that bile data serves as a valuable tool to assess the potential exposure to steroidal estrogens. Higher concentrations are expected in bile when compared to other matrices because it is the primary excretory route for steroidal estrogens. Bile sample processing that includes deconjugation provides information on the total estrogen concentration, which includes the free estrogen and conjugated forms. Including the conjugated forms may overestimate the overall estrogenic impact because the bioactivity of conjugated EDCs is generally much lower than the free form. However, some conjugated EDCs in bile are likely to be converted to the free form in the gut, due to bacterially-mediated deconjugation reaction which can then enter blood plasma. To assess potential activity from exogenous EDCs, levels in the free, unbound form should be measured in fish blood plasma and serum. However, these data are not currently available. With advanced technologies, hormones can be detected in plasma and serum at detection levels as low as picograms per mL (0.001 ug/L) (Nouri et al., 2020).

An additional challenge to estimating potential estrogenic activity from exogenous hormones is the biological and reversible interconversion between E1 and E2 (Yu et al., 2019). E1 can be converted *in vivo* to E2; where E2 is considered the most potent of the natural estrogens and therefore likely to pose a higher hazard than E1 (Ankley et al., 2017; Dammann et al., 2011; Tapper et al., 2020; Thorpe et al., 2003; Yu et al., 2019). In support of this, environmental data used in our work shows that E1 concentrations were greater than E2 in WWTP effluent, whereas E2 concentrations were higher than E1 in fish bile.

Because the organism experiences more E2 (as E1-derived E2; (Ankley et al., 2017) than would be measured in their aquatic environment, the mixture effects with E1 and E2 may be

different from simple additivity. Additionally, it is important to consider the potential for other chemicals to impact estrogen signaling synergistically or antagonistically. For example, one of the *Low Priority* chemicals identified in this study is trenbolone acetate which is fed to livestock to promote growth and is associated with agricultural runoff (Bartelt-Hunt et al., 2012). The metabolite, 17β-trenbolone is a weak estrogen agonist but acts as a stronger estrogen antagonist (Judson et al., 2015) in part by increasing the rate of E2 clearance (Schultz et al., 2013). This results in significant decreases of endogenous E2 levels in female fish (Schultz et al., 2013) and antagonism for the estrogenic response.

Table 2. Chemical classification, minimum and maximum concentrations, and detection frequency (DF) of e-EDCs detected in Puget Sound. ND is non-detect and "-" indicates that the chemical was not analyzed.

Chemical Name	CAS Number	Chemical Class	Category	Water Min - Max (ng/L)	Water DF	WWTP Min - Max (ng/L)	WWTP DF	Mussel Min - Max (ng/g)	Mussel DF	Fish Min - Max (ng/g)	Fish DF	Bile Min - Max (ng/mL)	Bile DF
17a-Estradiol	57-91-0	Hormones	Low Priority	-	-	1.8 - 1.8	14%	-	-	-	-	-	-
4-n-Octylphenol (OP)	1806-26-4	Commercial	Low Priority	-	-	-	-	0.55 - 2.65	64%	0.49 - 2.63	16%	ND	-
4-Nonylphenol (NP)	104-40-5	Commercial	Low Priority	13.6 - 41.4	67%	180 - 1690	33%	10.3 - 34.8	97%	3.12 - 75.6	91%	ND	-
4-tert-Octylphenol (tOP)	140-66-9	Commercial	Low Priority	-	-	-	-	-	-	-	-	30.0 - 220	15%
Androsterone	53-41-8	Hormones	Low Priority	-	-	0.45 - 0.45	14%	-	-	-	-	-	-
Betamethasone	378-44-9	Hormones	Low Priority	ND	-	-	-	ND	-	3.58 - 3.58	0.6%	-	-
Bisphenol A[1] (BPA)	80-05-7	Industrial	High Priority	2.8 - 4.3	3.7%	350 - 6200	56%	3.07 - 4.09	5.5%	2.52 - 40.7	5.1%	4.6 - 810	79%
Bisphenol AF (BPAF)	1478-61-1	Industrial	Low Priority	-	-	-	-	-	-	-	-	0.76 - 0.76	0.4%
Bisphenol F (BPF)	620-92-8	Industrial	Low Priority	-	-	-	-	-	-	-	-	4.6 - 88.0	5.2%
Bisphenol S (BPS)	80-09-1	Industrial	Low Priority	0.44 - 13.6	100%	-	-	-	-	-	-	0.76 - 30.0	37%
Butyl benzyl phthalate	85-68-7	Phthalates	Low Priority	-	-	1700 - 1700	14%	-	-	-	-	-	-
Desogestrel	54024-22-5	Hormones	Low Priority	-	-	1.01 - 7.04	29%	-	-	-	-	-	-
Dibutyl phthalate	84-74-2	Phthalates	Low Priority	-	-	920 - 920	14%	-	-	-	-	-	-
Estradiol[2] (E2)	50-28-2	Hormones	High Priority	-	-	11.9 - 11.9	14%	-	-	-	-	0.99 - 2700	93%
Estriol[3] (E3)	50-27-1	Hormones	High Priority	-	-	ND	-	-	-	-	-	0.86 – 540	26%
Estrone[4] (E1)	53-16-7	Hormones	High Priority	-	-	20.2 - 1000	29%	-	-	-	-	0.96 - 1600	100%
Perfluorooctanesulfonamide (PFOSA)	754-91-6	PFAS	Low Priority	ND	-	ND	-	0.62 - 0.62	5.6%	0.10 - 3.38	47%	-	-
Prednisone	53-03-2	Hormones	Low Priority	ND	-	-	-	ND	-	26.9 - 26.9	0.6%	-	-
Trenbolone acetate	10161-34-9	Hormones	Low Priority	ND	-	-	-	ND	-	0.23 - 0.31	1.3%	-	-

[1] *High Priority* based on bile and WWTP effluent. BPA was a contributor in samples classified as high potential for estrogenic effects based on bile and WWTP effluent. In some WWTP effluent samples, BPA was also a driver in samples classified as medium potential for estrogenic effects.

[2] *High Priority* based on bile and WWTP effluent. E2 was a contributor to samples classified as high potential for estrogenic effects based on WWTP effluent and was a driver based on bile.

[3] *High Priority* based on bile. E3 was a driver in samples classified as high potential for estrogenic effects based on bile.

[4] *High Priority* based on bile and WWTP effluent. E1 was a driver in samples classified as high potential for estrogenic effects based on bile and WWTP effluent.

3.3.4.4 Considerations for Low Priority and Insufficient Information Chemicals

Fifteen chemicals were categorized as *Low Priority* because they contributed less than 1% toward estrogenic response in mixtures that exceeded the screening values (Table 2). This could be caused by low concentrations (e.g.,17α-Estradiol (αE2); a stereoisomer of E2) and/or low potency, compared to the *High Priority* chemicals. Although E2 has been considered by most authorities to be the most potent, according to ToxCast αE2 has an ACC5 that is three times lower than E2 and is therefore the most potent chemical detected in any matrix. αE2 was also more likely to interact with the ER receptor, with a higher AUC value than E2 (Judson et al., 2015). This is contrary to another study that found αE2 has between two times and 10 times lower affinity for the ER than E2 (Kuiper et al., 1997). In environmental samples, αE2 was only analyzed in WWTP effluent samples and was not measured in fish bile. Since it was only detected at trace concentrations in one WWTP effluent sample, it did not meet the criterial for *High Priority* or *Watch List.* Due to the potential interconversion among αE2, E2, and E1 (Zheng et al., 2012), the simultaneous quantitation of these natural estrogens poses a challenge (Prokai-Tatrai et al., 2010). Therefore, improved analytical methods are recommended to distinguish these natural estrogens accurately, along with expanded monitoring in additional matrices to ensure αE2 does not significantly contribute toward estrogenic response.

The 14 *Low Priority* chemicals with lower potencies based on Judson et al. (2015) are alkylphenols (e.g., NP and OP), alternative bisphenols (e.g., BPS, BPF, and BPAF), phthalates, PFAS, and other classes of hormones besides estrogens. Compared to E2, these ACC5s were 69 to almost 200,000 times greater, illustrating the lower estrogenic potency of these chemicals. Although results suggest that e-EDCs in this category are not drivers or major contributors, it is possible that the analytical frequency contributes towards them not being identified as such. For

example, alkylphenols were only analyzed in 36% of bile samples while alternate bisphenols were analyzed in 64% of bile samples, compared to BPA and natural estrogens that were analyzed in all male bile samples. *Low Priority* chemicals may also be present in other matrices and at higher concentrations, and with other chemicals that may contribute to the mixture response such that they should continue to be included in monitoring programs.

In some instances, chemicals are a lower priority here than in James et al. (2023) where the mode of action was not a focus. Categorized as *Low Priority* in this work, betamethasone, prednisone, PFOSA, and butyl benzyl phthalate were previously prioritized as *High Priority* and αE2, NP, dibutyl phthalate, and trenbolone acetate, were classified as *Watch List* chemicals (James et al., 2023). This difference in categorization can be attributed to the ability of chemicals to elicit biological responses through multiple modes of action; James et al. (2023) considered this by using all ToxCast information for a chemical regardless of the mode of action. With conflicting prioritizations, our recommendation is to retain these chemicals in the highest prioritization category.

Chemicals categorized as *Low Priority,* which exhibit lower estrogenic activity, may still impact aquatic organisms at environmental concentrations through alternative modes of action. When considering endocrine disruption, estrogen agonism is only one of many modes of action to consider. For instance, among the *Low Priority* chemicals, betamethasone and prednisone are synthetic corticosteroids frequently used to treat a variety of inflammatory and immune diseases in human and veterinary medicine (Macikova et al., 2014). These chemicals can weakly bind to the estrogen receptor, though their endocrine disrupting activity is primarily through glucocorticoid receptor (GR) agonism (Macikova et al., 2014). As another example, anti-androgenic chemicals can induce similar endocrine-disrupting effects as estrogenic chemicals

(Filby et al., 2007). Future work should consider additional modes of action beyond estrogen agonism. This work can be conducted with ToxCast based on the methods outlined here and elsewhere.

Chemicals in the *Insufficient Information* category were not evaluated for estrogenic potential in Puget Sound. This included 61 chemicals that have not been evaluated in ToxCast ER agonist assays (Table B5). These 61 chemicals should be re-evaluated as more information becomes available. Additionally, there were chemicals that were active in ER agonist assays but were not detected in the environment above the limit of quantification (LOQ), but may be of concern at levels below the LOQ. One such chemical is EE2, one of the most potent estrogenic chemicals (MIyagawa et al., 2021). Recent monitoring of EE2 in marine water had an LOQ of approximately 5 ng/L. If EE2 were present at levels just below the LOQ the EAR_{ACC5} would exceed the $NOEC_{EAR}$ and the chemical could contribute toward or drive estrogenic response in mixtures. Therefore, continued improvements to analytical techniques are necessary, and chemicals like EE2 should continue to be monitored in the environment.

While not part of the *Insufficient Information* category, the results obtained from the ER pathway model (Judson et al., 2015) offer an opportunity to assess chemicals that were not analyzed in Puget Sound samples but have the potential to act as estrogen agonists. Chemicals such as plastic precursors (e.g., 1,1,1-Tris(4-hydroxyphenyl)ethane)) and UV- filters present in sunscreens (e.g., Benzophenone-1, 2, and 3) have demonstrated estrogenic activity *in vitro*. UV-filters enter aquatic systems either directly or through WWTP effluent and are reportedly ubiquitous in Swiss surface waters (Fent et al., 2010; Wang et al., 2021), suggesting their potential presence in Puget Sound and contribution to estrogenic responses. As this example shows, not all e-EDCs may be accounted for in this work, partly due to the lack of analytical

methods available to measure them in the environment, thus hindering a complete understanding. Furthermore, legacy chemicals were not considered, despite having known estrogenic effects (e.g., organochlorine pesticides). In some cases, estrogenic effects from legacy pollutants may be greater than that associated with the CECs evaluated here (Matthiessen et al., 2018). For example, 2-bis(p-Hydroxyphenyl)-1,1,1-trichloroethane (HPTE), a metabolite of the legacy pesticide methoxychlor, has demonstrated impact on ER signaling *in vivo* (Hewitt and Korach, 2011) and has greater potency than BPA with an ACC_5 an order of magnitude lower. Therefore, it is probable that legacy pollutants are present and contributing toward estrogenic response in the marine environment.

3.3.5 Classification of Sample Locations Using Bile Data

A total of 18 English sole bile sampling locations were assessed (Fig. 8), with 12 sites classified as low potential sites, indicating a low likelihood for estrogenic activity in the sampled area. Six sites were classified as medium potential sites, including Commencement Bay (Hylebos Waterway and Blair Waterway), Vendovi Island, Anderson Island, Port Susan, and Strait of Georgia. Two sites were classified as high potential sites, Seattle Waterfront and Carr Inlet. Seattle Waterfront in Elliott Bay is part of the most highly-developed urbanized shoreline in the Puget Sound watershed. Carr Inlet is situated in a low-development area, characterized by minimal impervious surfaces and the absence of known point sources. A higher median EAR_{mix} value suggests that these locations are more likely to experience estrogen signaling disruptions and related consequences, and altered reproductive timing has been consistently observed at this site likely resulting from exposure to e-EDCs (Johnson et al., 2008; O'Neill et al., 2016).

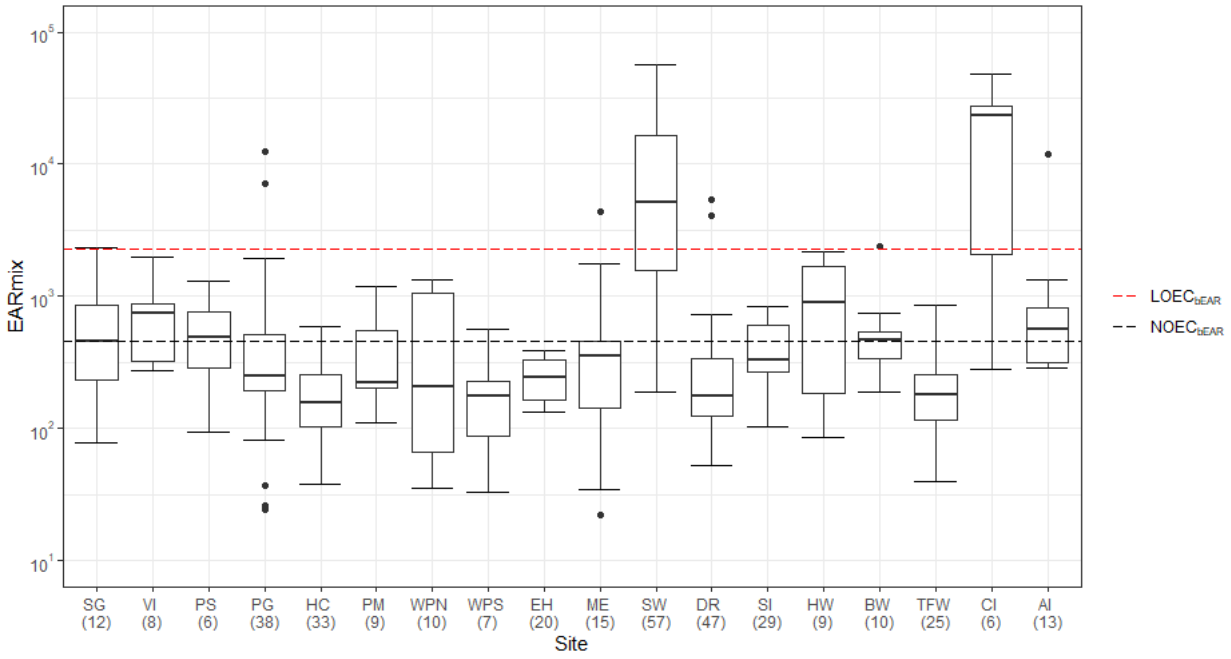

Fig. 8. Distribution of EAR_{mix} values for each site. Box and whisker plot shows the median, first/third quartile, and a distance of 1.5 times the interquartile range Data points outside the box and whisker plot are outliers. Site abbreviations are included in Fig. 1. Number in parentheses in axis label represent the total samples collected at that site.

3.3.5.1 Validation of Screening Values with Field-Based Data

Bile screening values derived from field observations resulted in an unimpacted UCL of 742 and an impacted LCL of 2931. The unimpacted UCL is within a factor of 1.6 from the $NOEC_{bEAR}$, and the impacted LCL is within a factor of 1.3 from the $LOEC_{bEAR}$ (Fig. 9). The value of using field-based measurements is that it allows for use of multiple individuals to understand baseline conditions and account for endogenous levels of estrogens present in male English sole. It also allows for previous knowledge of the sites to be included in the form of best professional judgement. By considering two distinct threshold derivation methods as independent lines of supporting evidence, an increased level of confidence is achieved in the application of NOEC and LOEC-based screening values.

Site selection for the derivation of field-based screening values depended, in part, on Vtg concentrations in male English sole plasma. However, a notable finding in regional studies found no correlation between plasma Vtg and English sole bile concentrations of xenoestrogens (King County, n.d.; O'Neill et al., 2016). This observation aligns with a previous study by Scott et al. (2006), which demonstrated no significant relationship between plasma Vtg and plasma E2 in male flounder. One plausible explanation is the temporal discrepancy between the measurable presence of xenoestrogens in bile and the presence of Vtg in plasma. Xenoestrogens in the bile are short-lived, typically spanning a few days, and represent relatively short-term exposures (O'Neill et al., 2016). Conversely, Vtg response is slower; significant increase may occur up to a week following exposure and continue to rise for an additional week after exposure, contingent upon the magnitude of the exposure (Craft et al., 2004; Hemmer et al., 2002; Schmid et al., 2002). The temporal nature of fish bile samples further emphasizes the need to include data from multiple individuals at a particular site. Moreover, it is important to recognize that this approach relies heavily on Vtg induction as an endpoint of interest, which may not fully account for chronic exposures. Fish may be experiencing other unmeasured estrogenic effects, which highlights the need for further research that considers alternate endpoints.

NOECs and LOECs are commonly used in setting benchmarks for chemicals in the environment (Tanaka et al., 2018), but have recognized limitations (Landis and Chapman, 2011; Murado and Prieto, 2013; Warne and van Dam, 2008). For one, NOECs and LOECs are equated to the specific concentrations used in toxicity tests and therefore dependent on test design as different test concentrations may yield different results (Tanaka et al., 2018). Despite the potential variability associated with NOEC and LOEC generation methods, their accessibility

and application in similar work (e.g., Baldwin et al., 2016; Jobling et al., 2006) make them a practical choice for deriving screening values.

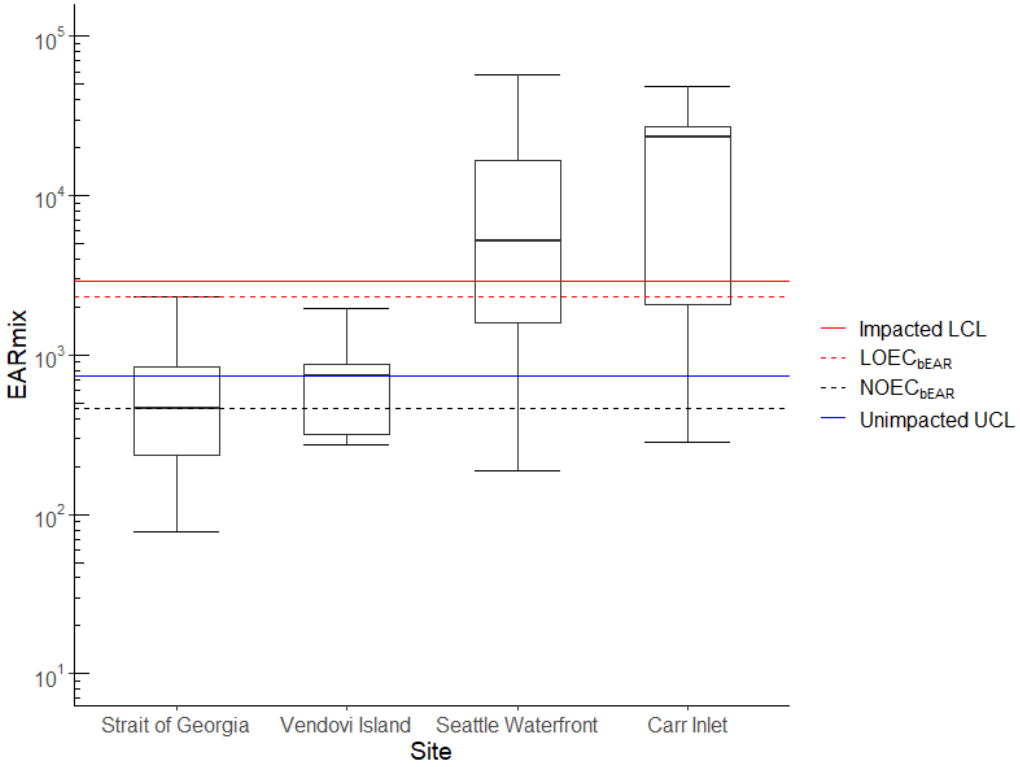

Fig. 9. Distribution of EARmix for samples collected at unimpacted sites (Strait of Georgia and Vendovi Island) and to impacted sites (Carr Inlet and Seattle Waterfront). Box and whisker plot shows the median, first/third quartile, and a distance of 1.5 times the interquartile range. Screening values based on NOEC and LOEC values are represented by dotted lines and screening values based on field data are represented by solid lines.

3.3.6 Utility and Implications of this Work

As described above, one main objective of this study was to expand the use and application of NAMs to a broader range of biological tissues, and to validate that application using field data and observations from a complex, urbanized estuary. Developing and validating screening values for a range of tissue types is important because exogenous chemicals are often unequally distributed throughout an organism and so a specific tissue might provide the opportunity to characterize exposures to CECs that would not otherwise be observed in whole

bodies (Lv et al., 2019). The total concentration (i.e., free and conjugated) of exogenous estrogens in fish bile has been shown to be a good way to characterize recent environmental exposures (Leppänen et al., 1998; Yang et al., 2014). This, combined with the broad information potential of NAMs (e.g., there are currently many large *in vitro* testing programs and the number will likely grow as regulatory preference turns away from traditional whole body testing) provides a clear justification for developing such an approach. However, there were not yet validated screening values that could be used to provide biological context to the results of a chemical analysis.

The validation exercise described above was performed based on traditional measures of toxicity (i.e., NOECs and LOECs; used as benchmarks for endocrine disruption; Baldwin et al., 2016), as well as biological observations of two important measures of exposure to estrogenic chemicals (i.e., Vtg production and altered reproductive timing). That there was agreement between the approaches, with comparable screening values (Figure 9), suggests that it is possible to establish meaningful biological thresholds for endocrine disruption with measures of e-EDCs in bile and demonstrates that measurements of e-EDCs in tissues is a valid means by which to measure the potential impacts of CEC exposures on marine species.

There are several points that should be considered when evaluating and applying such an approach. The first is that we currently have incomplete data by which to fully evaluate the extent of exposure to endocrine disrupting chemicals. For example, PCBs and hydroxylated-PAHs, which have been documented in tissue samples from the region (West et al., 2017) and have been shown to weakly induce vitellogenesis (Andersson et al., 1999; Nomiyama et al., 2010), were not included in this evaluation. In this exercise, we focused on e-EDCs that were

both measured in bile, and had relevant *in vitro* information in the ToxCast system, which does not capture the entire exposome.

A second point that may be illustrated by this case study is that land use profiles near the sampling sites are not always sufficient to inform potential chemical exposures and biological impacts. System hydrodynamics can also affect exposures, and the potential for exposure associated with sources such as WWTP outfalls. To illustrate, there are approximately 99 WWTP outfalls that enter Puget Sound with flow distribution roughly proportional to the population distribution. However, due to the complex geometry and bathymetry, the WWTP effluent distribution is not well aligned with population (James et al., 2020) but heavily influenced by factors such as stratification and the hydraulic residence time in embayments. As such, WWTP-associated contaminants such as E1 and E2 may be present at relatively high concentrations in deep embayments that otherwise receive little direct wastewater inputs via WWTP outfalls. Carr Inlet, one of the two impacted sites, may be illustrative of this although this site may also receive non-point source wastewater via on-site sewage systems (Guyader et al., 2018).

This case study demonstrates the importance of natural estrogens (E1, E2, and E3) as endocrine disruptors to aquatic resources. The presence of these chemicals in the environment is often sufficient to lead to measurable biological impacts. Wastewater is typically the primary pathway by which these natural estrogens enter the aquatic environment and so alterations to WWTP (Liu et al., 2015) and on-site sewage systems (Guyader et al., 2018) operation and infrastructure may be one approach to reduce their environmental impacts.

3.4 Conclusion

In this study, we investigated the estrogenic potential of mixtures of e-EDCs in Puget Sound. By employing a novel methodology that incorporates data from the ToxCast *in vitro* HTS database, we expanded the assessment of estrogenic activity to chemicals beyond the scope of traditional water quality benchmarks. Out of the 286 CECs analyzed for and detected in Puget Sound, 19 were identified as estrogen receptor agonists. Notably, E1, E2, and E3 were categorized as *High Priority* chemicals due to their ability to drive estrogenic response, in addition to BPA because of its substantial contribution to overall estrogenic response. By incorporating bile data in our analysis, we strengthen the prior prioritization of E1 and E2 as *High Priority* chemicals, previously based on their detections in WWTP effluent alone, and add E3, which was detected exclusively in bile. Bile serves as a robust matrix that provides biological evidence of exposure to estrogenic compounds. Considering the transformation of E1, E2, and E3 in the environment and within organisms, we recommend managing these steroidal estrogens collectively to effectively address their impacts. Furthermore, the findings of this study indicate that a small number of chemicals are responsible for the majority of the combined estrogenic response within environmental mixtures.

The integration of data from multiple matrices that were not synoptically collected, facilitated by our developed methodology, represents an advancement in this field. Moreover, we provided EAR-based estrogenic screening values for water and fish bile mixtures, which had not been established previously. We also successfully demonstrated the utilization of bile data, which included the validation of the NOEC and LOEC based screening values using field observations such as vitellogenin induction and altered reproductive timing. The results of our

study not only strengthened the previous prioritization efforts but also improved understanding of chemicals related to a specific mode of action, estrogen agonism.

Identifying priority chemicals and sites provides valuable insights for informing subsequent investigations and effective environmental management decisions. The methodology developed in Puget Sound can be applied to understand the impacts of mixtures in other ecosystems and regions. Additionally, this approach can be further adapted to investigate the impacts of mixtures with different modes of action, thus broadening its utility.

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4.0 Synthesis and Recommendations for Future Work

4.1 Summary of Findings

The presence of CECs in Puget Sound, stemming from everyday human activities, raises concerns regarding potential risks to aquatic wildlife. This study has made significant contributions to our understanding of CEC occurrence and the impacts of CECs on the marine environment. Through screening-level evaluations, this work allows us to better gauge the potential for biological response associated with CEC exposures measured in the environment. The improved understanding of CECs can help focus future monitoring and environmental management and recovery activities to minimize impacts.

This study included the development and application of a method for utilizing multiple lines of evidence to screen and prioritize CECs in the marine environment. This included the use of readily-available, traditional ecotoxicological data in combination with NAMs to maximize the use of available data and provide a measure of certainty of outcomes. Environmental monitoring data was compiled from 17 regional studies and used to identify *High Priority*, *Watch List*, and *Low Priority* compounds. The 57 compounds identified as *High Priority* are those which were in environmental samples and exceeded thresholds for expected biological response. These should be the focus of future monitoring and management campaigns. To our knowledge, this is the first application of the use of *in vitro* effects information with tissue monitoring data to identify priority CECs in marine systems.

The mixtures work resulted in the successful utilization of fish bile data in the screening of environmental contaminants; some CECs are preferentially sequestered in bile and so it is a valuable tissue type for environmental monitoring. This work represents a significant milestone

as it is the first to employ fish bile for screening and provides insights in how to interpret bile chemistry. The bile data were used to specifically understand the potential impacts of mixtures of e-EDCs through the application of NAMs. The use of the field data to validate screening values, combined with the use of NAMs is an important advancement. It also demonstrated that mainly natural exogenous estrogens, but also some industrial chemicals (such as BPA), in mixtures can impact estrogen receptor signaling with potential implications for fish reproduction. We advocate for the prioritization of these chemicals by environmental managers.

4.2 Recommendations for Future Work

This research constitutes the first CEC prioritization study of a large marine ecosystem, harnessing both *in vivo* and *in vitro* HTS data to identify CECs with the potential to impact aquatic organisms. The intent of this work is to serve as a guiding tool for future research efforts which can expand upon these findings. By focusing monitoring efforts in areas flagged for high potential CEC impacts, we can pinpoint sources and source areas for cleanup and management. For instance, bile data evaluation identified certain areas, such as Carr Inlet and Elliott Bay, as having substantial potential for CEC impacts. These sites could benefit from further source investigation through targeted sampling campaigns.

This research also results in priority lists of CECs with potential effects on individual organisms. Lists were informed by *in vitro* HTS data which are conducted at the cellular and subcellular level using primarily mammalian cells and cell components. To gain a more comprehensive understanding of the *in vivo* effects of priority CECs on key aquatic species, additional biological monitoring can be employed to validate *in vitro* HTS data. Observations of changes to key biomarkers, like Vtg induction, combined with experimental work focused on apical effects, can provide insights on the impacts of CEC exposures.

To expand the work from a focus on individual organisms, future research should also explore how CEC exposures can impact populations, which is vital for effective environmental management. Future studies can concentrate on developing and implementing models to better grasp the population-level impacts of CECs on important marine species. Beyond informing future research, this study also presents a methodology and framework that can serve as a blueprint for similar efforts in other regions and watersheds impacted by CECs.

List of Acronyms and Abbreviations[1]

Acronym	Definition
AC50	half-maximal activity concentration
ACC	activity concentration at cutoff
AOP	adverse outcome pathway
AUC	area-under-the-curve
BPA	bisphenol A
BPAF	bisphenol AF
BPF	bisphenol F
BPS	bisphenol S
BRR	biological response ratio
CEC	contaminants of emerging concern
CERAPP	Collaborative Estrogen Receptor Activity Prediction Project
CR	cumulative ratio
CWA	The U.S. Clean Water Act
DES	Diethylstilbestrol
EAR	exposure-activity ratio: the quotient of the measured concentration of a chemical in a sample and the activity concentration at cut-off (ACC)[2]
EAR_{AOP}	exposure-activity ratio by adverse outcome pathway: the sum of EAR values for each assay-chemical combination associated with an individual AOP[2]
EAR_{Chem}	exposure-activity ratio by chemical; the sum of EAR values from all assays for an individual chemical[2]
$EAR_{Mixture}$	exposure-activity ratio by mixture: the sum of EAR values for each individual assay endpoint across all chemicals for a given sample[2]
EAR_{Mix}	exposure-activity ratio by estrogenic mixture: the quotient of the measured concentration of a chemical in a sample and the 5^{th} percentile of ACCs for estrogenic assays summed across all chemicals in a given sample
E1	estrone
E2	17β-estradiol
αE2	17α-estradiol

E3	estriol
EDCs	endocrine disrupting chemicals
e-EDCs	estrogenic endocrine disrupting chemicals
EE2	17α-ethynylestradiol
EEF	estradiol equivalency factor
EEQ	estradiol equivalency quotient
ER	estrogen receptor
ER-CALUX	ER-mediated chemically activated luciferase gene expression
ESR1(2)	estrogen receptor 1(2)
HC_5	fifth percentile hazard concentration
HPTE	2-bis(p-Hydroxyphenyl)-1,1,1-trichloroethane
HRMS	high resolution mass spectrometry
HTS	high throughput screening
LCL	lower confidence limit
LOEC	lowest observed effect concentration
LOQ	limit of quantification
NAM	new approach methodology
NOEC	no observed effect concentration
NP	4-nonylphenol
NRC	National Research Council
OP	octylphenol
PAHs	polycyclic aromatic hydrocarbons
PFOSA	perfluorooctanesulfonamide
PNEC	predicted no effects concentration
POD	point of departure
POPs	persistent organic pollutants
QSAR	quantitative structure-active relationship
SSD	sensitive species distributions
SV	screening values
TQ	toxicity quotient
UCL	upper confidence limit

Vtg	vitellogenin
WDFW	Washington Department of Fish and Wildlife
WWTP	wastewater treatment plant
YES	yeast estrogen screen

[1]List only includes acronyms included in Chapter 1 and Chapter 3

[2] As defined in Corsi et al. (2019)

Appendix A: Analysis

A1.1. Data QAQC Review Notes

A1.2 ER Agonist Manual Evaluation

A1.1 Data QAQC Review Notes

Data were obtained from published reports, publications in the peer-reviewed literature, and analytical laboratory reports. In all cases, measured concentrations (not averages or estimates) for individual samples were used and data reporting was accompanied by laboratory quality assurance information, and sample information such as date and location of sample collection, sample matrix, species (as applicable), sample mass or volume, whether a sample was and individual or composite, and number of individuals in a composite (as appropriate). All data were compiled into a single spreadsheet and reviewed for quality assurance prior to use.

Data quality assurance included a review of the laboratory flags, which are notations provided by analytical laboratories to provide the user information necessary for use evaluation. Laboratory flags and the use interpretation are shown below.

Flags	Description	Included (Y/N)
D	Dilution data	Y
J	Concentration less than LMCL	Y
B	Found in sample blank	Y (with blank corrections as indicated below)
N	Recovery not within limits	Y (after review)
V	Surrogate recovery not within limits	Y (after review)
G	Lock max interference	Y
NA*		N
NQ*	Data not quantifiable	N
OLR*	Outside linear calibration range	N
H	Estimate	Y
Max*	Concentration is estimated maximum value	N
U*	Not detected	N
K*	Peak detected but did not meet quantification criteria, result reported represents the estimated maximum possible concentration	N

Based on our data review, there were some compounds that were detected in the laboratory blanks (which are also known more generally as “control samples”). Blanks are essentially clean samples (either pure water from the laboratory pure water system, or vegetable oil that has been certified to be clean by a test lab) that are run through the same processes as the field samples that provide information on systematic laboratory contamination. The presence of a compound in

the blanks creates uncertainty as to whether a detection in a field sample is "real" or just a result of lab processing. In order to ensure that detections in field samples are "real," we did the following: 1) compiled analytical results for all blanks, 2) for those compounds present in three or more blanks, we estimated a distribution of concentrations using mean and standard deviation, 3) we calculated the 95th percentile confidence interval of the concentration in the blanks, and 4) adjusted the analyte reporting limit to be the upper 95th percentile of the distribution. If a field sample concentration was above the upper 95th percentile concentration for the blanks, we deemed that a "real" occurrence. If a field sample concentration was below the upper 95th percentile concentration for the blanks, we deemed that a non detect.

A1.2 ER Agonist Manual Evaluation

Twenty chemicals without available AUC scores were manually evaluated to predict interaction with the ER, with a positive call indicating likely interaction and a negative call indicating unlikely interaction. The following considerations were taken into account to make a putative call:

Description	Putative Call
Collaborative Estrogen Receptor Activity Prediction Project (CERAPP) is positive	Positive
Only Attagene (ATG) ER assays were active, no positive CERAPP	Negative
Activity in assays outside of the ATG platform and the T-value for chemical-assay response is <50, and/or response is associated with excluded lab flags*	Negative
Activity in assays outside of the ATG platform and the T-value is >50, and the response is not associated with excluded lab flags	Positive

*Excluded lab flags as applied in Corsi et al. (2019):

Flag	Description
Borderline Active	Assay is active with borderline activity
Only highest conc above baseline, active	Single point hit with activity only at the highest concentration tested
Gain AC50 < lowest conc & loss AC50 < mean conc	Gain-loss model won, though the gain concentration at half-maximal activity (AC50) is less than the minimum tested concentration, and the loss AC50 is less than the mean tested concentration
AC50 less than lowest concentration tested	Activity concentration at half-maximal activity is less than the lowest concentration tested

Appendix B: Tables and Figures

Tables

Figures

Table B1. EAR and EEQ correlation data.

Chemical Name	CAS	Median EEF	ACC5	Concentration (ug/L)	EEQ	EAR(ACC5)
4-Nonylphenol monoethoxylate (NP1EO)	104-35-8	0.0000038	489.92	100.00	0.00038	0.20
4-Nonylphenol	104-40-5	0.00023	29.39	100.00	0.02	3.40
Estriol	50-27-1	0.05	0.07	100.00	4.50	1469.39
17beta-Estradiol	50-28-2	1.00	0.05	100.00	100.00	1989.36
Estrone	53-16-7	0.05	0.07	100.00	5.00	1430.21
Diethylstilbestrol	56-53-1	2.60	0.02	100.00	260.00	6243.04
17alpha-Ethinylestradiol	57-63-6	1.20	0.02	100.00	119.50	4721.33
Bisphenol A	80-05-7	0.000036	9.30	100.00	0.00	10.76

Table B2. ER agonist determinations for chemicals detected in Puget Sound with activity in ToxCast ER agonist assays.

Chemical Name	CAS	AUC	CERAPP	Putative Call	Supporting evidence
Trenbolone acetate	10161-34-9	-	Active	Positive	Active in multiple assay platforms and CERAPP positive
4-Nonylphenol	104-40-5	0.088	Active	Positive	AUC > 0.01
Phenol	108-95-2	0	Negative	Negative	AUC <0.01; CERAPP negative
Di(2-ethylhexyl) phthalate	117-81-7	0	Negative	Negative	AUC <0.01; CERAPP negative
Vanillin	121-33-5	0	Active	Negative	AUC < 0.01; AUC trumps CERAPP
Triisopropanolamine	122-20-3	0	Negative	Negative	AUC <0.01; CERAPP negative
Atorvastatin	134523-00-5	-	Negative	Negative	Only active in ATG platform and CERAPP negative
4-tert-Octylphenol	140-66-9	0.393	Active	Positive	AUC > 0.01
Oxolinic Acid	14698-29-4	-	Negative	Negative	Active in 1 assay (not ATG platform), T-value <50; CERAPP negative
Bisphenol AF	1478-61-1	0.552	Active	Positive	AUC > 0.01
Thiabendazole	148-79-8	0	Negative	Negative	AUC <0.01; CERAPP negative
2-Mercaptobenzothiazole	149-30-4	0	Negative	Negative	AUC <0.01; CERAPP negative
Ibuprofen	15687-27-1	-	Negative	Negative	Active in 1 assay (besides ATG), Tvalue is <50 and CERAPP negative
Perfluorooctanesulfonic acid (PFOS)	1763-23-1	0.000259	Negative	Negative	AUC <0.01; CERAPP negative
4-n-Octylphenol	1806-26-4	0.118	Active	Positive	AUC > 0.01
Dacthal	1861-32-1	0	Negative	Negative	AUC <0.01; CERAPP negative
2,6-Dichlorobenzamide	2008-58-4	-	Negative	Negative	Active in 1 assay, Tvalue < 50; CERAPP negative
Perfluoroundecanoic acid (PFUnA)	2058-94-8	0.00337	Negative	Negative	AUC <0.01; CERAPP negative

Clotrimazole	23593-75-1	0.000434	Negative	Negative	AUC <0.01; CERAPP negative
Propamocarb	24579-73-5	-	Negative	Negative	Active in 1 assay, Tvalue >50 but assocaited with excluded lab flag "only highest concentration above baseline active", CERAPP negative
Gemfibrozil	25812-30-0	0	Negative	Negative	AUC <0.01; CERAPP negative
Atenolol	29122-68-7	0	Negative	Negative	AUC <0.01; CERAPP negative
Carbamazepine	298-46-4	0	Negative	Negative	AUC <0.01; CERAPP negative
Hexa(methoxymethyl)melamine	3089-11-0	-	Negative	Negative	Only active in ATG platform and CERAPP negative
N-Methylperfluorooctanesulfonamide (MeFOSA)	31506-32-8	-	Negative	Negative	Only active in ATG platform and CERAPP negative
2,4,6-Tribromophenylallyl ether	3278-89-5	-	Negative	Negative	Only active in ATG platform and CERAPP negative
Perfluorooctanoic acid (PFOA)	335-67-1	0	Negative	Negative	AUC <0.01; CERAPP negative
Perfluorodecanoic acid (PFDA)	335-76-2	0.0000142	Negative	Negative	AUC <0.01; CERAPP negative
Triclosan	3380-34-5	0	Negative	Negative	AUC <0.01; CERAPP negative
Tebuthiuron	34014-18-1	0	Negative	Negative	AUC <0.01; CERAPP negative
Perfluorohexanesulfonic acid (PFHxS)	355-46-4	-	Negative	Negative	Only active in ATG platform and CERAPP negative
Perfluorobutanoic acid (PFBA)	375-22-4	-	Negative	Negative	Only active in ATG platform and CERAPP negative
Perfluoroheptanoic acid (PFHpA)	375-85-9	0	Negative	Negative	AUC <0.01; CERAPP negative
Perfluorononanoic acid (PFNA)	375-95-1	0.00751	Negative	Negative	AUC <0.01; CERAPP negative
Betamethasone	378-44-9	-	Active	Positive	Active in multiple assay platforms and CERAPP positive
Triamterene	396-01-0	-	Negative	Negative	Active in more than ATG assay, Tvalue >50, CERAPP negative; Assay activity falls within the cytotoxicity region
Hydrocortisone	50-23-7	-	Active	Negative	Glucocorticoid agonist, shows activity 2 orders of magnitude below ER activity, which is inconsistent
Estriol	50-27-1	0.786	Active	Positive	AUC > 0.01
Estradiol	50-28-2	0.935	Active	Positive	AUC > 0.01
2,4-Dinitrophenol	51-28-5	0	Negative	Negative	AUC <0.01; CERAPP negative
Permethrin	52645-53-1	0	Negative	Negative	AUC <0.01; CERAPP negative
Prednisone	53-03-2	0.0991	Active	Positive	AUC > 0.01
Estrone	53-16-7	0.807	Active	Positive	AUC > 0.01
Androsterone	53-41-8	-	Active	Positive	Possible ER activity, main target is AR though included to be conservative; CERAPP is positive
Desogestrel	54024-22-5	-	Active	Positive	Possible ER activity, main target is progesterone receptor though included to be conservative; CERAPP is positive
17a-Estradiol	57-91-0	1.06	Active	Positive	AUC > 0.01
Caffeine	58-08-2	0	Negative	Negative	AUC <0.01; CERAPP negative

Iopamidol	60166-93-0	-	Negative	Negative	Active in 1 assay, Tvalue <50, associated with multiple excluded lab flags; inactive CERAPP
Tetracycline [TC]	60-54-8	0	Negative	Negative	AUC <0.01; CERAPP negative
Desethylatrazine	6190-65-4	0	Negative	Negative	AUC <0.01; CERAPP negative
Bisphenol F	620-92-8	0.0303	Active	Positive	AUC > 0.01
Colchicine	64-86-8	0	Negative	Negative	AUC <0.01; CERAPP negative
Benzoic Acid	65-85-0	0	Negative	Negative	AUC <0.01; CERAPP negative
Ranitidine	66357-35-5	0	Negative	Negative	AUC <0.01; CERAPP negative
Perfluorooctanesulfonamide (PFOSA)	754-91-6	0.024	Negative	Positive	AUC > 0.01
Triethyl citrate	77-93-0	0.0000604	Negative	Negative	AUC <0.01; CERAPP negative
Tris(2-butoxyethyl) phosphate	78-51-3	0.000118	Negative	Negative	AUC <0.01; CERAPP negative
Tetrabromobisphenol A	79-94-7	0	Negative	Negative	AUC <0.01; CERAPP negative
Bisphenol A	80-05-7	0.45	Active	Positive	AUC > 0.01
Bisphenol S	80-09-1	0.263	Active	Positive	AUC > 0.01
Roxithromycin	80214-83-1	-	Negative	Negative	Only active in ATG platform and CERAPP negative
Sulfachloropyridazine	80-32-0	-	Negative	Negative	Active in 1 assay (not ATG platform), T-value <50; CERAPP negative
2H, 2H, 3H, 3H-perfluorodecanoic acid	812-70-4	-	-	Negative	Only active in ATG platform and assocaited with excluded lab flag; "only highest conc above baseline, active"; CERAPP negative
Dibutyl phthalate	84-74-2	0.0265	Negative	Positive	AUC > 0.01
Butyl benzyl phthalate	85-68-7	0.179	Negative	Positive	AUC > 0.01
2,4,6-Trichlorophenol	88-06-2	0	Negative	Negative	AUC <0.01; CERAPP negative
1,2,3-Benzotriazole	95-14-7	0	Negative	Negative	AUC <0.01; CERAPP negative
1,2-Dichlorobenzene	95-50-1	0.00153	Negative	Negative	AUC <0.01; CERAPP negative

Table B3. Median Chemical Concentration per Chemical per Species.

CEC Name	CEC Use Categor	CAS	Bay mussel whole	Brown rockfish whole	English sole bile	English sole muscle	juvenile Chinook salmon whole	juvenile Chinook salmon WholeMinusGutsBrains	juvenile Chinook salmon WholeMinusGutsGills	juvenile Chinook salmon WholeMinusOrgs	Pacific herring whole	Pacific sand lance whole	Pacific staghorn sculpin whole	Quillback rockfish whole	resident Chinook salmon muscle	Smallmouth bass whole
4-Nonylphenol	Commercial	104-40-5	19.5	6.005		14	41.75	23.825	34.25	18.4	12.4		29.3	7.61	13.5	6.34
4-tert-Octylphenol	Commercial	140-66-9			57.000											
Bisphenol AF	Industrial	1478-61-1			0.760											
4-n-Octylphenol	Commercial	1806-26-4	0.846	0.635					0.6565	0.972						
Betamethasone	Hormones	378-44-9									3.580					
Estriol	Hormones	50-27-1			14.000											
Estradiol	Hormones	50-28-2			14.000											
Prednisone	Hormones	53-03-2									26.900					
Estrone	Hormones	53-16-7			12.000											
Bisphenol F	Industrial	620-92-8			8.300											
Perfluorooctanesulfonamide (PFOSA)	PFAS	754-91-6	0.618					0.452	0.6315	0.178	0.784		1.51		0.668	
Bisphenol A	Industrial	80-05-7	3.74		19		5.5			2.965			4.035			
Bisphenol S	Industrial	80-09-1			1.200											

Conditional Formatting applied to each chemical row

Color Scale	Percentile	
	0	
	10	
	20	
	30	
	40	
	50	Or if single value
	60	
	70	
	80	
	90	
	100	

Table B4. Number of total unique chemicals and unique e-EDCs in each sample.

SAMPLE_ID	Matrix	Species	Tissue Type	Detected CAS per sample	ER - Detected CAS per sample
11CB-ESB01	Bile	English sole	bile	1	1
11CB-ESB02	Bile	English sole	bile	2	2
11CB-ESB5841	Bile	English sole	bile	3	3
11CB-ESB5842	Bile	English sole	bile	3	3
11CB-ESB5856	Bile	English sole	bile	3	3
11CB-ESB5864	Bile	English sole	bile	5	4
11CB-ESB5874	Bile	English sole	bile	3	3
11CB-ESB5876	Bile	English sole	bile	3	3
11DU-ESB01	Bile	English sole	bile	1	1
11DU-ESB02	Bile	English sole	bile	1	1
11DU-ESB5644	Bile	English sole	bile	3	3
11DU-ESB5653	Bile	English sole	bile	3	3
11DU-ESB5657	Bile	English sole	bile	3	3
11EB-ESB5481	Bile	English sole	bile	5	5
11EB-ESB5482	Bile	English sole	bile	1	1
11EB-ESB5484	Bile	English sole	bile	5	5
11EB-ESB5485	Bile	English sole	bile	4	4
11EB-ESB5489	Bile	English sole	bile	4	4
11EB-ESB5491	Bile	English sole	bile	5	5
11EB-ESB5494	Bile	English sole	bile	4	4
11EB-ESB5497	Bile	English sole	bile	5	5
11EB-ESB5500	Bile	English sole	bile	6	5
11EB-ESB5501	Bile	English sole	bile	5	5
11EH-ESB02	Bile	English sole	bile	1	1
11EH-ESB03	Bile	English sole	bile	1	1
11EH-ESB6098	Bile	English sole	bile	3	3
11HC-ESB5242	Bile	English sole	bile	2	2
11HC-ESB5260	Bile	English sole	bile	2	2

11HC-ESB5268	Bile	English sole	bile	3	3
11HC-ESB5276	Bile	English sole	bile	2	2
11HC-ESB5277	Bile	English sole	bile	3	3
11HC-ESB5282	Bile	English sole	bile	2	2
11HC-ESB5288	Bile	English sole	bile	2	2
11HC-ESB5289	Bile	English sole	bile	2	2
11HC-ESB5296	Bile	English sole	bile	2	2
11NQ-ESB01	Bile	English sole	bile	2	2
11NQ-ESB6009	Bile	English sole	bile	3	3
11NQ-ESB6011	Bile	English sole	bile	6	5
11PG-ESB01	Bile	English sole	bile	1	1
11PG-ESB02	Bile	English sole	bile	1	1
11PG-ESB5380	Bile	English sole	bile	3	3
11PG-ESB5387	Bile	English sole	bile	3	3
11PG-ESB5398	Bile	English sole	bile	3	3
11PG-ESB5403	Bile	English sole	bile	3	3
11PG-ESB5417	Bile	English sole	bile	2	2
11PG-ESB5420	Bile	English sole	bile	5	4
11SG-ESB01	Bile	English sole	bile	1	1
11SG-ESB5055	Bile	English sole	bile	2	2
11SG-ESB5059	Bile	English sole	bile	2	2
11SG-ESB5060	Bile	English sole	bile	4	4
11SI-ESB5732	Bile	English sole	bile	3	3
11SI-ESB5739	Bile	English sole	bile	4	4
11SI-ESB5744	Bile	English sole	bile	3	3
11SI-ESB5774	Bile	English sole	bile	2	2
11VD-ESB01	Bile	English sole	bile	1	1
13CB-ESB01	Bile	English sole	bile	1	1
13CB-ESB3841	Bile	English sole	bile	2	2
13CB-ESB3842	Bile	English sole	bile	1	1
13CB-ESB3846	Bile	English sole	bile	1	1
13CB-ESB3860	Bile	English sole	bile	1	1
13CB-ESB3863	Bile	English sole	bile	1	1

13CPS_CBTF-MTW01	Mussel	Bay mussel	whole	7	2
13CPS_DM-MTW01	Mussel	Bay mussel	whole	8	2
13CPS_DP-MTW01	Mussel	Bay mussel	whole	9	2
13CPS_SB-MTW01	Mussel	Bay mussel	whole	6	3
13DUE01-TW04	Tissue	juvenile Chinook salmon	WholeMinusGutsGills	10	2
13DUE02-TW01	Tissue	juvenile Chinook salmon	WholeMinusGutsGills	15	1
13DU-ESB01	Bile	English sole	bile	1	1
13DU-ESB02	Bile	English sole	bile	2	1
13DU-ESB3602	Bile	English sole	bile	1	1
13DU-ESB3604	Bile	English sole	bile	3	3
13DU-ESB3605	Bile	English sole	bile	1	1
13DU-ESB3611	Bile	English sole	bile	1	1
13DU-ESB3612	Bile	English sole	bile	1	1
13DU-ESB3614	Bile	English sole	bile	1	1
13DU-ESB3616	Bile	English sole	bile	2	2
13DU-ESB3619	Bile	English sole	bile	5	4
13DU-ESB3627	Bile	English sole	bile	6	5
13DU-ESB3628	Bile	English sole	bile	4	3
13DU-ESB3630	Bile	English sole	bile	3	3
13DUR-TW04	Tissue	juvenile Chinook salmon	WholeMinusGutsGills	25	3
13EB_ME-MTW01	Mussel	Bay mussel	whole	8	2
13EB_P17-MTW01	Mussel	Bay mussel	whole	9	2
13EB-ESB01	Bile	English sole	bile	2	2
13EB-ESB3481	Bile	English sole	bile	6	5
13EB-ESB3483	Bile	English sole	bile	6	5
13EB-ESB3486	Bile	English sole	bile	5	5
13EB-ESB3487	Bile	English sole	bile	6	5
13EB-ESB3488	Bile	English sole	bile	6	5
13EB-ESB3489	Bile	English sole	bile	5	5

13EB-ESB3490	Bile	English sole	bile	6	5
13EB-ESB3491	Bile	English sole	bile	6	5
13EB-ESB3492	Bile	English sole	bile	2	2
13EB-ESB3494	Bile	English sole	bile	6	5
13EB-ESB3495	Bile	English sole	bile	5	5
13EB-ESB3496	Bile	English sole	bile	5	5
13EB-ESB3535	Bile	English sole	bile	2	2
13EB-ESB3539	Bile	English sole	bile	1	1
13EH-ESB9429	Bile	English sole	bile	1	1
13EH-ESB9431	Bile	English sole	bile	1	1
13EH-ESB9432	Bile	English sole	bile	3	3
13EH-ESB9433	Bile	English sole	bile	1	1
13EH-ESB9434	Bile	English sole	bile	1	1
13EH-ESB9437	Bile	English sole	bile	3	3
13EH-ESB9461	Bile	English sole	bile	3	3
13EH-ESB9464	Bile	English sole	bile	4	3
13EH-ESB9468	Bile	English sole	bile	2	2
13EH-ESB9475	Bile	English sole	bile	2	2
13HC_HO-MTW01	Mussel	Bay mussel	whole	5	2
13HC-ESB3244	Bile	English sole	bile	2	2
13HC-ESB3247	Bile	English sole	bile	3	2
13HC-ESB3253	Bile	English sole	bile	1	1
13HC-ESB3256	Bile	English sole	bile	2	2
13HC-ESB3258	Bile	English sole	bile	2	2
13HC-ESB3263	Bile	English sole	bile	2	2
13HYLE1-TW02	Tissue	juvenile Chinook salmon	WholeMinusGutsGills	16	1
13HYLE2-TW05	Tissue	juvenile Chinook salmon	WholeMinusGutsGills	14	1
13HYLR-TW01	Tissue	juvenile Chinook salmon	WholeMinusGutsGills	7	0
13NPS_BPP-MTW01	Mussel	Bay mussel	whole	8	2

13NPS_CPAR3-MTW01	Mussel	Bay mussel	whole	9	2
13NQE1-TW04	Tissue	juvenile Chinook salmon	WholeMinusGutsGills	9	1
13NQE2-TW02	Tissue	juvenile Chinook salmon	WholeMinusGutsGills	14	3
13NQ-ESB01	Bile	English sole	bile	1	1
13NQ-ESB9302	Bile	English sole	bile	1	1
13NQ-ESB9303	Bile	English sole	bile	2	2
13NQ-ESB9315	Bile	English sole	bile	1	1
13NQ-ESB9347	Bile	English sole	bile	3	3
13NQR-TW02	Tissue	juvenile Chinook salmon	WholeMinusGutsGills	9	1
13PG-ESB3363	Bile	English sole	bile	1	1
13PG-ESB3366	Bile	English sole	bile	6	5
13PG-ESB3389	Bile	English sole	bile	3	3
13PG-ESB3396	Bile	English sole	bile	2	2
13PG-ESB3398	Bile	English sole	bile	4	3
13PG-ESB3406	Bile	English sole	bile	2	2
13SG-ESB3003	Bile	English sole	bile	1	1
13SG-ESB3008	Bile	English sole	bile	1	1
13SG-ESB3052	Bile	English sole	bile	3	3
13SI-ESB3724	Bile	English sole	bile	1	1
13SI-ESB3726	Bile	English sole	bile	1	1
13SI-ESB3730	Bile	English sole	bile	1	1
13SI-ESB3731	Bile	English sole	bile	1	1
13SI-ESB3734	Bile	English sole	bile	4	3
13SI-ESB3735	Bile	English sole	bile	2	2
13SI-ESB3736	Bile	English sole	bile	1	1
13SI-ESB3738	Bile	English sole	bile	5	5
13SI-ESB3739	Bile	English sole	bile	1	1
13SI-ESB3741	Bile	English sole	bile	1	1
13SI-ESB3743	Bile	English sole	bile	4	4

13SI-ESB3746	Bile	English sole	bile	4	4
13SI-ESB3747	Bile	English sole	bile	5	5
13SI-ESB3748	Bile	English sole	bile	1	1
13SI-ESB3755	Bile	English sole	bile	4	4
13SJI_SJFH-MTW01	Mussel	Bay mussel	whole	5	2
13SKE01-TW02	Tissue	juvenile Chinook salmon	WholeMinusGutsGills	7	1
13SKES02-TW03	Tissue	juvenile Chinook salmon	WholeMinusGutsGills	11	3
13SKGR-TW01_04	Tissue	juvenile Chinook salmon	WholeMinusGutsGills	9	1
13SNME01-TW01	Tissue	juvenile Chinook salmon	WholeMinusGutsGills	9	2
13SNME02-TW04	Tissue	juvenile Chinook salmon	WholeMinusGutsGills	29	3
13SNMR-TW01_04	Tissue	juvenile Chinook salmon	WholeMinusGutsGills	6	0
13SPS_KSP-MTW01	Mussel	Bay mussel	whole	7	2
13SPS_OBI-MTW01	Mussel	Bay mussel	whole	14	2
13SPS_SBP-MTW01	Mussel	Bay mussel	whole	7	1
13VD-ESB3121	Bile	English sole	bile	2	1
13VD-ESB3127	Bile	English sole	bile	1	1
13VD-ESB3148	Bile	English sole	bile	3	3
13VD-ESB3152	Bile	English sole	bile	3	3
13WB_EF-MTW01	Mussel	Bay mussel	whole	9	1
13WB_EH-MTW01	Mussel	Bay mussel	whole	13	2
13WB_MBG-MTW01	Mussel	Bay mussel	whole	7	2
13WPS_BSCB-MTW01	Mussel	Bay mussel	whole	12	2
13WPS_BSF-MTW01	Mussel	Bay mussel	whole	9	2
15CB-ESB8467	Bile	English sole	bile	3	3
15CB-ESB8481	Bile	English sole	bile	3	3
15CB-ESB8482	Bile	English sole	bile	3	3

15CB-ESB8489	Bile	English sole	bile	3	3
15DU-ESB8133	Bile	English sole	bile	3	3
15DU-ESB8142	Bile	English sole	bile	3	3
15DU-ESB8143	Bile	English sole	bile	3	3
15DU-ESB8144	Bile	English sole	bile	3	3
15DU-ESB8145	Bile	English sole	bile	1	1
15DU-ESB8149	Bile	English sole	bile	3	3
15DU-ESB8157	Bile	English sole	bile	3	3
15DU-ESB8169	Bile	English sole	bile	3	3
15DU-ESB8177	Bile	English sole	bile	3	3
15DU-ESB8178	Bile	English sole	bile	3	3
15DU-ESB8180	Bile	English sole	bile	3	3
15DU-ESB8183	Bile	English sole	bile	3	3
15DU-ESB8184	Bile	English sole	bile	3	3
15DU-ESB8190	Bile	English sole	bile	3	3
15EB-ESB8011	Bile	English sole	bile	3	3
15EB-ESB8012	Bile	English sole	bile	4	4
15EB-ESB8036	Bile	English sole	bile	3	3
15EB-ESB8037	Bile	English sole	bile	5	5
15EB-ESB8039	Bile	English sole	bile	4	4
15EB-ESB8044	Bile	English sole	bile	4	4
15EB-ESB8047	Bile	English sole	bile	3	3
15HC-ESB7773	Bile	English sole	bile	3	3
15HC-ESB7777	Bile	English sole	bile	3	3
15HC-ESB7801	Bile	English sole	bile	3	3
15HC-ESB7817	Bile	English sole	bile	3	3
15HC-ESB7820	Bile	English sole	bile	3	3
15HC-ESB7823	Bile	English sole	bile	3	3
15HC-ESB7827	Bile	English sole	bile	3	3
15PG-ESB7915	Bile	English sole	bile	3	3
15PG-ESB7932	Bile	English sole	bile	3	3
15PG-ESB7936	Bile	English sole	bile	3	3
15SG-ESB7541	Bile	English sole	bile	3	3

15SG-ESB7571	Bile	English sole	bile	3	3
15SI-ESB8311	Bile	English sole	bile	3	3
15SI-ESB8322	Bile	English sole	bile	3	3
15SI-ESB8325	Bile	English sole	bile	3	3
15SI-ESB8331	Bile	English sole	bile	3	3
15SI-ESB8332	Bile	English sole	bile	3	3
15SI-ESB8342	Bile	English sole	bile	3	3
15SI-ESB8355	Bile	English sole	bile	1	1
15SI-ESB8365	Bile	English sole	bile	3	3
15VD-ESB7687	Bile	English sole	bile	3	3
16DK-TW_Dp01r5	Tissue	juvenile Chinook salmon	WholeMinusGutsBrains	8	1
16DN-TW_Dp02r4	Tissue	juvenile Chinook salmon	WholeMinusGutsBrains	3	0
16LKW-TW_Dp01r8	Tissue	juvenile Chinook salmon	WholeMinusGutsBrains	21	2
16MA6-TM9831	Tissue	resident Chinook salmon	muscle	6	1
16MA6-TM9835	Tissue	resident Chinook salmon	muscle	5	1
16MA6-TM9839	Tissue	resident Chinook salmon	muscle	5	1
16MA8_2-TM9844	Tissue	resident Chinook salmon	muscle	2	0
16ST-TW_Dfp01r2	Tissue	juvenile Chinook salmon	WholeMinusGutsBrains	7	0
16ST-TW_Dfp02	Tissue	juvenile Chinook salmon	WholeMinusGutsBrains	8	0
16ST-TW_Dfp03	Tissue	juvenile Chinook salmon	WholeMinusGutsBrains	7	0
16ST-TW_Dfp04r1	Tissue	juvenile Chinook salmon	WholeMinusGutsBrains	6	0

16ST-TW_Dfp04r2	Tissue	juvenile Chinook salmon	WholeMinusGutsBrains	5	0
16ST-TW_Hp01	Tissue	juvenile Chinook salmon	WholeMinusGutsBrains	9	0
16ST-TW_Nfp01	Tissue	juvenile Chinook salmon	WholeMinusGutsBrains	8	0
16ST-TW_Nfp02	Tissue	juvenile Chinook salmon	WholeMinusGutsBrains	5	0
16ST-TW_Nfp03	Tissue	juvenile Chinook salmon	WholeMinusGutsBrains	4	0
16ST-TW_Nfp04	Tissue	juvenile Chinook salmon	WholeMinusGutsBrains	10	0
16ST-TW_Rfp01	Tissue	juvenile Chinook salmon	WholeMinusGutsBrains	8	0
16ST-TW_Tp01	Tissue	juvenile Chinook salmon	WholeMinusGutsBrains	10	1
17CB-ESB0122	Bile	English sole	bile	3	3
17CB-ESB0124	Bile	English sole	bile	5	5
17CB-ESB0126	Bile	English sole	bile	2	2
17CB-ESB0130	Bile	English sole	bile	4	4
17CB-ESB0134	Bile	English sole	bile	4	4
17CB-ESB0137	Bile	English sole	bile	4	4
17CB-ESB0149	Bile	English sole	bile	4	4
17CB-ESM01	Tissue	English sole	muscle	11	1
17CB-ESM02	Tissue	English sole	muscle	2	0
17DU-ESB0482	Bile	English sole	bile	4	4
17DU-ESB0499	Bile	English sole	bile	5	5
17DU-ESB0500	Bile	English sole	bile	4	4
17DU-ESB0502	Bile	English sole	bile	4	4
17DU-ESB0506	Bile	English sole	bile	4	4
17DU-ESB0512	Bile	English sole	bile	4	4
17DU-ESB0521	Bile	English sole	bile	1	1
17DU-ESB0526	Bile	English sole	bile	4	4

17DU-ESB0537	Bile	English sole	bile	3	3
17DU-ESM01	Tissue	English sole	muscle	5	1
17EB4-ESB1563	Bile	English sole	bile	3	3
17EB4-ESB1567	Bile	English sole	bile	1	1
17EB4-ESB1573	Bile	English sole	bile	2	2
17EB4-ESB1579	Bile	English sole	bile	1	1
17EB4-ESB1580	Bile	English sole	bile	2	2
17EB4-ESB1581	Bile	English sole	bile	3	3
17EB4-ESB1582	Bile	English sole	bile	3	3
17EB4-ESB1583	Bile	English sole	bile	1	1
17EB4-ESB1588	Bile	English sole	bile	1	1
17EB4-ESB1589	Bile	English sole	bile	1	1
17EB4-ESB1592	Bile	English sole	bile	3	3
17EB4-ESB1604	Bile	English sole	bile	6	5
17EB4-ESB1605	Bile	English sole	bile	3	3
17EB4-ESB1610	Bile	English sole	bile	6	5
17EB4-ESB1612	Bile	English sole	bile	3	3
17EB4-ESM01	Tissue	English sole	muscle	6	1
17EB-ESB0614	Bile	English sole	bile	3	3
17EB-ESB0623	Bile	English sole	bile	3	3
17EB-ESB0624	Bile	English sole	bile	4	4
17EB-ESB0629	Bile	English sole	bile	4	4
17EB-ESB0632	Bile	English sole	bile	5	4
17EB-ESB0634	Bile	English sole	bile	3	3
17EB-ESB0639	Bile	English sole	bile	4	4
17EB-ESM01	Tissue	English sole	muscle	7	1
17EH-ESB0362	Bile	English sole	bile	1	1
17EH-ESB0371	Bile	English sole	bile	3	3
17EH-ESB0380	Bile	English sole	bile	4	4
17EH-ESB0386	Bile	English sole	bile	3	3
17EH-ESB0408	Bile	English sole	bile	3	3
17EH-ESB0409	Bile	English sole	bile	6	5
17EH-ESB0416	Bile	English sole	bile	4	4

17EH-ESB0418	Bile	English sole	bile	4	4
17EH-ESM01	Tissue	English sole	muscle	7	1
17HC-ESB0722	Bile	English sole	bile	2	2
17HC-ESB0725	Bile	English sole	bile	2	2
17HC-ESB0726	Bile	English sole	bile	2	2
17HC-ESB0728	Bile	English sole	bile	2	2
17HC-ESB0752	Bile	English sole	bile	2	2
17HC-ESB0764	Bile	English sole	bile	3	3
17HC-ESM01	Tissue	English sole	muscle	6	1
17MA13-TM1754	Tissue	resident Chinook salmon	muscle	5	1
17MA13-TM1757	Tissue	resident Chinook salmon	muscle	2	1
17MA13-TM1762	Tissue	resident Chinook salmon	muscle	7	2
17MA13-TM1763	Tissue	resident Chinook salmon	muscle	8	2
17NQ-ESB0003	Bile	English sole	bile	2	2
17NQ-ESB0006	Bile	English sole	bile	1	1
17NQ-ESB0018	Bile	English sole	bile	1	1
17NQ-ESB0028	Bile	English sole	bile	3	3
17NQ-ESB0064	Bile	English sole	bile	3	3
17NQ-ESM01	Tissue	English sole	muscle	7	1
17PG-ESB0849	Bile	English sole	bile	4	4
17PG-ESB0855	Bile	English sole	bile	4	4
17PG-ESB0865	Bile	English sole	bile	3	3
17PG-ESB0877	Bile	English sole	bile	2	2
17PG-ESB0880	Bile	English sole	bile	3	3
17PG-ESB0882	Bile	English sole	bile	2	2
17PG-ESB0887	Bile	English sole	bile	3	3
17PG-ESB0899	Bile	English sole	bile	3	3
17PG-ESM01	Tissue	English sole	muscle	7	1
17PS-ESB0961	Bile	English sole	bile	4	4

17PS-ESB0963	Bile	English sole	bile	3	3
17PS-ESB0964	Bile	English sole	bile	2	2
17PS-ESB0968	Bile	English sole	bile	3	3
17PS-ESB0970	Bile	English sole	bile	3	3
17PS-ESB0973	Bile	English sole	bile	5	5
17PS-ESM01	Tissue	English sole	muscle	5	1
17SG-ESB1324	Bile	English sole	bile	2	2
17SG-ESB1332	Bile	English sole	bile	3	3
17SG-ESB1353	Bile	English sole	bile	1	1
17SG-ESB1357	Bile	English sole	bile	2	2
17SG-ESB1376	Bile	English sole	bile	2	2
17SG-ESM01	Tissue	English sole	muscle	5	1
17SI-ESB0244	Bile	English sole	bile	3	3
17SI-ESB0248	Bile	English sole	bile	3	3
17SI-ESB0253	Bile	English sole	bile	1	1
17SI-ESB0273	Bile	English sole	bile	3	3
17SI-ESB0281	Bile	English sole	bile	3	3
17SI-ESB0292	Bile	English sole	bile	3	3
17SI-ESB0295	Bile	English sole	bile	3	3
17SI-ESM01	Tissue	English sole	muscle	7	1
17SI-ESM02	Tissue	English sole	muscle	1	0
17SI-ESM03	Tissue	English sole	muscle	1	0
17VD-ESB1224	Bile	English sole	bile	3	3
17VD-ESB1244	Bile	English sole	bile	3	3
17VD-ESM01	Tissue	English sole	muscle	3	1
17WPD01-ESM01	Tissue	English sole	muscle	5	1
17WPD02-ESM01	Tissue	English sole	muscle	8	1
17WPD1-ESB1442	Bile	English sole	bile	1	1
17WPD1-ESB1443	Bile	English sole	bile	1	1
17WPD1-ESB1444	Bile	English sole	bile	2	2
17WPD1-ESB1453	Bile	English sole	bile	1	1
17WPD1-ESB1456	Bile	English sole	bile	3	3
17WPD1-ESB1457	Bile	English sole	bile	2	2

17WPD1-ESB1465	Bile	English sole	bile	1	1
17WPD1-ESB1474	Bile	English sole	bile	1	1
17WPD1-ESB1475	Bile	English sole	bile	4	4
17WPD1-ESB1484	Bile	English sole	bile	1	1
17WPD1-ESB1485	Bile	English sole	bile	1	1
17WPD1-ESB1492	Bile	English sole	bile	1	1
17WPD1-ESB1494	Bile	English sole	bile	1	1
17WPD2-ESB1515	Bile	English sole	bile	3	3
17WPD2-ESB1522	Bile	English sole	bile	3	3
17WPD2-ESB1523	Bile	English sole	bile	4	3
17WPD2-ESB1532	Bile	English sole	bile	1	1
17WPD2-ESB1533	Bile	English sole	bile	1	1
17WPD2-ESB1538	Bile	English sole	bile	1	1
17WPD2-ESB1542	Bile	English sole	bile	4	3
17WPD2-ESB1546	Bile	English sole	bile	1	1
17WPD2-ESB1549	Bile	English sole	bile	2	2
17WPD2-ESB1553	Bile	English sole	bile	1	1
17WPD2-ESB1554	Bile	English sole	bile	1	1
17WPD2-ESB1555	Bile	English sole	bile	1	1
17WPD2-ESB1559	Bile	English sole	bile	3	3
18CP-PHW01	Tissue	Pacific herring	whole	7	2
18CP-PHW02	Tissue	Pacific herring	whole	6	2
18CP-PHW03	Tissue	Pacific herring	whole	7	2
18EB02-TW_H02	Tissue	juvenile Chinook salmon	WholeMinusOrgs	17	2
18EB02-TW_W02	Tissue	juvenile Chinook salmon	WholeMinusOrgs	16	2
18EB02-TW_W03	Tissue	juvenile Chinook salmon	WholeMinusOrgs	17	2
18EB03-TW_H01	Tissue	juvenile Chinook salmon	WholeMinusOrgs	14	2
18EB03-TW_H02	Tissue	juvenile Chinook salmon	WholeMinusOrgs	18	2

18EB03-TW_W02	Tissue	juvenile Chinook salmon	WholeMinusOrgs	17	2
18EB04-TW_H02	Tissue	juvenile Chinook salmon	WholeMinusOrgs	12	3
18EB04-TW_H03	Tissue	juvenile Chinook salmon	WholeMinusOrgs	13	3
18EB04-TW_W03	Tissue	juvenile Chinook salmon	WholeMinusOrgs	12	1
18EB04-TW_W05	Tissue	juvenile Chinook salmon	WholeMinusOrgs	7	1
18EB06-TW_H02	Tissue	juvenile Chinook salmon	WholeMinusOrgs	11	2
18EB06-TW_W03	Tissue	juvenile Chinook salmon	WholeMinusOrgs	12	1
18EB06-TW_W04	Tissue	juvenile Chinook salmon	WholeMinusOrgs	18	2
18GR02-TW_H04	Tissue	juvenile Chinook salmon	WholeMinusOrgs	17	3
18GR02-TW_H07	Tissue	juvenile Chinook salmon	WholeMinusOrgs	19	3
18GR02-TW_H10	Tissue	juvenile Chinook salmon	WholeMinusOrgs	21	3
18LDR01-TW_H01	Tissue	juvenile Chinook salmon	WholeMinusOrgs	18	2
18LDR01-TW_H03	Tissue	juvenile Chinook salmon	WholeMinusOrgs	17	3
18LDR01-TW_H04	Tissue	juvenile Chinook salmon	WholeMinusOrgs	19	3
18LDR03-TW_H01	Tissue	juvenile Chinook salmon	WholeMinusOrgs	21	3
18LDR03-TW_H04	Tissue	juvenile Chinook salmon	WholeMinusOrgs	16	3

18LDR03-TW_H06	Tissue	juvenile Chinook salmon	WholeMinusOrgs	20	3
18LDR05-TW_H02	Tissue	juvenile Chinook salmon	WholeMinusOrgs	19	3
18LDR05-TW_H03	Tissue	juvenile Chinook salmon	WholeMinusOrgs	17	3
18LDR05-TW_H06	Tissue	juvenile Chinook salmon	WholeMinusOrgs	21	3
18LDR06-TW_H01	Tissue	juvenile Chinook salmon	WholeMinusOrgs	12	1
18LDR06-TW_H02	Tissue	juvenile Chinook salmon	WholeMinusOrgs	20	4
18LDR06-TW_H03	Tissue	juvenile Chinook salmon	WholeMinusOrgs	15	3
18LDR07-TW_H01	Tissue	juvenile Chinook salmon	WholeMinusOrgs	16	3
18LDR07-TW_H02	Tissue	juvenile Chinook salmon	WholeMinusOrgs	15	3
18LDR07-TW_H03	Tissue	juvenile Chinook salmon	WholeMinusOrgs	12	1
18LDR08-TW_H01	Tissue	juvenile Chinook salmon	WholeMinusOrgs	15	4
18LDR08-TW_H02	Tissue	juvenile Chinook salmon	WholeMinusOrgs	16	3
18LDR08-TW_H03	Tissue	juvenile Chinook salmon	WholeMinusOrgs	13	2
18LDR09-TW_H01	Tissue	juvenile Chinook salmon	WholeMinusOrgs	18	2
18LDR09-TW_H03	Tissue	juvenile Chinook salmon	WholeMinusOrgs	15	3
18LDR09-TW_H04	Tissue	juvenile Chinook salmon	WholeMinusOrgs	11	2
18PO-PHW01	Tissue	Pacific herring	whole	8	2

18PO-PHW02	Tissue	Pacific herring	whole	9	2
18PO-PHW03	Tissue	Pacific herring	whole	8	3
18QB-PHW01	Tissue	Pacific herring	whole	6	2
18QB-PHW02	Tissue	Pacific herring	whole	8	1
18QB-PHW03	Tissue	Pacific herring	whole	7	1
18SM-PHW01	Tissue	Pacific herring	whole	8	2
18SM-PHW02	Tissue	Pacific herring	whole	7	2
18SM-PHW03	Tissue	Pacific herring	whole	4	1
18SQ-PHW01	Tissue	Pacific herring	whole	9	2
18SQ-PHW02	Tissue	Pacific herring	whole	7	2
18SQ-PHW03	Tissue	Pacific herring	whole	8	2
19BLL-ESB1626	Bile	English sole	bile	4	4
19BLL-ESB1627	Bile	English sole	bile	4	4
19BLL-ESB1628	Bile	English sole	bile	5	5
19BLL-ESB1629	Bile	English sole	bile	4	4
19BLM-ESB1623	Bile	English sole	bile	5	5
19BLM-ESB1625	Bile	English sole	bile	4	4
19BLU-ESB1618	Bile	English sole	bile	2	2
19BLU-ESB1620	Bile	English sole	bile	4	4
19BLU-ESB1678	Bile	English sole	bile	4	4
19BLU-ESB1681	Bile	English sole	bile	2	2
19CB-ESB0121	Bile	English sole	bile	3	3
19CB-ESB0131	Bile	English sole	bile	4	4
19CB-ESB0147	Bile	English sole	bile	4	4
19CB-ESB0149	Bile	English sole	bile	4	4
19CB-ESB0168	Bile	English sole	bile	3	3
19CB-ESB0174	Bile	English sole	bile	3	3
19CIM-ESB1447	Bile	English sole	bile	5	5
19CIM-ESB1449	Bile	English sole	bile	4	4
19CIM-ESB1451	Bile	English sole	bile	4	4
19CIM-ESB1460	Bile	English sole	bile	4	4
19CIM-ESB1474	Bile	English sole	bile	4	4
19CIM-ESB1478	Bile	English sole	bile	3	3

19CWU-ESB1653	Bile	English sole	bile	3	3
19CWU-ESB1655	Bile	English sole	bile	4	4
19DU-ESB0482	Bile	English sole	bile	4	4
19DU-ESB0483	Bile	English sole	bile	4	4
19DU-ESB0492	Bile	English sole	bile	5	5
19DU-ESB0497	Bile	English sole	bile	4	4
19DU-ESB0499	Bile	English sole	bile	4	4
19DU-ESB0503	Bile	English sole	bile	4	4
19DU-ESB0504	Bile	English sole	bile	4	4
19DU-ESB0506	Bile	English sole	bile	4	4
19DU-ESB0507	Bile	English sole	bile	4	4
19DU-ESB0513	Bile	English sole	bile	4	4
19DU-ESB0516	Bile	English sole	bile	4	4
19DU-ESB0517	Bile	English sole	bile	4	4
19DU-ESB0518	Bile	English sole	bile	4	4
19DU-ESB0521	Bile	English sole	bile	4	4
19DU-ESB0528	Bile	English sole	bile	4	4
19DU-ESB0530	Bile	English sole	bile	4	4
19DU-ESB0531	Bile	English sole	bile	4	4
19DU-ESB0536	Bile	English sole	bile	3	3
19EB4-ESB1561	Bile	English sole	bile	3	3
19EB4-ESB1564	Bile	English sole	bile	4	4
19EB4-ESB1570	Bile	English sole	bile	4	4
19EB4-ESB1572	Bile	English sole	bile	3	3
19EB4-ESB1577	Bile	English sole	bile	4	4
19EB4-ESB1578	Bile	English sole	bile	3	3
19EB-ESB0602	Bile	English sole	bile	5	5
19EB-ESB0603	Bile	English sole	bile	5	5
19EB-ESB0605	Bile	English sole	bile	5	5
19EB-ESB0606	Bile	English sole	bile	5	5
19EB-ESB0608	Bile	English sole	bile	5	5
19EB-ESB0609	Bile	English sole	bile	5	5
19EB-ESB0610	Bile	English sole	bile	5	5

19EB-ESB0611	Bile	English sole	bile	5	5
19EB-ESB0612	Bile	English sole	bile	5	5
19EB-ESB0613	Bile	English sole	bile	5	5
19EB-ESB0617	Bile	English sole	bile	4	4
19EB-ESB0618	Bile	English sole	bile	5	5
19EB-ESB0619	Bile	English sole	bile	5	5
19EB-ESB0620	Bile	English sole	bile	5	5
19EB-ESB0621	Bile	English sole	bile	4	4
19EB-ESB0623	Bile	English sole	bile	4	4
19EB-ESB0624	Bile	English sole	bile	4	4
19EB-ESB0625	Bile	English sole	bile	4	4
19EB-ESB0629	Bile	English sole	bile	5	5
19EB-ESB0632	Bile	English sole	bile	5	5
19EB-ESB0634	Bile	English sole	bile	4	4
19EB-ESB0635	Bile	English sole	bile	4	4
19EH-ESB0377	Bile	English sole	bile	3	3
19EH-ESB0408	Bile	English sole	bile	3	3
19EH-ESB0423	Bile	English sole	bile	3	3
19EH-ESB0424	Bile	English sole	bile	4	4
19EH-ESB0425	Bile	English sole	bile	3	3
19EH-ESB0426	Bile	English sole	bile	3	3
19HC-ESB1084	Bile	English sole	bile	3	3
19HC-ESB1098	Bile	English sole	bile	2	2
19HC-ESB1114	Bile	English sole	bile	3	3
19HC-ESB1122	Bile	English sole	bile	2	2
19HC-ESB1131	Bile	English sole	bile	3	3
19HC-ESB1132	Bile	English sole	bile	3	3
19HYL-ESB1614	Bile	English sole	bile	4	4
19HYL-ESB1615	Bile	English sole	bile	4	4
19HYM-ESB1607	Bile	English sole	bile	4	4
19HYM-ESB1610	Bile	English sole	bile	4	4
19HYU-ESB1601	Bile	English sole	bile	3	3
19HYU-ESB1602	Bile	English sole	bile	4	4

19HYU-ESB1604	Bile	English sole	bile	4	4
19HYU-ESB1605	Bile	English sole	bile	3	3
19HYU-ESB1676	Bile	English sole	bile	3	3
19MWW-ESB1646	Bile	English sole	bile	2	2
19NQ-ESB0006	Bile	English sole	bile	5	5
19NQ-ESB0026	Bile	English sole	bile	4	4
19NQ-ESB0035	Bile	English sole	bile	4	4
19NQ-ESB0056	Bile	English sole	bile	6	6
19NQ-ESB0059	Bile	English sole	bile	4	4
19NQ-ESB0061	Bile	English sole	bile	4	4
19NQ-ESB0081	Bile	English sole	bile	4	4
19OMK-ESB1658	Bile	English sole	bile	3	3
19OMW-ESB1649	Bile	English sole	bile	3	3
19OTW-ESB1662	Bile	English sole	bile	3	3
19OTW-ESB1665	Bile	English sole	bile	3	3
19PG-ESB0962	Bile	English sole	bile	3	3
19PG-ESB0968	Bile	English sole	bile	2	2
19PG-ESB0983	Bile	English sole	bile	3	3
19PG-ESB0990	Bile	English sole	bile	3	3
19PG-ESB1000	Bile	English sole	bile	3	3
19PG-ESB1008	Bile	English sole	bile	3	3
19PG-ESB1011	Bile	English sole	bile	3	3
19PG-ESB1012	Bile	English sole	bile	3	3
19PG-ESB1017	Bile	English sole	bile	3	3
19PG-ESB1018	Bile	English sole	bile	3	3
19PG-ESB1021	Bile	English sole	bile	3	3
19PG-ESB1022	Bile	English sole	bile	3	3
19PG-ESB1023	Bile	English sole	bile	4	4
19PG-ESB1041	Bile	English sole	bile	3	3
19PG-ESB1042	Bile	English sole	bile	3	3
19PG-ESB1043	Bile	English sole	bile	3	3
19PM-ESB0841	Bile	English sole	bile	3	3
19PM-ESB0850	Bile	English sole	bile	3	3

19PM-ESB0851	Bile	English sole	bile	1	1
19PM-ESB0853	Bile	English sole	bile	3	3
19PM-ESB0868	Bile	English sole	bile	4	4
19PM-ESB0875	Bile	English sole	bile	4	4
19PM-ESB0886	Bile	English sole	bile	3	3
19PM-ESB0888	Bile	English sole	bile	3	3
19PM-ESB0898	Bile	English sole	bile	3	3
19PM-ESB0900	Bile	English sole	bile	3	3
19RUS-ESB1668	Bile	English sole	bile	3	3
19RUS-ESB1670	Bile	English sole	bile	3	3
19SG-ESB1336	Bile	English sole	bile	1	1
19SG-ESB1342	Bile	English sole	bile	4	4
19SG-ESB1349	Bile	English sole	bile	2	2
19SG-ESB1351	Bile	English sole	bile	1	1
19SG-ESB1359	Bile	English sole	bile	1	1
19SG-ESB1378	Bile	English sole	bile	4	4
19SI-ESB0254	Bile	English sole	bile	4	4
19SI-ESB0261	Bile	English sole	bile	4	4
19SI-ESB0267	Bile	English sole	bile	3	3
19SI-ESB0268	Bile	English sole	bile	3	3
19SI-ESB0282	Bile	English sole	bile	3	3
19SI-ESB0285	Bile	English sole	bile	4	4
19STC-ESB1633	Bile	English sole	bile	3	3
19STC-ESB1635	Bile	English sole	bile	3	3
19VD-ESB1226	Bile	English sole	bile	2	2
19VD-ESB1281	Bile	English sole	bile	3	3
19VD-ESB1282	Bile	English sole	bile	3	3
19WPD01-ESB0738	Bile	English sole	bile	2	2
19WPD01-ESB0739	Bile	English sole	bile	5	5
19WPD01-ESB0740	Bile	English sole	bile	3	3
19WPD01-ESB0758	Bile	English sole	bile	4	4
19WPD01-ESB0766	Bile	English sole	bile	4	4
19WPD01-ESB0772	Bile	English sole	bile	3	3

20AI_MMB-MTW01	Mussel	Bay mussel	whole	2	0
20AI_OB-MTW01	Mussel	Bay mussel	whole	1	0
20BBSM-MTW01	Mussel	Bay mussel	whole	5	0
20CB_CBSW-MTW01	Mussel	Bay mussel	whole	14	3
20CB_CBTF-MTW01	Mussel	Bay mussel	whole	9	2
20CB_MW-MTW01	Mussel	Bay mussel	whole	15	2
20CBTP-MTW01	Mussel	Bay mussel	whole	4	0
20CPS_EMB-MTW01	Mussel	Bay mussel	whole	8	2
20CPS_KM-MTW01	Mussel	Bay mussel	whole	6	2
20CPS_MASO-MTW01	Mussel	Bay mussel	whole	8	2
20CPS_QMH-MTW01	Mussel	Bay mussel	whole	7	2
20CPS_SB-MTW01	Mussel	Bay mussel	whole	7	1
20CPS_SQSO-MTW01	Mussel	Bay mussel	whole	8	2
20EB_ME-MTW01	Mussel	Bay mussel	whole	8	1
20EB_P59-MTW01	Mussel	Bay mussel	whole	8	1
20EBDH-MTW01	Mussel	Bay mussel	whole	4	0
20EBFR-MTW01	Mussel	Bay mussel	whole	4	0
20HC_HO-MTW01	Mussel	Bay mussel	whole	6	1
20NPS_BLSC-MTW01	Mussel	Bay mussel	whole	7	2
20NPS_FBAR-MTW01	Mussel	Bay mussel	whole	4	0
20PSEF-MTW01	Mussel	Bay mussel	whole	6	0
20PSEH-MTW01	Mussel	Bay mussel	whole	4	0
20PSEM-MTW01	Mussel	Bay mussel	whole	4	0
20PSHC-MTW01	Mussel	Bay mussel	whole	2	0
20PSKP-MTW01	Mussel	Bay mussel	whole	6	0
20PSMF-MTW01	Mussel	Bay mussel	whole	5	0
20PSPT-MTW01	Mussel	Bay mussel	whole	6	0
20PSTB-MTW01	Mussel	Bay mussel	whole	4	0
20SAM004-MTW01	Mussel	Bay mussel	whole	6	1

20SAM006-MTW01	Mussel	Bay mussel	whole	4	1
20SAM011-MTW01	Mussel	Bay mussel	whole	5	1
20SAM013-MTW01	Mussel	Bay mussel	whole	7	1
20SAM014-MTW01	Mussel	Bay mussel	whole	8	1
20SAM017-MTW01	Mussel	Bay mussel	whole	6	2
20SAM019-MTW01	Mussel	Bay mussel	whole	5	1
20SAM021-MTW01	Mussel	Bay mussel	whole	3	1
20SAM025-MTW01	Mussel	Bay mussel	whole	13	2
20SAM029-MTW01	Mussel	Bay mussel	whole	5	2
20SAM030-MTW01	Mussel	Bay mussel	whole	9	2
20SAM031-MTW01	Mussel	Bay mussel	whole	7	2
20SAM034-MTW01	Mussel	Bay mussel	whole	5	2
20SAM037-MTW01	Mussel	Bay mussel	whole	8	2
20SAM039-MTW01	Mussel	Bay mussel	whole	9	2
20SAM042-MTW01	Mussel	Bay mussel	whole	6	2
20SAM043-MTW01	Mussel	Bay mussel	whole	7	0
20SAM049-MTW01	Mussel	Bay mussel	whole	6	2
20SAM056-MTW01	Mussel	Bay mussel	whole	7	2
20SIWP-MTW01	Mussel	Bay mussel	whole	4	0
20SJD_JSK-MTW01	Mussel	Bay mussel	whole	5	2
20SJD_NMB-MTW01	Mussel	Bay mussel	whole	6	2
20SPS_PBL-MTW01	Mussel	Bay mussel	whole	5	1
20SPS_SH-MTW01	Mussel	Bay mussel	whole	5	2
20SSBI-MTW01	Mussel	Bay mussel	whole	7	0
20WB_PCB1-MTW01	Mussel	Bay mussel	whole	9	1
20WB_PCR-MTW01	Mussel	Bay mussel	whole	5	1
20WBNA-MTW01	Mussel	Bay mussel	whole	2	0
20WPS_SVD-MTW01	Mussel	Bay mussel	whole	6	2
8344181	WWTP	N/A	N/A	43	2
8344184	WWTP	N/A	N/A	55	6
8344193	WWTP	N/A	N/A	50	2
8344194	WWTP	N/A	N/A	47	0
8344197	WWTP	N/A	N/A	15	0

8344198	WWTP	N/A	N/A	20	0
8344202	WWTP	N/A	N/A	28	3
AGT_20120618	Tissue	Pacific sand lance	whole	5	0
Brem WWTP Eff	WWTP	N/A	N/A	28	2
BuddInle_20180417	Water	N/A	N/A	7	0
BuddInle_20180509	Water	N/A	N/A	12	0
BuddInle_20180618	Water	N/A	N/A	15	0
BuddInle_20180822	Water	N/A	N/A	12	0
BuddInle_20181030	Water	N/A	N/A	9	0
CLAY 2010_20101205	Tissue	Pacific sand lance	whole	5	0
CLAY 2011_20110216	Tissue	Pacific sand lance	whole	4	0
CommBayS_20180509	Water	N/A	N/A	12	1
CommBayS_20180618	Water	N/A	N/A	12	1
CommBayS_20180822	Water	N/A	N/A	11	1
CommBayS_20181030	Water	N/A	N/A	14	1
CommBayT_20180509	Water	N/A	N/A	16	1
CommBayT_20180618	Water	N/A	N/A	11	1
CommBayT_20180822	Water	N/A	N/A	8	1
CommBayT_20181030	Water	N/A	N/A	13	1
EAG LG_20120509	Tissue	Pacific sand lance	whole	5	0
EAG REP_20120509	Tissue	Pacific sand lance	whole	5	0
EAG_20120509	Tissue	Pacific sand lance	whole	2	0
EdmondsF_20180509	Water	N/A	N/A	11	1
EdmondsF_20180618	Water	N/A	N/A	11	1
EdmondsF_20180822	Water	N/A	N/A	9	0
EdmondsF_20181030	Water	N/A	N/A	8	1
EverettB_20180509	Water	N/A	N/A	8	0
EverettB_20180618	Water	N/A	N/A	11	0
EverettB_20180822	Water	N/A	N/A	8	0
EverettB_20181030	Water	N/A	N/A	6	0
Evergree_20180417	Water	N/A	N/A	8	1

Evergree_20180509	Water	N/A	N/A	10	1
Evergree_20180618	Water	N/A	N/A	7	0
Evergree_20180822	Water	N/A	N/A	6	0
Evergree_20181030	Water	N/A	N/A	9	0
Hammersl_20180417	Water	N/A	N/A	5	0
Hammersl_20180509	Water	N/A	N/A	7	0
Hammersl_20180618	Water	N/A	N/A	8	0
Hammersl_20180822	Water	N/A	N/A	5	0
Hammersl_20181030	Water	N/A	N/A	8	0
HoodCana_20180417	Water	N/A	N/A	7	0
HoodCana_20180509	Water	N/A	N/A	9	0
HoodCana_20180618	Water	N/A	N/A	8	0
HoodCana_20180822	Water	N/A	N/A	3	0
HoodCana_20181030	Water	N/A	N/A	7	0
JackPerr_20180509	Water	N/A	N/A	7	1
JackPerr_20180618	Water	N/A	N/A	3	0
JackPerr_20180822	Water	N/A	N/A	5	0
JackPerr_20181030	Water	N/A	N/A	6	0
JoeBlock_20180509	Water	N/A	N/A	4	1
JoeBlock_20180618	Water	N/A	N/A	4	0
JoeBlock_20180822	Water	N/A	N/A	4	0
JoeBlock_20181030	Water	N/A	N/A	7	0
L76915-10	Tissue	Smallmouth bass	whole	10	1
L76915-2	Tissue	Smallmouth bass	whole	7	1
L76915-3	Tissue	Smallmouth bass	whole	7	1
L76915-4	Tissue	Smallmouth bass	whole	9	1
L76915-5	Tissue	Smallmouth bass	whole	9	1
L76915-6	Tissue	Smallmouth bass	whole	9	1
L76915-7	Tissue	Smallmouth bass	whole	8	1
L76915-8	Tissue	Smallmouth bass	whole	7	1
L76915-9	Tissue	Smallmouth bass	whole	8	1
LIB_20120617	Tissue	Pacific sand lance	whole	2	0
LOP_20140814	Tissue	Pacific sand lance	whole	3	0

NIS_20140618	Tissue	Pacific sand lance	whole	3	0
Nisq Estaury	Water	N/A	N/A	5	1
Nisq La 2013	Tissue	Pacific staghorn sculpin	whole	7	1
Nisq La 2014	Tissue	Pacific staghorn sculpin	whole	6	2
Nisq Ot 2014	Tissue	juvenile Chinook salmon	whole	13	2
P61010-1 0512 1m	Water	N/A	N/A	7	0
P61010-2 0518 1m	Water	N/A	N/A	6	0
P61010-3 0536 1m	Water	N/A	N/A	5	0
P61010-4 A522 1m	Water	N/A	N/A	7	0
P61010-5 A522 10m	Water	N/A	N/A	7	0
P61162-1 LSCW02 1m	Water	N/A	N/A	7	0
P61162-10 LTUM03 1m	Water	N/A	N/A	5	0
P61162-11 LTUM03 4m	Water	N/A	N/A	4	0
P61162-12 LTXQ01 1m	Water	N/A	N/A	6	0
P61162-2 LSCW02 173m	Water	N/A	N/A	4	0
P61162-3 LTED04 1m	Water	N/A	N/A	5	0
P61162-4 LTED04 74m	Water	N/A	N/A	7	0
P61162-5 LTEH02 1m	Water	N/A	N/A	11	0
P61162-6 HNFD01 1m	Water	N/A	N/A	5	0
P61162-7 HNFD01 14m	Water	N/A	N/A	3	0
P61162-8 LTKE03 1m	Water	N/A	N/A	5	0
P61162-9 LTKE03 9m	Water	N/A	N/A	5	0
P61478-1 0512 1m	Water	N/A	N/A	9	0
P61478-2 0518 1m	Water	N/A	N/A	11	0
P61478-3 0536 1m	Water	N/A	N/A	6	0
P61478-4 A522 1m	Water	N/A	N/A	8	0

P61478-5 A522 10m	Water	N/A	N/A	9	0
P61478-6 LTEH02 1m	Water	N/A	N/A	8	0
P61478-7 LTXQ01 1m	Water	N/A	N/A	5	0
P61647-1 LSCW02 1m	Water	N/A	N/A	5	0
P61647-10 LTUM03 6m	Water	N/A	N/A	7	0
P61647-2 LSCW02 175m	Water	N/A	N/A	7	0
P61647-3 LTED04 1m	Water	N/A	N/A	7	0
P61647-4 LTED04 75m	Water	N/A	N/A	5	0
P61647-5 HNFD01 1m	Water	N/A	N/A	5	0
P61647-6 HNFD01 7m	Water	N/A	N/A	6	0
P61647-7 LTKE03 1m	Water	N/A	N/A	6	0
P61647-8 LTKE03 11m	Water	N/A	N/A	7	0
P61647-9 LTUM03 1m	Water	N/A	N/A	5	0
P61856-1 0512 1m	Water	N/A	N/A	7	0
P61856-2 0518 1m	Water	N/A	N/A	7	0
P61856-3 0536 1m	Water	N/A	N/A	6	0
P61856-4 A522 1m	Water	N/A	N/A	7	0
P61856-5 A522 10m	Water	N/A	N/A	7	0
P61856-6 LTEH02 1m	Water	N/A	N/A	8	0
P61856-7 LTXQ01 1m	Water	N/A	N/A	1	0
P61858-1 LSCW02 1m	Water	N/A	N/A	6	0
P61858-10 LTUM03 4m	Water	N/A	N/A	6	0
P61858-2 LSCW02 175m	Water	N/A	N/A	5	0
P61858-3 LTED04 1m	Water	N/A	N/A	6	0
P61858-4 LTED04 75m	Water	N/A	N/A	7	0

P61858-5 HNFD01 1m	Water	N/A	N/A	7	0
P61858-6 HNFD01 15m	Water	N/A	N/A	6	0
P61858-7 LTKE03 1m	Water	N/A	N/A	6	0
P61858-8 LTKE03 7m	Water	N/A	N/A	8	0
P61858-9 LTUM03 1m	Water	N/A	N/A	4	0
P76888-1	Tissue	Brown rockfish	whole	13	1
P76888-2	Tissue	Brown rockfish	whole	9	1
P76888-3	Tissue	Brown rockfish	whole	12	1
P76888-4	Tissue	Brown rockfish	whole	13	1
P76888-5	Tissue	Brown rockfish	whole	10	1
P76888-6	Tissue	Brown rockfish	whole	10	2
P76888-7	Tissue	Brown rockfish	whole	10	1
P76888-8	Tissue	Brown rockfish	whole	13	1
P76888-9	Tissue	Quillback rockfish	whole	6	1
Point_No_20180417	Water	N/A	N/A	6	0
Point_No_20180509	Water	N/A	N/A	9	1
Point_No_20180618	Water	N/A	N/A	6	0
Point_No_20180822	Water	N/A	N/A	3	0
Point_No_20181030	Water	N/A	N/A	6	0
Port_Tow_20180509	Water	N/A	N/A	10	0
Port_Tow_20180618	Water	N/A	N/A	6	0
Port_Tow_20180822	Water	N/A	N/A	4	1
Port_Tow_20181030	Water	N/A	N/A	6	0
PT MON_20120615	Tissue	Pacific sand lance	whole	5	0
Puyullap Estuary	Water	N/A	N/A	10	2
Puyullap La 2013	Tissue	Pacific staghorn sculpin	whole	9	3
Puyullap La 2014	Tissue	Pacific staghorn sculpin	whole	9	1
Puyullap Ot 2014 A	Tissue	juvenile Chinook salmon	whole	26	1
Puyullap Ot 2014 B	Tissue	juvenile Chinook salmon	whole	24	1

SalmonBa_20180509	Water	N/A	N/A	18	0
SalmonBa_20180618	Water	N/A	N/A	13	0
SalmonBa_20180822	Water	N/A	N/A	16	0
SalmonBa_20181030	Water	N/A	N/A	10	0
SalmonBe_20180417	Water	N/A	N/A	6	1
SalmonBe_20180509	Water	N/A	N/A	7	0
SalmonBe_20180618	Water	N/A	N/A	11	1
SalmonBe_20180822	Water	N/A	N/A	7	1
SalmonBe_20181030	Water	N/A	N/A	10	1
SaltarsP_20180417	Water	N/A	N/A	15	0
SaltarsP_20180509	Water	N/A	N/A	8	0
SaltarsP_20180618	Water	N/A	N/A	8	0
SaltarsP_20180822	Water	N/A	N/A	3	0
SaltarsP_20181030	Water	N/A	N/A	9	0
Silverda_20180417	Water	N/A	N/A	7	0
Silverda_20180509	Water	N/A	N/A	8	0
Silverda_20180618	Water	N/A	N/A	9	0
Silverda_20180822	Water	N/A	N/A	6	0
Silverda_20181030	Water	N/A	N/A	9	0
Sinclair Estuary	Water	N/A	N/A	10	1
Sinclair La 2014 A	Tissue	Pacific staghorn sculpin	whole	15	2
Sinclair Ot 2014 A	Tissue	juvenile Chinook salmon	whole	13	1
Sinclair Ot 2014 B	Tissue	juvenile Chinook salmon	whole	12	2
SmithCov_20180509	Water	N/A	N/A	24	0
SmithCov_20180618	Water	N/A	N/A	16	0
SmithCov_20180822	Water	N/A	N/A	19	0
SmithCov_20181030	Water	N/A	N/A	11	0
Tac WWTP Eff	WWTP	N/A	N/A	30	2
VC Hatch Ot 2014	Tissue	juvenile Chinook salmon	whole	7	2
WestPoin_20180509	Water	N/A	N/A	9	1

WestPoin_20180618	Water	N/A	N/A	7	0
WestPoin_20180822	Water	N/A	N/A	3	0
WestPoin_20181030	Water	N/A	N/A	6	0

Table B5. Chemicals with insufficient information for evaluation as ER agonists

Chemical name	**CAS**	**Notes**
4-Nonylphenol monoethoxylate (NP1EO)	104-35-8	Not evaluated in ToxCast ER assays
Clinafloxacin	105956-97-6	Not evaluated in ToxCast ER assays
10-Hydroxyamitriptyline	1159-82-6	Not evaluated in ToxCast ER assays
alpha-HBCDD	134237-50-6	Not evaluated in ToxCast ER assays
gamma-HBCDD	134237-52-8	Not evaluated in ToxCast ER assays
4-Epioxytetracycline [EOTC]	14206-58-7	Not evaluated in ToxCast ER assays
4-Epichlortetracycline [ECTC]	14297-93-9	Not evaluated in ToxCast ER assays
Lincomycin	154-21-2	Not evaluated in ToxCast ER assays
Beta-Stigmastanol	19466-47-8	Not evaluated in ToxCast ER assays
4-Nonylphenol diethoxylate (NP2EO)	20427-84-3	Not evaluated in ToxCast ER assays
Virginiamycin M1	21411-53-0	Not evaluated in ToxCast ER assays
Tetrabromo-p-xylene	23488-38-2	Not evaluated in ToxCast ER assays
2-(N-Methylperfluorooctanesulfonamido)acetic acid (MeFOSAA)	2355-31-9	Not evaluated in ToxCast ER assays
dicyclohexyl urea	2387-23-7	Not evaluated in ToxCast ER assays
4-hydroxy-chlorothalonil	28343-61-5	Not evaluated in ToxCast ER assays
2-(N-Ethylperfluorooctanesulfonamido)acetic acid (EtFOSAA)	2991-50-6	Not evaluated in ToxCast ER assays
Amphetamine	300-62-9	Not evaluated in ToxCast ER assays
Perfluorododecanoic acid (PFDOA)	307-55-1	Not evaluated in ToxCast ER assays
Dechlorane 602	31107-44-5	Not evaluated in ToxCast ER assays
Desmosterol	313-04-2	Not evaluated in ToxCast ER assays
Perfluorodecanesulfonic acid (PFDS)	335-77-3	Not evaluated in ToxCast ER assays
Coprosterol	360-68-9	Not evaluated in ToxCast ER assays
Methyl perfluoro-3,6-dioxaheptanoate	39187-41-2	Not evaluated in ToxCast ER assays
Tetrabromo-o-chlorotoluene	39569-21-6	Not evaluated in ToxCast ER assays

Anhydrochlortetracycline [ACTC]	4497-08-9	Not evaluated in ToxCast ER assays
Propoxyphene	469-62-5	Not evaluated in ToxCast ER assays
Campesterol	474-62-4	Not evaluated in ToxCast ER assays
Cyclophosphamide	50-18-0	Not evaluated in ToxCast ER assays
Cocaine	50-36-2	Not evaluated in ToxCast ER assays
Amitriptyline	50-48-6	Not evaluated in ToxCast ER assays
2-Hydroxy-ibuprofen	51146-55-5	Not evaluated in ToxCast ER assays
Amsacrine	51264-14-3	Not evaluated in ToxCast ER assays
4-Nonylphenol Triethoxylate	51437-95-7	Not evaluated in ToxCast ER assays
Methamphetamine	537-46-2	Not evaluated in ToxCast ER assays
Doxycycline	564-25-0	Not evaluated in ToxCast ER assays
Chlortetracycline [CTC]	57-62-5	Not evaluated in ToxCast ER assays
Ergosterol	57-87-4	Not evaluated in ToxCast ER assays
1,2-Dibromobenzene	583-53-9	Not evaluated in ToxCast ER assays
Citalopram	59729-33-8	Not evaluated in ToxCast ER assays
Pentabromobenzene	608-90-2	Not evaluated in ToxCast ER assays
Penicillin G	61-33-6	Not evaluated in ToxCast ER assays
Cloxacillin	61-72-3	Not evaluated in ToxCast ER assays
Cefotaxime	63527-52-6	Not evaluated in ToxCast ER assays
Oxacillin	66-79-5	Not evaluated in ToxCast ER assays
Norverapamil	67018-85-3	Not evaluated in ToxCast ER assays
Dehydronifedipine	67035-22-7	Not evaluated in ToxCast ER assays
Oxycodone	76-42-6	Not evaluated in ToxCast ER assays
Sertraline	79617-96-2	Not evaluated in ToxCast ER assays
4-Epitetracycline [ETC]	79-85-6	Not evaluated in ToxCast ER assays
Cholestanol	80-97-7	Not evaluated in ToxCast ER assays
4-Epianhydrochlortetracycline [EACTC]	81163-11-3	Not evaluated in ToxCast ER assays
Stigmasterol	83-48-7	Not evaluated in ToxCast ER assays
Norfluoxetine	83891-03-6	Not evaluated in ToxCast ER assays
Desmethyldiltiazem	86408-45-9	Not evaluated in ToxCast ER assays
Penicillin V	87-08-1	Not evaluated in ToxCast ER assays
Pentabromotoluene	87-83-2	Not evaluated in ToxCast ER assays

Amlodipine	88150-42-9	Not evaluated in ToxCast ER assays
O-desvenlafaxine	93413-62-8	Not evaluated in ToxCast ER assays
Benzothiazole-2-sulfonic acid	941-57-1	Not evaluated in ToxCast ER assays
Lomefloxacin	98079-51-7	Not evaluated in ToxCast ER assays
Isochlortetracycline [ICTC]	514-53-4	Not evaluated in ToxCast ER assays

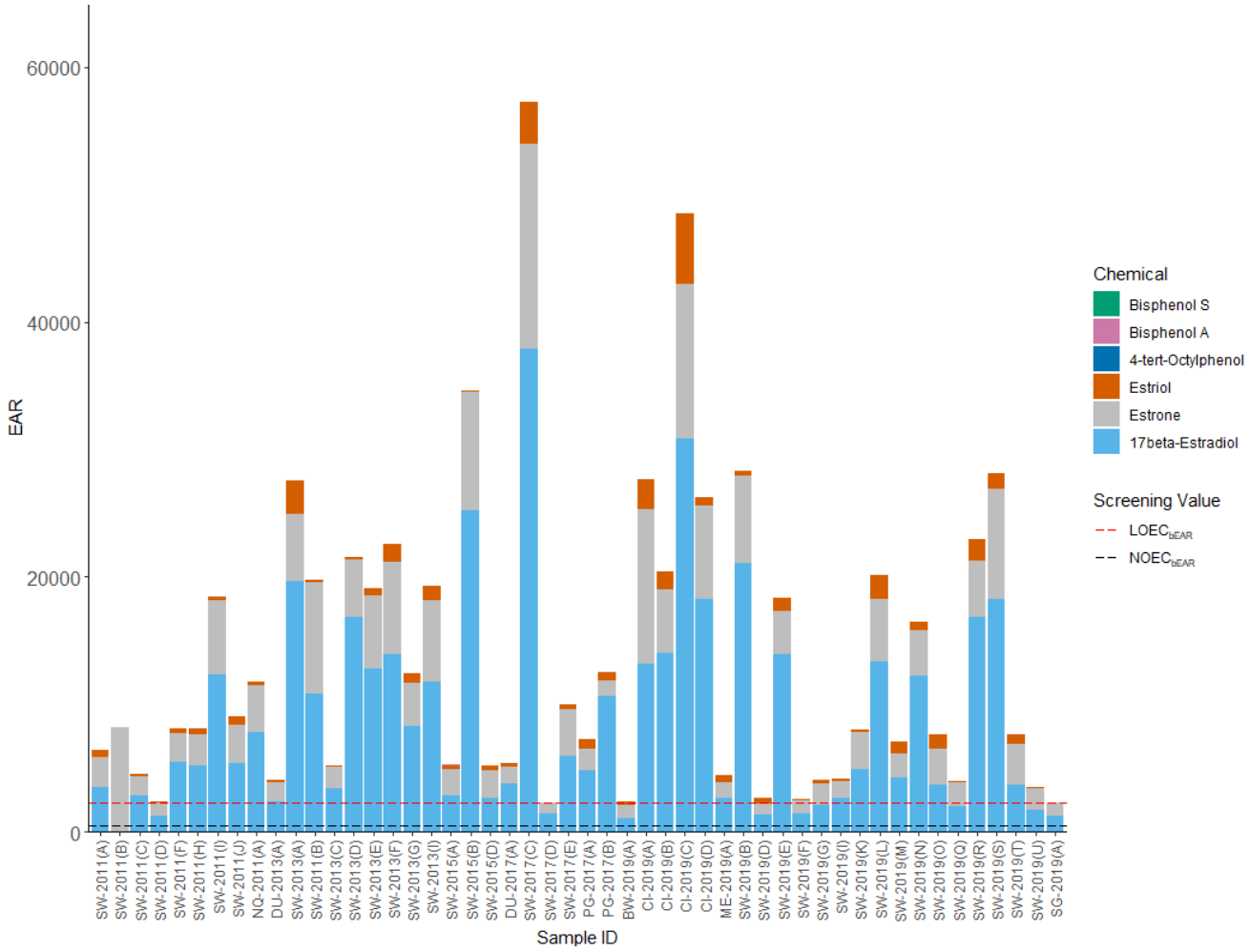

Fig. B1. Stacked barplot with exposure-activity ratios (EARs) for each bile sample with EARs exceeding the LOEC$_{bEAR}$, represented by the red dashed line. The sample labels include the site ID abbreviation (as described in Fig. 1), collection year, and a letter to distinguish samples. The EAR$_{mix}$ is the sum of all EARs in a sample, where each component of the stacked bar is the EARA$_{CC5}$ for that chemical. Samples with EAR$_{mix}$ above the LOEC$_{bEAR}$ have high potential for estrogenic effects.

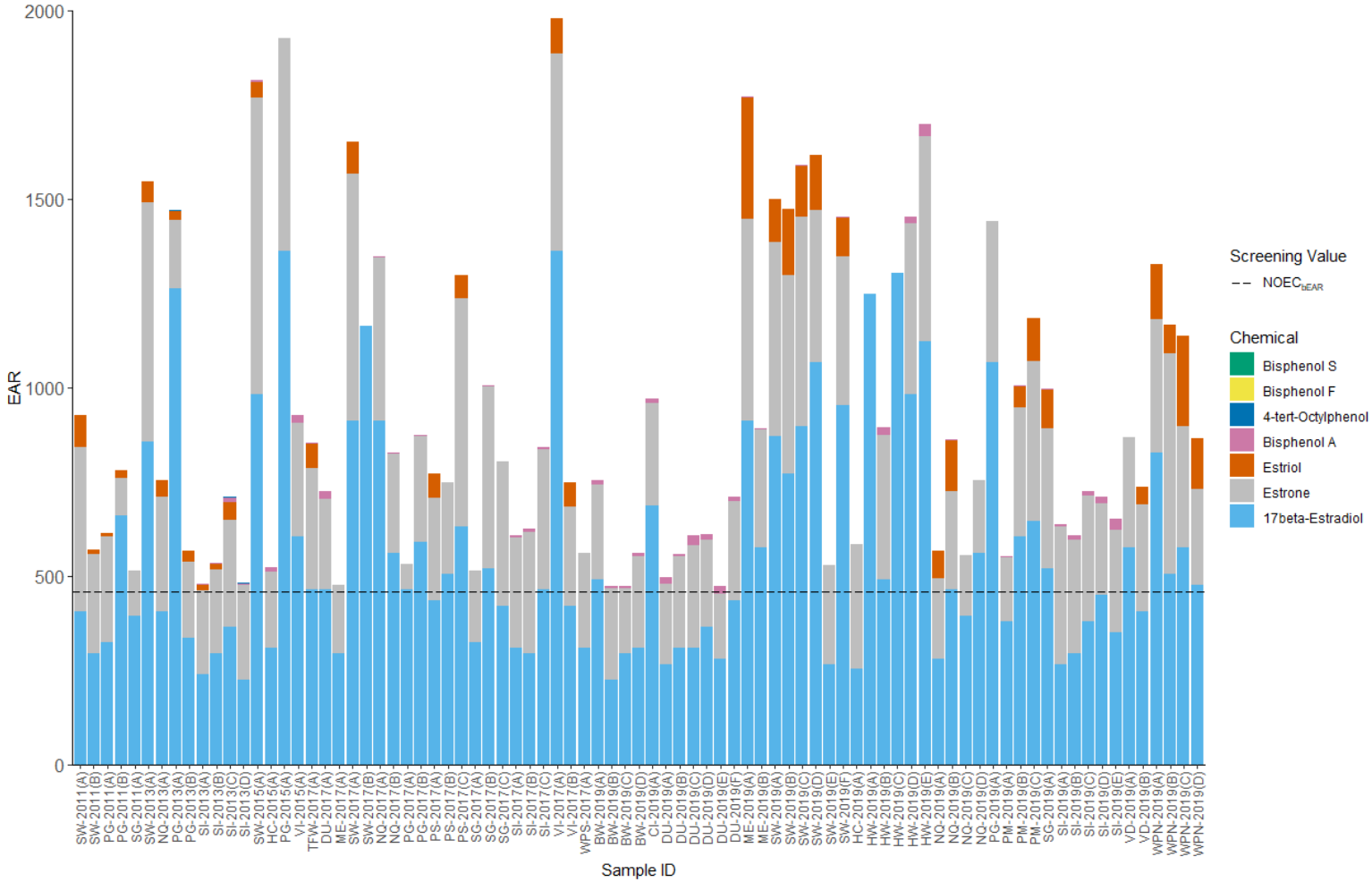

Fig. B2. Stacked barplot with exposure-activity ratios (EARs) for each bile sample with EARs between the $LOEC_{bEAR}$ and the $NOEC_{bEAR}$, represented by the black dashed line. The sample labels include the site ID abbreviation (as described in Fig. 1), collection year, and a letter to distinguish samples. The EAR_{mix} is the sum of all EARs in a sample, where each component of the stacked bar is the EAR_{ACC5} for that chemical. Samples with EAR_{mix} between the $LOEC_{bEAR}$ and $NOEC_{bEAR}$ have medium potential for estrogenic effects.