Modulation of mitochondrial function as an indicator for sublethal effects of contaminants of emerging concern

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Abstract

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Environmental and Occupational Health Sciences

Effluent from wastewater treatment plants (WWTPs) are a primary source of a wide range of structurally-diverse compounds into Puget Sound, WA. These contaminants of emerging concern (CECs) include personal care products and pharmaceuticals (PPCPs), perfluorinated compounds, natural and synthetic hormones, alkylphenol and alkylphenol ethoxylate surfactants, current-use pesticides, and polybrominated diphenyl ether (PBDE) flame retardants. Currently, the types and concentrations of these emerging contaminants entering Puget Sound, and their effects on aquatic receptors, are poorly understood. Recent reports in numerous species of fish indicate exposure to CECs can cause sublethal, detrimental effects on growth, reproduction, and behavior, warranting development of biomarkers of exposure and effect of CECs in relevant biomonitoring species. There is evidence to suggest that numerous CECs negatively affect mitochondrial function in humans and laboratory animal models. Hence, the overarching hypothesis of my doctoral research

was that mitochondrial dysfunction can serve as an indicator of sublethal effect, and exposure to, emerging contaminants.

Both human health- and aquatic health-related effects of CECs on mitochondrial function were addressed in my dissertation project. Aim 1 of my project was an oceans and human health study concerning the safety of salmon consumption by humans. The tested hypothesis was that the antioxidant effects of omega-3 polyunsaturated fatty acids found in salmon chemoprotect against mitochondrial and oxidative injury from a common "emerged" contaminant detected in Pacific salmon, the PBDE flame retardant 2,2',4,4'-tetrabromodiphenyl ether (BDE 47). HepG2 cells were treated for 12 h with a mixture of omega-3s relevant to salmon consumption (oxEPA/oxDHA), followed by exposure to BDE 47 (100 μM) for 24 h. Pretreatment with oxEPA/oxDHA prevented BDE 47-induced production of reactive oxygen species (ROS) and depletion of GSH, significantly increased expression of protective cellular antioxidant response genes, and protected against BDE 47-induced loss of viability and mitochondrial membrane potential. Analysis of mitochondrial electron transport system (ETS) revealed extensive inhibition of state 3 respiration and maximum respiratory capacity by BDE 47 were partially reversed by treatment with oxEPA/oxDHA. These findings indicated that the antioxidant effects of salmon omega-3s protected against short exposures to BDE 47, including a protective role of these compounds on maintaining cellular and mitochondrial function.

The subsequent goal of my research was to examine the potential impacts of CECs in Puget Sound aquatic organisms. Thus, studies in Aim 2 involved an analytical field study to determine the specific types of CECs present in Puget Sound, and their potential to bioaccumulate in

representative biomonitoring species. Samples of estuary water, WWTP effluent, and whole-bodies of Chinook salmon and staghorn sculpin were collected from three sites: two estuaries that receive effluent from WWTPs and a minimally-polluted estuarine reference site. Samples were analyzed for 150 chemical analytes by HPLC/MS/MS. In total, 81 analytes were detected in effluent samples in the ng/L to low µg/L range, 25 analytes were detected in estuary water samples at ng/L concentrations, and 42 analytes were detected in fish whole-bodies at ng/g concentrations. Noteworthy findings included high concentrations of CECs measured in WWTP effluent relative to other major U.S. WWTPs, greater-than-expected contamination of the reference site, and preferential bioaccumulation of CECs in Chinook salmon relative to sculpin. These results can inform environmental risk assessment or ecological studies at other sites in Puget Sound.

The analytical field study provided crucial data that was required to conduct subsequent experiments in Aim 3 that investigated the association between exposure to CECs and liver mitochondrial dysfunction in fish. Mitochondrial function was assessed in feral juvenile Chinook from the analytical field study, and in hatchery-reared juvenile Chinook exposed in the laboratory to a dietary mixture of CECs representative of the predominant contaminants detected in the field. Liver mitochondrial content was reduced in fish exposed to CECs in the laboratory, which occurred concomitantly with a reduction in expression of peroxisome proliferator-activated receptor (PPAR)-γ coactivator-1a (*pgc-1α*), a positive transcriptional regulator of mitochondrial biogenesis. The laboratory exposures also caused elevation of state 4 respiration per unit mitochondria, which drove a reduction of efficiency of oxidative phosphorylation relative to controls. The mixture-induced elevation of respiration was associated with increased oxidative injury as evidenced by increased mitochondrial protein carbonyls, elevated expression of

glutathione (GSH) peroxidase 4 (*gpx4*), a mitochondrial-associated GSH peroxidase that protects against lipid peroxidation, and reduction of mitochondrial GSH. Juvenile Chinook sampled in from a WWTP effluent-impacted estuary with demonstrated releases of CECs showed similar trends toward reduced liver mitochondrial content and elevated respiratory activity per mitochondria (including state 3 and uncoupled respiration). Interestingly, respiratory control ratios were greater in fish from the contaminated site relative to fish from the reference site, which may have been due to differences in the timing of exposure to CECs under laboratory and field conditions. Collectively, these results demonstrate that exposure to environmentally-relevant mixtures of CEC can cause sublethal impacts on the bioenergetics of fish, including modulation of both quantity and quality of liver mitochondria.

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This work is dedicated to my fiancée, Michelle Redhair.

Introduction

Contaminants of emerging concern (CECs) in the Puget Sound aquatic environment

The Puget Sound of Washington state is an estuary surrounded by populated urban areas. As a result, these estuaries are receiving waters for a broad range of anthropogenic contaminants and pollutants. Several estuaries in Puget Sound have been designated as federal Superfund sites due to industrial activity resulting in environmental contamination with heavy metals (Williams et al., 2016), and persistent organic pollutants (POPs) (PSAT, 2016). An additional source input of chemical contaminants into the aquatic environment is the effluent from wastewater treatment plants (WWTPs). Each day, an estimated 1,347 million liters of WWTP effluent is discharged into the Puget Sound of Washington state (Washington Department of Ecology and Herrera Environmental Consultants, 2010). Many of the chemicals present in WWTP effluent are a result of the elimination and excretion of medicines, or the usage of common household products. Primary and secondary treatment processes remove or reduce the concentrations of many chemicals present in WWTP effluent. However, not all chemicals are removed completely, resulting in chemical concentrations in effluent at the parts per billion (ppb) or parts per trillion (ppt) level.

A broad range of structurally-diverse compounds, with varying modes of action within biological organisms, are present in WWTP effluent. These include personal care products and pharmaceuticals (PPCPs), perfluorinated compounds, synthetic and natural hormone-like compounds, alkylphenols and alkylphenol ethoxylate surfactants, current-use pesticides, and polybrominated diphenyl ether (PBDE) flame retardants. The aforementioned classes of

compounds are termed “contaminants of emerging concern” (CECs) due to critical data gaps regarding the ecological toxicity of these chemicals, and that they are currently unregulated and unmonitored in the environment. Although most state and federal agencies have reached consensus on which classes of chemicals are designated as CECs, the prioritization of specific compounds for monitoring is subjective (Diamond et al., 2011; Lubliner et al., 2010) and a unified monitoring program for CECs currently remains elusive.

Few published studies exist regarding the types, occurrence, and concentrations of CECs in the Puget Sound aquatic environment (Johnson A, 2004; Lubliner et al., 2010; Lower Columbia River Esuary Partnership, 2007; Pickett, 1997; Washington Department of Ecology and Herrera Environmental Consultants). State and federal agencies in this region have measured concentrations of PPCPs in WWTP influents, effluents, and aqueous and sediment samples from receiving waters (de Voogt et al., 2009; Johnson A, 2011; Lubliner et al., 2010). To a lesser extent, the concentrations of perfluorinated compounds (PFCs) have also been studied, but are still poorly characterized in Puget Sound (Furl and Meredith, 2010; Long et al., 2013).

In contrast to other classes of CECs, the toxicological effects and environmental concentrations of the highly lipophilic PBDE flame retardants have been well characterized (Donohue et al., 2008; Hale et al., 2003; Hites et al., 2004). PBDEs are a class of environmental contaminant which are of high priority in environmental monitoring programs conducted by NOAA, and EPA, among other regulatory groups (Environmental Protection Agency, 2010; Anderson et al., 2012). The most prevalent and persistent PBDE congener often detected in both wildlife and human samples is 2,2’,4,4’-tetrabromodiphenyl ether, or BDE 47 (Donohue et al., 2008), a known developmental

and neurological toxicant (Costa and Giordano, 2007; Giordano et al., 2008; Herbstman et al., 2010). Over the last decade, high levels of PBDEs have been detected in the edible portions of fish tissues, including Pacific salmon (Hites et al., 2004; S.M. O'Neill, 2004; Schecter et al., 2003; Sloan et al., 2010). Sloan *et al.* (2010) detected particularly high levels of PBDEs in juvenile Chinook salmon (*Oncorhynchus tshawytscha*) from the Puget Sound, and up to 65% of the total PBDE body burden was comprised of BDE 47 (Sloan et al., 2010). Thus, BDE 47 is an excellent model of an "emerged" contaminant that is currently monitored and relevant to the Puget Sound aquatic environment.

Effects of CECs in aquatic organisms

Hydrophobic CECs discharged in WWTP effluent can associate with sediment or suspended organic matter in the water column and ultimately accumulate in biological tissues. As the primary site of xenobiotic metabolism, and due to the high perfusion rate and exposure to chemicals, the liver is a target tissue of many CECs. All major classes of CECs have been reported to accumulate substantially in fish livers (Bay SM, 2011; Brooks et al., 2005; Hagenaars et al., 2008a; Hagenaars et al., 2008b; Lower Columbia River Esuary Partnership, 2007; Shao et al., 2010). Numerous hydrophobic CECs including PBDE flame retardants, hormones, certain PPCPs, and perfluorinated and industrial compounds, can also impact metabolic and endocrine function via interactions at cellular receptors in species ranging from invertebrates to mammals (Grun and Blumberg, 2007). Specifically in fish, perfluorinated compounds and the industrial compound bisphenol A are known to affect growth and adipogenesis. Impacts of emerging contaminants on these aforementioned physiological parameters can have implications on population fitness for certain aquatic species, including Pacific salmon in Puget Sound. For salmonids, body mass has

been linked to survival in wild fish (Meador, 2014; Spromberg and Meador, 2005). Significant reduction of growth was observed in juvenile rainbow trout (*Oncorhynchus mykiss*) exposed to ng/L concentrations of the alkylphenols nonylphenol and nonylphenol diethoxylate (Ashfield et al., 1998). Effluent-relevant concentrations of the antidiabetic medication metformin similarly causes significant growth reduction in male fathead minnow (Niemuth and Klaper, 2015), and acute exposure to 100 ng/L of the antidepressant pharmaceutical amitriptyline significantly reduces body length in larval zebrafish (Yang et al., 2014).

Hydrophilic CECs remain dissolved in the aqueous phase after discharged in WWTP effluent, and this could provide an exposure scenario for aquatic biota that is arguably equivalent to constant, low-level exposure (Connors et al., 2013). Hydrophilic PPCPs have been detected at ng/L concentrations in aqueous samples from WWTP effluent-impacted sites (Johnson A, 2012; Lubliner et al., 2010). Furthermore, several hydrophilic CECs including selective serotonin reuptake inhibitor (SSRI) pharmaceuticals have been detected at ng/g concentrations in fish obtained from field sites impacted by WWTP effluent (Brooks et al., 2005; Ramirez et al., 2009; Ramirez et al., 2007; Schultz et al., 2010). Importantly, modulation of behavioral responses has been reported in fish exposed to CECs at environmentally-relevant concentrations under controlled laboratory conditions. Reduced predator avoidance behavior was observed in larval fathead minnows (*Pimephales promelas*) exposed to ng/L concentrations of four antidepressant pharmaceuticals commonly detected in WWTP effluent (fluoxetine, sertraline, venlafaxine, and bupropion) (Painter et al., 2009). Exposure to ng/L concentrations of the antimicrobial personal care product triclocarban decreased aggression in adult male fathead minnow (Schultz et al., 2012). Increased activity, reduced sociality, and high feeding rates were observed in European perch

(*Perca fluviatilis*) exposed to 1.8 µg/L (representative of effluent concentrations) of the benzodiazepine oxazepam (Brodin et al., 2013). Exposure to 0.5-50 ng/L of the synthetic estrogen 17α-ethinylestradiol (EE_2), the active component in oral contraceptive pills, reduced aggression and courtship behaviors in adult male zebrafish (*Danio rerio*) (Colman et al., 2009). Adverse impacts on reproductive function in fish caused by CEC have also been reported, including increased estrogenic activity (Crago et al., 2016), altered gonad development (Niemuth and Klaper, 2015), and reduced fecundity (Lister et al., 2009). A recent report in juvenile coho salmon (*Oncorhynchus kisutch*) determined impacts on the hypothalamic-pituitary-gonad axis caused by exposure to 2 and 10 ng/L EE_2 and diluted WWTP effluent (Harding et al., 2016). Collectively, these results indicate that exposure to CECs can adversely affect behavior, reproductive function, and growth in fish. However, to date no biomarkers of exposure or effects of CECs have been established in any aquatic organism.

Mitochondrial toxicity of CECs

Mitochondria are essential energy-producing organelles present in all eukaryotic organisms, including ectotherms such as fish. Mitochondria are particularly enriched in high energy demand tissues like brain, heart, red muscle, kidney, and liver tissue due to their central role in cellular metabolism. In fish, mitochondria are also enriched in gill tissue, and are involved in swimming and motility, and growth and metabolism. Mitochondria play a critical role in numerous cellular pathways including fatty acid oxidation, regulation of intracellular calcium, and apoptosis. Arguably the most well-known function of mitochondria is in the generation of cellular ATP through the process of oxidative phosphorylation, which occurs in the inner mitochondrial membrane via function of the electron transport system (ETS) (**Figure i**).

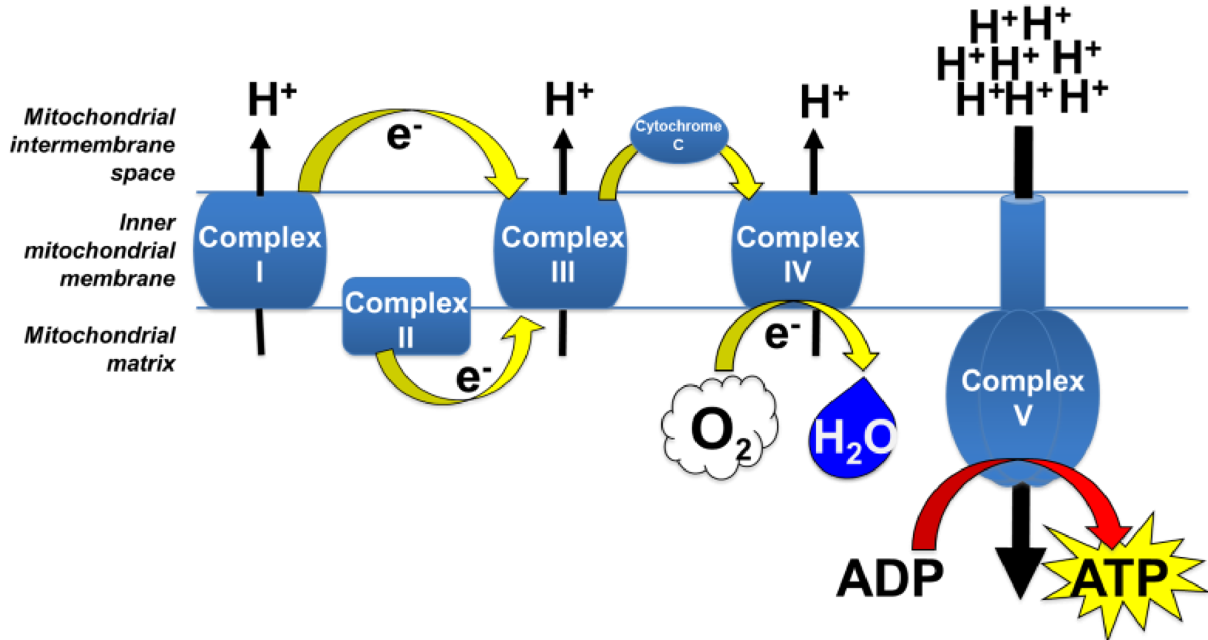

Figure i. Diagram of the mitochondrial electron transport system (ETS)

Although, as discussed, there are currently no established biomarkers of exposure of effect of CECs in fish, a number of CECs share a common mechanism of toxicity via mitochondrial dysfunction. For example, in human and fish liver cells, our laboratory has reported that BDE 47 induces cellular oxidative stress in association with significant loss of mitochondrial membrane potential ($\Delta\Psi_m$) (Shao et al., 2008a; Shao et al., 2008b). The hydroxylated BDE 47 metabolite 6-hydroxy-BDE 47 (6-OH-BDE47) decreases maximum respiratory capacity in isolated zebrafish mitochondria, suggesting toxicity to ETS components (van Boxtel et al., 2008). The perfluorinated compound N-acetyl-perfluorooctanesulfonamide (FOSAA) also inhibits maximum uncoupled respiration in isolated rat liver mitochondria, similarly suggesting functional impairment of the ETS (O'Brien and Wallace, 2004). The pharmaceutical ibuprofen specifically inhibits activity of complex I of the ETS in rat and human cell models (Sandoval-Acuna et al., 2012). Coupled respiration driven by complexes I and II (i.e., maximally-induced state 3 respiration) is

significantly decreased in isolated rat liver mitochondria by exposure to fluoxetine (Souza et al., 1994). Decreased state 3 respiration has also been reported in mouse, rat, and human cell models exposed to bisphenol A (Moon et al., 2012; Nakagawa and Tayama, 2000). Exposure of rat liver mitochondria to the pharmaceutical triclosan increased respiration due to proton leak across the mitochondrial membrane (i.e., state 4 respiration) (Newton et al., 2005), and potently uncouples mitochondria as demonstrated in studies involving human cells and zebrafish (Shim et al., 2016; Weatherly et al., 2016). Collectively, candidate indicators of mitochondrial dysfunction in aquatic organisms induced by CECs may include: oxidative stress, decreased $\Delta\Psi_m$, inhibition of specific respiratory complexes of the ETS, and modulation of respiratory control (e.g., state 3, state 4, and uncoupled respiration).

Summary

Unregulated and/or unmonitored environmental contaminants, termed CECs, originate from common household products and are discharged into receiving waters via WWTP effluents. Locally, there is poor understanding of: 1) the types and concentrations of CEC accumulating in Puget Sound ecosystems, and 2) the impacts of the predominant CEC on aquatic biota. Arguably, among the major classes of CECs, the PBDE flame retardants are the most well-characterized with respect to toxicological effects and environmental concentrations. BDE 47 is the predominant PBDE congener detected in Puget Sound Chinook salmon, making it a suitable model of an "emerged" contaminant relevant to Puget Sound. Mitochondrial dysfunction is a mechanism of toxicity shared among several high-priority CECs including BDE 47. Numerous reports in human and mammalian models suggest that examination of mitochondrial function, particularly that of

the ETS, may be a critical aspect in understanding the impacts of BDE 47 and other CECs on aquatic organisms.

Chapter 1

Effect of omega-3 fatty acid oxidation products on the cellular and mitochondrial toxicity of BDE 47

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Abstract

High levels of the flame retardant 2,2',4,4'-tetrabromodiphenyl ether (BDE 47) have been detected in some Pacific salmon sampled near urban areas, raising concern over the safety of salmon consumption. However, salmon are also enriched in the antioxidants eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), and the oxidation products of EPA and DHA are critical mediators of protective cellular antioxidant responses. Because oxidative stress is a mechanism of BDE 47 toxicity, we tested the hypothesis that oxidized EPA and DHA can ameliorate the cellular and mitochondrial toxicity of BDE 47. HepG2 cells were treated with a mixture of oxidized EPA and DHA (oxEPA/oxDHA) at a ratio relevant to salmon consumption (1.5/1 oxEPA/oxDHA) to induce a maximal cellular antioxidant response as assessed by glutathione (GSH) biosynthesis, followed by exposure to BDE 47 (100 μM) for 24 h. Pretreatment with oxEPA/oxDHA for 12 h

prior to BDE 47 exposure prevented BDE 47-induced depletion of GSH, and significantly increased expression of protective cellular antioxidant response genes. The antioxidant response was associated with partial but significant protection against BDE 47-induced loss of viability, and mitochondrial membrane potential. Examination of mitochondrial electron transport system function revealed extensive inhibition of State 3 respiration and maximum respiratory capacity by BDE 47 were partially reversed by oxEPA/oxDHA. Additionally, oxEPA/oxDHA significantly prevented BDE 47-induced ROS production for 12 h, but no longer attenuated ROS production by 24 h BDE 47 exposure. Our findings indicate that the antioxidant effects of oxEPA/oxDHA protect against short exposures to BDE 47, including a protective role of these compounds on maintaining cellular and mitochondrial function.

1.0 Introduction.

Polybrominated diphenyl ether (PBDE) flame retardants are common lipophilic organic contaminants present in environmental media and human samples (Gomara et al., 2011; Hale et al., 2003; Miller et al., 2012). Despite governmental bans on the new manufacture of these compounds (Legislatures, 2012), most PBDE congeners are resistant to degradation and continue to persist in the environment (Darnerud, 2003). The fact that PBDEs are often detected in human blood, adipose tissue, and breast milk underscores the risk of potential toxic effects (Donohue et al., 2008; Herbstman et al., 2010; Jakobsson et al., 2012). Uptake of PBDEs from household dust appears to be the primary route of exposure for humans (Johnson et al., 2010; Meeker et al., 2009; Stapleton et al., 2012), but significant exposures can also occur through dietary consumption of contaminated meat, poultry, and especially seafood (Hites et al., 2004; Lee et al., 2013; Ohta et al., 2002; Schecter et al., 2003; Tittlemier et al., 2004). Elevated levels of PBDEs have been detected in the edible tissues of several food fish, including Pacific salmon (Hites et al., 2004; S.M. O'Neill, 2004; Schecter et al., 2003; Sloan et al., 2010). This may pose a particular problem for certain populations, including Tribal Nations, and Asian and Pacific Islander populations, whose dietary customs of high rates of fish consumption put them at particularly high risk of significant exposures to PBDEs and other persistent contaminants (Ecology, 2013). For this reason, a major focus for assessing the safety of human consumption of marine seafood species is to measure the toxicological effects of PBDEs found in fish.

The most prevalent and persistent PBDE congener detected in human and wildlife samples is 2,2’,4,4’-tetrabromodiphenyl ether (BDE 47) (Donohue et al., 2008; Hites et al., 2004), which may

comprise up to 65% of the total PBDE body burden in salmon (Sloan et al., 2010). The dominance of BDE 47 among other congeners in salmon is likely a result of several factors, including its bioaccumulation potential, resistance to debromination, and that it can occur as a debromination product of other higher molecular weight PBDE congeners (Browne et al., 2009; Stapleton et al., 2004). BDE 47 is a developmental and neurological toxicant (Costa and Giordano, 2007; Giordano et al., 2008; Herbstman et al., 2010) and is reported to disrupt thyroid hormone status (Branchi et al., 2003). The mechanisms of BDE 47 toxicity are poorly understood, but substantial evidence indicates the induction of cellular oxidative stress, via generation of reactive oxygen species (ROS), may play a major role (An et al., 2011; He et al., 2008a; He et al., 2008b; Shao et al., 2010; Yan et al., 2011). Importantly, BDE 47-induced oxidative stress is associated with a loss of cellular viability and proliferation, and a loss of mitochondrial membrane potential (Park et al., 2014; Shao et al., 2008a; Shao et al., 2008b; Yan et al., 2011).

Despite concerns about the presence of lipophilic toxicants, consumption of most marine fish is considered to be highly beneficial to human health. Salmon, in particular, are rich dietary sources of high quality protein, vitamins, and the beneficial antioxidant micronutrients known as omega-3 polyunsaturated fatty acids. The two major omega-3 fatty acids in marine fish oil are eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). Salmon fillets rank among the highest EPA+DHA/weight ratios among food species of fish (Kris-Etherton et al., 2000), and are estimated to contain 4.75 g of omega-3s in an average 180 g meal (Domingo et al., 2007; Palacios-Pelaez et al., 2010). Omega-3s are highly labile, and auto-oxidation of these compounds occurs readily under ambient environmental conditions both *in vitro* and *in vivo* (Musiek et al., 2008). Peroxidation of EPA and DHA by free radicals and ROS generates electrophilic cyclopentenone

isoprostanes (Gao et al., 2007; Musiek et al., 2008). Recent evidence suggests these products of oxidized EPA and oxidized DHA (oxEPA and oxDHA) may be critical mediators of the beneficial human health effects of fish oil omega-3s (Anderson and Taylor, 2012; Majkova et al., 2011), as oxEPA and oxDHA can activate nuclear factor erythroid 2-related factor 2 (Nrf2), leading to the upregulation of a suite of antioxidant genes that function to maintain cellular redox status (Kensler et al., 2007) and also glutathione (GSH). In this regard, GSH has been shown to modulate the toxicity of PBDEs, including BDE 47 (Giordano et al., 2008; He et al., 2008a; Shao et al., 2008b). Hence, it has been proposed that the activation of Nrf2-regulated cellular antioxidant responses via oxEPA and oxDHA can be a protective mechanism against the progression of diseases with a cellular oxidative stress etiology (Bousquet et al., 2009; Palacios-Pelaez et al., 2010).

The fact that fish omega-3s are potent cellular antioxidants, whereas a major mechanism of BDE 47 cell injury is oxidative damage, provides a scenario in which omega-3s may chemoprotect against the toxicity of co-consumed PBDEs. This hypothesis is supported by studies showing that induction of intracellular GSH by the antioxidant *N*-acetylcysteine (NAC), a GSH precursor, protects human fetal hematopoietic stem cells (Shao et al., 2008b), human hepatocytes (An et al., 2011), and T lymphocyte cell lines (Yan et al., 2011) against BDE 47 toxicity. Others have demonstrated that oxidative stress-mediated inflammation resulting from *in vitro* exposure to BDE 47 (Park et al., 2014; Park and Loch-Caruso, 2014) and similar persistent organic contaminants (Majkova et al., 2011) can be reduced by treatment with dietary antioxidant compounds, including via activation of Nrf2 by free radical-oxidized EPA and DHA.

In the present study we investigated the hypothesis that the activation of cellular antioxidant responses by a mixture of oxidized omega-3s relevant to dietary exposures (1.5/1 oxEPA/oxDHA) can ameliorate the toxicity of BDE 47. Among other relevant target systems of PBDE toxicity, including cells of the developmental (Schreiber et al., 2010) and nervous systems (Giordano et al., 2009; Giordano et al., 2008), the liver is a major target organ of BDE 47 toxicity and receives extensive PBDE exposures through dietary routes. Hence, chemoprotection against BDE 47-induced cellular toxicity and mitochondrial dysfunction were investigated in the human hepatocellular carcinoma cell line HepG2, a model in which activation of the Nrf2 antioxidant response via oxEPA and oxDHA has been previously characterized (Gao et al., 2007). Our approach involved characterizing the modulatory effects of oxEPA/oxDHA on specific functional components of the mitochondrial electron transport system under conditions of BDE 47 exposure.

2.0 Methods and Materials.

2.1. Chemicals and reagents

BDE 47 (2,2′,4,4′-tetrabromodiphenyl ether, >99% purity) was obtained from AccuStandard, Inc. (New Haven, CT, USA). 5-Sulfosalicylic acid dehydrate (SSA), Napthalinedicarboxyaldehyde (NDA), dimethyl sulfoxide (DMSO), eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA) were obtained from Sigma-Aldrich Corp (St. Louis, MO, USA). Sulforaphane (SFN) (R-Sulforaphane) was obtained from LKT Laboratories, Inc. (St. Paul, MN, USA). 2,2'-azobis-2-methyl-propanimidamide, dihydrochloride (AAPH) was obtained from Cayman Chemical (Ann Arbor, MI, USA). 2',7'-dichlorodihydrofluorescein diacetate (H_2DCFDA) was obtained from Life

Technologies (Grand Island, NY, USA). All cell culture reagents were obtained from Invitrogen, GIBCO (Carlsbad, CA, USA).

2.2. Preparation of oxidized omega-3s

The ratios of omega-3s reported in salmon fillets (1.5/1 EPA/DHA) are relatively consistent and are often reflected in the ratio of EPA/DHA in many commercially-available omega-3 dietary supplements. A ratio of 1.5/1 EPA/DHA is often used in clinical trials investigating the effects of omega-3 dietary supplementation (Jackson et al., 2012; Viral Brahmbhatta, 2013; Wojtowicz et al., 2011), and this ratio was also used in our experiments. EPA, DHA, and SFN were dissolved in DMSO, and frozen stocks were held at -20 °C. Methods for preparation and dosing with oxEPA/oxDHA were conducted as described by Majkova *et al.*, 2011 (Majkova et al., 2011), with minor modifications. For each experiment, EPA and DHA were oxidized separately by diluting the fatty acid to 1 mM in PBS containing 2 mM of the free radical generator AAPH, and incubated at 37 °C for 16 h. After oxidation, oxEPA and oxDHA were combined as a 1.5/1 mixture, sterile filtered, and diluted in Minimum Essential Media α (MEMα) containing 5% FBS such that the final concentrations in the pretreatment media were 60 and 40 µM of oxEPA and oxDHA, respectively.

2.3. Cell culture, and in vitro exposures

HepG2 human hepatocellular carcinoma cells were purchased from American Type Culture Collection (ATCC, Rockville, MD, USA). HepG2 cells were cultured in MEMα media supplemented with 5% fetal bovine serum (FBS), 1% HEPES buffer, and 1% penicillin-streptomycin. Cells were maintained in 75 cm^2 flasks at 37 °C and 95% CO_2 and split at 90-100%

confluency every five days. For all experiments, cells were seeded into wells at a density such that confluency was ≥80% at time of dosing (48 h after seeding). MEMα pretreatment media contained 5% FBS and the oxEPA/oxDHA mixture, SFN, or an equal volume of DMSO (vehicle control). For all experiments, the final volume of DMSO in media was <0.4%. Cells were treated with oxEPA/oxDHA for a time period to induce a maximal antioxidant response. Pretreatment media was then aspirated and cells were washed with PBS. Cells were then exposed to 50 or 100 μM BDE 47 (or DMSO) in serum-free MEMα media for a period of 24 h, unless specified otherwise. These toxicant concentrations were selected based on our previous studies in human cells (Shao et al., 2008b), as well as range-finding experiments in HepG2 cells, to elicit a moderate 50-75% loss in cell viability. The effect of oxEPA/oxDHA on BDE 47 cell toxicity was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cell viability assay (Mosmann, 1983) in three separate experiments, each containing three replicate treatments.

2.4. Glutathione status

Intracellular GSH was measured after experimental treatment with DMSO or antioxidants to determine an exposure time to elicit a robust antioxidant response in HepG2 cells. Following these optimization experiments, GSH was then measured in cells following pretreatment with DMSO or oxEPA/oxDHA for the determined exposure period, and subsequent toxicant challenge with BDE 47 (100 μM) for 24 h. Briefly, cells were washed with PBS, detached with 0.25% trypsin-EDTA (Invitrogen), and pelleted by centrifugation at 800 rpm for 5 min at 4 ˚C. Cells were resuspended in ice-cold PBS and lysed on ice by sonication. Redox status of reduced glutathione (GSH) was ensured by acidification of cell lysates in 5% SSA as described (Giordano et al., 2011), and lysates were immediately stored at -80 °C. GSH concentrations in acidified lysates were determined by

conjugation with NDA, which generates a stable fluorescent product detected at excitation 472 nm/emission 528 nm (Giordano et al., 2011). GSH concentrations were normalized to total protein by the Bio-Rad Protein Assay (Bio-Rad, Hercules, CA, USA).

2.5. Quantitative PCR analysis of oxidative stress marker genes

Two inducible antioxidant genes under Nrf2 regulation were selected as markers of oxidative stress elicited by BDE 47: glutamate-cysteine ligase catalytic subunit (*GCLC*) and NAD(P)H-quinone oxidoreductase 1 (*NQO1*). After pretreatment with or without oxEPA/oxDHA (12 h), followed by exposure to BDE 47 or DMSO (24 h), mRNA transcript levels of *gclc* and *nqo1* were measured in cells. Cells were harvested in TRIzol® reagent and immediately stored at -80°C. Procedures for isolation of total RNA, cDNA synthesis, PCR primer product validation, and subsequent quantitative PCR analyses with SYBR Green were conducted as described (Espinoza et al., 2012). Primer sequences and information are presented in **Table 1**. Expression of glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) did not differ between treatment groups and was used for normalization purposes. Measurements were carried out in three separate experiments, each containing three replicate treatments.

2.6. Mitochondrial membrane potential assay

Mitochondrial membrane potential ($\Delta\Psi_m$) was assessed using the fluorescent probe JC-1 (Cayman Chemical, Ann Arbor, MI, USA). Following pretreatment with or without oxEPA/oxDHA (12 h), followed by exposure to BDE 47 or DMSO (24 h), cells were then incubated for 1 h with JC-1 probe (diluted 1:10 in the media of each well). Quantification of $\Delta\Psi_m$ was based on the ratio of red fluorescent J-aggregates to green fluorescent J-monomers, detected at 560 nm/595 nm and 485

nm/535 nm excitation/emission, respectively. Measurements were carried out in three separate experiments each containing three replicate treatments using a 96-well fluorescent plate reader.

2.7. Assessment of electron transport system function

Electron transport system (ETS) functional parameters were measured in saponin-permeabilized HepG2 cells by high-resolution respirometry using the Oxygraph-2k (Oroboros Instruments, Innsbruck, Austria). After BDE 47 exposure, with or without pretreatment with oxEPA/oxDHA, cells were harvested and resuspended in 1 mL of ice-cold respiration buffer: distilled H_2O, 0.25 M mannitol, 10 mM $MgCl_2$, 10 mM $KHPO_4$ buffer, pH=7.2. Solubility of oxygen in respiration buffer was assumed to be 0.920 µmol/L, and the oxygraph chambers were set to 30 ˚C and calibrated by determining dissolved oxygen in 2.2 mL respiration buffer under ambient atmospheric conditions as described by Gnaiger *et al.* (Gnaiger, 2009). Cells were permeabilized on ice with 12 µL of permeabilization buffer (50 µg/mL saponin in respiration buffer, 20 min, 4 °C) prepared prior to each experiment.

Respiratory substrates and inhibitors were added sequentially in the following order: 5 mM pyruvate, 2 mM malate, 10 mM glutamate, and 2.5 mM ADP (to induce State 3 respiration with complex I substrates only); 10 mM succinate (to induce maximum State 3 respiration with substrates of complexes I and II); 2.5 µM oligomycin (to induce proton leak, or State 4 respiration); 2.5 µM CCCP (to induce maximum respiratory capacity, or uncoupled respiration); 0.5 µM rotenone (to measure uncoupled respiration with complex I inhibition, i.e., uncoupled flux through complex II); 2.5 µM antimycin A (to determine non-mitochondrial respiration); 0.5 mM N,N,N′,N′-tetramethyl-ρ-phenylenediamine (TMPD), 2 mM ascorbate (to determine flux through

complex IV); 1 mM potassium cyanide (normalization by inhibition of complex IV) (Siegel et al., 2011). The non-mitochondrial rate of oxygen consumption was subtracted from all measured functional parameters before reporting. Similarly, the rate of oxygen consumption after addition of potassium cyanide was subtracted from the rate of flux through complex IV for normalization purposes. Respiratory control ratios (RCRs) were determined for all treatment groups as the ratio of maximally-induced State 3 respiration/State 4 respiration.

2.8. Reactive oxygen species (ROS) production

Generation of ROS was evaluated using the fluorescent probe 2',7'-dichlorodihydrofluorescein diacetate (H_2DCF-DA) (Life Technologies, Carlsbad, CA, USA). Cells were plated in black 96-well plates, pretreated with or without oxEPA/oxDHA, and subsequently exposed to BDE 47 (or DMSO) for: 0.5 min, 1.5 min, 3, 6, 12, and 24 h. Cells pretreated with DMSO (12 h) and subsequently exposed to hydrogen peroxide (250 μM) were used as a positive control for ROS production at each time point. After each exposure period, cells were directly incubated with 10 μM H_2DCF-DA for 30 min at 37 °C. Intracellular ROS production is correlated to cellular oxidation of H_2DCF-DA to the highly fluorescent 2,7-dichlorofluorescein (DCF). Oxidation of H_2DCF-DA to DCF was measured in a fluorescence plate reader at 485 excitation/530 emission. Fluorescence was normalized to total protein by the Bio-Rad Protein Assay. Measurements were carried out in three separate experiments, each containing three replicate treatments.

2.9. Statistical analyses.

All statistical analyses were conducted in GraphPad Prism Ver. 5.0 (Graph Pad Software Inc., San Diego, CA, USA). The effect of treatments on cellular GSH status, gene expression, cell viability,

mitochondrial membrane potential, mitochondrial ETS functional parameters, and ROS production were assessed by One-way ANOVA with a Bonferonni's Multiple Comparisons post-test. Differences between means of control and treatment groups were considered significant at $p \leq 0.05$, and homogeneity of variances was confirmed by F test. All data sets were examined for potential outliers using the Grubb's test (Grubbs, 1969), and outlier values were excluded at significance level $p < 0.05$.

3.0 Results.

3.1. Glutathione status

Treatment of HepG2 cells with oxEPA/oxDHA for 12 h significantly increased intracellular GSH to two-fold control levels **(Fig. 1A**). The level of GSH induction at 12 h by oxEPA/oxDHA exceeded that observed for SFN, a positive control of GSH induction, although the potency of GSH induction by oxEPA/oxDHA relative to SFN was not statistically different ($p > 0.05$). Because the elevation in intracellular GSH by oxEPA/oxDHA was consistent over 12-18 h of incubation, we selected a 12 h oxEPA/oxDHA pretreatment for all subsequent chemoprotection experiments. GSH was measured in cells after pretreatment with or without oxEPA/oxDHA for 12 h, and subsequent exposure to BDE 47 (or DMSO) for 24 h. After oxEPA/oxDHA treatment, and in the absence of toxicant, GSH levels were 1.8-fold above those of control cells (**Fig. 1B)**. Exposure to BDE 47 in the absence of antioxidants elicited a 39% increase in GSH relative to controls, although the level of induction was not statistically significant. By contrast, GSH levels were 2.2-fold above control levels in cells pretreated with oxEPA/oxDHA before BDE 47 exposure (**Fig. 1B**).

3.2. Effect of treatments on antioxidant gene expression

As observed in **Figure 2**, exposure to BDE 47 caused a significant modulation of *gclc* and *nqo1* mRNA expression. Specifically, BDE 47 increased *gclc* expression to 3.3-fold control levels (**Fig. 2A**), and also reduced *nqo1* expression by 25% relative to controls (**Fig. 2B**). By contrast, both *gclc* and *nqo1* were significantly induced in cells pretreated with oxEPA/oxDHA prior to BDE 47 exposure. Specifically, a 4-fold induction of *gclc* relative to controls (**Fig. 2A**), and a minor 24% increase in *nqo1* (**Fig. 2B**) was observed in cells pretreated with oxEPA/oxDHA and challenged with BDE 47. Expression of *gclc* and *nqo1* in cells treated with oxEPA/oxDHA before incubation in the absence of toxicant for 24 h did not differ from controls.

3.3. Effect of treatments on HepG2 cell viability

Treatment of cells with oxEPA/oxDHA under conditions that elevated intracellular GSH concentrations (i.e. 12 h pretreatment) was associated with a minor, albeit significant 13% increase in cell viability in the absence of toxicant **(Fig. 3)**. As observed, pretreatment with oxEPA/oxDHA prior to exposure to BDE 47 provided partial protection against BDE 47-induced loss of cell viability **(Fig. 3)**. Specifically, the 50 µM dose of BDE 47 caused a 55% loss of viability in HepG2 cells, and oxEPA/oxDHA pretreatment reduced the observed loss in viability to 48%, which was not statistically significant (p=0.052). At the 100 µM dose, BDE 47 caused a 67% loss of viability, and oxEPA/oxDHA pretreatment provided a minor, albeit significant 6% protection of viability **(Fig. 3)**.

3.4. Effect of treatments on mitochondrial membrane potential and electron transport system function

Exposure to BDE 47 (100 μM) for 24 h resulted in a significant 22% loss of mitochondrial membrane potential ($\Delta\Psi_m$) **(Fig. 4).** By contrast, pretreatment of HepG2 cells with oxEPA/oxDHA fully protected against the loss of $\Delta\Psi_m$ by the toxicant. Pretreatment of HepG2 cells with oxEPA/oxDHA in the absence of BDE 47 had no effect on $\Delta\Psi_m$. The loss of $\Delta\Psi_m$ elicited by BDE 47 was accompanied by a decline in mitochondrial respiratory capacity **(Fig. 5)**. As observed in the $\Delta\Psi_m$ experiments, the decline in mitochondrial respiratory capacity was partially mitigated by pretreatment with oxEPA/oxDHA. Mitochondrial State 3 respiration using complex I substrates was reduced by 54% following BDE 47 exposure, and oxEPA/oxDHA pretreatment reduced the magnitude of loss by 33% **(Fig. 5A)**. Maximally-induced State 3 respiration using complex I+II substrates was also reduced by 59% following BDE 47 exposure **(Fig. 5B)**, with oxEPA/oxDHA attenuating this reduction by 20% **(Fig. 5B)**. Maximum respiratory capacity as measured under conditions of uncoupled mitochondrial respiration was decreased by 66% following BDE 47 exposure, and oxEPA/oxDHA attenuated the BDE 47-associated decrease in maximum uncoupled respiration by 14% **(Fig. 5C)**. State 4 respiration was reduced by 25% following BDE 47 exposure, but this effect was not statistically significant **(Fig. 5D)**. No modulation of State 4 respiration was observed in the group pretreated with oxEPA/oxDHA prior to BDE 47 exposure. Exposure to BDE 47 reduced oxygen flux capacity through complexes II and IV by 32% **(Fig. 5E)**, and 28% **(Fig. 5F)**, respectively. Pretreatment with oxEPA/oxDHA partially prevented the loss of flux through complexes II and IV by 9%, and 20%, respectively. BDE 47 exposure reduced the respiratory control ratios (RCR) of HepG2 cells by 41%, and pretreatment with oxEPA/oxDHA prior to BDE 47 exposure did not prevent loss of RCR **(Fig. 5G)**. **Table 2** summarizes the effects on mitochondrial ETS functional parameters cause by BDE 47 exposure, with and without oxEPA/oxDHA pretreatment.

3.5. ROS production

Treatment with oxEPA/oxDHA in the absence of toxicant did not modulate ROS production relative to control cells. By contrast, exposure of cells to BDE 47 in the absence of antioxidants caused a significant 60% increase in ROS production relative to controls by 24 h exposure. As observed in **Figure 6**, the greatest increase in BDE 47-stimulated ROS generation (31% increase) occurred rapidly after 1.5 h exposure to BDE 47 (**Fig. 6**). By contrast, oxEPA/oxDHA pretreatment prior to BDE 47 exposure prevented the BDE 47 stimulated ROS production observed at 1.5 h, and significantly limited BDE 47-induced ROS generation for 12 h such that levels were not statistically different from controls (**Fig. 6**). By 24 h, however, the protective effect of the antioxidants diminished and there were no differences in ROS production among antioxidant pretreated and control cells in the presence of BDE 47.

4.0. Discussion.

In the present study we have demonstrated the protective interaction among the major antioxidants and a major contaminant present in Pacific salmon, relevant to a scenario involving salmon consumption. Our results are consistent with reports that oxidized omega-3s play a critical role in activating beneficial cellular antioxidant responses (Gao et al., 2007; Majkova et al., 2011; Musiek et al., 2008). The fact that oxEPA/oxDHA induced intracellular GSH in HepG2 cells more effectively than treatment with sulforaphane, a model dietary isothiocyanate and a positive control for Nrf2 activation and GSH induction (Ahn et al., 2010; Majkova et al., 2011), and that oxEPA/oxDHA increased overall cell viability in the absence of toxicant, are noteworthy.

Treatment with oxEPA/oxDHA stimulated a significant increase of GSH that remained stably elevated in cells for 24 h after removal of the treatment media, whereas expression of *gclc* and *nqo1* were similar to control levels 24 h after initial treatment. This finding suggests oxEPA/oxDHA stimulates a biphasic effect on expression of Nrf2-regulated genes, inducing an initial increase in expression that eventually returns to near control levels. Indeed, similar studies using HepG2 cells treated with dietary antioxidants are consistent with the observed effects of oxEPA/oxDHA, reporting a biphasic increase and subsequent decrease toward control levels of Nrf2-mediated antioxidant response gene expression, including *gclc* (Gong and Cederbaum, 2006; Huerta-Olvera et al., 2010; Kay et al., 2010), by 8-12 h after initial treatment. Taken together, the lasting stimulatory effect on GSH status, and protection against BDE 47-induced depletion of GSH, indicates the activation of cellular antioxidant responses by oxEPA/oxDHA is associated with protective functional impacts in cells.

We hypothesized a more dramatic chemoprotective effect of oxidized omega-3s on cell viability than the partial protection observed in the current study. However, the partial protection of the oxidized omega-3s against cell toxicity is consistent with the extent of antioxidant-mediated amelioration reported in previous studies of human cells exposed to BDE 47. For example, pretreatment of human fetal hematopoietic stem cells with 8 mM NAC induced a significant, but partial 20% protection of viability against 50 μM BDE 47 (Shao et al., 2008b). Similarly, co-treatment of Jurkat cells with 5 mM NAC has been shown to reduce the rate of apoptosis induced by 50 μM BDE 47 from 14% to 3% (Yan et al., 2011). The partial chemoprotective effect of oxEPA/oxDHA against 50 and 100 μM BDE 47-induced loss of viability observed in HepG2 cells

may reflect the fact that BDE 47 cell toxicity involves multiple mechanisms, including those other than oxidative stress, and are thus not mitigated by antioxidant responses.

As discussed, a major goal of this study was to better understand the mechanisms of BDE 47-mediated mitochondrial injury. Previous studies in our laboratory using both human and fish cells indicated BDE 47-induced generation of ROS is associated with a loss of $\Delta\Psi_m$ (Shao et al., 2008a; Shao et al., 2008b). Significant loss of $\Delta\Psi_m$ can activate mitochondrial-regulated apoptosis pathways or cellular necrosis via opening of the mitochondrial permeability transition pore (MPTP) (Lemasters et al., 2009). Interestingly, the onset of MPTP opening can be delayed by dietary supplementation with DHA (Khairallah et al., 2010; O'Shea et al., 2009), supporting a role of DHA in stabilizing $\Delta\Psi_m$. Dietary supplementation with omega-3s, particularly DHA, is reported to have significant beneficial effects on mitochondrial membranes and function (Khairallah et al., 2012). Our results suggest the oxidation products of omega-3s may mediate some of the beneficial effects on mitochondrial membranes by significantly limiting loss of $\Delta\Psi_m$ from BDE 47 exposure.

An interesting finding from our study was that exposure of HepG2 cells to BDE 47 reduced all electron transport system functional parameters with the possible exception of State 4 respiration, although we observed a similar trend toward reduction of State 4 respiration in the presence of 100 μM BDE 47 as well. The fact that extensive reduction of State 3 and maximally-induced State 3 respiration was observed following BDE 47 exposure, suggests inhibition of the ETS within complexes I-V, or a loss of electron transfer capacity of the ETS. Although we did not determine the effect of BDE 47 on complex V, we observed significant loss of maximum uncoupled respiration, which indicates impairment within complexes I-IV. In this regard, a reduction of

oxygen flux capacity through complexes I, II, and IV by BDE 47 was verified in our experiments. Additionally, the significant reduction of uncoupled respiration supports the hypothesis that BDE 47 causes a loss of respiratory capacity of the ETS.

A previous report in isolated zebrafish mitochondria exposed to the hydroxylated BDE 47 metabolite 6-hydroxy-BDE 47 (6-OH-BDE47) showed a significant inhibition of complex II function and uncoupling of mitochondrial respiration (van Boxtel et al., 2008). By contrast, our data did not support a specific antagonistic effect of BDE 47 against a particular ETS complex, as we observed loss of respiratory capacity occurring at several complexes. Rather, BDE 47 caused a general inhibition of the ETS in HepG2 cells, significantly reducing State 3 respiration and maximum uncoupled respiratory capacity. A previous report in isolated rat mitochondria exposed to 25 and 50 μM BDE 47 similarly demonstrated a reduction of State 3 respiration using complex I and complex II substrates (Pazin et al., 2014), consistent with our findings. Further experiments are needed to elucidate the mechanisms underlying the observed effects on complexes III and V in BDE 47-induced loss of ETS function. The observed protection of State 3 and maximally-induced State 3 respiration suggests the antioxidant effects of oxEPA/oxDHA play a beneficial role of preserving coupling of oxidative phosphorylation in mitochondria. Also, the protection of maximum uncoupled respiration indicates oxEPA/oxDHA can prevent against loss of function within complexes I-IV. Indeed, partial protection of flux capacity through complexes II and IV was observed, and the most extensive protective effect of oxEPA/oxDHA was observed for complex I (i.e. rescue of State 3 respiration using complex I substrates). Rescue of respiratory coupling by oxEPA/oxDHA may also involve beneficial protective effects on complexes III and V, but this was not determined in the current study.

The significant reduction of maximally-induced State 3 respiration in BDE 47-exposed HepG2 cells resulted in significant reduction of the RCR, a measure of coupling of oxidative phosphorylation (Brand and Nicholls, 2011). Compared to this treatment group, nearly identical RCRs were calculated in our study in cells pretreated with oxEPA/oxDHA before BDE 47 treatment. This suggests that oxEPA/oxDHA did not prevent loss of overall mitochondrial respiratory function caused by BDE 47, despite significant protection of critical respiratory parameters in mitochondria. The lack of a more marked protective effect on mitochondrial function by oxEPA/oxDHA supports the hypothesis that other mechanisms unrelated to oxidative stress may contribute to BDE 47 mitochondrial toxicity in HepG2 cells. However, the fact that oxEPA/oxDHA antioxidant induction was associated with some preservation of respiratory function in HepG2 cells strongly suggests a role of oxidative stress in the toxicity of BDE 47 to the mitochondrial ETS.

A limitation of our study is that we did not conduct the relevant co-treatment of cells with oxEPA/oxDHA and BDE 47, as this exposure scenario can be similarly representative of dietary intake of contaminated seafood. However, our approach was to better understand if an antioxidant response elicited by the dietary antioxidants would be sufficient to ameliorate BDE 47 toxicity. Thus, we preferred to pretreat cells with oxEPA/oxDHA to elicit the antioxidant response and subsequently challenge with BDE 47 as in the context of a chemoprevention study. We also wanted to minimize the potential for artifacts from co-administration, specifically with regards to the presence of serum in the treatment media, which can decrease the bioavailability of lipophilic toxicants including BDE 47 *in vitro* (Hestermann et al., 2000; Mundy et al., 2004).

Supplementation of the pretreatment exposure media with 5-10% FBS was required for administration of oxEPA/oxDHA to HepG2 cells (Gao et al., 2007), whereas BDE 47 exposure media utilized in similar *in vitro* studies are supplemented with very low concentrations of serum (1%) (Park et al., 2014; Park and Loch-Caruso, 2014), or is serum-free (He et al., 2008b; Tagliaferri et al., 2010). Thus, co-treatment of cells with oxEPA/oxDHA and BDE 47 should be addressed in future experiments, but was somewhat out of the scope of the current manuscript due to reasons including the aforementioned differences in experimental exposure methods that are required for administering these compounds to cells.

It is important to note that the doses of BDE 47 utilized in the current study were selected to induce significant cellular and mitochondrial toxicity in HepG2 cells based on previous studies in human cells (An et al., 2011; Shao et al., 2008b; Yan et al., 2011). In human (Park et al., 2014; Park and Loch-Caruso, 2014), fish (Shao et al., 2010; Shao et al., 2008a), and rodent cells (He et al., 2008a) exposed to BDE 47, oxidative injury is not typically observed below concentrations in the 20-50 μM range. Hence, it is plausible that the antioxidant effects of oxEPA/oxDHA may provide greater protection against, or ameliorate, the toxicity of lower doses of BDE 47 representative of environmental exposures. Additionally, the fact that oxEPA/oxDHA significantly attenuated BDE 47-induced ROS production for 12 h suggests that cellular toxicity resulting from a dose of 100 μM BDE 47 for an exposure period of 24 h selected in our experiments may have overwhelmed the protective antioxidant effects of oxEPA/oxDHA. Therefore, we hypothesize that oxEPA/oxDHA pretreatment can ameliorate or have a greater protective effect against shorter subsequent exposure periods to BDE 47 (i.e. 0.5-12 h) than assessed in the current study.

While experimental PBDE doses in the micromolar range are relevant to toxicological studies, these doses likely far exceed PBDE levels reaching target human tissues from fish consumption (Hayward et al., 2007; Hites et al., 2004; Jenssen et al., 2007; Pulkrabova et al., 2007; Stone, 2006). The fact that an average meal size of 227 grams of salmon contains approximately $1x10^6$-fold more omega-3s on a wet weight basis than persistent organic such as PBDEs (Domingo et al., 2007) suggests that *in vitro* results from studies such as ours likely underestimate the chemoprotection elicited by omega-3s *in vivo*. In this regard, it is of importance that risk assessment paradigms addressing the adverse effects of persistent compounds in seafood that elicit oxidative stress as a mechanism of toxicity account for the protective antioxidant effects from omega-3s and other micronutrients.

Tables.

Table 1. Sequences of gene-specific primers used in quantitative PCR experiments

Gene	Primers (5'—3')	Accession #/reference
GCLC	Forward: AGGCATTGATCATCTCCTGG Reverse: AGGAGGGGGCTTAAATCTCA	NM_001498.3
NQO1	Forward: ACTGCCCTCTTGTGGTGCAT Reverse: GCTCGGTCCAATCCCTTCAT	NM_001025434
GAPDH	Forward: TCCTGCACCACCAACTGCTT Reverse: GAGGGGCCATCCACAGTCTT	BC08351/Shao et al., (2007)

Table 2. Summary of effects of oxEPA/oxDHA and BDE 47 on mitochondrial ETS complexes

Mitochondrial ETS Complex	Effects of tested compounds on specified ETS complex	
	BDE 47	**oxEPA/oxDHA**
Complex I	Dysfunction suggested by reduction of State 3 respiration with complex I substrates (**Fig. 5A**), and maximum uncoupled respiration (which involves complexes I-IV; **Fig. 5C**).	Protection suggested by protection of State 3 respiration with complex I substrates, and maximum uncoupled respiration.
Complex II	Decreased function by 32% (**Fig. 5E**). Dysfunction also suggested by reduction of State 3 respiration with complex I+II substrates (**Fig. 5B**), and maximum uncoupled respiration.	Protected function by 9% (**Fig. 5E**). Protection also suggested by protection of State 3 respiration with complex I+II substrates, and maximum uncoupled respiration.
Complex III	Not determined in current study. However, reduction of maximum uncoupled respiration suggests an impact on complex III is possible.	Not determined in current study. However, protection of maximum uncoupled respiration suggests an effect on complex III is possible.
Complex IV	Decreased function by 28% (**Fig. 5F**). Dysfunction also suggested by reduction of State 3 respiration, and maximum uncoupled respiration.	Protected function by 20% (**Fig. 5F**). Protection also suggested by protection of State 3 respiration, and maximum uncoupled respiration.
Complex V	Not determined in current study. However, reduction of State 3 respiration (which involves complexes I-V) suggests an impact on complex V is possible.	Not determined in current study. However, protection of State 3 respiration suggests an effect on complex V is possible.

Figures.

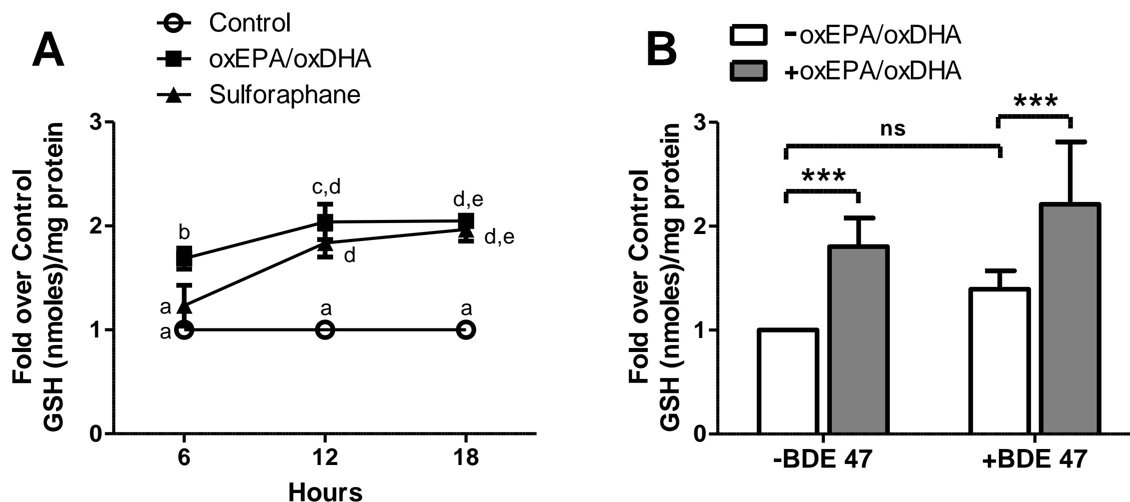

Figure 1. Effect of oxEPA/oxDHA and BDE 47 on GSH status. (**A**) Induction of GSH was measured in HepG2 cells exposed to a 1.5/1 mixture of oxEPA/oxDHA (60 µM oxEPA/40 µM oxDHA), sulforaphane (SFN, 40 µM), or equal volume of DMSO (vehicle control). Alphabetical letters are used to indicate statistically significant differences between means at $p \leq 0.05$. Letters shared in common between or among the groups indicate no significant difference at $p > 0.05$. (**B**) GSH status in cells pretreated with oxEPA/oxDHA (or DMSO, denoted as "-oxEPA/oxDHA in figure legend) for 12 h, prior to exposure to 100 µM BDE 47 (or DMSO, denoted as "-BDE 47" in figure legend) for 24 h. ***$p \leq 0.001$ relative to corresponding controls. Data are expressed as fold over control values and are means ± SEM of n=3 experiments.

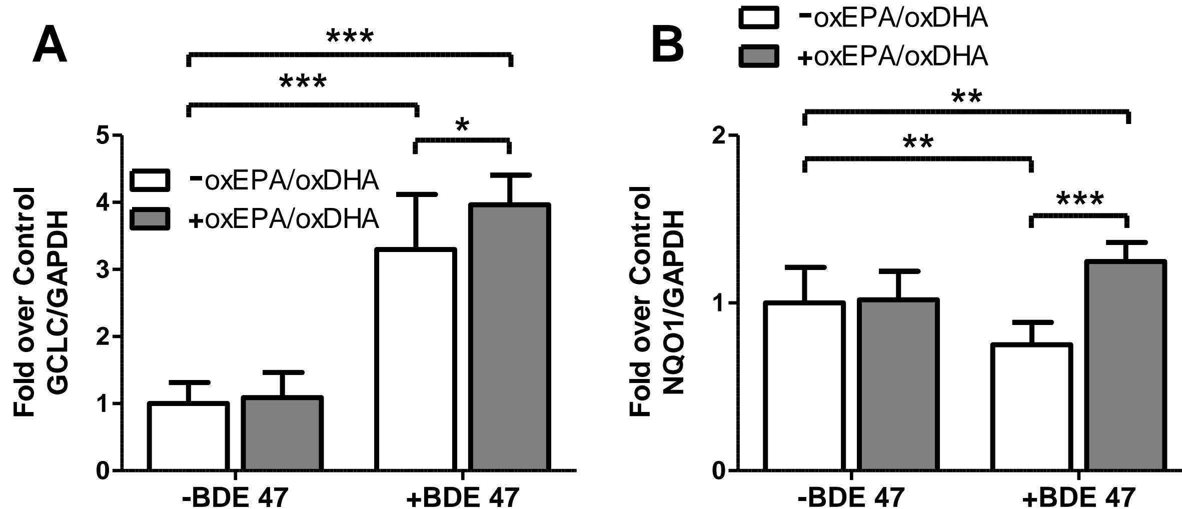

Figure 2. Effect of oxEPA/oxDHA and BDE 47 on oxidative stress marker genes. Fold induction relative to controls of **(A)** *gclc*, or **(B)** *nqo1* mRNA transcripts was measured in cells pretreated with or without oxEPA/oxDHA for 12 h, followed by exposure to 100 µM BDE 47 for 24 h. *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$ relative to corresponding controls. Data are expressed as fold over control values and are means ± SEM of n=3 experiments.

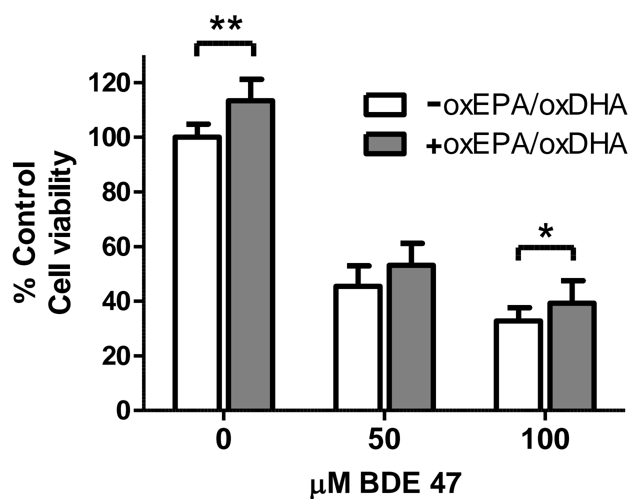

Figure 3. Effect of oxEPA/oxDHA on loss of cellular viability from BDE 47 exposure. HepG2 cells were treated with oxEPA/oxDHA for 12 h to induce intracellular GSH prior to 24 h exposure to BDE 47 (50 or 100 µM). Cellular viability was determined by MTT assay. *$p \leq 0.05$, **$p \leq 0.01$ relative to corresponding controls. Data are expressed as percent of control values and are means ± SEM of n=3 experiments.

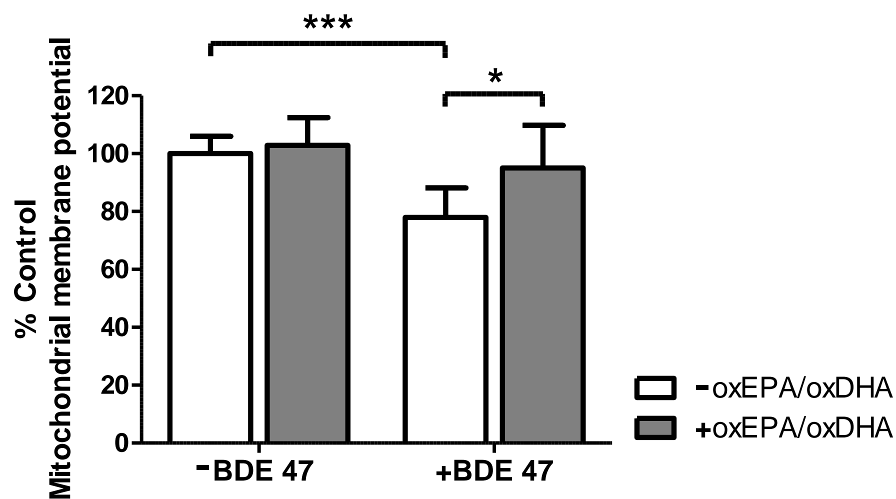

Figure 4. Effect of oxEPA/oxDHA on loss of mitochondrial membrane potential from BDE 47 exposure. Mitochondrial membrane potential ($\Delta\Psi_m$) was quantified in cells by measuring JC-fluorescence in cells pretreated with, or in the absence of oxEPA/oxDHA for 12 h, followed by 24 h exposure to 100 µM BDE 47 (or DMSO). *$p \leq 0.05$, ***$p \leq 0.001$ relative to the corresponding control group. Data are expressed as percent of control values and are means ± SEM of n=3 experiments.

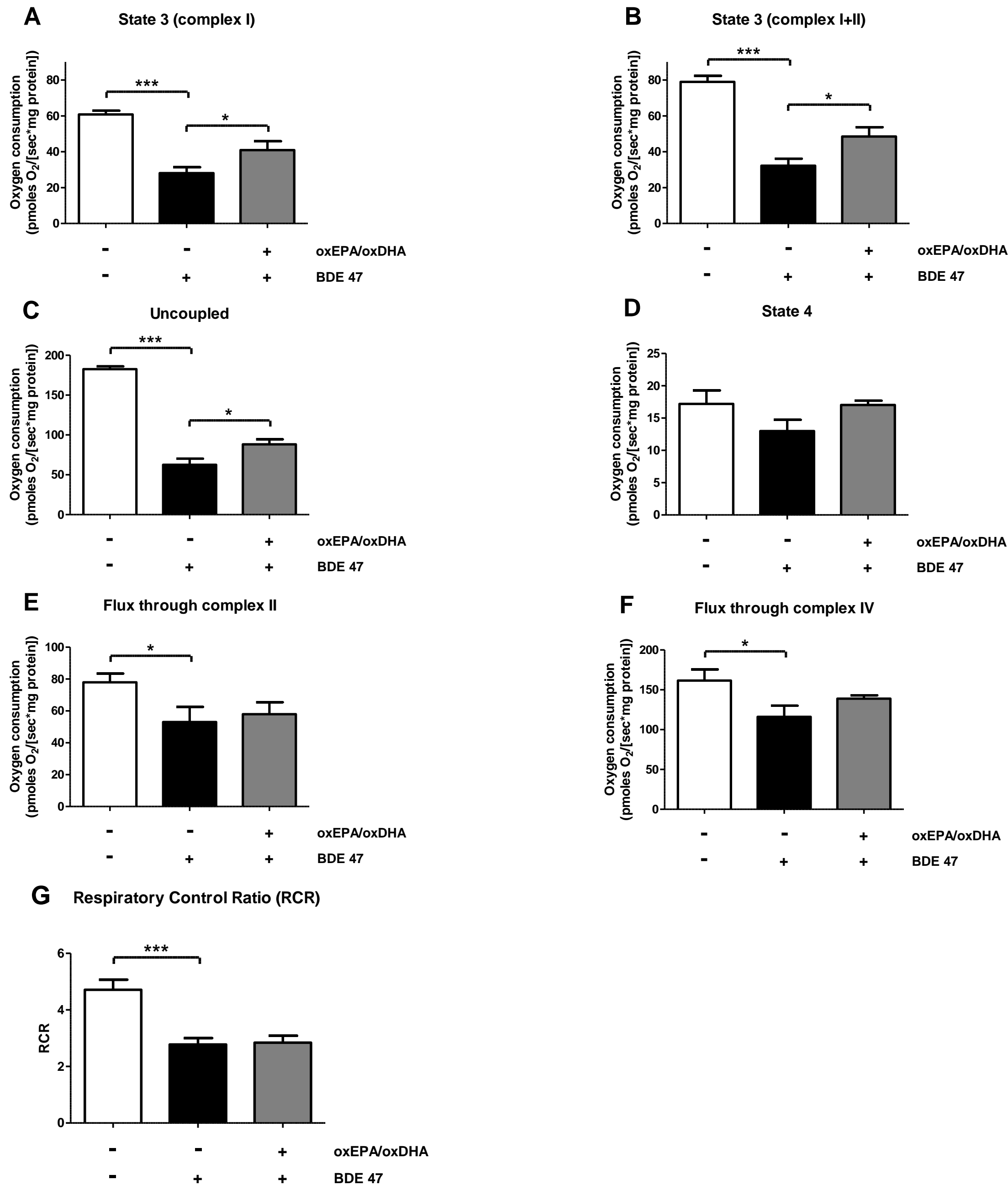

Figure 5. Effect of oxEPA/oxDHA on loss of mitochondrial electron transport system function from BDE 47 exposure. Oxygen consumption (flux) was determined in permeabilized HepG2 cells exposed to: 1) vehicle control (DMSO, 36 h), 2) DMSO for 12 h followed by BDE 47 (100 μM) for 24 h, or 3) pretreated with oxEPA/oxDHA for 12 h before BDE 47 exposure for 24 h. Experimental conditions included: **(A)** State 3 respiration with complex I substrates only; **(B)** maximally-induced State 3 respiration with substrates of complex I and II; **(C)** maximum uncoupled respiration using the uncoupling agent CCCP; **(D)** State 4 respiration induced by oligomycin. Oxygen flux capacity through **(E)** complex II, and **(F)** complex IV were also determined. **(G)** The respiratory control ratios (RCRs) were calculated as the ratio of maximally-induced State 3 respiration/State 4 respiration. *$p \leq 0.05$, ***$p \leq 0.001$ relative to control group. All data are means ± SEM of n=4 experiments.

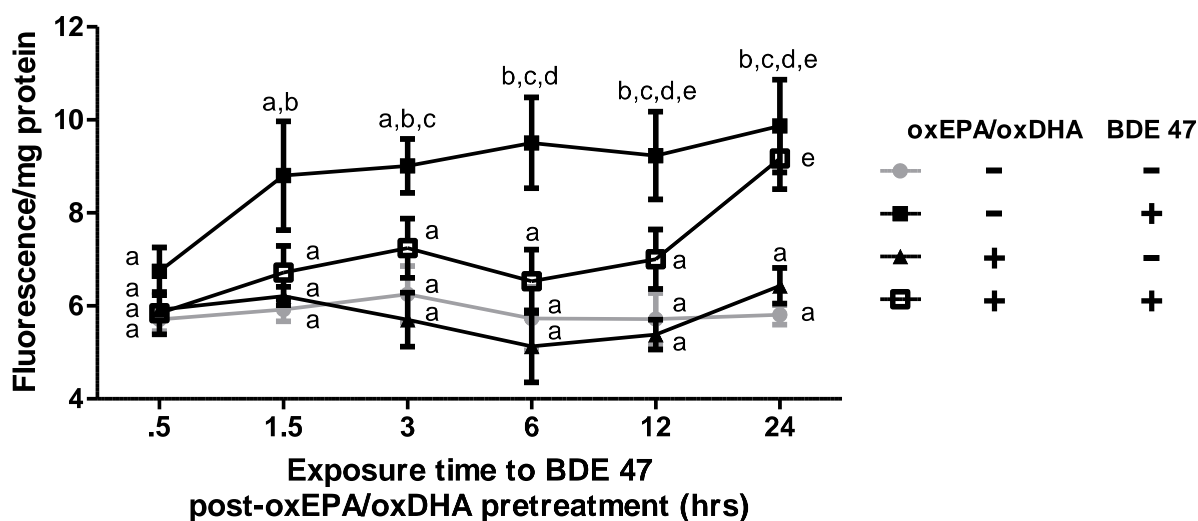

Figure 6. Effect of oxEPA/oxDHA on BDE 47-induced ROS production. Production of ROS was measured in HepG2 cells pretreated with (or without) oxEPA/oxDHA prior to exposure to 100 μM BDE 47 (or DMSO). Alphabetical letters are used to indicate statistically significant differences between means at $p \leq 0.05$. Letters shared in common between or among the groups indicate no significant difference at $p > 0.05$. Data are means ± SEM of n=3 experiments.

Chapter 2

Contaminants of emerging concern detected in Puget Sound water and fish samples

Andrew Yeh, James P. Meador, Graham Young, and Evan P. Gallagher

Portions of this chapter were published: Meador, J.P., Yeh, A., Young, G., and Gallagher, E.P. (2016). "Contaminants of emerging concern in a large temperate estuary." Environmental Pollution (213): 254-267.

Abstract

The purpose of this study was to determine the specific types of CECs that are present in Puget Sound, and their potential to bioaccumulate in representative biomonitoring species. This analytical field study focused on three sites: two estuaries that receive effluent from WWTPs (Puyallup River estuary, and Sinclair Inlet) and a minimally-polluted estuarine reference site (Nisqually River estuary). Samples of estuary water, WWTP effluent, and whole-bodies of Chinook salmon and staghorn sculpin were analyzed for concentrations of 150 chemical analytes by HPLC/MS/MS techniques. The analytes selected for analysis were determined *a priori* and represented understudied compounds that were also of interest to regulatory agencies. 81 analytes were detected in effluent samples in the ng/L to low µg/L range, with 31 of the measured concentrations ranking in the 95th percentile or higher relative to reports from 50 of the largest US

WWTPs. Additionally, 25 analytes were detected in estuary water samples at ng/L concentrations, and 42 analytes were detected in fish whole-bodies at ng/g concentrations. Reference site samples of water and fish had greater-than-expected contamination with CECs, indicative of the ubiquitous presence of these emerging contaminants in the aquatic environment. Also, preferential bioaccumulation of the contaminants was observed in Chinook salmon relative to sculpin, suggesting potential species differences. This study generated a robust dataset representing a snap-shot of aqueous and whole-body concentrations of emerging contaminants present in Puget Sound water and biota, respectively. These results can inform environmental risk assessment or ecological studies at other sites in Puget Sound, and provided crucial data that was required to conduct a subsequent laboratory exposure study modeled on environmentally-relevant concentrations and mixtures of Puget Sound CECs.

1.0. Introduction

Contaminants of emerging concern (CECs) are environmental contaminants for which there is little data regarding occurrence, environmental fate, and toxicity. A broad range of structurally-diverse chemicals are defined as CECs, including pharmaceuticals and personal care products (PPCPs), polybrominated diphenyl ethers (PBDEs), perfluorinated compounds (PFCs), alkylphenols, bisphenol A, phthalates, and current-use pesticides. Many of these compounds are components of common household chemicals that have been approved for public consumption or usage, and are generally considered to be non-toxic. As a result, CECs enter municipal wastewater from anthropogenic activities, and are subsequently discharged into aquatic environments via wastewater treatment plant (WWTP) effluent. Further investigation into the potential adverse impacts of CECs on non-target organisms are warranted by the frequent detection of these emerging contaminants in environmental samples, and critical data gaps regarding risk assessment and toxicity (Roos et al., 2012; Arnold et al., 2014).

Few studies to date have conducted robust analyses of a large array of CECs in marine or estuarine ecosystems in the United States (Hedgespeth et al., 2012; Klosterhaus et al., 2013; Scott et al., 2016; Vidal-Dorsch et al., 2012). Similarly, in the U.S. Pacific Northwest region, few studies have assessed the types and occurrences of emerging contaminants in Puget Sound estuaries. A study conducted by the Washington State Department of Ecology and the Environmental Protection Agency (EPA) assessed concentrations of 24 PPCP analytes in WWTP influent and effluent, reclaimed water, and biosolids from five WWTPs in the Pacific Northwest (Lubliner et al., 2010). More recently, concentrations of 119 PPCPs and 13 perfluorinated compounds were measured in

marine sediments from 40 Puget Sound field sites (Long et al., 2013), and the aqueous concentrations of 18 CEC analytes were measured at 20 shoreline field sites in Puget Sound (James et al., 2016). However, none of the aforementioned studies measured concentrations of CECs in animal tissue or whole-body samples. Accordingly, bioaccumulation and comparative toxicity constitute the largest data gaps in assessing the ecological impact of CECs in aquatic organisms.

The goals of the present study were to determine which CECs are relevant to the Puget Sound, and to describe their environmental levels and potential for bioaccumulation. A review of the literature resulted in a prioritized list of 102 PPCPs, 17 hormones, and 31 industrial compounds to serve as a representative subset of CECs identified as being of potential concern in Puget Sound estuaries. The data generated in this analytical field study provided rationale and aided in the study design of subsequent laboratory experiments that assessed the biological impacts associated with exposure to CECs.

2. Methods

2.1. Selection of field sites and aquatic biomonitoring species

Figure 1 is a map showing the locations of three estuaries that were the focus of the present study: two estuaries that receive effluent from WWTPs (Sinclair Inlet and the Puyallup River estuary), and a reference site that is not known to have direct inputs from WWTPs (Nisqually River estuary). The Sinclair Inlet receives effluent from the Bremerton Westside WWTP which has an effluent outfall located approximately 170 meters from shore at a depth of 10 meters below mean lower low water, and the South Kitsap Water Reclamation Facility which has a design flow of 16 million

liters/d (South Kitsap Water Reclamation Facility, 2013). Similarly, the Puyallup River estuary receives effluent from the Tacoma Central WWTP which has a discharge outfall at 40 meters below mean lower low water approximately 370 meters northwest from the mouth of the Blair Waterway in Commencement Bay, and eight additional WWTPs with a combined permitted effluent volume of 63 million liters/d (Pierce County, 2010). The Nisqually estuary was selected as a minimally-contaminated reference site and has been used in numerous studies as a reference site (Meador, 2014). **Table 1** contains additional details for each site including GPS location data.

Two fish species that commonly occur in Puget Sound estuaries were selected for assessing bioaccumulation of CECs. Pacific staghorn sculpin (*Leptocottus armatus*) was selected due to its ubiquitous presence in Puget Sound and U.S. west coast temperate waters, and that this species generally exhibits high site fidelity and may reside in estuaries for extended periods (Tasto, 1976). Additionally, juvenile Chinook salmon (*Oncorhynchus tshawytscha*) were selected based on their residence time of up to several weeks in local estuaries where contaminants are often concentrated (Healey, 1991). Chinook salmon were selected over other salmonids that do not exhibit this life history trait (Meador, 2014). We also collected hatchery-reared juvenile Chinook salmon from the Voight's Creek hatchery on the Puyallup River for comparison to fish collected in the estuary. Fish were collected under a Washington State Scientific Collection Permit 13—046 and ESA Section 10(a)(1)(A) permit 17798. All methods for obtaining, transporting, and tissue sampling of fish were approved by the University of Washington Institutional Animal Care and Use Committee (protocol number 4096-01). Details of all sampling methods used in this study were reported in Yeh et al., (2013), a Quality Assurance Project Plan written for the Washington state Department of Ecology.

2.2. Sampling for CEC analytes in WWTP effluents and water

The effluent from Bremerton West WWTP was sampled on September 9, 2014 and the effluent flow was 13.2 MLD. The maximum monthly design flow from October-April is stated to be 58.7 MLD and permitted at 86 MLD (Bremerton Westside Factsheet, 2013). The effluent from Tacoma Central WWTP, Tacoma, WA was collected on September 17, 2014 and the flow on that day was 56.8 MLD. The maximum month design flow for wet weather is listed as 143.8 MLD (Tacoma Central WWTP Factsheet, 2004) and the permitted capacity is 228 MLD (Pierce County, 2010). These values do not include secondary treatment bypass during high volume flows or peak flows, which may exceed average flows by 2-fold. For the two-week period prior to sampling, Tacoma experienced 0.03 inches of rain and Bremerton received 0.25 inches of rain (Weatherunderground, 2014).

At each WWTP, a total of 11 one-liter amber glass bottles were filled with effluent sampled at the final stage of processing, just before discharge into the outfall leading to the estuary. Similarly, at each field site a total of 11 one-liter amber glass bottles were filled with estuarine water at a depth of 2 m below the surface with a swing-sampling pole designed to collect water below the surface. We followed the protocol of Washington Department of Ecology (2006) for obtaining water samples. Estuary water quality parameters including dissolved oxygen, conductivity, salinity, and temperature of the water column were measured at a depth of 2 m below the surface using the YSI Model 85 handheld probe (YSI Incorporated, Yellow Springs, OH). Similarly, the pH of the water column was measured using the Eutech Multi-Parameter PCSTestr 35 (Oakton Instruments, Vernon Hills, IL). One water sample was taken at each site and the estuary parameters were

measured within minutes of water collection. No field blanks were collected. Estuarine water quality parameters for each field site are reported in **Table 1**.

2.3. Fish sampling

Juvenile Chinook salmon were obtained at each field site with a beach seine and were categorized as wild or hatchery origin based on the presence of an adipose fin. Artificially-reared salmon are marked by removal of the adipose fin by each hatchery. Staghorn sculpin were also obtained by beach seining; however, 4-5 individuals from the Puyallup estuary were obtained by shrimp traps set at 8-9 m below the surface. Each species was collected as close as possible to the outfall area (**Figure 1**), which in most cases was several hundred meters away. Fish were kept alive after collection in the field and transported to the laboratory for processing. Fish were transported in site water that was aerated and temperature was maintained at 11 °C with ice packs. Samples were taken approximately 3-6 hours after capture, and whole bodies of all fish were frozen at -80 °C after processing.

Fish were euthanized with tricaine methanesulfonate (MS-222; Argent Chemical Laboratories, Redmond, WA) for processing. To avoid analysis of stomach contents that were considered external to the fish, the entire alimentary canal and stomach contents of all fish analyzed for chemistry were cleaned of material by rinsing with distilled water. The contents were discarded and the cleaned tissue included with the whole bodies for analysis. Chemical analyses for CECs were conducted on composite samples consisting of 3-12 whole-body salmon or 3-5 whole-body sculpin.

Juvenile Chinook salmon from the nearby Gorst Creek rearing ponds that empty directly into the head of Sinclair Inlet (far west end) were released unusually early in the year (Mike Huff, hatchery manager, personal communication) and were probably out of the area at the time of sampling. As a result, the juvenile salmon sampled were likely from outside the area, as noted in previous studies of this local estuary (Fresh et al., 2006), but were nonetheless exposed to WWTP effluent while residing in Sinclair Inlet. All collected fish were scanned for the presence of coded wire tags (CWTs) by personnel from the U.S. Fish and Wildlife Service (USFWS). Heads of fish with detected CWTs were removed and read by USFWS personnel. Only four CWTs were found in Chinook salmon obtained from Sinclair Inlet and all were from nearby Grover's Creek Hatchery. Two CWTs were detected in Chinook salmon obtained from Puyallup estuary and both were from the White River Hatchery. Two CWTs were also detected in Chinook salmon from the Nisqually estuary, which indicated the Kalama Creek and Clear Creek Hatcheries as the source.

2.4. Analytical methods

Concentrations of CECs were determined by AXYS Analytical, Ltd. (Sidney, British Columbia, Canada) using LC/MS/MS techniques. **Table 2** lists full-length chemical names, detection methods, and reporting limits (RL) for all 150 analytes assessed in the current study. 147 analytes were analyzed in water samples and 122 were analyzed in fish whole-body samples. All analytes were measured in water and tissue, except hormones, hexabromocyclododecanes (HBCDDs), and phthalate esters. Hormones were only determined in water because many of these compounds occur naturally in tissue and the available phthalate ester method was developed for water. Because phthalates are difficult to quantify in various matrices due to high control and analytical blank values, the ester metabolites of these compounds were quantified, which are less problematic.

HBCDDs were analyzed in tissue only. Two of the compounds (bisphenol A and triclosan) were determined by two different analytical methods, once as part of a general analytical method and again by a compound-specific method (**Table 2**). No corrections were applied to the analytical values (e.g. percent recovery).

3.0. Results

3.1. Occurrence and concentrations of CECs in WWTP effluents

Table 3 reports the concentrations and ranges of CECs detected in WWTP effluent samples. In total, 81 analytes were detected in effluent samples, representing 55% of the total analyzed. Fifteen analytes were detected at concentrations greater than 1,000 ng/L (i.e., in the low ppb range); specifically, the PPCPs atenolol, caffeine, dimehylxanthine 1,7, diphenhydramine, furosemide, gemfibrozil, ibuprofen, 2-OH-ibuprofen, metformin, sulfamethoxazole, and valsartan, and the industrial compounds bisphenol A, 4-NP, NP1EO, and NP2EO (**Table 3**). Among the aforementioned CECs, the diabetes medication metformin was detected at the highest concentration (29,300-82,700 ng/L). In general, the types and occurrences of CECs in effluent from the two sampled WWTPs were similar.

3.2. Occurrence and concentrations of CECs in estuary water

In total, 25 analytes were detected in samples of estuarine waters (**Table 3**). The concentrations of the detected CECs measured at each field site are reported in **Table 4**. Briefly, 16-17 analytes were detected in Sinclair Inlet and the Puyallup estuary water samples, and 10 analytes were detected in water from the Nisqually reference site. In general, aqueous concentrations of CECs were

detected in the low ng/L concentrations (<10 ng/L), with the exception of metformin and 4-NP which were detected at relatively high concentrations (105-832 ng/L and 14-41 ng/L, respectively).

3.3. Bioaccumulation of CECs in sculpin and salmon

Collectively, 42 compounds were detected in whole-body samples of fish (**Table 3** and **Table 5**). In general, concentrations of the detected analytes were in the low ng/g range (<10 ng/g). However, 14 of the detected CECs were measured at concentrations exceeding 10 ng/g, specifically, the PPCPs amphetamine, caffeine, ciprofloxacin, metformin, ormetoprim, sertraline, sulfadimethoxine, triclosan, and virginiamycin M1, and the industrial compounds bisphenol A, 4-NP, NP1EO, NP2EO, and PFOS. The range of concentrations of detected analytes in fish whole-bodies from each site are reported in **Table 4**. Juvenile Chinook from the contaminated Puyallup River estuary and Sinclair Inlet sites bioaccumulated 25 and 19 analytes, respectively, and Chinook from the Nisqually reference site bioaccumulated 13 analytes. Fifteen analytes were detected in sculpin from both the Puyallup and Sinclair Inlet sites, and 9 analytes in Nisqually sculpin. Comparison of the occurrence of CECs in the two species of fish determined that CECs were detected at greater frequency and higher concentrations in juvenile Chinook salmon relative to staghorn sculpin (**Table 5**).

4.0. Discussion

The current study represents the most robust assessment of a large suite of CECs in Puget Sound to date. Although other studies of Puget Sound CECs performed a greater number of sampling events and focused on more field sites (James et al., 2016; Lubliner et al., 2010), those studies

were limited with respect to the number of analytes that could be assessed by analytical chemistry. Hence, the approach of the current study was to focus on a minimal number of sites in order to maximize the scope of the analytical chemistry analysis allowed by budgetary constraints.

The effluent concentrations of CECs in the current study were compared to effluent concentrations reported by Kostich et al. (2014) for the 50 largest WWTPs in the United States, none of which are in the Pacific Northwest region (**Table 3**). Among the 45 CECs shared in common between these studies, 31 compounds detected in the Puget Sound WWTP effluent samples were greater than the 95th percentile for values from the 50 U.S. WWTPs. Additionally, in general, the effluent concentrations detected in the current study were higher than most values for a given compound reported by Kostich et al. (2014). These findings provide further evidence of the loading of CECs into the Puget Sound aquatic environment, and suggest that input of CECs into Puget Sound may be greater relative to other areas.

Water and fish whole-body samples from the reference site in the Nisqually River estuary were more contaminated with CECs than expected, highlighting the difficulty of establishing suitable non-polluted reference sites for these ubiquitously-distributed emerging contaminants (Ferguson et al., 2013). Although the source of these compounds to the Nisqually estuary was not known, recent studies have implicated leaking septic systems as a potential source (James et al., 2016). Despite this, the analytical chemistry results confirmed that fish residing in receiving waters of WWTP effluent are indeed exposed to a greater number of CECs, and at higher concentrations, relative to fish from sites that do not receive inputs from WWTPs. A potential species difference with respect to bioaccumulation of CECs was also observed, as evidenced by the detection of

greater numbers and concentrations of analytes in juvenile Chinook relative to sculpin. Although the mechanism underlying this difference was not elucidated in the current study, rates of metabolism and respiration are known to vary between species. Indeed, high rates of ingestion and gill ventilation in Chinook salmon have been reported (Meador, 2014).

The fact that a diverse range of compounds with varying mechanisms of action were detected in fish underscores the need to investigate potential adverse effects in aquatic organisms resulting from exposure to mixture of these emerging contaminants. The data presented herein will aid in the development and application of mechanism-based biomarkers linking physiological effects with exposure to CECs, and help close critical data gaps regarding the ecological impacts of CECs.

Tables.

Table 1. Sampling locations, water and fish collection data, composition of chemistry composites, and estuary parameters.

	Puyallup estuary	Sinclair Inlet	Nisqually estuary	Voight's Creek Hatchery
Collection data				
Coordinates	47°16'35.4"N 122°24'58.0"W	47°32'24.4"N 122°39'44.3"W	47°05'56.4"N 122°42'01.8"W	47°04'58.8"N 122°10'40.8"W
Sample dates fish	21 Aug 2013, and 4 Sept 2013 (La); 16 June 2014 (Ot); 29 June 2014, and 7 and 13 Aug 2014 (La)	9 and 11 June 2014 (Ot); 27 July 2014 (La)	27 Aug 2013 (La); 19 June 2014 (Ot); 4 Aug 2014 (La)	29 May 2014 (Ot)
n fish collected	Ot: 75 La: 18§ and 31^	Ot: 38 La: 40^	Ot: 72 La: 24§ and 47^	Ot: 56
Mean (SD) salmon wt. (g)	5.4 (2.4)	13.4 (8.2)	6.8 (1.5)	5.4 (0.9)
Mean (SD) sculpin wt. (g)	La§: 60.7 (29.4) La^: 22.7 (20.5)	La^: 18.8 (6.6)	La§: 36.7 (14.3) La^: 16.1 (5.0)	N/A
% hatchery Chinook salmon	70%	71%	100%	100%
Salmon CF mean (sd)	0.94 (0.14)	0.90 (0.19)	0.96 (0.12)	1.09 (0.12)
Sample dates water	21 Aug 2013 (EW); 17 Sept 2014 (EF)	22 July 2014 (EW); 9 Sept 2014 (EF)	27 Aug 2013 (EW)	N/A
Chemistry composites				
N Fish / chem composite, lipids %	Ot A: 10, 4.3% Ot B: 12, 3.2% La§: 3, 1.6% La^: 5, 1.9%	Ot A: 3, 3.3% Ot B: 3, 1.5% La^: 3, 1.7%	Ot: 9, 2.5% La§: 4, 2.1% La^: 3, 1.6%	Ot: 12, 5.1%
Mean (SD) salmon wt. (g)	Ot A: 5.5 (1.3) Ot B: 4.1 (0.6)	Ot A: 14.1 (4.7) Ot B: 16.9 (9.0)	5.6 (0.7)	5.4 (0.9)
Mean (SD) sculpin wt. (g)	La§: 47.5 (50.2) La^: 9.4 (1.6)	La^: 30.9 (6.2)	La§: 48.1 (31.8) La^: 16.8 (2.8)	N/A
Estuary parameters				
pH	8.04	8.45	7.62	–
Salinity (ppt)	23.5	27	15.5	0
Temp (°C)	12.5	12.5	13.5	10
Oxygen (mg/L)	8.2	15	10.6	12

EW= estuary water, EF=effluent, Ot= *Oncorhynchus tshawytscha* (Chinook salmon), La= *Leptocottus armatus* (staghorn sculpin). §=2013 sampling year sculpin, ^=2014 sampling year sculpin. CF is condition factor (=weight $(g)^2$ / length $(mm)^3$). Percent hatchery fish based on the presence of an adipose fin. Estuary parameters determined at time of water sampling. SD is standard deviation.

Table 2. List of all CEC analytes measured, and their method of detection and matrix reporting limits.

Class	Analyte class	Method water	Method tissue	Analyte	Abbrev	Common name	Water RL (ng/L)	Tiss RL (ng/g)
1	AP and APE	MLA 004	MLA 080	4-Nonylphenols	4-NP		9.1	0.46
1				4-Nonylphenol diethoxylates	NP2EO		14.8	0.46
1				4-Nonylphenol monoethoxylates	NP1EO		18.6	0.46
1				Octylphenol	4-OP		2.35	0.46
2	BPA (specific method)	MLA 082	MLA 084	Bisphenol A	BPA		1.92	1.01
3	HBCDD flame retardants		MLA 070	alpha-Hexabromocyclododecane	α -HBCDD			0.10
3				beta-Hexabromocyclododecane	β -HBCDD			0.10
3				gamma-Hexabromocyclododecane	γ -HBCDD			0.10
4	Hormones (negative)	MLA 072		17 alpha-Dihydroequilin			4.33	
4				17 alpha-Estradiol			4.33	
4				17 alpha-Ethinyl-Estradiol	EE2		6.05	
4				17 beta-Estradiol	E2		6.09	
4				Equilenin			0.87	
4				Equilin			10.6	
4				Estrone	E1		4.33	
5	Hormones (positive)	MLA 072		Allyl Trenbolone			1.02	
5				Androstenedione			2.16	
5				Androsterone			28.0	
5				Desogestrel			254	
5				Estriol			20.3	
5				Mestranol	MeEE2		54.1	
5				Norethindrone			4.33	
5				Norgestrel			4.33	
5				Progesterone			0.87	
5				Testosterone			1.04	
6	Perfluorinated compounds	MLA 060	MLA 043	Perfluorobutanoate	PFBA		0.95	0.50
6				Perfluorobutanesulfonate	PFBS		1.90	1.01
6				Perfluorodecanoate	PFDA		0.95	0.50
6				Perfluorododecanoate	PFDoA		0.95	0.50
6				Perfluoroheptanoate	PFHpA		0.95	0.50
6				Perfluorohexanoate	PFHxA		0.95	0.50
6				Perfluorohexanesulfonate	PFHxS		1.90	1.01
6				Perfluorononanoate	PFNA		0.95	0.50
6				Perfluorooctanoate	PFOA		0.95	0.50
6				Perfluorooctanesulfonate	PFOS		1.90	1.01
6				Perfluorooctane sulfonamide	PFOSA		0.95	0.60
6				Perfluoropentanoate	PFPeA		0.95	0.50
6				Perfluoroundecanoate	PFUnA		0.95	0.50
7	Phthalate ester metabolites	MLA 059		Mono-methyl phthalate	MMP		0.20	
7				Mono-n-butyl phthalate	MBP		0.20	
7				Mono-benzyl phthalate	MBzP		0.20	
7				Mono-cyclohexyl phthalate	MCHP		0.20	
7				Mono-3-carboxypropyl phthalate	MCPP		0.20	
7				Mono-(2-ethyl-5-hydroxyhexyl) phthalate	MEHHP		0.20	
7				Mono-2-ethylhexyl phthalate	MEHP		0.20	
7				Mono-(2-ethyl-5-oxohexyl) phthalate	MEOHP		0.20	
7				Mono-ethyl phthalate	MEP		0.20	
7				Mono-isononyl phthalate	MiNP		0.20	
8	PPCP (ANEG)	MLA 075	MLA 075	Bisphenol A	BPA		541	195
8				Furosemide		Lasix	43.3	15.6
8				Gemfibrozil		Lopid	1.62	0.58
8				Glipizide		Glucotrol	6.49	2.33
8				Glyburide (Glibenclamide)		Glynase	3.24	1.17

Class	Analyte class	Method water	Method tissue	Analyte	Abbrev	Common name	Water RL (ng/L)	Tiss RL (ng/g)
8				Hydrochlorothiazide		Esidrix	16.0	5.76
8				Ibuprofen		Advil	16.2	5.84
8				2-Hydroxy-ibuprofen	Ibuprofen 2OH		86.5	31.1
8				Naproxen		Aleve	3.24	1.17
8				Triclocarban			3.24	1.17
8				Triclosan			64.9	23.3
8				Warfarin		Coumadin	1.62	1.55
9	PPCP (APOS)	MLA 075	MLA 075	Acetaminophen		Tylenol	16.2	5.84
9				Azithromycin		Zithromax	2.04	1.23
9				Caffeine			16.2	5.84
9				Carbadox			1.62	0.58
9				Carbamazepine		Tegretol	1.62	0.58
9				Cefotaxime		Claforan	6.49	2.33
9				Ciprofloxacin	Cipro	Cipro	6.49	5.58
9				Clarithromycin		Biaxin	1.62	0.58
9				Clinafloxacin			13.9	11.1
9				Cloxacillin			3.24	3.60
9				Dehydro Nifedipine			0.65	0.23
9				Digoxigenin			130	2.33
9				Digoxin		Lanoxin	6.49	2.81
9				Diltiazem		Cardizem	0.32	0.63
9				1,7-Dimethylxanthine (paraxanthine)		Theobromine	64.9	23.3
9				Diphenhydramine		Benadryl	0.65	0.23
9				Enrofloxacin		Baytril	3.24	1.17
9				Erythromycin-H2O		Ilotycin	2.49	0.90
9				Flumequine			1.62	1.41
9				Fluoxetine		Prozac	1.62	0.58
9				Lincomycin		Lincocin	3.24	1.36
9				Lomefloxacin		Maxaquin	3.53	1.50
9				Miconazole		Monistat	1.62	1.14
9				Norfloxacin		Noroxin	18.4	18.6
9				Norgestimate			6.75	16.5
9				Ofloxacin		Ocuflox	1.62	0.58
9				Ormetoprim			0.65	0.23
9				Oxacillin		Bactocill	3.24	4.99
9				Oxolinic Acid			2.44	0.32
9				Penicillin G			3.24	1.20
9				Penicillin V			3.24	2.03
9				Roxithromycin			0.32	0.14
9				Sarafloxacin			16.2	5.84
9				Sulfachloropyridazine			1.62	0.67
9				Sulfadiazine			1.62	0.58
9				Sulfadimethoxine			0.32	0.18
9				Sulfamerazine			0.65	0.60
9				Sulfamethazine			1.34	0.89
9				Sulfamethizole			0.65	0.80
9				Sulfamethoxazole			0.75	0.68
9				Sulfanilamide			16.2	5.84
9				Sulfathiazole			1.62	0.96
9				Thiabendazole		Mintezol	1.62	0.58
9				Trimethoprim		Proloprim	1.62	0.58
9				Tylosin			6.49	2.33
9				Virginiamycin M1			3.68	3.23
10	PPCP (APOSX)	MLA 075	MLA 075	Alprazolam		Xanax	0.32	0.12
10				Amitriptyline		Elavil	0.37	0.12
10				10-hydroxy-amitriptyline	10-OH amitrip		0.16	0.06
10				Amlodipine		Norvasc	1.62	0.58
10				Benzoylecgonine		Esterom	0.32	0.17
10				Benztropine		Cogentin	0.54	0.20

Class	Analyte class	Method water	Method tissue	Analyte	Abbrev	Common name	Water RL (ng/L)	Tiss RL (ng/g)
10				Betamethasone		Luxiq	2.42	1.36
10				Cocaine			0.16	0.10
10				*N*,*N*-Diethyl-*meta*-toluamide	DEET	DEET	0.87	0.31
10				Desmethyldiltiazem			0.16	0.06
10				Diazepam		Valium	0.32	0.46
10				Fluocinonide		Lidex	6.49	5.94
10				Fluticasone propionate		Flonase	2.68	5.28
10				Meprobamate		Miltown	4.33	2.30
10				Methylprednisolone		Medrol	6.79	6.52
10				Metoprolol		Lopressor	3.01	1.81
10				Norfluoxetine		Seproxetine	1.62	0.58
10				Norverapamil			0.16	0.09
10				Paroxetine		Paxil	4.33	1.56
10				Prednisolone		Orapred	8.53	13.0
10				Prednisone		Deltasone	36.9	112
10				Promethazine		Phenergan	1.44	0.52
10				Propoxyphene		Darvon	0.32	0.12
10				Propranolol		Inderal	2.16	0.78
10				Sertraline		Zoloft	0.43	0.16
10				Simvastatin		Zocor	21.6	7.78
10				Theophylline		Elixophyllin	107	89.3
10				Trenbolone			4.33	1.56
10				Trenbolone acetate			0.38	0.25
10				Valsartan		Diovan	4.33	1.56
10				Verapamil		Calan	0.16	0.06
11	PPCP (BPOS)	MLA 075	MLA 075	Albuterol (salbutamol)		Proventil	3.05	0.28
11				Amphetamine			15.2	12.1
11				Atenolol		Tenormin	6.09	0.57
11				Atorvastatin		Lipitor	15.2	1.42
11				Cimetidine		Tagamet	6.09	0.57
11				Clonidine		Kapvay	15.2	1.42
11				Codeine			30.5	2.83
11				Cotinine			15.2	1.42
11				Enalapril		Vasotec	3.05	0.28
11				Hydrocodone			15.2	1.42
11				Metformin		Glucophage	30.5	2.83
11				Oxycodone		Oxycontin	6.09	0.69
11				Ranitidine		Rantacid	6.09	0.59
11				Triamterene		Dyrenium	3.05	0.28
12	Triclosan (specific method)	MLA 083		Triclosan			5.08	

RL is approximate reporting limit for each analyte in water or tissue. Method refers to the analytical method used for extraction and analytical determination for each compound by AXYS Analytical Services LTD, Sidney, B.C., Canada.

Table 3. Range of observed concentrations for CEC detected in water or fish.

Analytes	Range for effluent (ng/L)	Range for estuary water (ng/L)	Range for salmon (ng/g)	Range for sculpin (ng/g)	WWTP output (g/d)[#]		Percentile ranking for effluent
					Bremerton	Tacoma	
Albuterol	36 – 41	12			0.54	2.03	> 90th
Alprazolam	3.0 – 4.0			0.38	0.04	0.23	> 95th
Amitriptyline	88 – 119		0.58 – 0.68		1.58	4.97	> 99th
10-OH-amitriptyline	43 – 60	0.19 – 0.21	0.09	0.13	0.80	2.43	*> 99th
Amlodipine	9.7 – 26		0.62 – 1.0		0.13	1.49	> 99th
Amphetamine	67 – 164	2.2 – 29	3.4 – 25	7.3 – 25	2.17	3.81	> 99th
Androstenedione	8.4				0.11		
Atenolol	1,700 – 2,440	3 – 22			22.5	138.5	> 95th
Atorvastatin	68					3.87	*> 99th
Azithromycin	261 – 629	2.2	1.7		8.33	14.8	
Benzoylecgonine	151 – 293	0.50 – 0.80			3.88	8.57	
Benztropine	0.57 – 0.93		0.20		0.01	0.03	*> 99th
Bisphenol A	350 – 4,290	2.8 – 4.3	3.3 – 41	3.6 – 4.5	4.64	243	
Caffeine	152 – 1170		18	13	15.5	8.63	
Carbamazepine	510 – 735	1.9			6.76	41.7	> 99th
Cimetidine	194					11.0	> 99th
Ciprofloxacin	158 – 192	7.3		17	2.54	8.97	> 80th
Clarithromycin	52 – 181				0.69	10.3	
Cocaine	9 – 59	0.30			0.78	0.48	
Codeine	290 – 178				2.36	16.5	
Cotinine	115 – 340				4.50	6.53	
DEET	23.3 – 684	2.4 – 5.3	0.39 – 1.6	0.41 – 2.2	9.06	1.32	
Diazepam	1.5 – 2.2		0.39	0.25	0.03	0.09	
Dehydronifedipine	13 – 15				0.20	0.73	
Diltiazem	390 – 425	0.52 – 0.75	1.4 – 1.6		5.17	24.1	> 99th
Diltiazem desmethyl	82 – 148		0.06 – 1.5	0.07 – 0.08	1.96	4.64	> 99th
Dimethylxanthine 1,7	873 – 2060				27.3	49.6	
Diphenhydramine	1030 – 1240	0.96 – 1.5	0.24 – 2.7	0.28	16.4	58.5	
Enalapril	5.9		1.2			0.34	> 80th
Erythromycin	87 – 138	3.3	0.90		1.83	4.96	
Estrone	4.5 – 58				0.77	0.25	> 85th
Fluocinonide			6.5				
Fluoxetine	57 – 60		4.9		0.75	3.38	> 99th
Furosemide	994 – 1290				17.1	56.4	> 95th
Gemfibrozil	1360 – 1640	3.4 – 4.5	1.3		21.7	77.2	> 90th
Glipizide	22 – 23				0.29	1.24	*> 99th
Glyburide	7.6 – 11				0.14	0.43	*> 99th
α -HBCDD			0.10 – 0.20				
γ- HBCDD			0.42				
Hydrochlorothiazide	411 – 578				7.66	23.3	≈ 5th
Hydrocodone	69 – 74				0.98	3.93	> 80th
Ibuprofen	116 – 1060				14.0	6.59	> 80th
2-OH-ibuprofen	1,160 – 4,550				60.3	65.9	> 95th
Lincomycin	27					1.55	*> 99th
MBP		289 – 491					
MEHP	0.40					0.02	
Meprobamate	513 – 623				8.25	29.1	
Metformin	29,300 – 82,700	105 – 832		28	388	4695	
Metoprolol	805 - 835				10.7	47.4	> 90th
Miconazole	4.9		1.8			0.28	
Naproxen	106 –701				9.29	6.02	
Norfluoxetine	17 – 28		0.68 – 3.2		0.37	0.97	> 99th
Norverapamil	13 – 14		0.12 – 0.47	0.20 – 0.30	0.17	0.77	> 95th
4-NP	506 – 1690	41	30 – 76	7.7 – 35	6.70	95.9	

Analytes	Range for effluent (ng/L)	Range for estuary water (ng/L)	Range for salmon (ng/g)	Range for sculpin (ng/g)	WWTP output (g/d)[#]		Percentile ranking for effluent
					Bremerton	Tacoma	
NP1EO	1,220 – 1,760		1.3 – 60	3 – 4.9	23.3	69.3	
NP2EO	1,690 – 2,610		1.4 – 51	1.9 – 17	34.6	95.9	
Ofloxacin	108 – 387				5.13	6.13	> 90th
Ormetoprim			44 – 1,600				
Oxycodone	158 – 231				2.09	13.1	> 95th
Paroxetine	6.6 – 42				0.56	0.37	*> 99th
PFBA	6.7					0.38	
PFBS	13				0.17		
PFDA			0.78				
PFHpA	3 – 7.5				0.10	0.17	
PFHxA	15 – 53				0.71	0.86	
PFHxS	55				0.73	0.00	
PFNA	2					0.11	
PFOA	7.6 – 12				0.16	0.43	
PFOS	461		1.2 – 34	1.1 – 1.4	6.11	0.00	
PFOSA				0.82 – 2.2			
PFPeA	3.4 – 4.7				0.06	0.19	
Promethazine	3.8					0.21	*> 99th
Propoxyphene	0.7 – 1.9				0.02	0.04	> 80th
Propranolol	76 – 109				1.00	6.19	> 95th
Ranitidine	494	0.75	0.82 – 1.1	0.97		28.1	> 95th
Roxithromycin	3.8					0.22	
Sertraline	89 – 116		17	0.20	1.54	5.05	> 95th
Simvastatin	34					1.95	*> 99th
Sulfadiazine			0.88				
Sulfadimethoxine	8.2	0.46	0.34 – 17			0.47	*> 99th
Sulfamerazine			0.51				
Sulfamethoxazole	1380	1.5 – 4.2				78.4	> 90th
Testosterone		1.9					
Thiabendazole	24 – 27				0.36	1.35	
Triamterene	151 – 156				2.00	8.86	> 95th
Triclocarban	12 – 17		6.5		0.16	0.96	
Triclosan	250 – 538	5.2	26		7.13	14.2	
Trimethoprim	742 – 852	2.3			9.83	48	> 99th
Valsartan	2010 – 3000	5.4			26.6	170	> 80th
Verapamil	40 – 44		0.30 – 0.60	0.07 – 0.27	0.54	2.52	> 80th
Virginiamycin M1			10	8 – 34			
Warfarin	6.2					0.35	*> 99th
Detected analytes	81	25	37	21			
Sum kg/d for sample flow					0.82	6.66	
kg/d at maximum flow					3.5	17	

All blank values indicate a concentration <RL. Range shows minimum and maximum for each matrix (effluent, estuary water, or fish tissue) and type (sculpin or salmon). All single values indicate at least one site with a quantifiable concentration. Tissue concentrations are whole-body wet weight. Grams/day (g/d) for each analyte shown based on measured concentration (Table S4) and flow rate on the date of collection (personal communication from plant operators). Also shown is predicted kg/d for flow at the time of sampling and maximum flow. Our effluent concentrations expressed as percentile ranking compared to Kostich et al. (2013, 2014) who analyzed 56 active pharmaceutical ingredients in the 50 largest WWTPs in the U.S. * = all values from Kostich et al. (2014) below detection but detected in the present study.

Table 4. Concentrations of detected analytes in both fish and water samples for all replicates.

	Sinclair Inlet					Puyallup						Nisqually				Hatch
	Eff	Est	salmon		sculp	Eff	Est	salmon		sculp		Est	salmon	sculp		salmon
			A	B				A	B	A	B			A	B	
	2014	2014	2014	2014	2014	2014	2013	2014	2014	2014	2013	2013	2014	2014	2013	2014
Analytes	ng/L	ng/L	ng/g	ng/g	ng/g	ng/L	ng/L	ng/g	ng/g	ng/g	ng/g	ng/L	ng/g	ng/g	ng/g	ng/g
Alprazolam	3.0					4.0					0.38					
Amitript	119					88		0.58	0.68							
10-OH-amitript	60	0.19			0.09	43	0.21		0.09	0.13						
Amlodipine	9.7					26		1	0.62							
Amphetamine	164	29		3.4	7.3	67	2.2	25	20	7.5						
Azithromycin	629	2.2				261		1.7								
Benztropine																0.2
Bisphenol A	350	2.8		5.5		4290	4.3				4.5		3.3		3.6	41
Caffeine	1170		18			152								13		
Ciprofloxacin	192					158						7.3			17	
DEET	684	2.4	0.56	0.76	0.65	23	5.3	0.44	0.39	2.2	0.42	8.4	0.50	0.67	0.41	1.6
Desmethyl-diltiazem	148			0.06	0.08	82		1.5	1.2	0.07						
Diazepam	2.2				0.25	1.5							0.39			
Diltiazem	390	0.75				425	0.52	1.6	1.4							
Diphen-hydramine	1240	1.5		0.24	0.28	1030	1.2	2.4	2.7	0.28		0.96				
Enalapril																1.2
Erythromycin	138		0.9			87	3.3									
Fluocinonide			6.5													
Fluoxetine	57					60		4.9	4.7							
Gemfibrozil	1640	3.4				1360	4.5	1.2	1.3							
∀-HBCDD			0.20	0.17				0.20	0.11							
(-HBCDD								0.42								
Metformin	29300	832				82700	105								28	
Miconazole						4.9		1.4	1.8							
Norfluoxetine	28					17		3.2	2.7				0.68			
Norverapamil	13		0.20		0.20	14		0.47	0.39				0.12	0.25		
4-NP	506		30	33	27	1690	41	76	33	35	35	14	76	29	7.7	51
NP1EO	1760		3.0	2.2	3.0	1220		57	60	4.9			1.3	2.2		1.3
NP2EO	2610		1.4	2.2	1.9	1690		49	51	17	6.2		2.1	7.5		3.1
Ormetoprim				44				1010	1600				642			
PFDA			0.78													
PFOS	461		34	1.4	1.4						1.1		1.2			
PFOSA					0.82						2.2					
Ranitidine					0.97	494		0.82				0.75	1.1			
Sertraline	116					89		17	4.9	0.21						
Sulfadiazine			0.88													
Sulfadimethoxine		0.46		0.34		8.2		16.2	9.8				17			
Sulfamerazine									0.51							
Triclocarban	12					17		4.0	6.5							
Triclosan	411	5.2				183		26	23							
Verapamil	41		0.30		0.25	44		0.60	0.54		0.07		0.12	0.27		
Virginiamycin					8.0			10			34					
N detected for sample			13	12	15			26	24	9	9		13	7	5	7
N detected for matrix and site			19					28		15				9		

Fish and effluent (Eff) sampled in 2013 and 2014. Estuary water (Est) sampled in 2013. Hatch = Voight's Creek Hatchery (Puyallup watershed) sampled on 29 May 2014. Salmon is Chinook salmon (*Oncorhynchus tshawytscha*) and Sculp = staghorn sculpin (*Leptocottus armatus*).

Table 5. Comparison of bioaccumulated concentrations of CECs in Chinook salmon and sculpin

		Chinook salmon	Staghorn sculpin
Class	**CECs**	**ng/g**	**ng/g**
Antianxiety	Diazepam	0.4	0.3
	Alprazolam	-	0.4
Antibiotics	Azithromycin	1.7	-
	Ciprofloxacin	-	16.6
	Erythromycin	0.9	-
	Miconazole	1.8	-
	Ormetoprim	1,600	-
	Sulfadiazine	0.9	-
	Sulfadimethoxine	16.8	-
	Sulfamerazine	0.5	-
	Triclocarban	6.5	-
	Triclosan	26.4	-
	Virginiamycin M1	10.2	33.9
Antidepressants	Amitriptyline 10-OH	0.1	0.1
	Amitriptyline	0.7	-
	Fluoxetine	4.9	-
	Norfluoxetine	3.2	-
	Sertraline	17	0.2
Antihistamine	Diphenhydramine	2.7	0.3
	Ranitidine	1.1	0.97
Blood pressure	Enalapril	1.2	-
Diabetes	Metformin	-	27.8
Ca^{2+} channel blockers	Amlodipine	1.0	-
	Diltiazem	1.6	-
	Diltiazem desmethyl	1.5	0.08
	Norverapamil	0.5	0.3
	Verapamil	0.6	0.3
Glucocorticoids	Fluocinonide	6.5	-
Industrial compounds	Bisphenol A	40.7	4.5
	α-HBCDD	0.2	-
	γ-HBCDD	0.4	-
	4-NP	75.6	35.2
	NP1EO	60	4.9
	NP2EO	51	16.5
	PFDA	0.8	0.8
	PFOS	33.7	-
Insect repellants	DEET	1.6	2.2
PPAR agonists	Gemfibrozil	1.3	-
Stimulants	Amphetamine	24.5	7.5
	Caffeine	18.3	12.9
Parkinson’s	Beztropine	0.2	-

Values represent mean concentrations of compounds detected in whole-body samples of fish.

Figures.

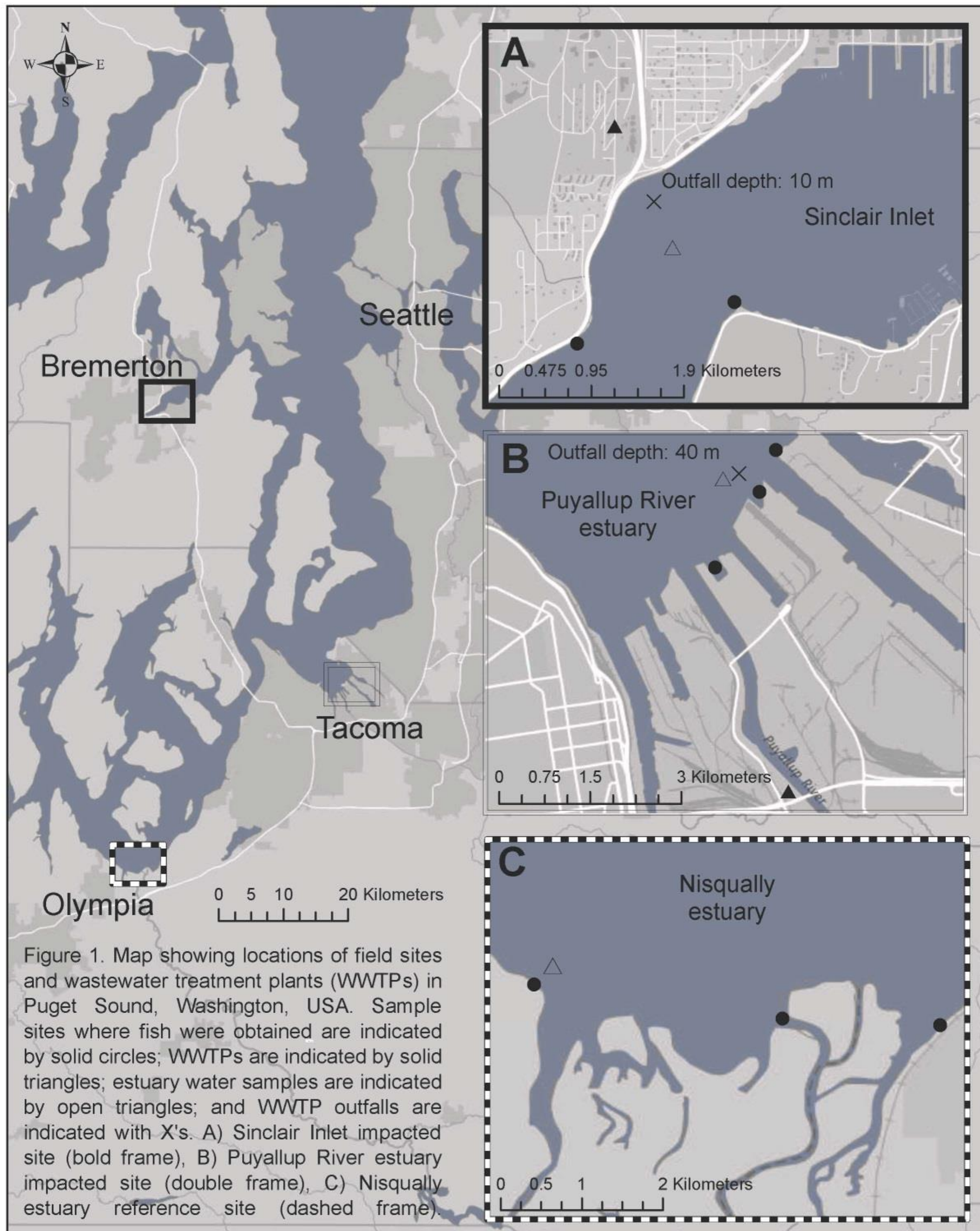

Figure 1. Overview of Puget Sound field study locations. Map generated by ArcMap, ArcGIS for Desktop 10.2, 2013.

Chapter 3

Effect of contaminants of emerging concern on liver mitochondrial function in Chinook salmon

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Abstract

We previously reported the bioaccumulation of contaminants of emerging concern (CECs), including pharmaceuticals and personal care products (PPCPs) and perfluorinated compounds, in wild juvenile Chinook salmon from urban estuaries of Puget Sound, WA (Meador et al., 2016). Although the toxicological impacts of CECs on salmon are poorly understood, several of the detected contaminants disrupt mitochondrial function in other species. Here, we sought to determine whether environmental exposures to CECs are associated with hepatic mitochondrial dysfunction in juvenile Chinook. Fish were exposed in the laboratory to a dietary mixture of 16 analytes representative of the predominant CECs detected in our field study. Liver mitochondrial content was reduced in fish exposed to CECs, which occurred concomitantly with a 24-32% reduction in expression of peroxisome proliferator-activated receptor (PPAR) Y coactivator-1a (*pgc-1α*), a positive transcriptional regulator of mitochondrial biogenesis. The laboratory exposures also caused a 40-70% elevation of state 4 respiration per unit mitochondria, which drove a 29-38% reduction of efficiency of oxidative phosphorylation relative to controls. The mixture-induced elevation of respiration was associated with increased oxidative injury as evidenced by increased mitochondrial protein carbonyls, elevated expression of glutathione (GSH) peroxidase 4 (*gpx4*), a mitochondrial-associated GSH peroxidase that protects against lipid peroxidation, and reduction of mitochondrial GSH. Juvenile Chinook sampled in a WWTP effluent-impacted estuary with demonstrated releases of CECs showed similar trends toward reduced liver mitochondrial content and elevated respiratory activity per mitochondria (including state 3 and uncoupled respiration). Interestingly, respiratory control ratios were greater in fish from the contaminated site relative to fish from a minimally-polluted reference site, which may have been

due to differences in the timing of exposure to CECs under laboratory and field conditions. Our results indicate that exposure to CECs can affect both mitochondrial quality and content, and support the analysis of mitochondrial function as an indicator of the sublethal effects of CECs in wild fish.

1. Introduction.

Municipal wastewater enters wastewater treatment plants (WWTPs) to undergo primary, secondary, and sometimes tertiary treatment processes that target the removal of biosolids, dissolved organic matter, and microbial contamination. Following these processes, the treated effluent is discharged into receiving waters such as rivers, lakes, and estuarine aquatic environments. Despite these treatment processes, reports over the past decade have indicated that WWTP effluent contain a broad range of structurally-diverse compounds which are subsequently introduced into aquatic environments (Brooks et al., 2005). These include the chemical components of common household products such as pharmaceuticals and personal care products (PPCPs), natural and synthetic hormones from contraceptive medications, alkylphenol surfactants, and perfluorinated compounds (PFCs) (Dickenson et al., 2011; Jasinska et al., 2015; Kim et al., 2012; Lubliner et al., 2010; Vidal-Dorsch et al., 2012). Compounds within the aforementioned chemical classes are termed “contaminants of emerging concern” (CECs) as there is currently little regulation of these chemicals in the environment (Diamond et al., 2011; Halden, 2015; Naidu et al., 2016). These emerging contaminants are frequently detected in environmental water samples at low ng/L concentrations, but can bioaccumulate in aquatic organisms (Meador et al., 2016; Vidal-Dorsch et al., 2012). Reports of adverse biological effects in aquatic biota resulting from accumulation and exposure to effluent-borne CECs at these levels underscore the need to clarify critical data gaps regarding their toxicity in non-target organisms.

Studies involving fish have indicated that behavior can be adversely modified by exposures to CECs at environmentally-relevant concentrations under controlled laboratory conditions. These

adverse effects on behavior include reduced predator avoidance (Painter et al., 2009), decreased or increased aggression (Colman et al., 2009; Schultz et al., 2012), and reduced sociality (Brodin et al., 2013). Adverse impacts on fish reproductive function caused by CECs have also been reported (Crago et al., 2016; Lister et al., 2009; Niemuth et al., 2015). For example, a recent study in juvenile coho salmon (*Oncorhynchus kisutch*) demonstrated impacts on the hypothalamic-pituitary-gonad axis caused by exposure to 2 and 10 ng/L EE_2 and diluted WWTP effluent (Harding et al., 2016). Finally, exposure to CECs can inhibit metabolic function and rates of growth in several fish species (Ashfield et al., 1998; Niemuth and Klaper, 2015; Yang et al., 2014), which has implications on fish populations, as body mass has been linked to survival in wild fish (Meador, 2014; Spromberg and Meador, 2005). Despite these findings, however, there are currently no established biomarkers of exposure or effect of CECs on aquatic organisms (Harding et al., 2016; Jasinska et al., 2015).

A review of the literature led us to the *a priori* observation that some emerging contaminants cause dysfunction of mitochondria in humans and animal laboratory models. Mitochondria are sensitive targets of toxicity to numerous pharmaceuticals and environmental toxicants (Meyer et al., 2013; Wallace and Starkov, 2000). A common mechanism of mitochondrial toxicity is shared by a number of CECs, specifically, inhibiting function of the mitochondrial electron transport system (ETS). For example, the antidepressant medications fluoxetine (Souza et al., 1994) and sertraline (Li et al., 2012) inhibit mitochondrial state 3 respiration in isolated rat liver mitochondria and are priority CECs frequently detected in WWTP effluent (Kostich et al., 2014; Lubliner et al., 2010; Meador et al., 2016; Vidal-Dorsch et al., 2012). In general, a wide array of pharmaceutical medications, including anesthetics, antidiabetics, antidepressants, and nonsteroidal anti-

inflammatory drugs, specifically inhibit the mitochondrial ETS in mammals (Chan et al., 2005) and may enter WWTPs from hospital wastewater. Perfluorooctane sulfonamide (PFOSA) is a high-priority perfluorinated compound that potently uncouples the mitochondrial ETS in isolated rat kidney mitochondria (Schnellmann and Manning, 1990). Similarly, perfluorooctanoate (PFOA) and perfluorodecanoic acid (PFDA) elevate mitochondrial state 4 respiration and reduce state 3 respiration in isolated rat liver mitochondria (Keller et al., 1992; Langley, 1990) and are frequently detected in WWTP effluent. Finally, the personal care product triclosan is a potent mitochondrial uncoupler in rat and human mast cells, primary human keratinocytes (Weatherly et al., 2016), and in 24-hour post-fertilization zebrafish embryos (Shim et al., 2016). Although the aforementioned mitochondrial effects were mostly described in mammalian laboratory models, a recent study of 12 diverse fish species demonstrated that 65–86% of human drug targets were evolutionarily conserved in the studied fish species, suggesting that the mechanisms of action of many CECs may share commonalities with fish (Brown et al., 2014). Collectively, these reports warrant the investigation of mitochondrial toxicity as a potential indicator of exposure and effect of CECs in aquatic species.

In the present study, we hypothesized that environmental exposures to CECs result in dysfunction of liver mitochondria in fish. We utilized juvenile Chinook salmon, an ecologically- and economically-critical species in the Pacific Northwest, and focused on hepatic mitochondria, because the liver is the primary site of xenobiotic biotransformation and a target organ of toxicity for many CECs. Our approach was to characterize the potential impacts of exposure to CECs on mitochondrial ETS function and associated mitochondrial oxidative injury in a subchronic dietary study involving a complex mixture of the predominant analytes representative of field exposures.

In a parallel field study, we compared mitochondrial content and function in juvenile Chinook collected from an estuarine field site that receives WWTP effluent, with those from a minimally-polluted reference site.

2. Materials and Methods.

2.1. Chemicals and reagents

Amlodipine, azithromycin dehydrate, and diltiazem were purchased from Abcam (Cambridge, MA). Fluoxetine hydrochloride, gemfibrozil, metformin hydrochloride, miconazole nitrate, perluorodecanoic acid (PFDA), and sertraline hydrochloride were purchased from Cayman Chemical (Ann Arbor, MI). Diphenhydramine hydrochloride, fluocinonide, and heptadecafluorooctane sulfonic acid (PFOS) were purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX). Amitriptyline hydrochloride, perfluorooctane sulfonamide (PFOSA), 3,4,4'trichlorocarbanilide (triclocarban), and Irgasan (triclosan) were purchased from Sigma-Aldrich Corp. (St. Louis, MO). Ethanol was purchased from Decon Labs, Inc. (King of Prussia, PA). RNAlater® solution was purchased from Ambion (Austin, TX). TRIzol® reagent was purchased from Invitrogen (Carlsbad, CA). Quantitative real time PCR (qPCR) primers were purchased from Eurofins MWG Operon (Huntsville, AL). Tricaine methanesulfonate (MS-222) was purchased from Argent Chemical Laboratories (Redmond, WA).

2.2. Dietary subchronic exposures of juvenile Chinook to a mixture of CEC

All methods associated with salmon husbandry, collection, and exposures were approved by the University of Washington Institutional Animal Care and Use Committee. Juvenile Chinook

salmon (1 year of age, approximately 45 g) were obtained from the Wallace River Hatchery (Sultan, WA) under Washington State Scientific Collection Permit 13-046 and ESA Section 10(a)(1)(A) permit 17798. The hatchery-reared Chinook were housed in large cylindrical tanks (n=20 fish per tank) receiving ~12 °C recirculating freshwater from Lake Washington, Seattle, under a natural photoperiod at the UW fish hatchery. Following acclimation, the juvenile Chinook were exposed via diet to a mixture of 16 of the predominant CECs detected in field samples based on data from the analytical field study (Meador et al., 2016). The rationale used to prioritize the 16 mixture analytes for the feeding study is presented in **Table 1**. Calculated amounts of stock solutions of each analyte (dissolved in ethanol) were added to three separate volumes of 4 L of ethanol in order to generate three mixture concentrations intended to mimic 0.1-, 1-, and 10-fold concentrations of each analyte detected in field-collected fish. BioClark's Fry 2.5 mm low-fat food pellets (Bio-Oregon, Longview, WA) were dosed with the contaminant mixtures as described previously (Meador et al., 2006). The feeding study consisted of four groups, including the aforementioned three concentrations of the mixture of emerging contaminants, and solvent control diets. Replicates of the experimental treatments included 4 replicate tanks for solvent control feed (n=80 fish), 3 replicate tanks for 0.1x CEC feed (n=60 fish), 4 replicate tanks for 1x CEC feed (n=80 fish), and 3 replicate tanks for 10x CEC feed (n=60 fish). Twice daily feedings (morning and afternoon) were conducted five days per week, for a total of 50 feedings over the 32-day dosing period starting on February 16, 2015. The mass of mixture-treated feed administered to fish was increased each week by a quantity assuming a growth rate of 2.2% body weight (bw)•day^{-1} based on day 0 of the experiment (Meador et al., 2005). Following exposures, AXYS Analytical Services Ltd. (Sidney, British Columbia, Canada) employed multi-analyte HPLC/MS/MS techniques to measure whole-body concentrations of CECs in laboratory-exposed fish. Details regarding

analytical methods of whole body residue analysis with limits of detection of each compound were reported previously (Meador et al., 2016).

2.3. Field sampling of Chinook

Wild juvenile Chinook were collected from field sites as described previously (Meador et al., 2016). Briefly, fish were collected by beach seine from a WWTP effluent-impacted site and a reference site in Puget Sound, WA. The effluent-impacted Puyallup River estuary (PE) contains eight WWTPs with a combined permitted effluent volume of 63 million liters/day, with flows generally running much lower (Pierce County, 2010). By contrast, our reference site in the nearby Nisqually river estuary (NE) does not receive inputs of WWTP effluent, and has been utilized as a reference site in studies conducted in the Pacific Northwest (Mccain et al., 1990; Meador, 2014; Myers et al., 1994; Varanasi et al., 1993). Live Chinook were transported to the laboratory in aerated coolers filled with site water chilled with ice packs in order to maintain the temperature of the water column measured at the field sites (13 °C). Water quality parameters measured at each site on the date of sampling, as well as body weights, lengths, and condition factors of the field-sampled fish assessed in subsequent laboratory experiments are reported in **Table 2**.

2.4. Assessment of mitochondrial function in field-sampled and laboratory-exposed fish

Liver mitochondrial function was assessed in 6-8 individual Chinook salmon exposed to the mixture of CECs as part of the dietary exposure study, and six individual Chinook per field site. All fish from the field and laboratory studies were sacrificed using 250 mg/L MS-222 prior to removal of liver tissues. Oxygen consumption rates (OCRs) were measured in saponin-permeabilized liver samples via high-resolution respirometry (Oxygraph-2k, Oroboros

Instruments, Innsbruck, Austria). Briefly, approximately 20 mg of liver tissue was gently homogenized with a glass tissue homogenizer in 150 µL ice-cold respiration buffer (distilled H_2O, 0.25 M mannitol, 10 mM $MgCl_2$, 10 mM $KHPO_4$ buffer, pH=7.2). Homogenized liver samples were transferred to a 1.5 mL centrifuge tube containing an additional 135 µL ice-cold respiration buffer, and centrifuged at 400 rpm at 4 °C for 5 min. Centrifuged liver samples were then permeabilized on ice by addition of 15 µL permeabilization buffer (50 µg/mL saponin in respiration buffer, 20 min, 4 °C). The total volume of permeabilized liver sample in 300 µL buffer was then transferred to an oxygraph chamber for analysis.

Experimental procedures for the assessment of ETS functional parameters were performed as described (Yeh et al., 2015). Briefly, respiratory substrates and inhibitors were sequentially added to permeabilized liver samples in the following order: 1) 5 mM pyruvate, 2 mM malate, 10 mM glutamate, and 2.5 mM ADP to induce state 3 respiration with complex I substrates only, 2) 10 mM succinate to maximally induce state 3 respiration with substrates of complexes I and II, 3) 2.5 µM oligomycin to induce state 4, or proton leak, respiration, 4) 2.5 µM CCCP to induce maximum respiratory capacity, or uncoupled respiration, 5) 0.5 µM rotenone to measure uncoupled respiration with complex I inhibition, i.e., uncoupled flux through complex II, 6) 2.5 µM antimycin A to determine non-mitochondrial respiration, 7) 0.5 mM N,N,N′,N′-tetramethyl-ρ-phenylenediamine (TMPD) and 2 mM ascorbate to determine flux through complex IV, and 8) 1 mM potassium cyanide to inhibit of complex IV. The non-mitochondrial rate of oxygen consumption was subtracted from all measured functional parameters before reporting final values. Similarly, the rate of oxygen consumption after addition of potassium cyanide was subtracted from the rate of flux through complex IV for normalization purposes. All experimentally-determined

mitochondrial OCRs were measured as pmoles O_2/sec/mg wet weight of liver sample. All mitochondrial function experiments were conducted at 13 °C, the average temperature of the water column in the field sites (Meador et al., 2016), and comparable to that of the recirculating freshwater from Lake Washington used in the laboratory exposure study (~12 °C).

Livers from each fish assessed in the respirometry experiments were further divided into three sections of approximately 20 mg each. Two sections were snap frozen and stored at -80 °C for assessment of oxidative injury in isolated liver mitochondria (described in the subsequent section), and quantification of mitochondrial content. Liver mitochondrial content was assessed in whole tissue homogenates by measurement of activity of citrate synthase (an enzymatic marker of the mitochondrial matrix) in a spectrophotometric plate reader assay according to the manufacturer's protocol (Citrate Synthase Assay Kit, Sigma, St. Louis, MO). A third liver section was preserved in RNAlater® solution and stored at -80 °C for gene expression experiments (described in section 2.6).

2.5. Analysis of oxidative injury in liver mitochondria

In the fish exposed to the contaminant mixtures in the laboratory, mitochondrial fractions were isolated from liver tissue sections as previously described (Gallagher et al., 1992). Citrate synthase activity was measured in the subcellular fractions to verify purity of intact mitochondria. Oxidative modification of mitochondrial proteins was determined by measurement of protein carbonylation in a fluorescent plate reader assay according to the manufacturer's protocol (OxiSelect Protein Carbonyl Fluorometric Assay kit, Cell Biolabs, San Diego, CA). Protein concentrations in the mitochondrial samples were determined by the Bio-Rad Protein Assay (Bio-Rad, Hercules, CA).

Total mitochondrial GSH concentrations were assessed in a fluorescent plate reader assay as previously described (Yeh et al., 2015). Mitochondrial 4-HNE-protein adducts were assessed in a competitive ELISA assay according to the manufacturer's protocol (HNE Adduct Competitive ELISA, Cell Biolabs, San Diego, CA).

2.6. Measurement of antioxidant and mitochondrial function gene expression in laboratory-exposed fish

Procedures for isolation of total RNA from liver tissue, cDNA synthesis, and PCR primer product validation were conducted as previously described (Espinoza et al., 2012). Prior to gene expression determination, the concentrations and quality of isolated liver RNA were determined via Agilent 2100 Bioanalyzer system (Agilent Technologies, Santa Clara, CA). Quantification of RNA transcript levels for selected antioxidant and mitochondrial marker genes was then determined in 300 ng of RNA per fish (n=6-8 individuals per treatment) according to the manufacturer's recommended procedure for isolated RNA.

Expression of hepatic genes involved in the cellular antioxidant response and mitochondrial function were quantified in fish from the dietary exposure laboratory study using a customized Quantigene® plex 2.0 (QGP) panel (Affymetrix, Fremont, CA) (Mills and Gallagher, 2017). The antioxidant response genes included glutamate-cysteine ligase catalytic subunit (*GCLC*), phospholipid hydroperoxide glutathione peroxidase (GPx4), and mitochondrial superoxide dismutase (SOD2). Genes involved in mitochondrial function included peroxisome proliferator-activated receptor (PPAR) Y coactivator-1a (PGC-1α), and nuclear respiratory factor-1 (NRF-1). All target genes were normalized to the geometric mean of the expression of beta actin (β-actin),

and 60S ribosomal protein L8 (RPL8). Primer sequence information (**Table 3**) determined by preliminary PCR experiments was used by Affymetrix to design custom QGP probe sets against the antioxidant and mitochondrial function genes.

2.7. Statistical analyses

All data sets were examined for potential outliers using the Grubb's test, and outlier values were excluded at significance level $p<0.05$. In the laboratory study, data reflecting mitochondrial content, ETS functional parameters, concentrations of mitochondrial protein carbonyls, total glutathione, HNE-protein adducts, and expression of antioxidant and mitochondrial function genes were assessed for homogeneity of variance by Bartlett's test and comparison of means assessed by One-Way ANOVA followed by Fisher's Least Significant Difference post-hoc test. Differences were considered significant at $p<0.05$. In experiments involving wild juvenile Chinook collected from the field, morphological measurements, mitochondrial content, and ETS functional parameters were assessed for homogeneity of variance by F test, followed by comparison of means by Unpaired t-test. Differences between means measured in reference and polluted site fish were considered significant at $p<0.05$. All statistical analyses were conducted in GraphPad Prism Ver. 5.0 (Graph Pad Software Inc., San Diego, CA, USA), with the exception of Fisher's Least Significant Difference post-hoc test which was conducted using the Analysis ToolPak in Microsoft Excel (Microsoft ® Excel ® 2016).

3. Results

3.1. Results of the feeding study

Bioaccumulation of CECs in laboratory-exposed fish. A summary of concentrations of CECs measured in Chinook salmon fed the dietary mixtures is presented in **Table 4**. The analytes bioaccumulated at roughly the proportion intended between treatments, with 14 of the 16 CECs in the mixture bioaccumulating in Chinook salmon following the 32-day dietary exposure. Eleven of the compounds, including the PPCPs amitriptyline, amlodipine, azithromycin, diltiazem, diphenhydramine, fluoxetine, metformin, miconazole, and the perfluorinated compounds PFDA, PFOS, and PFOSA, were detected in whole-body samples from all three of the experimental contaminant mixture groups. Three other analytes, gemfibrozil, sertraline, and triclocarban, were detected in the high concentration (10x) treatment group only. Only two analytes, fluocinonide and triclosan, were below detection limits in all whole-body samples. None of the studied CECs were detected in control fish. No fish mortalities occurred as a result of the 32-day dietary exposure to the mixture of emerging contaminants.

Mitochondrial function and content in laboratory-exposed fish. Liver CS activity was significantly reduced by 29% relative to controls in fish exposed to the medium (1x) dose of the contaminant mixture (**Figure 1A**). Fish exposed to the low (0.1x) and high dose (10x) mixture diets also trended toward lower CS activity relative to controls, but values did not reach statistical significance at $p<0.05$. Respirometry experiments determined similar rates of state 3 respiration (induced by complex I, and complex I+II substrates), uncoupled respiration, and fluxes through complexes II and IV per liver homogenate in control and mixture-exposed fish (**Table 5**). By contrast, state 4 respiration was significantly elevated by all tested doses of the contaminant mixture; specifically, 40 and 70% elevation relative to controls in the low and medium dose groups, and high dose group, respectively (**Table 5**). Subsequently, OCRs measured in

respirometry experiments were normalized to CS activity, and indicated that exposure to CECs caused an overall elevation of respiration per unit mitochondria. State 3 respiration (complex I substrates only) was significantly elevated by 46% in fish exposed to the medium dose of the mixture (**Figure 2A**). Similarly, oxygen flux through complex II was significantly elevated by 42% in fish exposed to the high dose of the mixture (**Figure 2E**). Mixture-exposed fish also trended toward elevated rates of state 3 respiration with complex I+II substrates (**Figure 2B**), uncoupled respiration (**Figure 2C**), and flux through complex IV (**Figures 2 F**) per mitochondria. Moreover, the dietary exposure to CECs induced significant 40 and 70% increases in the rates of state 4 respiration per unit mitochondria in the medium and high dose groups, respectively (**Figure 2D**), which drove the significant reduction of RCR and RCRu in all mixture-exposed fish relative to controls (**Figures 2G and H**).

Effect of dietary exposure to CECs on mitochondrial oxidative injury. Mitochondrial protein carbonyl formation was significantly elevated by 27-39% in the livers of fish exposed to the medium and high doses of the contaminant mixture diets (**Figure 3A**). The increase in mitochondrial protein carbonyl formation occurred concomitantly with a 32% reduction in the liver mitochondrial GSH pool in fish exposed to the high dose mixture (**Figure 3B**). By contrast, the levels of HNE-protein adduct formation (**Figure 3C**) did not differ among control and mixture-exposed fish. Expression of the mitochondrial antioxidant response gene *gpx4* was significantly elevated by 85% in fish exposed to the high dose of the contaminant mixture (**Figure 4A**). However, expression of the other antioxidant response genes *gclc* and *sod2* was not different among control and exposed fish (**Figures 4B and 4C)**. Measurement of genes involved in mitochondrial function revealed significant 24-32% reduction of *pgc1-α* in the medium and high

dose-exposed fish (**Figure 4D)**. Expression of *nrf-1* also trended toward reduction in mixture-exposed fish, but did not reach statistical significance at $p<0.05$ (**Figure 4E)**.

3.2. Field study

Field site water quality parameters and morphological data for wild fish utilized in the respirometry experiments are reported in **Table 2**. Similar temperature, pH, and dissolved oxygen content of the water column were observed at the reference and contaminated sites. Salinity of the water column at the polluted site (23.5 ppt) was higher than that measured at the reference site (15.5 ppt), but values at both sites were within the range of salinity previously reported for Puget Sound estuaries (Department of Ecology, 2014). Condition factors of fish from the two field sites did not significantly differ (**Table 2**).

Fish from the contaminated site had 33% lower liver citrate synthase (CS) activity relative to reference site fish, but these differences were not statistically significant ($p=0.09$) (**Figure 1B**). Similarly, no significant site differences were observed with respect to rates of state 3 respiration (induced by complex I, and complex I+II substrates), uncoupled respiration, state 4 respiration, and fluxes through complexes II and IV per liver homogenate (**Table 6**). Subsequently, mitochondrial OCRs were normalized to CS activity to determine rates of respiratory activity per unit mitochondria. Upon normalization, contaminated site fish trended toward elevated respiratory activity per mitochondria relative to reference site fish. Specifically, fish from the contaminated site trended toward elevated State 3 (complex I, and complex I+II) and uncoupled respiration per unit mitochondria (**Figures 5A-C**), and elevated rates of flux through complexes II and IV per unit mitochondria (**Figures 5E-F**). By contrast, rates of State 4 respiration per mitochondria were

similar among reference and polluted site fish (**Figure 5D**). As a result, respiratory control ratios (RCR) tended to be higher in contaminated site Chinook relative to reference fish (p=0.053, **Figure 5G**). Uncoupled respiratory control ratios (RCRu) also tended to be higher in Chinook from the contaminated site as well (p=0.08, **Figure 5 H**).

4. Discussion.

Effects of exposure to CECs in wild Chinook salmon

In the field study, a trend toward reduced liver mitochondrial content was observed in fish collected from the contaminated field site. It is important to note that in addition to chemical exposures, nonchemical stressors such as temperature (Lucassen et al., 2006) and dissolved oxygen (DO) content (Cooper et al., 2002; Mandic et al., 2014; Zhou et al., 2000) can affect mitochondrial content in fish tissues. However, at the time of sampling, similar temperature and DO content of the water columns were measured at the reference and impacted field sites, and thus we can largely discount the potential effect of these variables on our results. By contrast, salinity at the CEC-impacted site was higher than that measured at the reference site, although within the range of normal values reported for Puget Sound. Relatively few studies have reported the effects of salinity on mitochondrial content in fish tissues (Marshall et al., 1999; Mccormick et al., 1989), and to our knowledge, the effects of salinity on CS activity specifically in liver tissue of fish have not been characterized, with the exception of a report involving hatchery-reared juvenile coho salmon (*Oncorhynchus kisutch*) exposed to increased salinity having elevated CS activity in gill tissues (Shrimpton et al., 1994). The fact that we observed a similar reduction in liver CS activity in Chinook exposed to the mixture of CECs strongly suggests that the observed field effects were due to chemical exposures.

As discussed, OCRs measured per liver homogenate were similar between fish from the two field sites, with the notable exception of lower rates of state 4 respiration in impacted site fish. As a result, the RCRs measured in impacted site fish were higher than those of reference fish, suggesting that mitochondrial function was more efficient in fish from the CEC-impacted site. This finding was unexpected, as we hypothesized that fish from the impacted site would present with elevated state 4 respiration and lower RCR, indicative of mitochondrial dysfunction. However, Du et al. (2015) compared liver mitochondrial ETS function of *Fundulus heteroclitus* from a field site historically-contaminated with persistent organic pollutants to fish collected from a reference site and observed lower rates of state 4 respiration in hepatocytes from reference site fish hepatocytes, but no difference in rates of state 3 respiration (Du et al., 2015). These findings are in concordance with the results of the current study, and may indicate a cellular or physiological adaptive response of fish in polluted environments toward more efficient mitochondrial respiration.

A critical aspect of our mitochondrial function analysis was the normalization of mitochondrial OCRs per homogenate to mitochondrial content, as this led to the finding that respiration per unit mitochondria tended to be greater in fish from the polluted site. Without normalization to mitochondrial content, mitochondrial respiratory functional parameters in liver homogenates were similar among fish from the reference and impacted sites. However, as discussed, mitochondrial content tended to be lower in fish from the contaminated site, suggesting that liver mitochondria of contaminated site fish were respiring at a greater rate than reference site fish. Our findings demonstrate the importance of measuring mitochondrial ETS function as well as mitochondrial content, as this methodology allows for the determination of whether a chemical exposure-induced

change in mitochondrial respiratory activity was caused by a change in ETS function, or rather a change in the quantity of mitochondria in tissue.

Effects of CECs in laboratory-exposed Chinook

In contrast to the somewhat ambiguous trends in certain mitochondrial function parameters observed in wild fish, dysfunction of liver mitochondria was more clearly evident upon exposure to CECs in the laboratory. Significant reduction of liver CS activity was observed upon exposure to the medium dose of the mixture of CECs, and gene expression data showed reduced *pgc1-* expression by dietary exposure to CECs, underlying inhibition of mitochondrial biogenesis, and reduced expression of *nrf-1*, which regulates the expression of mitochondrial ETS subunits. Collectively, these results strongly suggest that environmental exposures to mixtures of CECs can cause a reduction in liver mitochondrial content in juvenile Chinook salmon. Other molecular markers of mitochondrial content, such as mitochondrial DNA (mtDNA), cardiolipin content, protein expression of ETS complexes I-V, and activities of complexes I-IV are of utility in future studies to elucidate the impact of environmental exposure to CECs on mitochondrial content in other tissues and species.

Similar to wild fish, the laboratory-exposed fish fed the contaminant mixture diets had no difference in rates of mitochondrial state 3 respiration per liver homogenate relative to controls, but trended toward reduced liver CS activity. Hence, normalization of OCRs to CS activity determined that exposure to CECs caused the respiration rates per mitochondria in liver to increase. The fact that this trend was also observed in wild fish provides further evidence to suggest that

environmental exposures to mixtures of CECs may increase the respiratory activity per mitochondria, but additional analyses are warranted to verify this hypothesis.

The elevation of state 4 respiration elicited by the contaminant mixtures resulted in reduced efficiency of oxidative phosphorylation as measured by RCR and RCRu. The dietary exposure-induced elevation of state 4 respiration may have been due to modulation of pathways associated with regulated, inducible proton conductance via adenine nucleotide translocase, or uncoupling proteins, which were not investigated in the current study. However, several CECs in the mixture, including the pharmaceutical fluoxetine, (Souza et al., 1994), the PPCP triclosan (Shim et al., 2016), and the perfluorinated compound PFOSA (Schnellmann and Manning, 1990) uncouple mitochondrial respiration and increase rates of state 4 respiration in laboratory animal models. Both fluoxetine and PFOSA bioaccumulated in Chinook salmon; however, triclosan was below detection limits in whole-body samples but may have been present. All 16 CEC analytes comprising the experimental mixture were detected in field samples of wild fish, including the aforementioned chemicals that are potential mitochondrial uncouplers. Similar elevation of state 4 respiration may not have occurred in wild fish due to the controlled nature of the laboratory study, which provided a consistent, daily dietary exposure to the mixture of CECs. Differences in the timing of exposures may have also played a role in the differential effect on state 4 respiration. The elevation of state 4 respiration in laboratory-exposed fish may indicate an early response to exposure to CECs that was not observed in the field-exposed fish. Alternatively, the laboratory exposure involved a mixture of only 16 representative CECs, whereas a complex mixture of chemicals is present in the Puget Sound aquatic environment. Indeed, thousands of compounds

with varying modes of action are present that can ultimately cause protective or inhibitory effects on liver mitochondria.

The physiological consequences of elevated mitochondrial state 4 respiration, which is a measure of respiration attributable to proton leak across the inner mitochondrial membrane, has been an area of interest to several investigators (Jastroch et al., 2010; Rolfe et al., 1999). Proton leak across mitochondrial membranes can vary between cell and tissue types. Across species, leak respiration is influenced by body size and whether an organism is an endotherm or ectotherm (Hulbert et al., 2002). Our results demonstrated that laboratory exposure to the mixture of CECs increased the rates of state 4 respiration in liver mitochondria, suggesting a reduction in the synthesis of ATP via oxidative phosphorylation and reduced metabolic capacity in the liver. This could suggest effects on growth and metabolic deficits. Indeed, our results indicated that exposure to the emerging contaminants caused deficits in growth, over the 32-day exposure period (Meador et al., 2017, in preparation, **Figure 6**). One of the CECs in the experimental mixture was the pharmaceutical metformin, which is a known metabolic disruptor and inhibitor of ETS complex I. Recent reports suggest that metformin may impact growth in fish (Niemuth and Klaper, 2015). Other recent studies have demonstrated that survival of juvenile Chinook salmon, particularly during the first year spent in marine waters, is directly dependent on growth. Furthermore, juvenile Chinook salmon migrating through Puget Sound estuaries impacted by WWTP effluent exhibited significant reduction in survival compared to those migrating through uncontaminated estuaries (Meador, 2014). In this way, the sublethal effects of exposure to CECs may indirectly cause mortalities as a result of reduced population fitness (Spromberg and Meador, 2005).

Mitochondrial respiration is the primary source of intracellular reactive oxygen species (ROS). These ROS generated via oxidative phosphorylation by cross-reactions occurring at ETS complexes include superoxide, hydrogen peroxide, and hydroxyl radical, which can cause oxidative damage to proteins and nuclear and mitochondrial DNA, peroxidation of lipids, and depletion of mitochondrial and cytosolic glutathione. This is consistent with our laboratory study that showed an elevation of protein carbonylation in isolated liver mitochondria, induction of the mitochondrial-protective antioxidant gene *gpx4*, and a reduction of total mitochondrial glutathione in salmon exposed to emerging contaminants. The fact that expression of *gclc* mRNA, a driver of GSH biosynthesis, was not induced by exposure to CECs is consistent with the strict cellular control of mitochondrial GSH (Mari et al., 2009). However, we should also note that we did not discriminate reduced from oxidized mitochondrial GSH in the present study, which may better inform the effects of CECs on mitochondrial redox status. Interestingly, others have proposed that increased proton leak respiration is a cellular adaptive response against oxidative injury from mitochondrial ROS production, i.e., the "uncoupling to survive" hypothesis (Brand, 2000). Because oxidative injury was also associated with elevated state 4 respiration, our results may indicate a compensatory uncoupling of mitochondria in fish exposed to the mixture of emerging contaminants.

As discussed, the laboratory feeding study was able to approximate bioaccumulation of the contaminants at roughly their intended doses measured in wild fish, indicating that the exposure study was representative of environmental exposures. Of note was that the sum of all observed compounds in whole-body fish was relatively low ranging from 51 ng/g wet wt. for the low dose treatment to 795 ng/g ww for the high dose on day 32. It is important to note that hatchery-reared,

actively migrating Chinook salmon are different from laboratory-reared Chinook salmon with respect to a number of physiological parameters, including nutritional status (Congleton and Wagner, 2006). Despite these differences, both wild and laboratory-exposed fish exhibited similar trends and effects on liver mitochondrial content and with respect to a number of functional parameters.

Conclusion

In the present study we observed modulation of both the function and content of mitochondria in liver of juvenile Chinook salmon after exposure to a mixture of CECs at environmentally-relevant concentrations. We compared these liver mitochondrial functional parameters in wild specimen collected from reference and polluted field sites, and in fish under controlled laboratory conditions. As a critical aspect of molecular and physiological function, the liver mitochondrial OCRs reported herein may aid in future studies involving other salmonids or fish species. Importantly, the effects on liver mitochondrial content and function characterized in wild fish from the contaminated estuary were largely replicated under controlled laboratory conditions in an exposure study involving a representative mixture of CECs, underscoring the utility of Chinook salmon as a relevant biomonitoring species.

Tables.

Table 1. Criteria for selection of analytes comprising the CEC mixture

Selection criteria 1.	**Selection criteria 2.**	**Selection criteria 3.**	**Selection criteria 4.**
• Impacted site • Water: ☑ • Fish: ☑ • Reference site • Water: ☐ • Fish: ☐	• Impacted site • Water: ☐ • Fish: ☑ • Reference site • Water: ☐ • Fish: ☐	• Impacted site • Water: ☑ • Fish: ☑ • Reference site • Water: ☐ • Fish: ☑ (lower)	• Impacted site • Water: ☑ • Fish: ☐ • Reference site • Water: ☐ • Fish: ☐
1. Amitriptyline 2. Amlodipine 3. Azithromycin 4. Diltiazem 5. Diphenhydramine 6. Fluoxetine 7. Gemfibrozil 8. Miconazole 9. Sertraline 10. Triclocarban 11. Triclosan	12. Fluocinonide 13. PFDA 14. PFOSA	15. PFOS	16. Metformin

Generation of representative CEC mixture for dietary exposure study. The rationale used to select the 16 CEC mixture analytes were based on four selection criteria. Criteria 1 analytes were detected in water (effluent or estuary) and fish samples from the impacted site, but not in reference site fish. Eleven of the selected CEC were designated as criteria 1 compounds: amitriptyline, fluoxetine, and sertraline (selective serotonin re-uptake inhibitors), amlodipine and diltiazem (Ca^{2+} channel blockers), azithromycin (antibiotic), diphenhydramine (antihistamine), gemfibrozil (lipid regulating agent), miconazole (antifungal agent), and triclocarban and triclosan (personal care products). Criteria 2 analytes were detected in impacted site fish but not water (effluent or estuary), and not in reference site fish; criteria 2 analytes were fluocinonide (topical glucocorticoid), and the perfluorinated compounds perfluorodecanoic acid and perfluorooctane sulfonamide (PFDA and PFOSA, respectively). The perfluorinated compound perfluorooctane sulfonate (PFOS) was a criteria 3 analyte detected in both impacted site water and fish, and detected at lower concentrations in reference site fish. The pharmaceutical metformin (diabetes medication) was a criteria 4 analyte based on its detection in water and whole-body of staghorn sculpin obtained from the reference site (Meador et al., 2016), and because it is one of the most widely prescribed antidiabetic drugs in the world that is frequently detected in environmental samples (Niemuth et al., 2015).

Table 2. Field site estuarine water parameters and morphological parameters of wild juvenile Chinook salmon

		Reference site	**Impacted site**	**p-value**
Estuary parameters	Temperature (°C)	13.5	12.5	-
	pH	7.62	8.04	-
	Dissolved oxygen (mg/L)	8.2	10.6	-
	Salinity (ppt)	15.5	23.5	-
Fish morphological parameters	Weight (g)	6.8 ± 1.5	5.4 ± 2.4	-
	Length (cm)	8.9 ± 0.6	8.2 ± 1.1	-
	Condition factor ([100*(weight/length^3)]	0.96 ± 0.12	0.94 ± 0.14	0.59

Estuary water parameters were determined at the time of sampling. Values for fish morphological parameters are means ± SD of n = 72-75 individuals collected as part of our analytical field study (Meador et al., 2016).

Table 3. Sequence-specific primers for antioxidant response and mitochondrial function-related genes in Chinook salmon

Gene function	Gene name	Primers (5'—3')	Accession # of gene sequence sharing 98-100% identity with primer product
Antioxidant response	*GCLC*	Forward: GTCACCGCTCAACTGGGAAG Reverse: GAACTCAACTCGCCATC	XM_014180126.1
	GPx4	Forward: GCCAAGGACATAGATGGTGA Reverse: TCTCCTGTGTTCTAACTGGG	JN967675.1
	SOD2	Forward: CTCCCTGACCTGACCTATGA Reverse: CCAGATGGCTTTGACGTAGT	XM_014145196.1
Mitochondrial function	*PGC-1*α	Forward: GCAGATGGGGACGTGACCAA Reverse: GAGGAGGAGGAGAGGGAGGA	FJ710605.1
	NRF-1	Forward: GGGGCAAGGAGAGCTGCAAG Reverse: ATGGTCATCTCCCCACCCTG	XM_014208801.1
Reference gene (Cell structure)	β-actin	Forward: AACGGATCCGGTATGTGCAA Reverse: TAGAAGGTGTGATGCCAGAT	FJ890357.1
Reference gene (Component of 60S subunit of ribosome)	*RPL8*	Forward: CGTCATGGTTACATCAAGGG Reverse: GGAAGCTTGACTCTGGACTT	FJ226373.1

Table 4. Whole body concentrations of CECs measured in laboratory-exposed Chinook salmon.

CEC analyte	**Field study data** (Meador *et al.*, 2016)	**Laboratory exposure study** Concentrations after day 32 of feeding study			
	Fish tissue ng/g	**Control** ng/g (RL)	**0.1x** ng/g (RL)	**1x** ng/g (RL)	**10x** ng/g (RL)
Amitriptyline	**0.63**	U (0.13)	**0.13** (0.13)	U (0.12)	**0.96** (0.1)
Amlodipine	**0.81**	U (0.59)	**1.2** (0.59)	**1.1** (0.59)	**20** (0.58)
Azithromycin	**1.7**	U (1.81)	**2.8** (2.42)	**1.8** (0.65)	**29** (0.733)
Diltiazem	**1.5**	U (0.24)	**17** (0.32)	**11** (0.23)	**100** (1.1)
Diphenhydramine	**2.6**	U (0.24)	**1.8** (0.23)	**1.2** (0.24)	**14** (0.23)
Fluocinonide	**6.5**	U (2.4)	U (2.9)	U (3.8)	U (3.0)
Fluoxetine	**4.8**	U (0.59)	**1.4** (0.59)	**1.0** (0.59)	**20** (0.58)
Gemfibrozil	**1.3**	U (0.59)	U (0.59)	U (0.59)	**3.3** (0.58)
Metformin	**28**	U (2.88)	**4.5** (3.03)	U (4.7)	**40** (2.61)
Miconazole	**1.6**	U (0.73)	**1.2** (0.75)	U (0.68)	**13** (0.63)
PFDA	**0.78**	U (0.50)	**1.3** (0.44)	**2.2** (0.50)	**31** (0.48)
PFOS	**35**	U (0.99)	**5.7** (0.88)	**7.5** (0.99)	**130** (0.95)
PFOSA	**2.2**	U (0.59)	**6.0** (0.53)	**9.1** (0.59)	**190** (0.57)
Sertraline	**11**	U (0.52)	U (0.52)	U (0.52)	**9.1** (0.52)
Triclocarban	**5.2**	U (1.18)	U (1.17)	U (1.18)	**8.7** (1.16)
Triclosan	**25**	U (23.5)	U (23.4)	U (23.5)	U (23.3)

Table legend. Fish tissue concentrations from the field study represent mean analyte concentrations measured in field samples. "RL" reporting limit; "U" below detection limit.

Table 5. Liver oxygen consumption rates (OCR) measured in laboratory-exposed juvenile Chinook salmon

	Liver oxygen consumption rates (pmoles O_2/[sec*mg tissue]) ± SEM			
ETS functional parameter	**Control**	**0.1x**	**1x**	**10x**
State 3 (complex I)	8.4 ± 0.3	8.1 ± 0.4	9.4 ± 0.5	8.6 ± 0.8
State 3 (complex I+II)	14 ± 0.6	12 ± 0.8	13 ± 0.4	14 ± 1.4
Uncoupled	15 ± 0.6	12 ± 1.0	13 ± 0.8	15 ± 1.2
State 4	1.0 ± 0.0	**1.4 ± 0.2**	**1.4 ± 0.1**	**1.7 ± 0.1**
Flux through complex II	9.1 ± 0.4	7.8 ± 0.6	8.6 ± 0.6	9.6 ± 0.8
Flux through complex IV	16 ± 1.2	12 ± 0.8	14 ± 1.6	15 ± 2.2

Values in bold indicate $p \leq 0.05$ relative to the control group. Data are mean liver mitochondrial oxygen consumption rates (OCR) ± SEM of n = 6-8 individuals per treatment group.

Table 6. Liver oxygen consumption rates (OCR) measured in wild juvenile Chinook salmon from the reference and CEC-impacted field sites

	Liver oxygen consumption rates (pmoles O_2/[sec*mg tissue]) ± SEM	
ETS functional parameter	**Reference site**	**Impacted site**
State 3 (complex I)	13 ± 0.8	13 ± 1.5
State 3 (complex I+II)	17 ± 0.9	16 ± 2.0
Uncoupled	17 ± 1.0	17 ± 2.1
State 4	1.9 ± 0.3	1.3 ± 0.2
Flux through complex II	6.9 ± 0.6	6.3 ± 1.1
Flux through complex IV	27 ± 1.8	28 ± 4.8

Data are mean liver mitochondrial oxygen consumption rates (OCR, pmoles O_2/[sec*mg tissue]) ± SEM of n = 6 individuals per field site.

Figures.

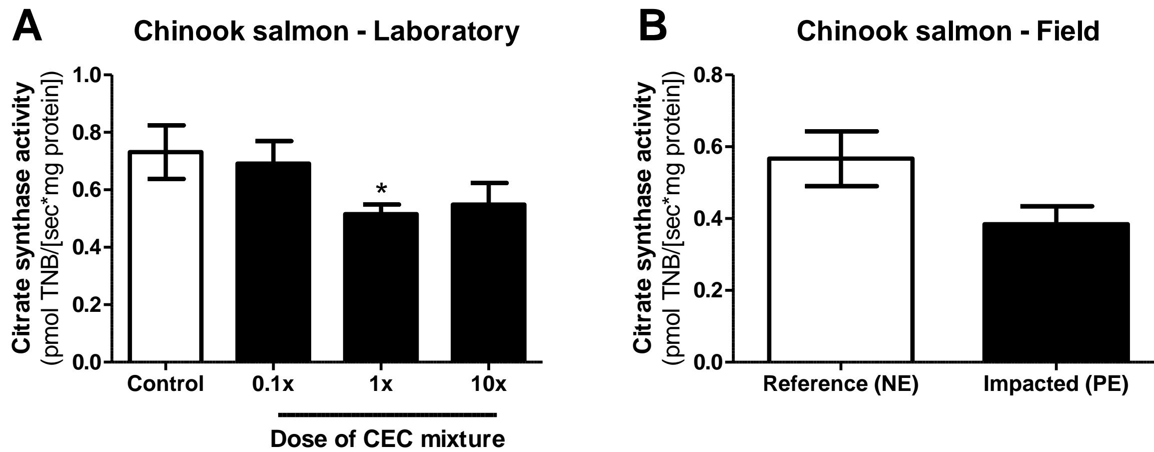

Figure 1. Mitochondrial content in livers of Chinook salmon as measured by activity of citrate synthase (CS) (pmoles TNB/[sec*mg tissue]). (A) CS activity measured in control and CEC-exposed Chinook salmon following 32-day dietary exposure in the laboratory. *$p \leq 0.05$ relative to control fish. Data are mean CS activity ± SEM of n = 6-8 individuals. (B) CS activity measured in livers of wild juvenile Chinook salmon from the NE reference site and PE CEC-impacted site. Data are mean CS activity ± SEM of n = 6 individuals.

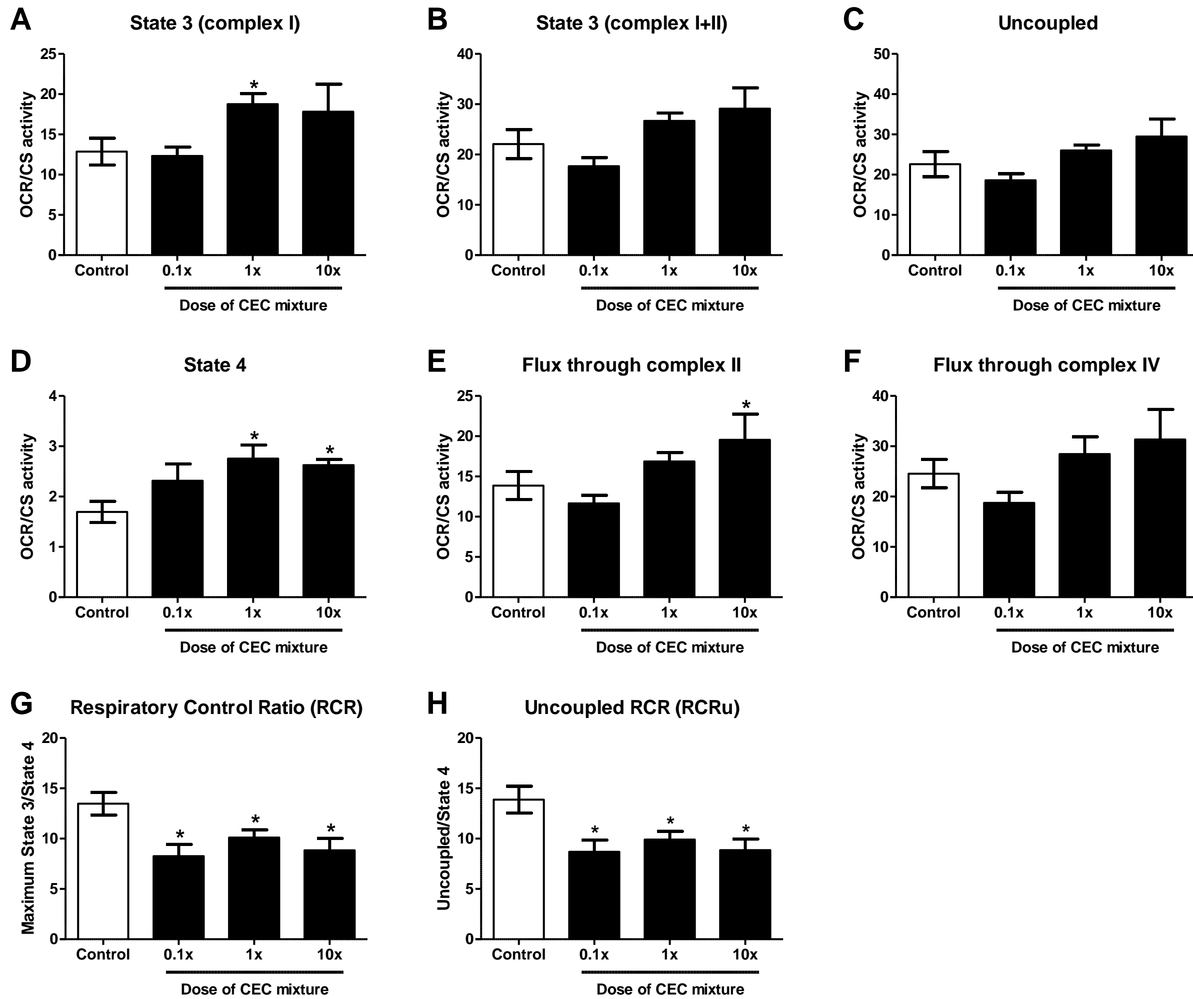

Figure 2. Liver mitochondrial oxygen consumption rate (OCR, pmoles O_2/[sec*mg tissue]) normalized to mitochondrial content as measured by citrate synthase activity (CS activity, pmoles TNB/[sec*mg tissue]) in liver of hatchery-reared juvenile Chinook salmon (*O. tshawytscha*) exposed to increasing dose of the Puget Sound CEC mixture for 30 days. Experimental conditions to measure mitochondrial OCR were: (A) State 3 respiration with complex I substrates; (B) maximal State 3 respiration with substrates of complexes I and II; (C) maximum uncoupled respiration; (D) State 4 respiration induced by oligomycin. Also, flux through (E) complex II and (F) complex IV were determined. Efficiency of oxidative phosphorylation as measured by (G) respiratory control ratio (RCR) and (H) uncoupled RCR (RCRu) were calculated as the ratio of maximal State 3/State 4 respiration, and uncoupled/State 4 respiration, respectively. *$p \leq 0.05$ relative to the control group. Data are means ± SEM of n = 6-8 individuals.

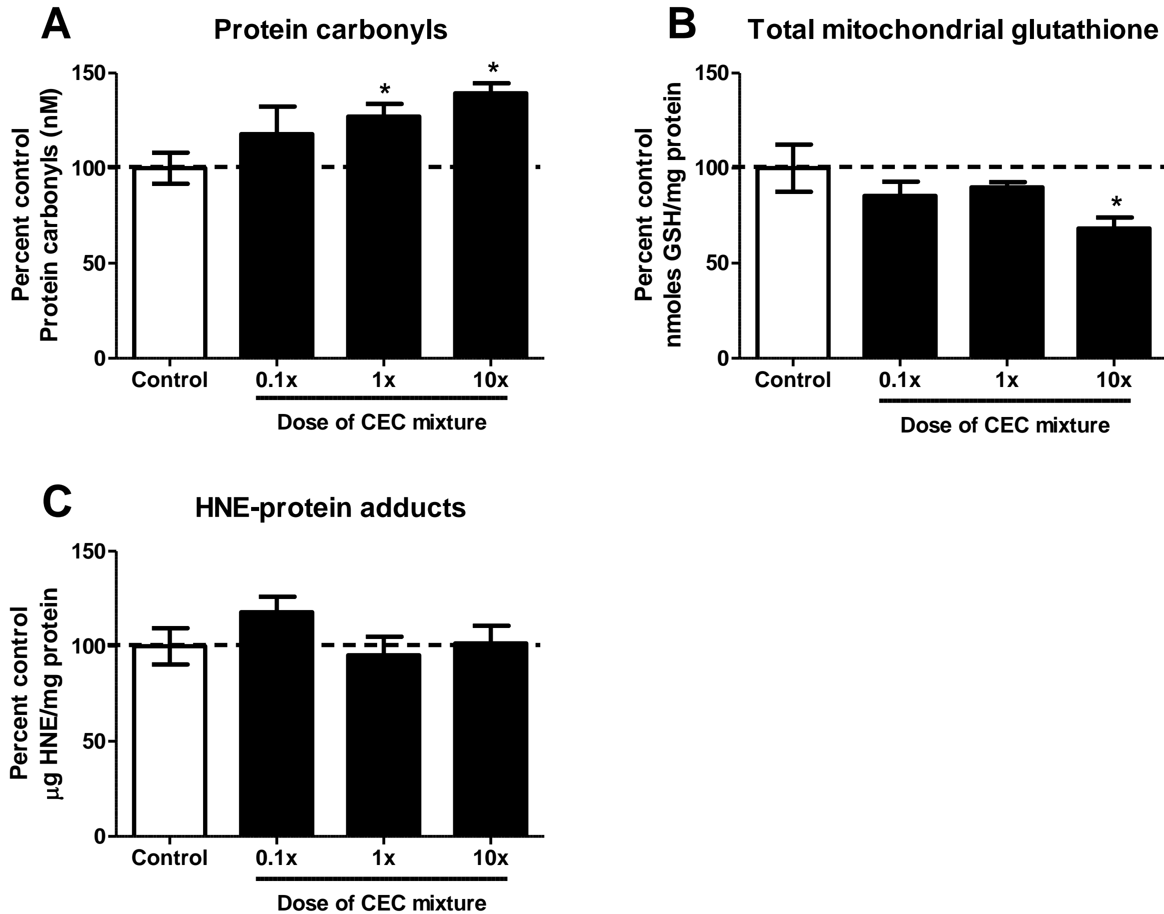

Figure 3. Indices of oxidative injury assessed in isolated liver mitochondria of CEC-exposed Chinook salmon. Concentrations of: (A) protein carbonyls, (B) total reduced and oxidized glutathione, and (C) HNE-protein adducts were determined in isolated liver mitochondria. *$p \leq 0.05$ relative to the control group. Data are expressed as percent of control values and are mean concentrations ± SEM for n=6-8 individuals.

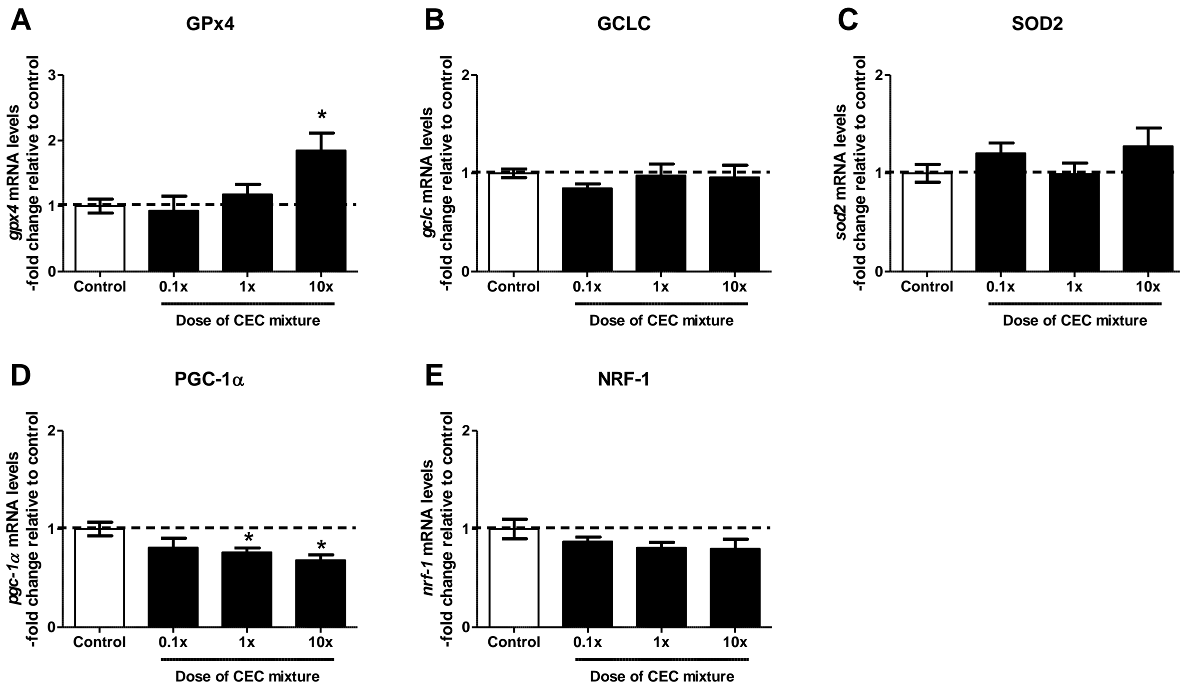

Figure 4. Effect of CEC mixture on antioxidant response and mitochondrial function-related genes. mRNA transcript levels of the antioxidant response genes (A) *gpx4*, (B) *gclc*, (C) *sod2*, and mitochondrial function genes (D) *pgc-1α*, and (E) *nrf-1* were measured in livers of juvenile Chinook salmon exposed to the CEC mixture for 32 days. Gene expression was normalized to the geometric mean of expression of reference genes *β-actin* and *rpl8*. *$p \leq 0.05$ relative to the control group. Data represent -fold change in mRNA levels normalized to the reference genes and are mean ± SEM of n=6-8 individuals.

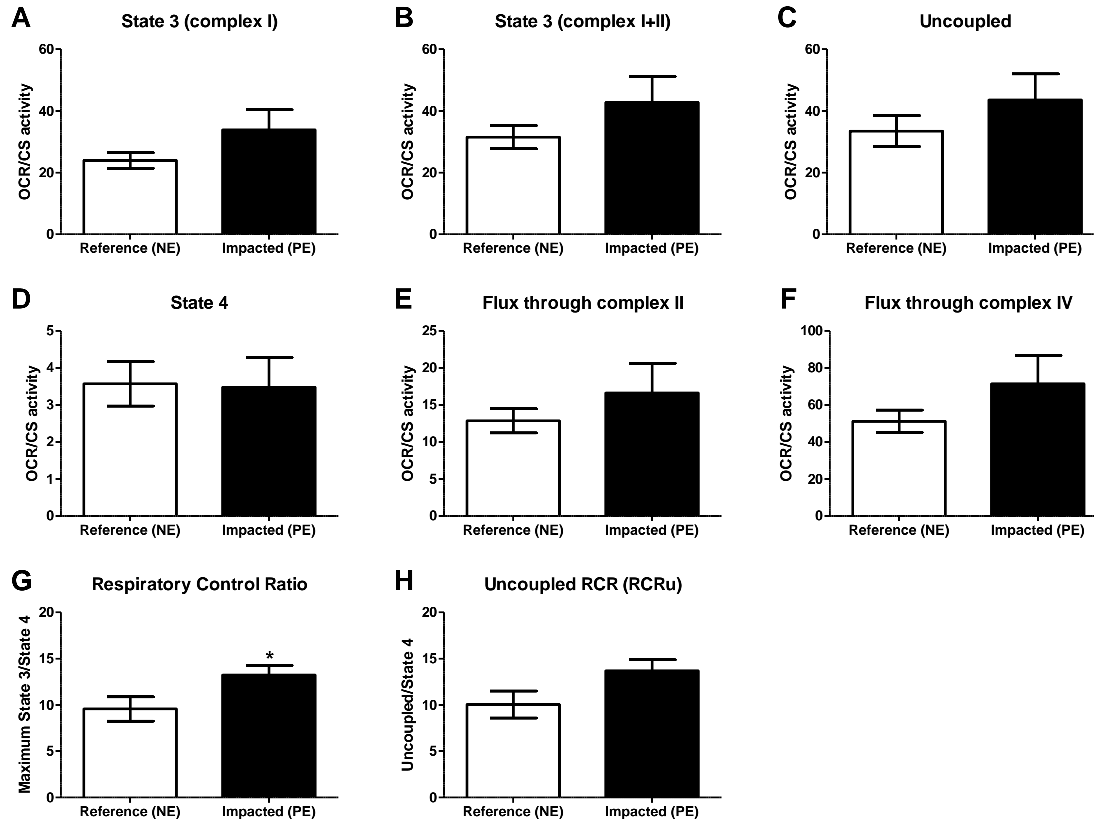

Figure 5. Liver mitochondrial oxygen consumption rate (OCR, pmoles O_2/[sec*mg tissue]) normalized to mitochondrial content as measured by citrate synthase activity (CS activity, pmoles TNB/[sec*mg tissue]) in liver of wild juvenile Chinook salmon (*O. tshawytscha*) from the reference Nisqually estuary (NE) and CEC-impacted Puyallup estuary (PE) field sites. Experimental conditions to measure mitochondrial OCR were: (A) State 3 respiration with complex I substrates; (B) maximal State 3 respiration with substrates of complexes I and II; (C) maximum uncoupled respiration; (D) State 4 respiration induced by oligomycin. Also, flux through (E) complex II and (F) complex IV were determined. Efficiency of oxidative phosphorylation as measured by (G) respiratory control ratio (RCR) and (H) uncoupled RCR (RCRu) were calculated as the ratio of maximal State 3/State 4 respiration, and uncoupled/State 4 respiration, respectively. Data are means ± SEM of n = 6 individuals.

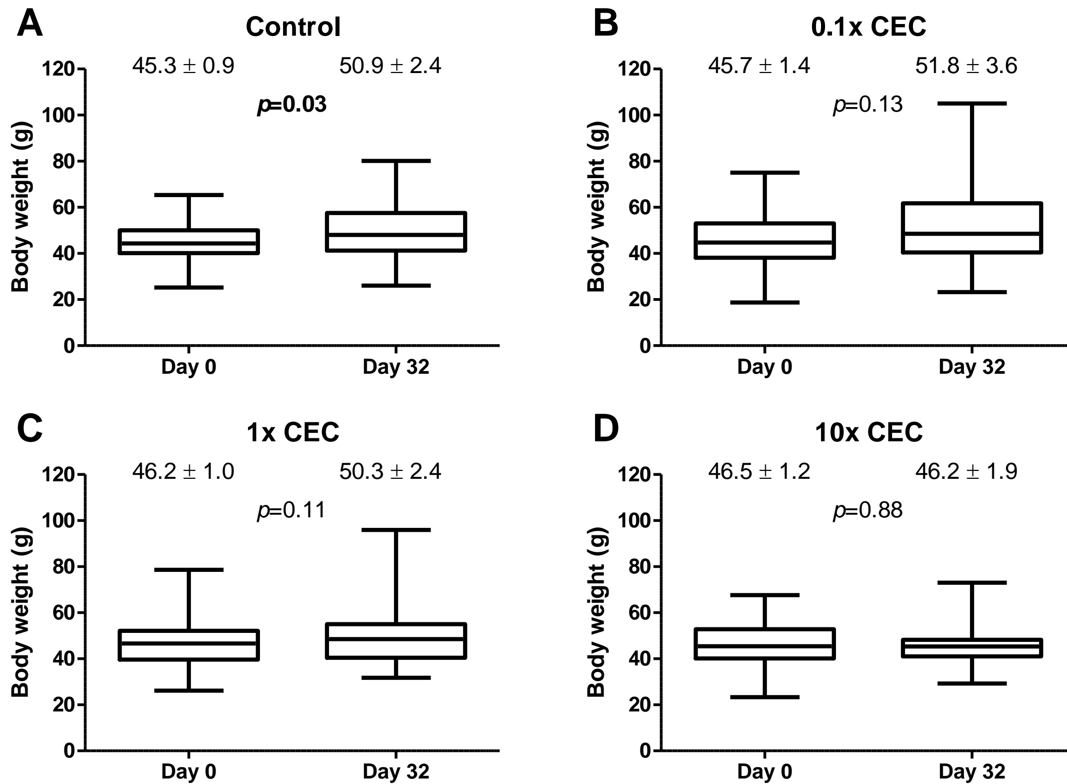

Figure 6. Growth of laboratory-exposed juvenile Chinook salmon. Body weights on days 0 and 32 of the exposure period are shown for (A) control, (B) 0.1x, (C) 1x, and (D) 10x CEC mixture dose-exposed fish. Data represent mean ± SEM of n=60-80 fish on day 0 and n=18-24 fish on day 32. Statistically significant difference of means was assessed by Unpaired t-test with Welch's correction at significant level $p<0.05$.

Conclusions

These studies demonstrate that mitochondrial function is an important aspect of environmental toxicology that should be considered in future human and ecological risk assessments of emerging contaminants. The flame retardant compound BDE 47, presently one of the most persistent and bioaccumulative "emerged" environmental contaminants detected in humans and Puget Sound Chinook salmon, elicited novel and unique effects on mitochondrial ETS function in a human liver cell line. These detrimental impacts on mitochondrial respiratory function were ameliorated by pretreatment with omega-3 fatty acids at levels that were conservative for human consumption of salmon. The data presented herein can better inform the human risk assessment regarding ingestion of contaminated salmon.

Mechanistic studies involving single compound exposures will always remain a necessity in assessing toxicological effects. However, as demonstrated by the analytical field study, the most environmentally-relevant exposure scenario for aquatic organisms is exposure to a mixture of structurally-diverse compounds. Barriers with respect to conducting analytical field studies for emerging contaminants remain. These include the monetary cost of analyses, and that current analytical methods cannot report concentrations of all compounds present within a mixture, but rather report concentrations of only those compounds that were of interest to researchers prior to the analyses. As a result, studies involving exposure to mixtures of environmental contaminants are difficult to conduct in the laboratory. Despite this, current analytical chemistry techniques and instruments are able to reliably detect part per trillion levels of emerging contaminants in environmental samples, and this technology is likely to continue to improve over time. The fact that CECs were detected at greater-than-expected concentrations and frequency in the reference

site, and that marked bioaccumulation of CECs was observed in juvenile Chinook relative to the benthic staghorn sculpin collected in the field, underscore the imperative need for continued biomonitoring for CECs and establishment of biomarkers of exposure and effect of CECs in aquatic organisms.

Bioaccumulation and exposure to emerging contaminants was associated with mitochondrial effects in wild juvenile Chinook, specifically, trends toward increased respiration per unit mitochondria, suggesting that the cause was related to exposure to CECs in the field. A controlled laboratory exposure to a mixture of Puget Sound CECs was able to replicate bioaccumulation of CECs in wild Chinook. An increased oxygen flux per unit mitochondria in Chinook liver was consistent between both the laboratory-exposed and wild fish, which removed the potential confounding effects of nonchemical stressors. The fact that some of the inhibitory mitochondrial effects lead to, or were associated with, oxidative damage to mitochondrial proteins and an increase in the mitochondrial protective antioxidant gene *gpx4*, implicates oxidative stress as a co-mechanism of CEC-induced mitochondrial toxicity. Collectively, these studies support analysis of mitochondrial content and function in wild fish as potential indicators linking exposure and effects of emerging contaminants.

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