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# Adverse metabolic effects in fish exposed to contaminants of emerging concern in the field and laboratory☆,☆☆

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ABSTRACT

Several metabolic parameters were assessed in juvenile Chinook salmon (*Oncorhynchus tshawytscha*) and staghorn sculpin (*Leptocottus armatus*) residing in two estuaries receiving wastewater treatment effluent and one reference estuary. We also conducted a laboratory study with fish dosed for 32 days with 16 of the most common contaminants of emerging concern (CECs) detected in feral fish. Several blood chemistry parameters and other indicators of health were measured in fish from the field and laboratory study that were used to assess potential metabolic disruption. The blood chemistry values observed in feral juvenile Chinook salmon were relatively consistent among fish collected from effluent-impacted sites and substantially different compared to reference site fish. These responses were more pronounced in Chinook salmon, which is supported by the disparity in accumulated CECs. The blood chemistry results for juvenile Chinook salmon collected at effluent-impacted sites exhibited a pattern generally consistent with starvation because of similarities to observations from studies of food-deprived fish; however, this response is not consistent with physical starvation but may be contaminant induced. The altered blood chemistry parameters are useful as an early indicator of metabolic stress, even though organismal characteristics (lipid content and condition factor) were not different among sites indicating an early response. Evidence of metabolic disruption was also observed in juvenile Chinook salmon that were exposed in the laboratory to a limited mixture of CECs; however, the plasma parameters were qualitatively different possibly due to exposure route, season, or the suite of CECs. Growth was impaired in the high-dose fish during the dosing phase and the low- and medium-dose fish assayed after 2 weeks of depuration. Overall, these results are consistent with metabolic disruption for fish exposed to CECs, which may result in early mortality or an impaired ability to compete for limited resources.

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## 1. Introduction

Contaminants of emerging concern (CECs) are frequently associated with endocrine disruption and reproductive effects because this broad class of compounds includes very potent natural and synthetic hormones in addition to hormone mimics (Harding et al., 2016). In addition to reproductive toxicants, several of these compounds are potential metabolic disruptors (Casals-Casas and Desvergne, 2011) and can also cause adverse behavioral and immune system responses in organisms.

Wastewater treatment plants (WWTPs) are known conduits to receiving waters for a variety of pharmaceuticals, personal care products, industrial compounds, metals, and legacy compounds. Several of these chemicals have been shown to affect fish at very low concentrations (Fairchild et al., 1999; Daughton and Brooks, 2011; Schultz et al., 2012; Saaristo et al., 2017); however, few data exist on toxic responses for most of these poorly studied chemicals, especially as mixtures. To date, most studies conducted on aquatic

☆ This paper has been recommended for acceptance by Dr. Sarah Michelle Harmon.

☆☆ In this study we examined organismal and physiological parameters for feral fish exposed to contaminants of emerging concern in WWTP impacted estuaries. The plasma chemistry parameters were consistent with metabolic disruption for juvenile Chinook and appear to be useful as early indicators of adverse effects.

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organisms have assessed reproductive effects or behavioral alterations and very few have focused on metabolic disruption. One study found reduced growth in male fathead minnow (*Pimephales promelas*) exposed to 40,000 ng/L metformin in addition to disruption of reproductive parameters (Niemuth and Klaper, 2015). Another study reported that alkylphenols (octylphenol, nonylphenol, and nonylphenol diethoxylate) inhibited growth in rainbow trout (*Oncorhynchus mykiss*) at relatively low concentrations in the range of ng/mL (Ashfield et al., 1998). Many of these compounds can affect multiple physiological pathways for a given exposure resulting in simultaneous impairment to reproductive, growth, or behavioral parameters. Additionally, these alterations may result in indirect effects such as abnormal behavior leading to reduced feeding and growth or increased predation (Painter et al., 2009).

For many endocrine disrupting compounds, there is a critical linkage between endocrine receptor agonism and activation of metabolic pathways, suggesting a commonality among metabolic abnormalities and classic endocrine disrupting responses (Chen et al., 2009). Certainly, many non-endocrine pathways may be impacted by CECs and other contaminants resulting is disruption of metabolic homeostasis. Indeed, these compounds may have fairly high specificity for their targets based on evolutionary conservation across vertebrates (Gunnarsson et al., 2008), suggesting conservation of function. Considering the range of compounds detected in WWTP effluent (Meador et al., 2016), a large number of these are potential metabolic disruptors in aquatic organisms and may act directly on metabolic or endocrine pathways, or indirectly via other receptors.

Our previous study reported a high percentage of analyzed CECs (61%, n = 150) in effluent, estuary water, or fish tissue collected from WWTP impacted estuarine sites (Meador et al., 2016). The current report presents an evaluation of the potential effects resulting from exposure to those compounds, which were selected as a representative group with little data available on occurrence in marine waters or toxicity for exposed fish. Our goal for this study was to assess several metabolic attributes in juvenile Chinook salmon (*Oncorhynchus tshawytscha*) and Pacific staghorn sculpin (*Leptocottus armatus*) exposed to WWTP effluent in the field and juvenile Chinook exposed in the laboratory to a model mixture of a select group of CECs quantified in whole-body feral fish inhabiting impacted sites. Our specific focus was on blood chemistry parameters as potentially useful metrics that could be utilized as early indicators of metabolic disruption.

## 2. Methods

### 2.1. Field study

Details regarding fish collections, physical-chemical parameters, and chemical analysis can be found in Meador et al. (2016) and Table S1. Briefly, fish were collected from three local Puget Sound, WA estuaries. Two of the sites (Sinclair Inlet and the Puyallup River estuary) receive effluent from WWTPs. A minimally contaminated site, the Nisqually River estuary, was selected for comparison and served as our reference site. Two fish species that commonly occur in Puget Sound estuaries were collected and sampled for blood plasma. One was a benthic species, Pacific staghorn sculpin, which is found widely in Puget Sound and U.S. west coast temperate waters. The other fish species was juvenile ocean-type Chinook salmon, which can reside for several weeks in nearshore estuaries where contaminants are often concentrated. We also collected several juvenile Chinook salmon from the Voights Creek Hatchery on the Puyallup River upstream of the estuary on 28 May 2014. As reported in Meador et al. (2016) only 7 analytes were detected in these fish, most at relatively low concentrations. All estuarine juvenile Chinook salmon were collected in the estuary within a 10 d period and were approximately the same age as most were released from upstream hatcheries approximately 3–5 weeks before capture (Table S1).

#### 2.1.1. Field samples

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 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 Meador et al. (2016).

Juvenile Chinook salmon and staghorn sculpin were obtained at each field site with a beach seine. Fish were kept alive after collection in the field and transported to the laboratory for processing in aerated site water that was maintained at 13 °C with ice packs. All samples for chemistry and plasma were taken approximately 3–6 h after capture. Fish were euthanized with tricaine methanesulfonate (MS-222; Argent Chemical Laboratories, Redmond, WA) for processing. Chemical analyses for CEC analytes were conducted on composite samples consisting of 3–12 whole-body salmon or 3–5 whole-body sculpin (Meador et al., 2016).

### 2.2. Laboratory study

The laboratory study was conducted at the University of Washington fish hatchery in Seattle, WA. Approximately 400 juvenile Chinook salmon were received from the Wallace Falls hatchery (Gold Bar, WA) on 9 Feb 2015. The average individual weighed approximately 45 g and water temperature at the hatchery was approximately 10 °C. These fish were yearling (1 + years) Chinook salmon that were a genetic cross between hatchery and wild fish (first generation) and were not treated with any chemicals at the hatchery. No mortality occurred due to handling or transit to our laboratory holding tanks. Fish (n = 20 per tank) were randomly distributed to 15 circular tanks each with a 500 L capacity. Lake Washington was the source of water to the tanks, which was supplied at approximately 1 L/m flow-through at 12 °C. Treatments were assigned at random to tanks and 3 or 4 tank replicates per CEC dose were tested. An additional tank of fish was not fed during the 32 d exposure period. Large windows allowed ambient light into the experimental area and tanks were partially covered with black plastic to give fish cover and shield them from artificial light.

The rationale for selection of the compounds comprising the CEC mixture was presented in Yeh et al. (2017) and in Table S2. Concentrated stock solutions for each CEC analyte were generated by dissolving the compounds in 50–100 mL of absolute ethanol. Calculated amounts of CEC stock solutions were added to three separate volumes of 4 L of 100% ethanol in order to generate the 0.3× (low), 1× (medium), and 10× (high) dose CEC mixtures, which were selected to mimic whole-body concentrations observed in our field-collected fish. BioClark's Fry 2.5 mm low-fat food pellets (BioOregon, Longview, WA) were dosed with the CEC mixtures by complete immersion with the ethanol mixture and taken to dryness under a fume hood. Previous studies have shown this to be an effective method for dosing fish pellets (Meador et al., 2005, 2006). The fish food used in the control diet was treated identically with the ethanol solvent minus the CEC mixture. Based on previous studies (Meador et al., 2005, 2006) fish were fed 2% body weight (bw)•day$^{-1}$ spread over 2 feedings per day, 5 days per week, from days 0–32 for a total of 25 daily feedings (50 total). Dietary exposure for toxicity studies is a well-supported approach that is widely used. The most important aspect for toxicity characterization is the

tissue concentration, not the ambient concentration or route of exposure (Meador et al., 2008).

The rate of dosing for each compound is listed in Table S3. An unquantified portion of the food was not consumed hence the true rate of ingestion by fish in each tank was unknown. Most fish actively fed during the application of food to tanks; however, the rate was less than anticipated based on previous studies, hence instead of pair-feeding the ration was considered as ad libitum. An additional tank of fish were not fed (food-deprived (fasted) treatment) during the first phase (days 0–32). After sampling on day 33, the remaining live fish were fed unadulterated fish pellets once/day over a two week period, days 34–50 (fed 10 times) at a rate of 2% bw•day$^{-1}$. The goal was to depurate the fish and resample for plasma and liver, which was completed on 6–7 April 2015. Fish in the food-deprived treatment were also fed this amount.

#### 2.2.1. Fish processing in the laboratory study

All fish in each tank were weighed individually to generate a tank mean for day 32 and approximately half of these fish were randomly sampled on days 32–33 for liver, plasma, and whole-body chemistry. The weights for fish not sampled were then used to determine the tank mean for the start of the depuration phase (day 34) and these fish were weighed again on day 50. For both sampling periods, concentrations of CECs and whole-body lipid content in lab fish tissue were determined on composite samples of 6–8 fish per treatment (2 fish from each replicate tank/treatment). The entire alimentary canal was opened and rinsed with distilled water of all undigested food to avoid bias in the chemical determinations.

### 2.3. Plasma samples and metabolic parameters

Whole blood was taken from several fish by transection of the caudal peduncle until approximately 1 mL was obtained to form one composite sample. Pooling samples was necessary due to low volumes of blood per fish; therefore, several individuals comprised each of multiple composites per field site (Table S4). For the lab study, composite samples containing 2 fish from each tank were formed for analysis of plasma chemistry that resulted in 3 or 4 independent replicates per treatment depending on the number of tanks per dose. For all composite samples, individual fish were essentially the same weight and contributed similar amounts of blood. Multiple composite samples containing several fish for each site or treatment was advantageous for characterizing the true mean, minimizing variance, and reducing analytical costs (Bignert et al., 2014; Heffernan et al., 2014).

Blood from each fish was collected in a heparinized 1.3 mL sample vial (heparin grade 1-A, 25 IU). Samples were immediately centrifuged at 18,800 rpm for 150 s in a Statspin veterinary centrifuge and then frozen at −80 °C until analyzed. Plasma samples were analyzed using an automated blood chemistry analyzer (Idexx VetTest 8008 Chemistry Analyzer, Idexx Labs, Westbrook, ME, USA). Blood plasma was analyzed for albumin (ALB), alkaline phosphatase (ALKP), alanine aminotransferase (ALT), amylase (AMYL), calcium (CA), cholesterol (CHOL), creatinine (CREA), glucose (GLU), lipase (LIPA), inorganic phosphate (PHOS), triacylglycerols (TAGs), and total protein (TP). This suite of selected blood chemistry parameters was based on previous studies demonstrating their utility for characterizing altered metabolic homeostasis (Wagner and Congleton, 2004; Meador et al., 2006; Congleton and Wagner, 2006). A quality control procedure (Idexx Vetrol control lot number J3910) was conducted prior to analysis of the test samples to verify both the VetTest optic groups and the integrity of the test slides. All samples were analyzed in random order.

### 2.4. Analytical methods

Concentrations of CEC analytes were determined by AXYS Analytical, Ltd. (Sidney, British Columbia, Canada) using LC/MS/MS techniques for the field and laboratory study fish. Meador et al. (2016) provides a complete list of the 150 different CEC analytes in feral fish with their analytical methods, reporting limits, and observed concentrations. Whole-body lipid content was determined by AXYS Analytical Ltd. using a Soxhlet extraction technique with dichloromethane as the extraction solvent. A portion of the extract was used to determine lipid content gravimetrically for the same composite samples as that for CEC chemistry.

### 2.5. Statistical analyses

Differences in treatment means for fish weights and blood chemistry were tested with Analysis of Variance (ANOVA). Tanks, as opposed to individual fish, were treated as the experimental unit for all ANOVA tests; therefore, treatment differences were based on mean values for replicate tanks. The hypothesis of equal variance among tank means (homoscedasticity) was tested with Levene's test.

Control versus treatment differences were determined with Fisher's Protected Least Significant Difference (PLSD) post-hoc test. We consider the p-values from the posthoc tests to be an important indicator of the relationship between observed data and the null hypothesis of no treatment effect (Hurlbert and Lombardi, 2009; Wasserstein and Lazar, 2016). The strongest response was noted for those p-values below 0.05. *P*-values between 0.05 and 0.15 were also shown because they may indicate biologically important trends, especially in light of occasional high variability.

A paired *t*-test was conducted on replicate tank values for average fish weight by treatment on days 0 and 32 and for days 34 and 50 to test the hypothesis of positive fish growth from one sampling period to the next. For all data, the standard error of the mean (SEM), a statistic of the mean, is reported to facilitate comparisons of mean of values. For some results, the standard deviation was shown to indicate a range in the data. Statistical analyses were conducted with SYSTAT 11 and Statview 5.0.

The plasma chemistry results were log transformed and autoscaled for multivariate analyses using MetaboAnalyst (Xia and Wishart, 2016). We generated heatmap plots, dendograms, and 2D scores plots using Partial Least Squares-Discriminant analysis. Dendograms and heatmaps were constructed using a Euclidean distance measure and the Ward clustering algorithm.

We calculated the apparent half-life for parent compounds during the depuration phase (days 34–50) in the lab study when data were available for both time points. A simple first order elimination equation, as described in Meador (1997), was used as an approximation for the half-life that was based on minimal data. Metabolites were not included because of the continual conversion from parent compound during the depuration phase. An additional analysis included applying the Fish Plasma Model (FPM) to these data as described in Meador et al. (2017). We predicted plasma concentrations with observed whole-body tissue concentrations and estimates for the volume of distribution. The estimated plasma concentration for each measured compound was compared to the 1%Cmax total and expressed as the Response Ratio ($RR_{tissue}$).

## 3. Results

### 3.1. Field study

Condition factor (CF) and lipid content were determined for fish from each site to assess general health. The CF for juvenile Chinook

among sites that was based on data from all fish collected ranged from 0.92 to 0.96 and while the values for fish from impacted sites were slightly lower, the p-values were relatively high (Table S5). The CF for sculpin was higher for the Puyallup estuary fish compared to the Nisqually estuary fish ($p = 0.045$) and the reference site fish were not different compared to the Sinclair Inlet fish. The observed whole-body lipid data indicated no differences between samples of juvenile Chinook from the Nisqually estuary and Sinclair Inlet; however, fish from the Puyallup River estuary exhibited a higher mean value (3.8%) (Table S5). The whole-body lipid content for staghorn sculpin was relatively constant among sites with values ranging from 1.7% to 1.9%.

### 3.1.1. Chinook salmon plasma chemistry

The plasma chemistry parameters measured in juvenile Chinook salmon indicated a number of strong differences ($p < 0.05$) between the reference site (Nisqually) and the two WWTP-impacted estuarine sites. For example, lower values were obtained for ALB, ALKP, ALT, AMYL, CHOL, GLU, LIPA, and TAGs compared to one or both impacted sites (Fig. 1, Table S6). TAGs were lower for the Sinclair fish, but highly elevated for fish from the Puyallup River Estuary. Creatinine, a breakdown product of creatine was elevated for the Puyallup fish as was CA, GLOB, and TP relative to the reference fish. The multivariate results indicate strong differences among sites as seen in the PLS-DA scores plot (Fig. 2), in addition to the heatmap and dendogram (Figs. S1 and S2).

### 3.1.2. Sculpin plasma chemistry

Insufficient plasma was obtained from sculpin collected in Sinclair Inlet precluding analysis for that site. The data for fish from the Nisqually and Puyallup River estuaries were analyzed separately by sex (Table S6, Fig. 1b and Fig. S3). Additionally, 2 large females (>100 g) were excluded because of concerns related to altered physiology during the reproductive phase and the remaining sculpin from both sites were pre-vitellogenic (Gallagher et al., 2016). The strongest response was for lower levels of albumin in the Puyallup female sculpin. Additional noteworthy differences include the generally lower values for GLU and TAGs for the Puyallup females, which is the same trend observed for juvenile Chinook. The multivariate results for sculpin indicate only weak separation among sites for plasma chemistry as seen in the PLS-DA scores plot (Fig. 3), in addition to the heatmap and dendogram (Figs. S4 and S5).

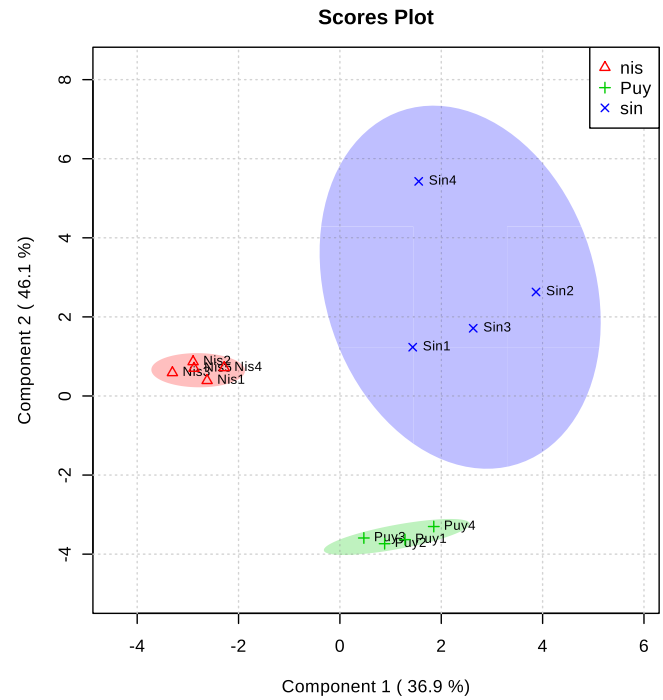


**Fig. 2.** Partial least-squares-discriminant analyses (PLS-DA) scores plots for the chemical profiles from plasma samples for juvenile Chinook salmon collected in the field. Each site highlighted by a 95% confidence ellipse. Nis is the Nisqually reference site, Sin in Sinclair Inlet, and Puy is the Puyallup River Estuary.

## 3.2. Laboratory exposure study

### 3.2.1. Bioaccumulation

Many of the CECs fed to fish were found in their tissue at concentrations relevant to those observed in field-collected juvenile Chinook salmon (Table 1). All administered CECs were detected in whole-body fish, except for fluocinonide and triclosan that may have been present but below their analytical reporting limits (Table 1). A comparison of the observed tissue concentrations for the laboratory study fish and those quantified in field-collected fish indicated a geometric mean ratio of 0.8, 1.2, and 7.5 when all individual compounds were summed, indicating relatively good

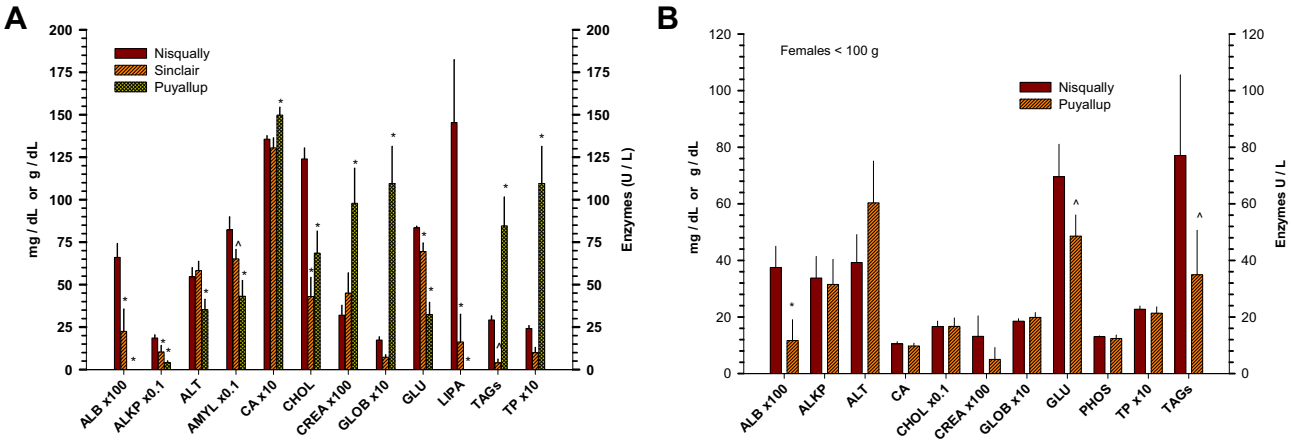


**Fig. 1.** Blood chemistry parameters. a. Juvenile Chinook salmon. b. Female sculpin <100 g. Mean and standard error of the mean values for plasma chemistry parameters measured in these species. * represents p-values <0.05 and ^ are p-values between 0.05 and 0.15. Parameters are albumin (ALB), alkaline phosphatase (ALKP), alanine transaminase (ALT), calcium (CA), cholesterol (CHOL), creatinine (CREA), total globulins (GLOB), glucose (GLU), inorganic phosphate (PHOS), lipase (LIPA), total proteins (TP), and triacylglycerols (TAGs). Nisqually is the reference site and the others are WWTP-effluent impacted. Composite sample sizes as follows; Nisqually Chinook (n = 5) and sculpin (n = 4), Puyallup Chinook (n = 4) and sculpin (n = 6), Sinclair Chinook (n = 4). CA, CHOL, CREA, GLU, and TAGs as mg/dL; ALB, GLOB, and TP as g/dL; ALKP, ALT, AMYL, and LIPA as U/L.

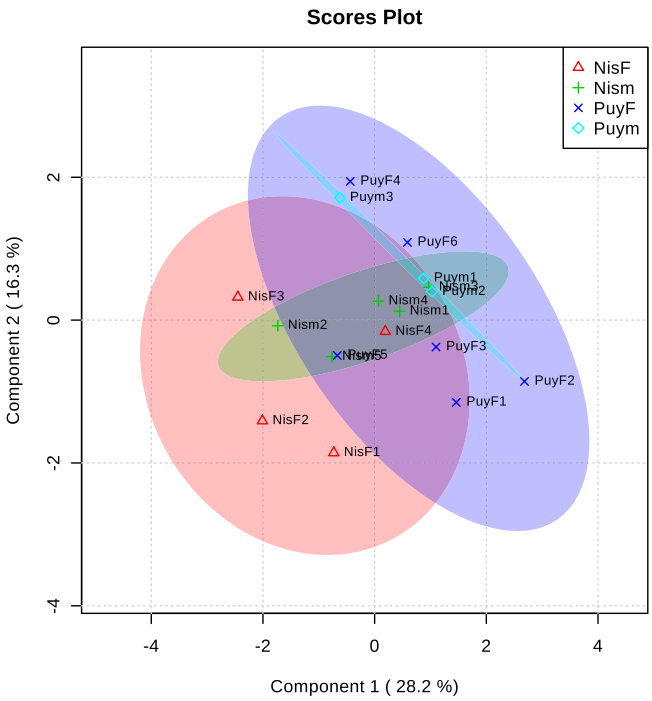


**Fig. 3.** Partial least-squares-discriminant analyses (PLS-DA) scores plots for the chemical profiles from plasma samples for staghorn sculpin salmon collected in the field. Each site highlighted by a 95% confidence ellipse. Nis is the Nisqually reference site and Puy is the Puyallup River Estuary. F is female and M is male fish.

agreement for the nominal (0.3×, 1×, and 10×) and observed ratios among treatments. Noteworthy values that were substantially higher than anticipated included diltiazem and its metabolite, PFDA, and PFOSA. It is noteworthy that the CEC metabolites from the medium dose treatment fish (desmethyl-diltiazem and norfluoxetine) exhibited increases in concentration during the depuration phase indicating continued metabolic conversion of the parent compound. The estimated half-lives for azithromycin, diltiazem, and diphenhydramine determined in the high dose treatment of the present study are similar to other values reported for fish (Meador et al., 2016, 2017). In addition, all half-lives reported in Table 1 are longer than those found in the literature for humans (except the perfluorinated compounds) as summarized in Meador et al. (2016, 2017). The sum of all observed compounds in whole-body fish was relatively low ranging from 51 ng/g wet wt. (ww) (0.14 nmol/g) for the low dose treatment to 794 ng/g ww (2.0 nmol/g) for the high dose treatment on day 32 (Table 1). Predicted plasma concentrations based on whole-body concentrations and the model presented in Meador et al. (2017) are shown in Table S7 along with predicted response ratios for each compound by treatment.

### 3.2.2. Plasma parameters

On day 32 (end of dosing) we observed a few changes in blood chemistry parameters (ALKP, GLOB, LIPA, PHOS, and TP), but generally in the opposite direction as the field-collected fish (Fig. 4, Table S8). Even after feeding the remaining fish clean food from day 34 to day 50 a number of parameters were substantially different (mostly elevated) on day 50 compared to control fish (ALB, ALKP, CA, CHOL, GLOB, and TP) (Fig. S6, Table S9).

### 3.2.3. Growth

After 32 days of dietary exposure, the CEC-treated fish accumulated concentrations of compounds that resulted in differential growth among treatments (Fig. 5). On day 32 the increase in average fish weight for the high dose treatment was substantially less than that for the control (0.7 g increase compared to 3.1 g, $p = 0.08$) (Table 2). A paired *t*-test comparing fish weight from day 0 to day 32 for each treatment indicates that the control, low, and medium dose fish increased substantially in mass over this time period, whereas the high dose fish exhibited weak increases in

**Table 1**
Whole-body fish concentrations and half-life for lab study.

| Chemical | Whole-body conc ng/g | | | | | | Half life (d) | | Max WB conc field | | WB conc. factor (lab/field) | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | D 32 Cont | D 32 Low | D 32 Med | D 32 High | D 50 Med | D 50 High | Med | High | Salm | Scul | D 32 Low | D 32 Med | D 32 High | D 50 Med | D 50 High |
| % Lipid | 5.26 | 5.51 | 5.26 | 5.74 | 5.19 | 3.5 | | | | | | | | | |
| Amitriptyline | <0.1 | **0.13** | <0.1 | **0.96** | <0.1 | <0.1 | – | – | 0.68 | – | 0.19 | – | 1.41 | – | – |
| Amlodipine | <0.6 | **1.15** | **1.12** | **19.5** | <0.6 | **2.51** | – | 6.1 | 1.00 | – | 1.15 | 1.12 | 19.5 | – | 1.0 |
| Azithromycin | <1.8 | **2.84** | **1.75** | **29** | **2.42** | **24.7** | – | 77.7 | 1.70 | – | 1.67 | 1.03 | 17.1 | 1.42 | 0.59 |
| Diltiazem | <0.1 | **17.1** | **10.5** | **102** | **3.79** | **12.9** | 12.2 | 6.0 | 1.60 | – | 10.7 | 6.56 | 63.8 | 2.37 | 0.63 |
| **Desmethyl-diltiazem** | <0.05 | **7.01** | **13.5** | **162** | **14.4** | **119** | – | – | 1.50 | 0.08 | 4.67 | 9.00 | 108 | 9.6 | 0.67 |
| Diphen hydramine | <0.2 | **1.81** | **1.16** | **14.5** | <0.2 | **0.58** | – | 3.9 | 2.70 | 0.28 | 0.67 | 0.43 | 5.37 | – | 0.37 |
| Fluocinonide | <2.2 | <2.0 | <3.8 | <3.0 | <2.2 | <2.3 | – | – | 6.50 | – | – | – | – | – | – |
| Fluoxetine | <0.6 | **1.4** | **1.04** | **19.6** | **0.91** | **5.61** | 91.2 | 10.0 | 4.90 | – | 0.29 | 0.21 | 4.00 | 0.19 | 0.20 |
| **Norfluoxetine** | <1 | **0.99** | **1.2** | **17.9** | **1.65** | **16.3** | – | – | 3.20 | – | 0.31 | 0.38 | 5.59 | 0.52 | 0.31 |
| Gemfibrozil | <0.6 | <0.6 | <0.6 | **3.34** | <0.6 | <0.6 | – | – | 1.30 | – | – | – | 2.57 | – | – |
| Metformin | <2.9 | **4.5** | <4.7 | **39.5** | <11 | <2.9 | – | – | – | 27.8 | 0.16 | – | 1.42 | – | – |
| Miconazole | <0.7 | **1.15** | <0.7 | **13.3** | <0.6 | <0.6 | – | – | 1.80 | – | 0.64 | – | 7.39 | – | – |
| PFDA | <0.5 | **1.31** | **2.15** | **31.1** | **2.05** | **14.4** | 262 | 16.2 | 0.78 | – | 1.68 | 2.76 | 39.9 | 2.63 | 1.28 |
| PFOS | <1 | **5.67** | **7.48** | **135** | **11.6** | **123** | – | 134 | 33.7 | 1.4 | 0.17 | 0.22 | 4.01 | 0.34 | 0.03 |
| PFOSA | <0.6 | **6.02** | **9.13** | **189** | **15.4** | **123** | – | 29.0 | – | 2.2 | 2.74 | 4.15 | 85.9 | 7.0 | 55.9 |
| **PFHxA** | <0.5 | <0.5 | <0.5 | <0.5 | **0.49** | **0.82** | – | – | – | – | – | – | – | – | – |
| Sertraline | <0.5 | <0.5 | <0.5 | **9.06** | <0.5 | <0.5 | – | – | 17.0 | 0.2 | – | – | 0.53 | – | – |
| Triclocarban | <1.2 | <1.2 | <1.2 | **8.72** | <1.2 | <1.2 | – | – | 6.50 | – | – | – | 1.34 | – | – |
| Triclosan | <24 | <24 | <24 | <23 | <23 | <23 | – | – | 26.4 | – | – | – | – | – | – |
| Sum (ng/g) | – | 51.1 | 49.0 | 794 | 52.7 | 443 | | | | | | | | | |
| Sum (nmol/g) | 0.14 | 0.11 | 2.0 | 0.11 | 0.95 | | | | Geomean | | 0.8 | 1.2 | 7.5 | 1.5 | 0.6 |
| | | | | | | | | | Median | | 0.7 | 1.1 | 5.5 | 1.9 | 0.6 |

Whole-body (WB) concentrations (bold) as ng/g wet weights based on composite samples containing 6–8 fish per treatment. Bold chemical names indicate metabolites detected, but not added to food. Reporting limits shown as < values. Geometric mean and median concentration factor ratios between observed values for lab and field fish. Nominal ratios for lab/field were 0.3× (Low), 1× (Med), and 10× (High).

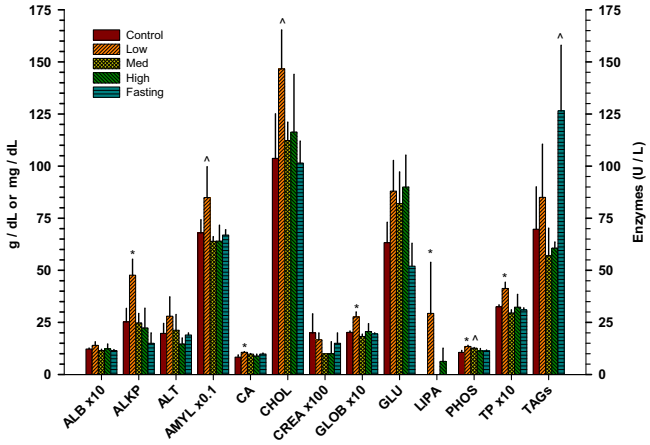


**Fig. 4.** Mean and standard error of the mean values for plasma chemistry parameters measured in juvenile Chinook salmon on day 32 in laboratory study. * represents p-values <0.05 and ˆ are p-values between 0.05 and 0.15. See Fig. 1 caption for abbreviations and units and Table 2 for composite sample sizes for plasma.

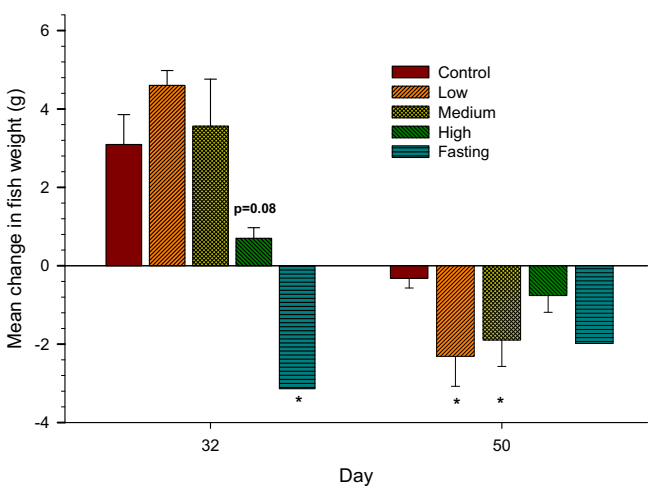


**Fig. 5.** Fish growth in laboratory study. Mean weight change in grams for each treatment based on tank replicates. Error bars are the standard error of the mean. Data for both sampling days (Day 32 and 50) shown. * indicates p-value <0.05 for treatment versus control posthoc comparisons. Additional statistical analysis shown in Table 2.

body mass ($p = 0.12$) (Table 2). A large reduction in mean weight was observed for the food-deprived treatment as compared to the control ($p = 0.006$). For most of the treatments, high variability and low sample size precluded lower p-values.

In general, the mean CF for each treatment was higher for the medium dose fish, which indicates these fish weighed more for a given length than fish in treatments with lower CF values (Table 2). During the depuration phase (days 34–50) the control fish exhibited essentially no growth over this period; however the low, medium, and starvation fish all showed substantial reductions in body mass (Fig. 5). Reductions in average fish mass were approximately 2 g for each treatment with low p-values (Table 2). Minimal growth was expected in these fish due to a lack of strong feeding, only 10 supplied food rations, and handling stress. The slight decline in control fish weight was within the variability expected due to repeated weighing and variable stomach contents. The paired *t*-test for day 50 shows that control and high dose fish weight did not change substantially over this period of time, whereas it did for the low and medium dose fish (Table 2). Also noteworthy is the reduction in whole-body lipid content for the high-dose fish on day 50 compared to the other treatments (Table 1).

## 4. Discussion

### 4.1. Effects on fish exposed to CECs in the field

In the current study, we measured a variety of plasma parameters as early indicators of metabolic status along with traditional fish health endpoints to ascertain the effects of emerging contaminants on two fish species with different life-history traits in Puget Sound. Condition factor (CF) and lipid content are two conventional indicators of fish health. In the current study, the mean CFs for juvenile Chinook did not differ among sites. This was also the case for lipid content for composite samples containing several fish from the Nisqually estuary and Sinclair Inlet (2.3 and 2.4%); while a much higher mean value was observed for the Puyallup River Estuary fish (3.8%). The mean values for these field-collected fish were less than observed for the upstream hatchery collected fish, which was expected because juvenile Chinook at this life stage use stored lipids for smoltification (Sheridan, 1988). Both of these parameters indicate that the fish appeared to be relatively healthy and exhibited no obvious site differences, which was also the case for staghorn sculpin for these two general health parameters.

For field studies, it is difficult to know with certainty the chemicals likely causing adverse effects. A recent study conducted one year before (2013) the present study reported relatively low whole-body concentrations for PCBs, PBDEs, DDTs, and other organochlorine compounds in juvenile Chinook salmon collected in

**Table 2**
Comparisons of fish weights and condition factor among treatments for laboratory study.

| Dose | Weight change (g) | Post-hoc p-value | T-test p-value | CF | Post-hoc p-value | N |
|---|---|---|---|---|---|---|
| | | | **Day 32** | | | |
| Control | 3.1 (0.76) | – | 0.03 | 1.08 (0.01) | – | 4 |
| Low | 4.6 (0.38) | 0.25 | 0.007 | 1.13 (0.05) | 0.29 | 3 |
| Medium | 3.5 (1.2) | 0.69 | 0.06 | 1.16 (0.04) | 0.08 | 4 |
| High | 0.71 (0.27) | 0.08 | 0.12 | 1.06 (0.02) | 0.72 | 3 |
| Starvation | −3.1 | 0.006 | – | – | – | 1 |
| | | | **Day 50** | | | |
| Control | −0.33 (0.25) | – | 0.28 | – | – | 4 |
| Low | −2.3 (0.76) | 0.02 | 0.09 | – | – | 3 |
| Medium | −1.9 (0.58) | 0.04 | 0.05 | – | – | 4 |
| High | −0.76 (0.43) | 0.57 | 0.22 | – | – | 3 |
| Starvation | −2.0 | 0.16 | – | – | – | 1 |

Change in mean fish weight and condition factor (CF) (weight (g)/length$^3$ (cm) *100) for each treatment and the standard error of the mean (SEM). Condition factor based on a random selection of 6 fish from each tank. *P*-value shows the post-hoc comparison after ANOVA between control and treatment for weight change and CF. N shows the number of replicate tanks per treatment. Paired *t*-test p-value based on comparing mean fish weight for replicate tanks within treatments for days 0–32 and days 34–50.

the Puyallup estuary and nearshore areas in addition to low levels of polycyclic aromatic hydrocarbons (PAHs) in stomach contents (O'Neill et al., 2015). These authors also reported no differences in gill metals (Cu, Cd, Ni, and Zn) compared to values observed for Nisqually juvenile Chinook; however, Pb levels were elevated in gill tissue of the Puyallup fish. A similar study by Olson et al. (2008) on juvenile Chinook salmon and staghorn sculpin collected in 2002 and 2003 in the Puyallup River estuary reported similar biliary concentrations of fluorescent aromatic compounds (FACs) in these two species, an indicator of PAH exposure. Olson et al. (2008) also reported higher levels of whole-body PCBs in sculpin (2–3 fold) compared to juvenile Chinook salmon at sampling sites comparable to our own. These results for the same species as the present study in the Puyallup River estuary support our contention that CECs are important constituents for the observed metabolic responses seen in juvenile Chinook salmon; hence, PAHs, metals, and legacy contaminants appear to be less of a factor.

#### 4.1.1. Chinook salmon plasma chemistry

Because most of the sampled juvenile Chinook were recently released from upstream hatcheries, it is important to note that hatchery practices for juvenile Chinook in Washington State are controlled and monitored (HSRG, 2004); hence, similar conditions were expected for these fish prior to release. In addition, these central Puget Sound Chinook are very similar genetically (Ruckelshaus et al., 2006) and were not expected to exhibit physiological differences. Blood chemistry parameters were not determined for hatchery fish because values would likely not be comparable between estuarine fish and freshwater fish. Because these fish were not exposed to CECs in the hatchery (Meador et al., 2016) and reared in the estuaries for only a few weeks, the observed differences are likely a result of estuarine conditions, including exposure to CECs and possibly other contaminants at impacted sites.

In general, we did not expect large differences in blood chemistry parameters for juvenile Chinook salmon among sites because all fish were collected at the same life stage and age, within a 10-day period, and basic water-quality parameters were similar and not unusual among estuaries (Table S1). Also, most were released from upstream hatcheries a few weeks prior to capture. In addition, a previous study (Meador, 2014) concluded that juvenile Chinook salmon in these estuaries were likely not food deprived, which was also noted by Olson et al. (2008) for the Puyallup estuary and Fresh et al. (2006) for Sinclair Inlet, who observed 82–89% of juvenile Chinook collected with full stomachs. Even though this parameter was not quantified in our study, most Chinook and sculpin we examined contained stomach contents.

Every plasma parameter assayed was altered in juvenile Chinook salmon from the two impacted sites in relation to the reference site and in most cases, similar patterns were observed for fish from the two impacted sites (i.e., a decrease over the corresponding reference site value). The altered parameters were more extreme for the Puyallup fish compared to the Sinclair fish, which was likely due to the higher accumulation of CECs in the Puyallup fish. Fish from the Puyallup estuary contained 27 detected analytes versus 13 for the Sinclair fish and total CEC concentrations that were 2–3 fold higher in the Puyallup fish (Table S5).

Interpreting changes for blood chemistry parameters is difficult because these can change temporally. For example, short-term responses may include a mobilization of stored lipids (e.g., TAGs) that occur in the plasma as they are broken down to free fatty acids for use in generating cellular energy (Sheridan and Mommsen, 1991). In time, this response would likely show reduced levels of TAGs in the blood as stored lipids are depleted. Any alteration as compared to the control or reference condition may result in potential metabolic disruption, which is likely disadvantageous for fish during normal seasonal cycles of growth and energy storage (Meador et al., 2011).

As far as we know there are no studies demonstrating high variability or directional changes in the blood parameters we measured for a given species when exposed to normal environmental conditions. Extreme values may cause physiological stress; however, we did not observe any such abnormal environmental or biological conditions or reason to believe these fish would exhibit differences. One study of blood chemistry parameters for rainbow trout over variable temperature, pH, conductivity, and several other water chemistry parameters found essentially no or low variation in 10 of the parameters in common with the present study (ALKP, ALT, ALB, AMYL, CA, CHOL, LIPA, PHOS, TP, TAGs) (Kopp et al., 2011). Blood chemistry values were obtained from fish over winter and summer months for temperatures ranging from 2.5 to 20 °C, pH values from 6.6 to 7.9, and conductivity from 17 to 60 mS $m^{-1}$. One higher temperature treatment (20 °C) did elicit AMYL values about 2× higher. Another study (Manera and Britti, 2006) observed only slight variation in 10 blood parameters overlapping with the present study in RBT sampled from March–June with temperatures ranging from 17 to 22 °C. Additionally, Hasler et al. (2011) reported low variation for CA, CHOL, GLU, PHOS, TAGs, and TP (and others) from adult migrating Chinook salmon sampled over 3 years at one location over a similar time frame (June–July). Also noteworthy here is the high degree of similarity for many of the blood chemistry parameters from the impacted and reference sites for staghorn sculpin (Fig. 3 and Fig. S2), which were of unknown age and previous exposure to environmental conditions.

The levels for many of the plasma parameters measured in Chinook collected from the impacted WWTP sites exhibited a similar pattern to that observed for food-deprived fish for various periods of time (Fig. 6, Table S10). In fact, data from several studies demonstrated significant declines in plasma levels for ALB, ALKP, ALT (liver), AMYL, CHOL, GLU, TP, and TAGs for fish starved from 7 to 72 days (Göran et al., 1975; Navarro and Gutierrez, 1995; Congleton and Wagner, 2006; Costas et al., 2011; Furné et al., 2012; Peres et al., 2014). Some parameters such as GLOB and TP exhibited increases due to fasting (Eslamloo et al., 2017) and a few (CHOL, GLU, LIPA, and TAGs) may exhibit increased levels over the short term, then decline over longer periods of time, indicating an initial physiological response of mobilized resources followed by depletion

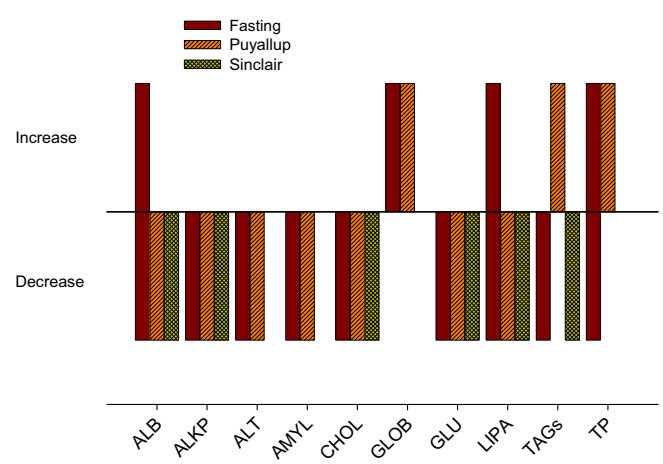


**Fig. 6.** Food-deprivation and fasting data are from published laboratory studies showing substantial ($p < 0.05$) changes in plasma levels (liver for ALT) from control values in fish that were food deprived. Values for WWTP effluent impacted sites (see Fig. 1a) show direction of change compared to fish from the reference site (Nisqually) at $p < 0.05$. See text for description and Table S10 for details.

(Sheridan and Mommsen, 1991; Furné et al., 2012). Also noteworthy is the increased total protein value for juvenile Chinook that was mostly a result of increased globulin levels, which has also been associated with food-deprivation (Eslamloo et al., 2017).

Levels of triacylglycerol lipase increased in plasma under food deprivation for yearling juvenile Chinook that were migrating (Congleton and Wagner, 2006); however, Mommsen (1998) noted that most forms of lipase are severely reduced (70–90%) in fish under fasting conditions, which was also determined by Black and Skinner (1986) and Albalat et al. (2006). Juvenile Chinook in the estuaries are actively growing and metabolizing stored lipids to enhance a growth spurt, which is normal for this life stage (Sheridan, 1988; Higgs et al., 1995). Thus, the high lipase value for the Nisqually fish is assumed to be an indication of healthy fish metabolizing stored lipids to free fatty acids to enhance rapid growth. An increase in TAGs can indicate mobilization of stored lipids for energy or growth and a decrease can result from inhibition of lipolysis or depletion due to food deprivation. Whole-body lipid content is known to decline in healthy juvenile Chinook during this smoltification phase while transitioning from freshwater to seawater (Sheridan, 1988). The lack of lipase in plasma for the Puyallup fish, coupled with high TAG levels and elevated whole-body lipid contents may be an indication of an impaired ability to utilize lipids at this critical life stage; however further experiments are needed to test this hypothesis. Overall, it is clear that fish from the WWTP effluent-impacted sites did not exhibit the same physiological profile as healthy actively growing fish from the reference site.

There are very few studies that examined blood chemistry constituents for fish exposed to contaminants, especially CECs. We compared our results to those of Rodrigues et al. (2015) and Gunnarsson et al. (2009) for CEC effluent, and Muthuviveganandavel et al. (2013) for cypermethrin, which were all short-term exposures (hours to 5 d). Our observed parameters generally followed the values reported by these authors, with consistent increases in CHOL, PHOS, and TAGs and a decrease in ALT.

An increase in plasma enzymes (e.g., ALKP, ALT, and AMYL) is usually associated with tissue damage or organ dysfunction (Boyd, 1983); however, declining levels in fish have rarely been addressed. Several studies indicate decreases in plasma levels of ALKP and AMYL (and others) associated with reduced growth or fasting (Hille, 1984; Sauer and Haider, 1979; Congleton and Wagner, 2006). Additionally, Sauer and Haider (1979) reported reductions in plasma levels of lactate dehydrogenase, glutamic oxaloacetic transaminase, and glutamate dehydrogenase in starved rainbow trout. Reduced ALT activity in liver was associated with food deprivation in fish (Vijayan et al., 1986; Pérez-Jiménez et al., 2012). Reductions in plasma enzymes such as ALT and AMYL have also been associated with the metabolic disruptors PAHs and TBT (Meador et al., 2006, 2011) and another study found large reductions in the plasma enzymes ALT, AMYL, and acid phosphatase in carp (*Catla catla*) exposed to the pesticide cypermethrin (Muthuviveganandavel et al., 2013).

We consider these altered blood chemistry parameters as early-warning indicators of metabolic disruption that may lead to declining vitality in these fish over time. This inhibition could result in decreased growth, which has been associated with decreased survival for salmonids at this life stage (Beamish and Mahnken, 2001). The reduction in many of these blood chemistry parameters are similar to that described by Meador et al. (2006) for juvenile Chinook exposure to high levels of PAHs, which was characterized as "toxicant-induced starvation".

#### 4.1.2. Sculpin plasma chemistry

The high variability among sites and sexes for the blood chemistry parameters for staghorn sculpin indicate no substantial differences in these parameters between reference and impacted sites. However, a few parameters (ALB, GLU, and TAGs) were lower in female fish from the Puyallup, which is similar to that observed for feral juvenile Chinook. These parameters were not consistent between sexes, which may have been a result of several factors including differential migration, feeding rate, or physiological condition.

Sculpin bioaccumulated less CECs compared to juvenile Chinook salmon at these sites (e.g., mean of 25 detected analytes for Puyallup Chinook compared to 9 analytes for sculpin at this site), which is likely attributed to location within the estuary, and lower rates of dietary uptake and water ventilation (Meador et al., 2016). Even though sculpin, a benthic fish, was expected to remain in these local estuaries for an extended period, males and females will migrate offshore into deeper water (Tasto, 1976) and they can exhibit differential movement. The lack of substantial differences in the plasma chemistry parameters for this species between sites was likely due to uncertain exposure and low concentrations of accumulated CECs, high variability in parameter responses, age, and uncertainty regarding their residence time in these estuaries.

The most important observation for sculpin is that there were only weak differences among sites for female fish as highlighted in the multivariate plots (Fig. 3, Figs. S4, and S5) collected from the same estuaries as juvenile Chinook salmon. This is striking given the unknown age and residence history for these fish compared to the well-characterized history for juvenile Chinook, which strongly supports the observation of site-induced differences for salmon exposed to these CECs.

### 4.2. Fish health in the field

For the estuaries studied, there are two hatcheries on the Nisqually and Puyallup systems and one hatchery on Sinclair inlet that can be used to assess survival as determined with the smolt to adult return ratio (SAR), of ocean-type Chinook as described in Meador (2014). The previous 12 years of available data were selected because these provided the greatest overlap among the hatcheries. The mean (SEM) SAR for the Clear Creek and Kalama hatcheries on the Nisqually River was 0.92 (0.10) % and 0.75 (0.11) % respectively for the release years 1999–2010 ($n = 12$ for both). The mean (SEM) SAR for the Voight's Creek and Puyallup Tribal hatcheries that release fish to the Puyallup River over this same period was 0.46 (0.11) % ($n = 8$) and 0.21 (0.09) % ($n = 3$). The mean (SEM) survival for the Gorst Creek Rearing pond fish transiting Sinclair Inlet was 0.45 (0.10) % ($n = 5$) (ANOVA $p = 0.007$). Based on posthoc tests at an alpha value of 0.05, the hatcheries were ranked by the SAR (high to low) as follows: Clear Creek = Kalma > Gorst Creek = Puyallup tribal = Voight's Creek.

A noteworthy observation from our study concerns the health of the fish sampled at these sites. The protocol for capturing and handling fish was the same for each site and transit time did not vary substantially among collection events; however, the Nisqually fish were subjected to one extra sorting step for the detection of coded wire tags. None of the Nisqually or Voight's Creek Hatchery Chinook died in transit during transport from the field to our laboratory. In contrast, approximately 50% of the Puyallup estuary and Sinclair Inlet fish died in the transport cooler. These results may be consistent with a study by Ings et al. (2012) who observed substantial physiological changes in rainbow trout subjected to acute handling stress after being exposed to WWTP effluent for 14 days. Fish that were handled exhibited strong increases in cortisol after 4 h and 24 h post handling (up to 60 ng/mL), whereas levels in

control fish rapidly declined after a few hours. Sustained elevated cortisol concentrations in Chinook salmon has been associated with increased disease and death (Fagerlund, 1967), which has important implications for feral fish avoiding predation or exposure to pathogens. Related to this is a study by Varanasi et al. (1993) who reported statistically significant increases in mortality for juvenile Chinook salmon collected in contaminated estuaries (Puyallup and Duwamish Rivers) compared to fish collected from upstream hatcheries held simultaneously in the laboratory for 40 d (86–88% survival for hatchery fish and 56–58% for estuary fish). No differences were observed between hatchery (88%) and estuary fish (81%) from the Nisqually River system, the reference site for this experiment and the present study.

### 4.3. Fish health and metabolic parameters due to CEC exposure in the laboratory

Although the laboratory-exposed fish consumed less food than expected, they still bioaccumulated most CEC analytes in the mixture in this strictly dietary study. The dietary route of exposure is generally not considered in other studies when evaluating CEC exposure for feral fish; however, data from the current study supports inclusion for any such assessment. More importantly, tissue concentrations of these CECs and plasma levels should be the focal dose metric for risk assessment (Meador et al., 2008, 2017).

From days 0–32 the high-dose fish exhibited essentially no increase in body mass relative to the control fish and were likely responding to increases in tissue CECs. Interestingly, the mean weight for the day 50 fish in the low and medium dose treatments was substantially lower relative to the control and high dose fish. Only minor changes in average fish mass were expected due to the short time frame for depuration, which was evident for the control fish. Additionally, a number of blood chemistry parameters that were altered in the day 32 fish were also altered for the day 50 sampling period including ALB, ALKP, CHOL, CREA, GLOB, TP, and TAGs. The aforementioned study by Ings et al. (2012) demonstrating increased cortisol after CEC exposure and handling stress may also be relevant here because fish in all treatments were handled (weighed) and increased cortisol is known to inhibit growth in rainbow trout (Gregory and Wood, 1999).

### 4.4. Extent of validation for field study results in the laboratory exposures

Comparing blood plasma parameters among fish under variable conditions is complex and usually subjected to confounding factors. In the present study, field-collected Chinook clearly exhibited a pattern of blood chemistry parameters that, in general, reflect that seen for food-deprived fish. Of the 11 blood chemistry parameters for feral fish from impacted sites exhibiting a difference compared to reference site fish, 8 of those parameters were consistent with a pattern indicating a starvation-like response. Fish in the laboratory study exhibited plasma chemistry values opposite to the field-exposed fish with many parameters elevated relative to the control fish. As reported by Congleton and Wagner (2006), TAGs and GLU can trend up or not change for the first several days of fasting, then decline as fish exhaust stored energy. Additionally, migrating fish may be somewhat energy depleted compared to laboratory-reared fish and show a stronger response in blood chemistry due to contaminant exposure, or fasting as noted by Congleton and Wagner (2006). In that study, laboratory-reared rainbow trout (RBT) and juvenile Chinook salmon exhibited large declines in body mass but little change in many of the same blood chemistry parameters as the present study after food deprivation during winter months (7–14 d or 35 d during January (RBT) and March, respectively), which was the time of year for our laboratory study. Contrary to this, Congleton and Wagner (2006) examined migrating juvenile Chinook salmon in the laboratory in the spring (May) and they exhibited strong declines in ALP, CHOL, GLU, TP, and TAGs, with an increase in LIPA after 8–16 days of food deprivation. Another important observation from Congleton and Wagner (2006) is that food-deprived migrating chinook can lose half of their whole-body lipid content within a few weeks, whereas fed fish can increase in lipid content. As seen in the present study, feral juvenile Chinook did not exhibit differences in whole-body lipids among sites as might be expected from food-deprived fish suggesting a contaminant-induced response rather than physical starvation.

Our field data strongly suggest a starvation-like physiological response in fish from contaminated estuaries, even though conventional metrics (e.g. whole-body lipid and CF) did not indicate impairment. The results are less clear for the laboratory-exposed fish, which exhibited obvious growth impairment and uncertain blood chemistry responses. Also noteworthy is the lack of a strong response in the blood chemistry parameters on day 32 from the food-deprived fish even though they lost considerable mass during this time.

Under conditions of field exposure, fish are exposed to a wide variety of contaminants that can bioaccumulate via gill ventilation and ingestion. The field-collected Chinook were exposed for a few weeks, which was similar to that for the lab fish. It is important to note that the lab fish were exposed via diet only and it would likely take considerably longer for tissue residues of these contaminants to accumulate compared to fish exposed to both aqueous and dietary levels. Additionally, the exposure mixture of contaminants for each group of fish was substantially different, with the field fish exposed to a far larger number of CECs (both quantified and unmeasured).

Noteworthy is a companion study by Yeh et al. (2017) who reported modulation of liver mitochondrial function in the Chinook salmon collected in the present study, which is consistent with a disruption of metabolic processes. Fish collected from the CEC-impacted field sites and exposed to CECs in the laboratory trended toward reduced liver mitochondrial content and elevated respiration per mitochondria relative to control and reference fish. Moreover, proton leak respiration (i.e. State 4 respiration induced by oligomycin) was increased by 40–70% relative to controls in the laboratory-exposed fish, indicative of mitochondrial uncoupling and reduced functional efficiency. Several of the CECs in this study including PFOSA and triclosan are known uncouplers of mitochondrial respiration (Schnellmann and Manning, 1990; Shim et al., 2016).

### 4.5. Effects of mixture exposures

Exposure to multiple pharmaceuticals at therapeutic doses can lead to adverse effects in humans due to multi-drug interactions. Although understudied in fish, it is possible that similar exacerbation of adverse physiological effects will also occur in fish from multiple exposures relative to those from single compounds. Given the large number of co-occurring compounds in WWTP effluent, the likelihood of adverse effects or negative interactions is increased. For the laboratory study, most of the Response Ratios were due to calcium channel blockers (amlodipine, diltiazem, and desmethyldiltiazem) and ranged from 87 to 95% of the value for these doses. There are few studies on the toxicity of these compounds to fish; however, Kim et al. (2007) observed a 96 h LC50 of 15 mg/L for diltiazem and Steinbach et al. (2016) reported no effects on growth for rainbow trout exposed at concentrations up to 30 ng/mL. It is not known if these compounds would impair fish growth or alter the plasma parameters examined in the present study. The

other compounds with elevated RR values include azithromycin, diphenhydramine, fluoxetine, metformin, and sertraline. Some of these (e.g. metformin) may inhibit growth and alter metabolic pathways. A recent study (Niemuth and Klaper, 2015) demonstrated reduced growth in male fathead minnow (*Pimephales promelas*) exposed to metformin at 40 ng/mL, which is essentially our predicted plasma concentration for the high dose fish and less than half the human Cmax value (Schulz et al., 2012). Additionally, tested compounds in the present study without Cmax values (e.g., perfluorocarbons) and those below detection but possibly occurring at concentrations considered adverse such as fluocinonide and triclosan may also cause adverse metabolic effects (Meador et al., 2017).

## 5. Conclusions

The present study suggests that exposure to CECs resulted in adverse physiological effects on the metabolic status of feral juvenile Chinook salmon as seen in blood chemistry parameters serving as early indicators. Despite the fact that we observed a number of potential emerging contaminants in the field, it is likely that the juvenile Chinook collected near the two WWTPs were exposed to a variety of other contaminants including a large number of unmeasured pharmaceuticals, personal care products, and industrial compounds. Based on recent studies, exposure to metals, PAHs, and organochlorine compounds were potentially minor for fish in the present study and may not have been contributory to the observed responses. Accordingly, it is unknown what combination of CECs and other contaminants would be causative for these responses; however, based on the obvious lack of overt impairment as measured by the condition factor and total lipid content, it appears that fish in the WWTP impacted estuaries exhibited metabolic dysfunction that appeared to mimic starvation conditions but was likely toxicant induced. Metabolic disruption was also observed in laboratory-exposed fish as noted by changes in fish mass and some plasma parameters; however, the responses were qualitatively different possibly due to differences in exposure route, mixture composition, life-cycle stage, seasonal changes in physiology, and the manifestation of responses over time.

A recent study concluded that juvenile Chinook salmon migrating through contaminated estuaries in Puget Sound exhibited a strong reduction in survival (two-fold) compared to those migrating through uncontaminated estuaries Meador (2014). Some of the lowest survival rates for juvenile Chinook occurred in estuaries that have WWTP discharges into the estuary or nearshore areas where Chinook reside before moving into open water. The aforementioned study provided data on a few well-known contaminants such as PAHs, butyltins, metals, and PCBs that were considered as markers of contaminant exposure for these impacted local estuaries, but not linked causally to adverse effects (Meador, 2014). Given the large number of compounds delivered to these estuarine areas from WWTPs and other sources and their potential for adverse effects on several physiological processes, a more detailed accounting of potential effects and rates of survival for all biota in these areas is warranted.

## Acknowledgements

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## Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.envpol.2018.02.007.

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