Environmental Science & Technology

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Article

Dietary Exposure to Environmentally Relevant Levels of Chemical Contaminants Reduces Growth and Survival in Juvenile Chinook Salmon

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Cite This: *Environ. Sci. Technol.* 2024, 58, 132−142

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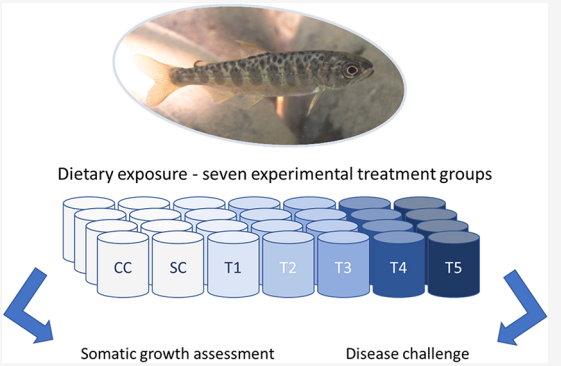
ABSTRACT: Chemical pollution can degrade aquatic ecosystems. Chinook salmon in contaminated habitats are vulnerable to health impacts from toxic exposures. Few studies have been conducted on adverse health outcomes associated with current levels and mixtures of contaminants. Fewer still address effects specific to the juvenile life-stage of salmonids. The present study evaluated contaminant-related effects from dietary exposure to environmentally relevant concentrations and mixture profiles in juvenile Chinook salmon from industrialized waterways in the U.S. Pacific Northwest using two end points: growth assessment and disease susceptibility. The dose and chemical proportions were reconstituted based on environmental sampling and analysis using the stomach contents of juvenile Chinook salmon recently collected from contaminated, industrialized waterways. Groups of fish were fed a mixture with fixed proportions of 10 polychlorinated biphenyls (PCBs), 3 dichlorodiphenyltrichloroethanes (DDTs), and 13 polycyclic aromatic hydrocarbons (PAHs) at five concentrations for 35 days. These contaminant compounds were selected because of elevated concentrations and the widespread presence in sediments throughout industrialized waterways. Fork length and otolith microstructural growth indicators were significantly reduced in fish fed environmentally relevant concentrations of these contaminants. In addition, contaminant-exposed Chinook salmon were more susceptible to disease during controlled challenges with the pathogen *Aeromonas salmonicida*. Our results indicate that dietary exposure to contaminants impairs growth and immune function in juvenile Chinook salmon, thereby highlighting that current environmental exposure to chemicals of potential management concern threatens the viability of exposed salmon.



KEYWORDS: *persistent organic pollutants (POPs), polychlorinated biphenyls (PCBs), polycyclic aromatic hydrocarbons (PAHs), dichlorodiphenyltrichloroethanes (DDTs), Chinook salmon, growth, disease challenge, Natural Resource Damage Assessment (NRDA)*

INTRODUCTION

The degradation of surface water habitats by historical and present-day chemical pollution remains an enduring global challenge for aquatic ecosystem conservation and restoration. In western North America, the challenge is increasingly central to the management of wild Pacific salmon (*Oncorhynchus* spp.), the majority of which are now listed as either threatened or endangered under the U.S. Endangered Species Act (ESA). Poor water quality, driven in part by toxic chemical contamination, remains a pervasive and highly complex limiting factor for recovery.[1] This complexity is due, in part, to the highly migratory life histories of most anadromous salmonids (spanning freshwater, estuarine, and marine habitats) and the sheer number of co-occurring chemicals that they are exposed to, particularly for stocks that must traverse urban waterways with a legacy of industrial pollution. Field and laboratory studies have demonstrated that Chinook and other salmonids are vulnerable to lethal and sublethal toxicity from exposures to real-world mixtures of these toxicants,[2,3] including subsequent reductions in estuarine survival following earlier life-stage exposures (i.e., delayed mortality).[4]

Received: August 4, 2023
Revised: November 29, 2023
Accepted: November 30, 2023
Published: December 28, 2023

https://doi.org/10.1021/acs.est.3c06330
Environ. Sci. Technol. 2024, 58, 132−142

In the Willamette River watershed in Oregon, juvenile Chinook salmon move downstream through Portland Harbor where they reside for weeks or longer to feed and grow before transitioning to the lower Columbia River estuary.[5,6] This journey through the historical working waterfront of the greater Portland metropolitan area, along the lower Willamette River, exposes these salmonids to a myriad of hazardous substances.[7] Out-migrating juvenile Chinook salmon experience similar exposures to contaminant mixtures in other industrialized waterways, such as the lower Duwamish River Superfund sites in Seattle, Washington. Nearly 10 miles of in-river area of the lower Willamette River was included in a National Priorities List (Superfund) designation in 2000, in part because of high levels of pollutants.[7] Because of elevated concentrations, the widespread presence in sediments throughout the Superfund site assessment area, and connection to industrial sources, the contaminants of concern for juvenile Chinook salmon for this study included polychlorinated biphenyls (PCBs), dichlorodiphenyltrichloroethanes (DDTs), and polycyclic aromatic hydrocarbons (PAHs).[8] In 2018, a field study in support of a Natural Resource Damage Assessment (NRDA) in Portland Harbor used whole body tissue metrics to evaluate contaminant exposures in juvenile Chinook salmon.[9] The NRDA process is intended to quantify natural resource injuries attributable to releases of hazardous substances into the environment and guide subsequent restoration to compensate for those injuries. Whole body and stomach content contaminant concentrations from the NRDA study demonstrated consistent trends by site, indicating consumption of contaminated prey as a likely exposure route for juvenile salmon.[10,11] In addition to profiling contaminant mixture exposures, the 2018 field assessment also provided evidence of reduced growth that was associated with hexa- through nonachlorinated PCBs and PAHs in the tissues of juvenile Chinook salmon prior to moving into estuarine habitats. Reduced growth, in turn, may lead to a loss of smaller fish from growth-dependent mortality, specifically in the form of elevated predation vulnerability and/or less effective competition for prey.[12,13]

The persistent organic pollutants PCBs and DDTs, although phased out from large-scale production in the 1970s,[14] are still manufactured globally and prevalent in aquatic systems around the world.[15,16] Present-day concentrations in many habitats are lower than peak historical levels, but the effects of these lingering exposures on protected salmon species are not well-known. This uncertainty in adverse effects is due to exposure studies at these lower concentrations being under-represented in the current literature, thereby challenging present-day estimates of contaminant-related losses at the scales of both individual juveniles and wild populations. Similarly, although many laboratory studies in recent decades have shown reduced growth effects in fish following dietary exposures to PCBs, PAHs, and DDTs, the experimental framing typically focused on larger fish, species other than salmonids, single-contaminant exposures (vs mixtures), and/or relatively high exposure concentrations.[3,17−21] For example, a recent meta-analysis explored the relationships between exposure to PCBs and growth effects in fish across 31 laboratory and 24 field studies.[18] The lowest tissue concentration in the aggregate data was 0.14 μg/g wet weight (ww), resulting in a PCB lowest-observed adverse effect concentration threshold estimation of 0.1 μg/g ww. This benchmark is higher than the average whole body PCB tissue concentration (0.04 μg/g ww) measured in juvenile Chinook salmon migrants from Portland Harbor, where reduced growth impact was observed.[9] Similarly, a reported adverse growth effect with dietary exposure to PAHs was previously observed on much larger Chinook salmon than the field-collected juveniles (∼13 vs ∼1 g, respectively); as such, the laboratory study's PAH effect level may not reflect the dose−response of smaller (∼1 g) out-migrating juvenile Chinook salmon.[21] The literature for DDT is largely from the 1960s−1970s[22] and is thus considerably dated and from an era when ongoing discharges resulted in higher environmental exposures.[23,24] A representative example is a feeding study by Buhler et al. (1969),[19] wherein the lowest dietary exposure of 6.25 μg of *p,p′*-DDT/g feed was nearly 300-fold above the mean concentration measured in the stomach contents of Portland Harbor juvenile Chinook (0.021 μg sum of 6 DDT metabolites/g ww stomach contents).[9] These examples highlight the need to address data gaps related to the effects of exposures from comparatively lower concentrations of contaminant mixtures that are representative of modern habitat conditions.

In addition to reduced growth, exposures to industrial chemicals can disrupt immune function in fish, including Chinook salmon.[2,3,25,26] Hence, a higher rate of infection and subsequent mortality among immunocompromised outmigrants represents another important consideration for managing ESA-listed stocks. Historically, disease-challenge assays have been used as holistic indicators of contaminant-driven immunotoxicity in salmonids. Several previous disease-challenge studies have used *Aeromonas salmonicida*,[27−29] a virulent bacterial agent in Pacific Northwest freshwater habitats that is known to cause furunculosis and acute mortality in Chinook salmon.[30]

This study provides insight into the effects of modern levels of contaminant mixtures on juvenile Chinook salmon rearing in freshwater habitat prior to further out-migration. Moreover, it isolates contaminant exposure from other environmental factors that have the potential to cause previously observed impacts on juvenile salmon health in industrialized rivers. The dietary exposure concentrations and mixture profile were derived from chemical analyses of the stomach contents of juvenile Chinook salmon collected from recent field sampling efforts of contaminated waterways. End points associated with growth and immune function were assessed in laboratory-reared juvenile Chinook salmon following a 5-week dietary exposure.

METHODS

Chinook Salmon Husbandry, Experimental Feed Preparation, and Dietary Exposures. *Juvenile Chinook Salmon Rearing.* Chinook salmon fry (∼15,000; yolk sacs completely absorbed) were obtained from the U.S. Fish and Wildlife Service's Little White Salmon National Fish Hatchery in Cook, Washington, USA, and transported to the National Oceanic and Atmospheric Administration (NOAA) Northwest Fisheries Science Center (NWFSC) Newport Research Station in Newport, Oregon, USA. Fish were placed into circular fiberglass tanks with flow-through, dechlorinated municipal freshwater and fed a dry pellet diet (Otohime; www.otohime.us). Conventional water quality parameters (e.g., pH, temperature, chlorine, ammonia, and dissolved oxygen) were routinely monitored (see Supporting Information (SI), Table S1).

https://doi.org/10.1021/acs.est.3c06330
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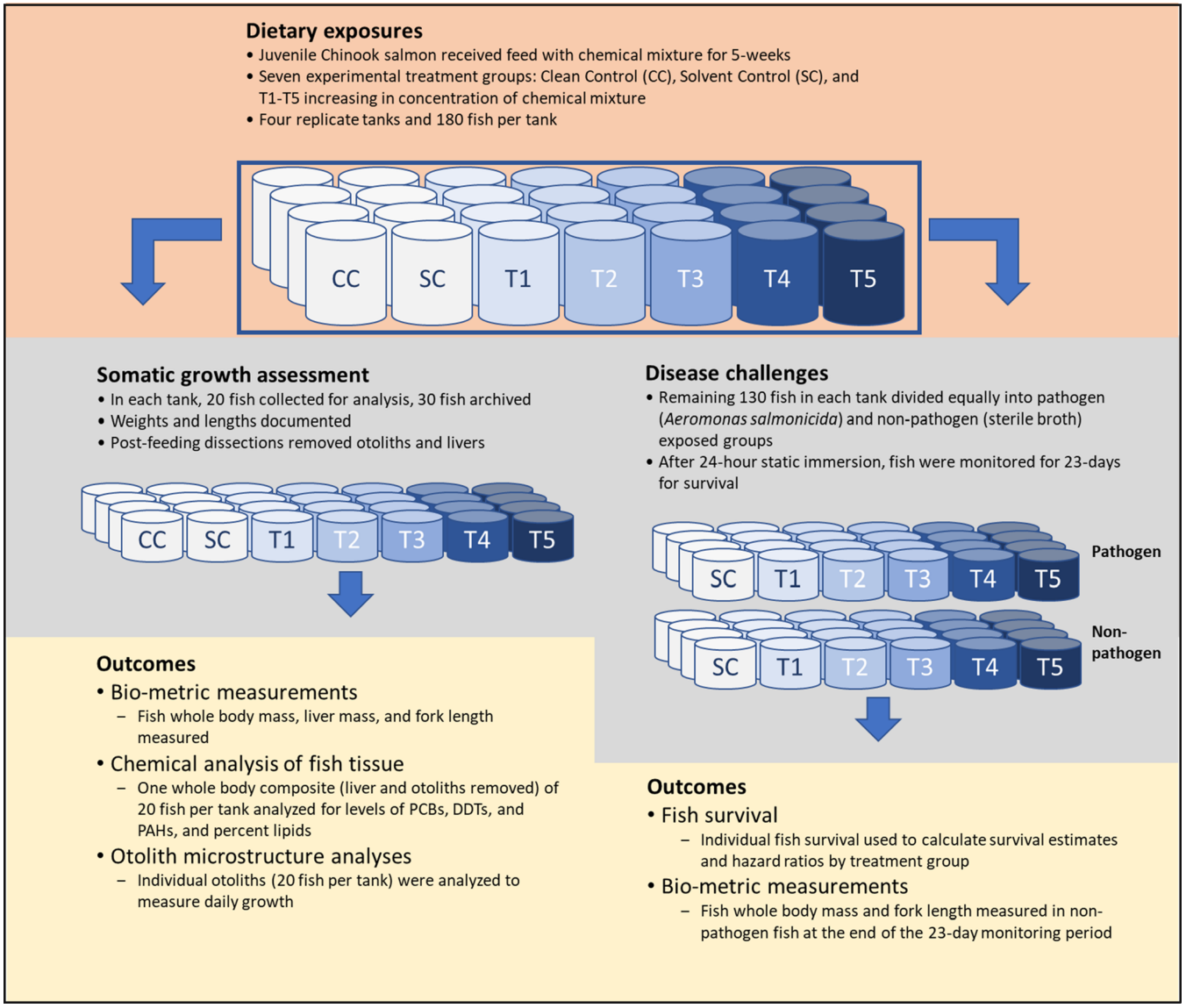

Figure 1. Study design, flow of study, chronology of events, and outcomes measured.

Custom Feed Formulation and Dietary Exposure Regimen. Five feed treatments (dietary formulations T1−T5) were prepared along a log-linear dosing profile, which allowed for injury response information across a range of exposure concentrations. The study objective was to recreate the contaminant mixture measured in stomach content samples from field observations to address the question of adverse health outcomes associated with mixtures (vs single contaminant exposures). Stomach content data from juvenile Chinook salmon collected within the boundaries of Superfund site areas (Portland Harbor, Portland, Oregon, USA; Duwamish River, Seattle, Washington, USA) were used to select the PCB, DDT, and PAH target analytes and concentrations (Table S2). The compounds added to the prepared diet included 3 DDTs (*p,p′*-DDD, *p,p′*-DDE, and *p,p′*-DDT), 10 PCBs (PCBs 28, 52, 191, 105, 118, 138, 153, 170, 180, and 187), and 13 PAHs (naphthalene, 2-methylnaphthalene, 2,6-dimethylnaphthalene, acenaphthene, fluorene, phenanthrene, 1-methylphenanthrene, fluoranthene, pyrene, benz[*a*]anthracene, chrysene, benzo[*a*]pyrene, and benzo[*b*]-fluoranthene. Treatment 2 (T2) was designed to reconstitute the level and proportion of contaminants based on recent field observations, intended to represent an environmentally relevant dietary exposure. The highest concentration (T5) was bound by previously reported growth effect concentrations for PAHs.[21] The T1 and T3−T5 treatment groups each contained contaminants in equal proportions to T2 but with concentrations along the intended logarithmic progression. See the SI for more details on the feed formulation, selection criteria for the analytes, and preparation of the dietary formulation. The specific list of compounds and relative proportions of each analyte added to each treatment diet are presented in Table S3.

Somatic Growth Assessment. *Experimental Treatments.* A flowchart of the experimental design and chronology is shown in Figure 1. Following initial rearing from fry (see above), fish were sorted using a floating fish grader to ensure that experimental fish were uniform in size prior to the start of the feeding. The mean fish mass at the start of feeding, determined from 100 size-sorted fish, was 2.2 g (standard deviation (SD), 0.4 g). The fish were transferred to 29 experimental tanks (180 fish per tank) that were randomly

https://doi.org/10.1021/acs.est.3c06330
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assigned to one of seven treatment groups [T1−T5, feed with nothing added (clean control; CC), and feed treated with dichloromethane solvent alone without the added contaminant mixture (solvent control; SC)], with four replicate tanks per group. An additional "growth tank" was included to independently monitor and adjust food rations to meet the target of 1.9% body-weight (bw) $tank^{-1}$ day^{-1} over the duration of the feeding study (described below). Fifty fish from the reference growth tank were weighed and then returned to the tank each week to assess growth. The average mass was used to adjust daily feed ration across all tanks. The residency time of juvenile Chinook salmon in industrialized waterways has been estimated to be weeks or more.[5,6] A 5-week study was selected to correspond with a realistic exposure duration. There were no mortalities during the 5-week dietary exposure. Thereafter, 50 fish were euthanized from each of the 28 treatment tanks and placed in individually labeled plastic bags. Twenty of these 50 individual fish per tank were randomly selected for individual growth and whole body analytical chemistry. The remaining 30 Chinook salmon were archived. The fish were frozen on dry ice and transferred to the NOAA NWFSC (Seattle, Washington USA) for storage at −80 °C. The remaining fish were used for the disease challenge (described below), targeting 124 fish per treatment replicate with excess fish sacrificed prior to challenge. Further details on the experimental treatments can be found in the SI.

Postfeeding Dissections. Juvenile Chinook salmon were partially thawed, weighed, and measured for the fork length. Otoliths were resected and placed in individual vials for microstructural analyses. Livers were removed and weighed to be consistent with treatment of field collected fish[9] and were archived. No stomach content was observed in any necropsied fish, which was expected because food was withheld on the day the fish were euthanized. The remaining whole bodies were combined into one 20-fish composite per tank for chemical contaminant analyses.

Otolith Microstructure Analysis. The otolith microstructure was examined to estimate recent somatic growth using methods described previously.[31] Otoliths were polished to visualize the core and associated daily growth increments. For each fish, the daily growth rate was measured as the distance in micrometers between daily increments starting at the edge and working toward the core (see SI). For this experiment, otolith increments were measured as far back in the study period as possible with all fish otolith growth increments distinguishable back 28 days, representing days 8 to 35 of feeding (Table S4). Otolith increments were visually identified back to day 4 of feeding for 60% of the total fish (22−92% of fish by the treatment group).

Chemical Analyses of Feed and Juvenile Chinook Tissue. Fish tissue and feed were analyzed at NOAA NWFSC for levels of PCBs, DDTs, and PAHs using a gas chromatography/mass spectrometry method (GC/MS).[32] The target analyte list included the 10 PCB congeners, 3 DDT isomers, and 13 PAH compounds added to the feed (Table S3). The full list of compounds measured and details of the methods and quality assurance program can be found in the SI. See Table S6 for summed concentrations from the target analyte list, all analytes measured, and values in nm/g. Concentrations of all measured analytes in the original feed, summed by contaminant class, were 2 ng/g dw DDTs, 11 ng/g dw PCBs, and 108 ng/g dw PAHs. These contaminant measurements in the original feed comprised 2% of DDTs, 6% of PCBs, and 2% of PAHs analytes measured in the T2 treatment group feed.

Disease Challenge. Fish that were not euthanized for the somatic growth assessment were used in a disease-challenge assay with *Aeromonas salmonicida*. Two days after the dietary exposure concluded, the fish in each tank were divided across two separate tanks (~62 fish each), which were randomly assigned to new locations. One of each pair of tanks for each pair was randomly selected for pathogen exposure. No fish from the CC treatment were used in the challenge because of limitations on the number of tanks available. Each of the SC and T1 through T5 was represented by eight tanks, four exposed to *A. salmonicida* in growth media [Brain Heart Infusion (BHI) broth] and four "no pathogen" (NP) tanks exposed to sterile BHI only. Each tank was administered feed from the SC dietary treatment (developed for the dietary exposure) each day, up to 1% body weight (i.e., 3 g/tank). The available feed amount was not adjusted for growth or mortalities during the disease challenge.

The static pathogen immersion challenge began 4 days after the last feeding of the experimental diet. At the outset, fish were removed from their flow-through tanks and placed into treatment-specific exposure vessels with 9.4 L of aerated, freshwater at an approximate density of 30 g fish/L. The exposure vessels were placed in individual water baths to maintain a stable mean water temperature, intentionally elevated to 15.6 °C to enhance pathogen virulence and infection. Aliquots (95 mL) of a lab-prepared stock suspension of *A. salmonicida* (details of preparation in the SI) were then dispensed into each of the pathogen-exposed vessels (final concentration of 1.45×10^7 cfu/mL), and corresponding aliquots of sterile BHI broth were dispensed into each of the NP control vessels. After 24 h, the fish were removed from the exposure vessels and placed back into their original aerated tanks. Fish survival was then monitored for the next 23 days (see the SI for more detail).

Statistics. Fish length, mass, and condition factor [K; fish mass (g) × 100/fork length $(cm)^3$] were evaluated at the end of the dietary exposure and disease challenge (NP only) using a generalized linear model. For the individual fish measurements listed above, the mean value for each replicate tank was used as the experimental unit. The analyses at the end of the dietary exposure evaluated each treatment group (T1−T5) relative to the combined control groups (SC + CC) after confirming that there was no significant difference between the control groups ($P > 0.05$ for length, mass, and K). Growth analyses of the NP fish after 23 days of receiving SC feed were evaluated relative to the SC group only because the CC group was not used for the disease challenge. The longitudinal analysis compared the difference in mean tank values in the NP fish at the end of the disease challenge to the measures at the end of the dietary exposure. Tissue contaminant values (total sum by treatment group and compound class), tissue lipid measures (percent total lipids), and hepatosomatic index (HSI; liver mass (g)/ fish mass (g) × 100) as tank mean were evaluated at the end of the dietary exposure only because the necropsies and the creation of tissue composites from 20 fish per tank for tissue contaminant chemistry were only conducted at this stage of the study.

The treatment group was tested as a predictor of variability in growth rate for fish after the dietary exposure using a linear mixed effects model (*lmer* package in R).[33] The treatment group replicate tank from which each fish was collected was

https://doi.org/10.1021/acs.est.3c06330
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included as a random effect. Individual growth rates, as determined from repeat measures from individual fish, were predicted by using daily growth measured from the otolith microstructure analysis across the days of receiving the contaminated diet. All fish had otolith measurements for days 8 to 35; just over half of the fish had otolith measurements for days 4 to 35 (Table S4). The two models showed similar results even with the reduced sample numbers for the latter (Table S5); as such, days 4 to 35 of receiving the dietary formulation are described here. Confidence limits were determined using the variance of the predicted growth values, calculated using the diagonal of the variance−covariance matrix. The two control groups, SC and CC, were combined for the otolith growth rate analyses after confirming that there was no significant difference in growth between them (p = 0.683).

Nonparametric Kaplan−Meier[34] survival estimates were determined following the disease challenge for each dietary treatment based on individual fish survival over the 23-day monitoring period. All surviving fish and any mortalities that did not have *A. salmonicida* confirmed by PCR were considered right-censored in the analysis. The Kaplan−Meier survival curves were generated and compared using the log-rank test and Benjamini−Hochberg adjusted p values within the *survminer* package in R (version 3.6.3). Treatment level hazard ratios were determined under a Cox mixed effects model framework to accommodate tank-specific variation within each treatment level. Specifically, the *coxme* package[35] was used with tank replicate as a random effect in the semiparametric Cox regression analysis. Proportional hazards assumptions of the Cox model were satisfied based on a log minus log (LML) plot and formal tests of Schoenfeld residuals within the *survival* R package.[36]

All analyses were conducted in R (version 4.1.0 unless noted).[37]

RESULTS

Analytical Confirmation of PCB, DDT, and PAH Mixtures in Experimental Diets. The experimental diet was designed to simulate contaminant mixture exposure (concentrations and relative proportions) based on the previous profiling of stomach contents in field-collected juvenile Chinook salmon from two industrialized waterways (Table S2). Consistent with this goal, feed and final whole body tissue concentrations in treatment group T2 were comparable to the targeted stomach content and whole body tissue contaminant concentrations from the field-collected fish (see SI for more details). The target dosing was successful, and thus, the diet and corresponding tissue contaminants for T2 are representative of current, real-world environmental exposures in industrialized waterways, and the measured tissue residues demonstrated reliable update of contaminants as expected. In addition, the measured tissue concentrations of PCBs, DDTs, and PAHs across all five treatment groups followed the intended logarithmic progression based on dietary dosing (Table S6) with consistent proportionality within and among chemical classes (Table 1, Figure S1). The resultant log-linear dosing profile of tissue contaminant values across treatment groups yielded injury response information across a range of exposure concentrations.

Total lipids in the whole body tissue composites ranged from 4.9 to 7.1% across all treatment groups (Table 2). Percent total lipids were significantly reduced in the highest dose treatment group (T5; mean, 4.9%; SD, 0.3) compared with the SC reference (mean, 6.8%; SD, 0.3; Table 2). The total whole body lipids from other treatment groups were not significantly different from the SC group. Lipid percentages in the chemical-laden feed ranged from 10.0 to 15.5%, with lower values in the T5 feed (10.0%) and T4 feed (11.5%) compared to all other treatment groups (14.0−15.5%; Table S6) (see the SI for more detail).

Table 1. Whole Body Concentrations of PCBs, DDTs, and PAHs; Mean Across Four Replicate Tanks (Standard Deviation)

	whole body concentrations ng/g wet weight, analytes added to feed[a]		
	PCBs	DDTs	PAHs
CC	3 (0.7)	1 (0.1)	1 (2)[b]
SC	3 (0.7)	1 (0.1)	0 (0.3)
T1	12 (0.5)	5 (0.3)	3 (0.4)
T2	68 (3)	29 (1)	19 (1)
T3	430 (8)	180 (7)	72 (4)
T4	3000 (67)	1400 (97)	180 (16)
T5	12400 (1300)	6100 (560)	1300 (560)

[a]Summed concentrations of 10 PCBs, 3 DDTs, 13 PAHs (see Table S3 for the full list). [b]One tank had 2.7 ng/g of NPH only, and all other PAH compounds were 0; all CC tanks had 0 PAHs for all replicate tanks; see Table S7 for summed concentrations from all analytes measured and for values in nm/g

Dietary Contaminant Exposure Reduces Growth. The influence of dietary contaminant exposure on growth was initially measured using whole body mass and fork length as end points. Overall, juvenile Chinook salmon SC + CC (hereafter referred to as controls unless otherwise noted) approximately doubled in size during the study, growing from an average mass of 2.2 g (SD, 0.4 g) at the outset of the experimental feeding interval to 4.7 g (SD, 0.2 g) on day 35 (Table 2). Among the contaminant-fed fish, the average mass of individuals from the T5 treatment (3.5 g) was significantly less than corresponding controls ($p < 0.05$) at the end of the exposure interval. No other treatment group was significantly different than controls. Fork length increased as well, with an average length of 58.3 mm (SD, 2.9 mm) at the beginning of the dietary exposure and 72.6 mm in controls after 35 days. The T5 fish were significantly shorter (mean, 66.9 mm) relative to controls ($p < 0.05$) after receiving the contaminated diet for 35 days. Also based on fork length, the fish from the T2, T3, and T4 groups (71.7, 72.0, and 72.0, respectively) were shorter relative to controls (T2, $p < 0.05$). The condition factor (K), percent lipids (whole body), and hepatosomatic index (HSI; Table 2, Table S8) were also all lower in T5 (K, 1.16; % lipids, 4.9; HSI, 1.4) compared to the controls (K, 1.23; % lipids, 6.8; HSI, 1.6; all $p < 0.05$). Therefore, dietary contaminant exposures significantly reduced juvenile Chinook growth, as evidenced by reductions in body mass (T5) as well as length (T2 and T5).

The growth rate of individual fish was evaluated by using otolith microstructural analysis (microns per day). As anticipated, the individual-based otolith growth rate was positively correlated with fork length (linear relationship of 7-day growth rate and fish length; R^2=0.74, p = 0.001). There were reduced overall growth rates and evidence of slower growth with progressing days of the study (i.e., change in slope of growth over time) for the T2 and T5 treatment groups

https://doi.org/10.1021/acs.est.3c06330
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Table 2. Descriptive Data of Fish Characteristics by Treatment Group; Mean (Standard Deviation) of the Four Replicate Tanks Averaged, *p < 0.05 (in Bold) SC + CC or SC as the Reference Group, †p < 0.05 (in Bold) Longitudinal Analysis

	end of dietary exposure (35-days contaminated feed)[a]					end of disease challenge (23-days SC diet, non-pathogen (NP) fish only)[b]		
	fish length (mm)	fish mass (g)	condition factor (K)	whole body % lipid		fish length (mm)	fish mass (g)	condition factor (K)
SC + CC	72.6 (0.8)	4.7 (0.2)	1.23 (0.03)	6.9 (0.3)	SC[c]	76.2 (0.3)	5.3 (0.03)	1.20 (0.005)
T1	72.5 (0.6)	4.6 (0.1)	1.20 (0.04)	6.8 (0.4)	T1	76.0 (0.4)	5.2 (0.1)	1.19 (0.03)
T2	**71.7 (0.8)***	4.7 (0.1)	1.26 (0.01)	7.1 (0.1)	T2	75.9 (0.2)	5.2 (0.1)	**1.18 (0.02)†**
T3	72.0 (0.7)	4.6 (0.1)	1.24 (0.03)	6.7 (0.1)	T3	75.7 (0.3)	5.2 (0.1)	1.19 (0.01)
T4	72.0 (0.6)	4.6 (0.1)	1.22 (0.03)	6.7 (0.2)	T4	75.5 (0.6)	5.0 (0.2)*	1.16 (0.02)
T5	**66.9 (1.0)***	**3.5 (0.1)***	**1.16 (0.01)***	**4.9 (0.3)***	T5	**68.9 (1.0)*,†**	**3.5 (0.2)*,†**	**1.06 (0.01)*,†**

[a]During the dietary exposure, the fish were fed a growth diet of 1.9% bw/day (treatment feed) with the amount of dispensed feed adjusted each week over the 5-week dosing period to account for growth. [b]During the disease challenge, the fish were fed a maintenance diet of 1% bw/day (SC feed) with the amount of dispensed feed based on the size and number of fish in each tank at the beginning of the disease challenge. [c]The CC treatment group was not carried through to the disease-challenge study.

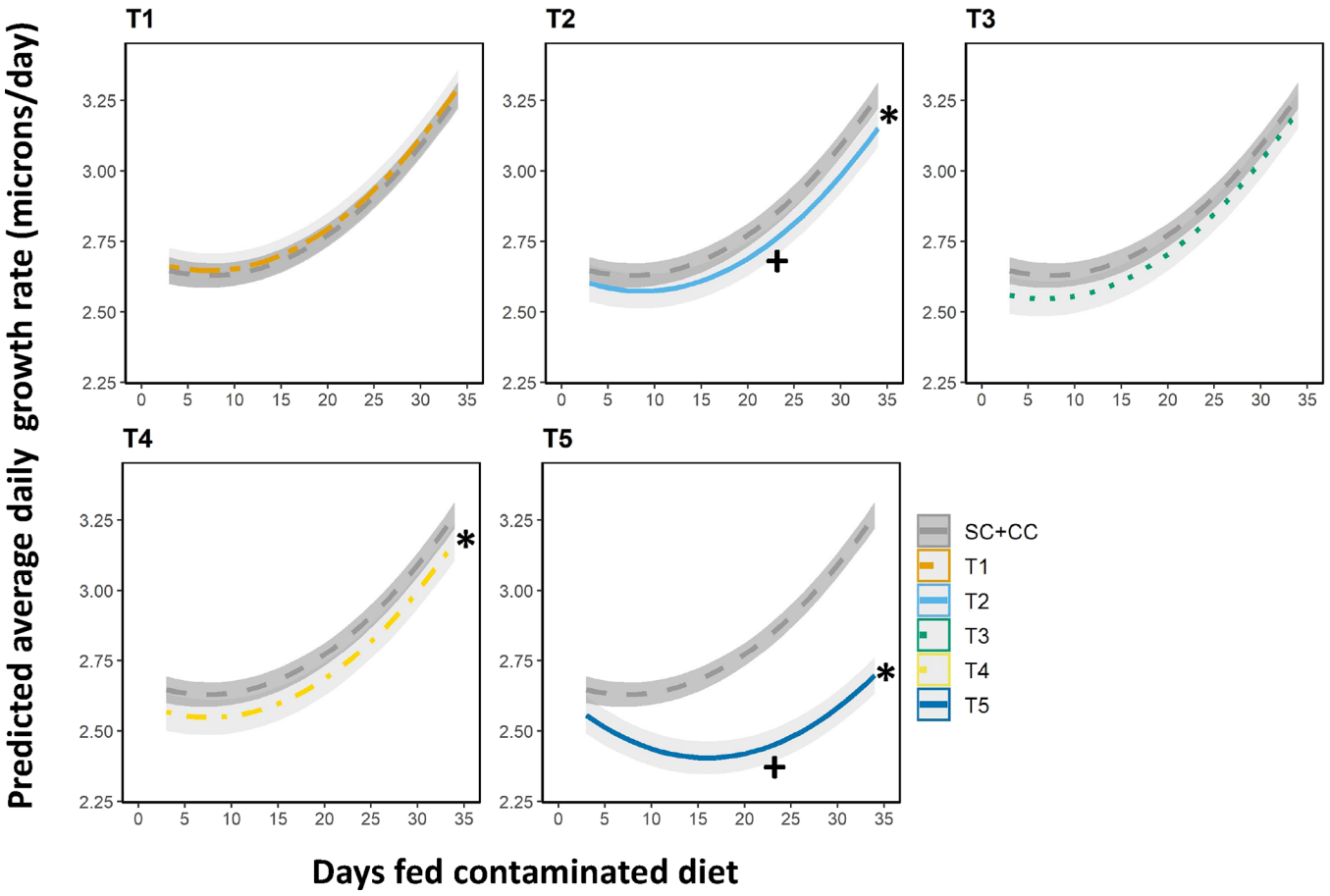

Figure 2. Plots of predicted daily growth rate (otolith microns/day) by days fed the contaminated diet (days 4−35) visualized by treatment group with repeat measures of individual fish and tank as random effects; bands show 95% confidence intervals; *p < 0.05 overall growth rate across days 4−35 of receiving contaminated feed relative to the control group, +p < 0.05 growth rate with progressing days of the study (i.e., slope of growth curve) relative to the control group.

relative to the controls (all p < 0.05; Figure 2; Table S5). The overall growth rates of T3 and T4 were also reduced (p = 0.21 and p < 0.05, respectfully), but unlike T2 and T5, their growth as factors of time (i.e., slope of growth curve) paralleled the control group. This indicates that a disruption to the growth rate occurred during the 35 day exposure followed by a return to the control group rate of growth. However, at the end of the dietary exposure, the fork lengths of fish from the T3 and T4 treatments groups were reduced compared to the controls, indicating that although fish from these treatment groups returned to the growth rate of the controls, they did not make up for the loss in growth.

The continued growth of juveniles during the disease challenge, which followed the 35 day feed exposure, provided an opportunity to revisit size differences among fish in the immune challenge reference group (NP fish). The NP fish, including SC and the T1−T5 treatment fish, were assessed for growth (fork length and fish mass measurements only) after 23 days of receiving a noncontaminated (SC) diet at 1% bw/day. The T5 NP fish showed no change in mass during this 23-day

https://doi.org/10.1021/acs.est.3c06330
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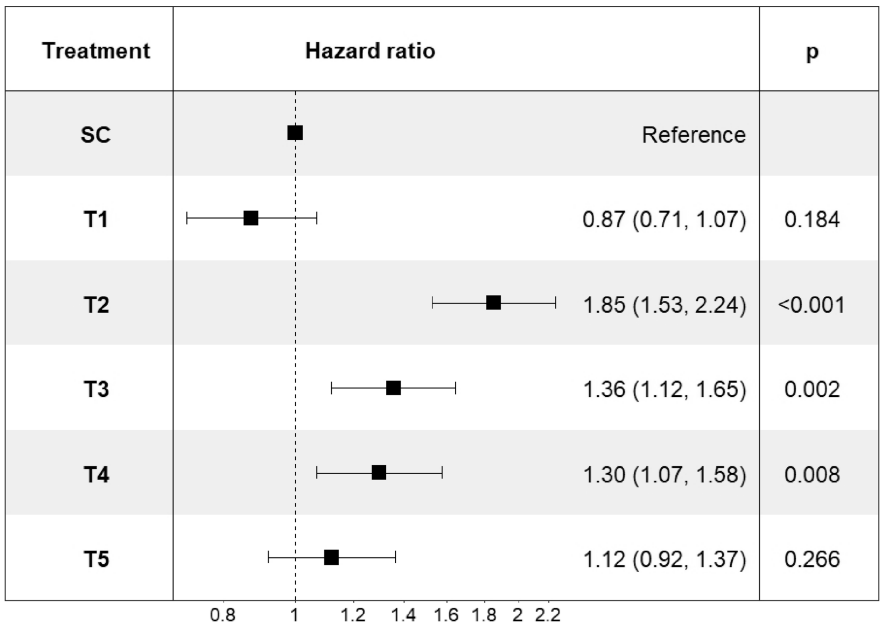

Figure 3. Forest plot of the hazard ratios (95% confidence interval) determined for each treatment (T1−T5) relative to the solvent control (SC) reference with a mixed effects Cox model of the salmon survival data. Hazard ratios greater than 1 indicate a greater risk of disease susceptibility during the challenge with *Aeromonas salmonicida*.

period compared to the end of the dietary exposure (longitudinal comparison), whereas fish with no prior exposure to the contaminated feed (SC group) continued to grow over the 23 day interval, a treatment effect that was significantly different ($p < 0.05$; Table 2). The T5 group also failed to keep pace on fork length growth and condition factor (longitudinal comparison, $p < 0.05$ for both; Table 2), a delayed effect on growth that persisted 3 weeks after the exposure was ended.

Dietary Contaminant Exposure Increases Disease Susceptibility. Following the 5-week dietary exposure interval, fish were assessed for vulnerability to a common infectious (pathogenic) bacterium in salmon habitats. Pairwise comparisons of the Kaplan−Meier survival estimates following a disease challenge with *A. salmonicida* indicated a non-monotonic response across the treatment groups (global $p < 0.001$; Figure S2). Specifically, survival in the T1 and T5 treatments did not differ from the (SC) control; $p = 0.199$ and $p = 0.359$, respectively). In contrast, the T2, T3, and T4 treatments all exhibited significantly lower survival ($p < 0.001$, $p < 0.001$, and $p < 0.006$, respectively; Figure S2). Consistent with this, the hazard ratios determined from the mixed effects Cox model indicated a significantly increased risk of disease mortality in the T2, T3, and T4 groups ($p < 0.008$; Figure 3). Apart from T1, all treatment groups had hazard ratios greater than 1, indicating a greater risk of mortality relative to the control. For example, the hazard ratio of 1.85 for T2 corresponds to an 85% increase in the risk of disease-related mortality. Nonmonotonicity was also evident in the hazard ratio, i.e., no increased hazard for T1, highest risk for T2, intermediate risk for T3 and T4, and no significant estimated hazard for T5. The present controlled study demonstrates increased disease mortality with a mixture of pollutants at contemporary exposure concentrations in industrialized waterways.

DISCUSSION

In the western United States, most Chinook salmon populations are at historic lows. Recovery efforts face numerous challenges related to the decades-long degradation and loss of supporting habitats, spanning physical (e.g., increasing temperature), biological (e.g., pathogens), and chemical (e.g., contaminants) habitat alterations.[38] Of these stressors, recent field sampling from a historically polluted industrialized waterway (Portland Harbor, OR) indicated that reduced growth during outmigration corresponded with increasing tissue contaminant levels.[9] Many of the chemicals in question (PCBs, DDTs) were phased out of large-scale production in the 1970s and 1980s, and risks posed by present-day exposures are underrepresented in the literature. To address this gap, we evaluated adverse health outcomes related to juvenile Chinook salmon growth and disease susceptibility following controlled dietary exposures to a contemporary environmental mixture. Our results indicate that current concentrations of a mixture of PCBs, DDTs, and PAHs, independent of other environmental costressors, impair both growth and pathogen resistance to an extent that could reduce survival among salmonids currently prioritized for recovery under the ESA.

Juvenile growth is a key determinant of individual fitness, in part because smaller fish are more vulnerable to predation and are compromised in their ability to compete for resources.[12] The reduced growth rate observed here following dietary exposures to PCBs, DDTs, and PAHs is consistent with previous findings from field-caught juvenile Chinook salmon[9] and also from studies on juvenile fish exposed to higher doses or to a single analyte or congener.[17−19,21] Specifically, we report reduced growth (Table 2; Figure 2) in fish receiving the environmentally relevant mixture (T2). This adverse effect in exposed salmon may compromise their ability to achieve sufficient length, mass, or lipid content necessary to survive their first year and beyond.[13,39−41] Following the additional 23 days of receiving SC feed (NP fish), there was reduced growth

https://doi.org/10.1021/acs.est.3c06330
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(fork length and fish mass) in the two highest treatment groups (T4 and T5). This delayed effect on growth may be further consequential for migratory juvenile Chinook salmon that reside temporarily in contaminated habitats before moving into less or noncontaminated areas.

Increased disease susceptibility among juvenile Chinook salmon was also significantly associated with dietary contaminant exposure. Specifically, the environmentally relevant dose (T2) resulted in the highest pathogen-associated mortality, with an elevated hazard ratio (>1) that also extended to the T3 and T4 treatments. The focal contaminants for this study (PCBs, DDTs, and PAHs) are well-known to compromise immune function and increase disease susceptibility in salmon and other fish following exposures to a single contaminant class.[3,26] The exact mechanisms that result in the reported effects are unclear but may be contaminants acting directly on immune system components. PAHs and PCBs have been shown to compromise the immune function of juvenile Chinook salmon by suppressing B-cell mediated immunity (as indicated by the plaque forming cell response of kidney and spleen leukocytes) when exposed as single contaminant classes.[26] Similarly, a review of studies evaluating the immune response of fish to contaminants reported that exposure to PCBs and DDTs modified the response of both B and T cells to mitogens as well as antibody production and activity.[3] Likewise, the observed impairment of juvenile Chinook salmon immune response is consistent with previous studies of salmon exposed to mixtures of industrial contaminants either under *in situ*[27] or under laboratory-controlled conditions.[29] Therefore, some degree of delayed mortality as a consequence of habitat stressor interactions (contaminants and pathogens) is possible for juvenile Chinook salmon that survive transient exposure to industrial toxics during their seaward migration phase.

The treatment group with the highest concentration of contaminants added to the diet (T5) had the most pronounced reduction in growth (Figure 2) but did not follow the same pathogen response trend as the other treatment groups (T2−T4). Specifically, this group did not show an increased risk of disease susceptibility compared with the reference group (SC) fish (Figure 3). Multiple mechanisms of action on immune function may be induced across the contaminant mixture and gradient of concentrations in this study, and each combination of effects may produce a different overall immune response.[42,43] The absence of increased pathogen vulnerability in the T5 group may be attributable to an overall activation of the immune system induced by the exposure concentration that minimized *A. salmonicida* infection and disease-associated mortality. *A. salmonicida* pathogenesis involves evading the host immune systems.[44,45] Preventing *A. salmonicida* outbreaks in aquaculture settings is managed, in part, by intentionally priming or activating the immune response with vaccines or feeding supplements that activate the immune system, e.g., macrophages, prior to pathogen exposure.[44,45] Such activation might have occurred in the current study for salmon receiving the highest concentration of contaminants in the feed. However, an activated immune system comes at a physiological cost.[43] Although there was no increased risk of disease and associated mortality following pathogen exposure within the challenge assay for fish in the T5 treatment group, the energetic demands of the contaminant exposure may have contributed to their reduced growth.

In conclusion, our findings describe the effects of exposure to current levels of a mixture of industrial pollutants on juvenile Chinook salmon, an area of study under-represented in the scientific literature. Previous studies have focused on larger fish, species other than salmonids, single-contaminant exposures, and/or relatively high concentrations. Here we demonstrated that juvenile Chinook salmon growth and disease susceptibility were adversely affected by a relevant mixture and concentration of pollutants (T2). Our results indicate that exposure to contemporary levels of chemicals of potential management concern is affecting Chinook salmon. Reducing the levels of these compounds in urbanized waterways may increase the salmon viability and recovery. Future studies may include variations on the chemical mixtures, longer exposures, more end points (such as reproductive success), and the trophic consequences of contaminants in aquatic ecosystems[46] to further improve the understanding of the effect of exposures to chemical mixtures on juvenile Chinook salmon.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.est.3c06330.

> The supporting text details the selection of exposure concentrations, exposure duration, somatic growth, experimental treatments, otolith microstructure analysis, water quality measurements, analytical chemistry methods and data handling, methods/quality assurance for fish tissue and stomach content composites, pathogenic bacterium preparation, monitoring tanks following static immersion challenge, mortalities during study, environmental relevance of final tissue concentrations, and lipid percentages in the chemical-laden feed; tables show water quality parameters, stomach contents contaminant concentrations, list of compounds and relative proportions of each analytes added to the prepared diet, number of fish with otolith microstructure data, linear mixed effect model output for predicted daily growth rate, target and measured concentrations in feed, whole body concentrations of total PCBs, total DDTs, and total PAHs, and hepatosomatic index descriptive data; and figures show whole body chemistry by treatment group and Kaplan−Meier survival probabilities (PDF)

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Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

The authors thank Kaitlin DeAeth and Erin Tyler for help with the lab and data management efforts and Daryle Boyd for assistance with feed prep. We also appreciate the PCR analysis completed by Mary Beth Rew Hicks and the chemical analyses and data calculations provided by Jennie Bolton, Jonelle Gates, and Kia Hayes. The authors thank Sean Sol, Dan Lomax, Mark Myers, Paul Olson, and Bernadita Anulacion for collecting fish used for the 2006 stomach composite sample from the Lower Duwamish River and Catherine Sloan, Jennie Bolton, Daryle Boyd, and Ron Pearce for related chemical analyses. We also thank Washington State Department of Fish and Wildlife Biologists for providing stomach content data from juvenile Chinook salmon hatchery fish collected in the Lower Duwamish River in May 2018. This work was performed while Jessica Lundin held a National Research Council Research Associateship award with the NOAA Northwest Fisheries Science Center. This work was funded by NOAA's Office of Response and Restoration, Assessment and Restoration Division. The findings and conclusions herein are those of the authors and do not necessarily represent the views of the sponsoring organizations.

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https://doi.org/10.1021/acs.est.3c06330
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