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EFFECTS OF DILUTED BITUMEN EXPOSURE ON JUVENILE SOCKEYE SALMON: FROM CELLS TO PERFORMANCE

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Abstract: Diluted bitumen (dilbit; the product of oil sands extraction) is transported through freshwater ecosystems critical to Pacific salmon. This is concerning, because crude oil disrupts cardiac development, morphology, and function in embryonic fish, and cardiac impairment in salmon can have major consequences on migratory success and fitness. The sensitivity of early life-stage salmon to dilbit and its specific cardiotoxic effects are unknown. Sockeye salmon parr were exposed to environmentally relevant concentrations of the water-soluble fraction (WSF) of dilbit for 1 wk and 4 wk, followed by an examination of molecular, morphological, and organismal endpoints related to cardiotoxicity. We show that parr are sensitive to WSF of dilbit, with total polycyclic aromatic hydrocarbon (PAH) concentrations of 3.5 μg/L sufficient to induce a liver biomarker of PAH exposure, and total PAH of 16.4 μg/L and 66.7 μg/L inducing PAH biomarkers in the heart. Furthermore, WSF of dilbit induces concentration–dependent cardiac remodeling coincident with performance effects: fish exposed to 66.7 μg/L total PAH have relatively fewer myocytes and more collagen in the compact myocardium and impaired swimming performance at 4 wk, whereas the opposite changes occur in fish exposed to 3.5 μg/L total PAH. The results demonstrate cardiac sensitivity to dilbit exposure that could directly impact sockeye migratory success. *Environ Toxicol Chem* 2016;9999:1–7. © 2016 SETAC

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INTRODUCTION

Canada has one of the largest proven crude oil reserves in the world, most of which exists as bitumen in the oil sands deposits of the Western Canada Sedimentary Basin. Bitumen extraction from these deposits has grown exponentially to over 300 million L/d [1], and most of this product is exported for refinement via an extensive transcontinental pipeline network. The high viscosity of bitumen necessitates dilution to permit flow, and diluted bitumen (70–80% bitumen diluted with 20–30% natural gas condensate [dilbit]) is the most common bitumen product transported in these pipelines. Although pipeline leaks into the aquatic environment are infrequent, those that have occurred highlight the novel complexity of dilbit spills compared with those of conventional crude oil. Namely, dilbit released into the aquatic environment can distribute throughout the water column and sediment because of its high density, challenging cleanup efforts and prolonging exposure of aquatic biota [1,2]. This is best demonstrated by a large spill in Michigan (USA) in 2010, when 3.2 million L of dilbit entered the Kalamazoo River following a pipeline failure, and as much as 30% of the spilled dilbit remained entrained in river sediments years later [2]. Importantly, proposals are under review to expand existing pipeline infrastructure in the Pacific Northwest of North America that would traverse critical freshwater habitats and migration routes of Pacific salmon, including the Fraser River, Skeena, and Kitimat Watersheds in British Columbia, Canada [3].

Pacific salmon, such as sockeye (*Oncorhynchus nerka*), are anadromous. Eggs and juveniles develop in freshwater streams and lakes usually for 1 yr but up to 3 yr before smolting and migrating to the ocean. These seaward migrations are rapid and active; telemetry estimates that smolts swim at nearly maximal sustainable rates (up to 50 cm/s), covering some 8 km/d and necessitating swimming up to 8.5 h/d [4]. Sockeye spend up to 4 yr maturing in the ocean, returning by the millions to their natal spawning grounds for a single opportunity to reproduce before dying [4], and cardiovascular performance is critical to the success of this upstream migration [5]. Diluted bitumen and other petrochemicals already pass through critical salmon freshwater habitat in the Trans Mountain Pipeline, and this pipeline route is slated for expansion to approximately 140 million L/d. Another pipeline proposal, the Northern Gateway, would transport up to 83.5 million L/d of dilbit through several salmon-bearing watersheds. This increased potential for dilbit release into sensitive aquatic ecosystems raises many concerns [2,3] including long-term conservation implications of sublethal physiological effects (e.g., cardiotoxicity) that could impact the migratory success of salmon.

Like other crude oils, dilbit contains numerous chemicals known to be toxic to fish, including metals, naphthenic acids, and polycyclic aromatic hydrocarbons (PAHs) [6]. Notably, oil- and PAH-induced cardiotoxicity in embryonic fish (e.g., pericardial edema, heart malformations, reduced heart rate [7]) can reduce future aerobic performance [7–9]. Few studies, however, have addressed the cardiotoxicity of oil in juvenile fish, and none have examined the consequences of dilbit exposure beyond the embryo stage. Critical swimming speed (U_{crit}) was reduced in juvenile Pacific herring (*Clupea pallasi*) exposed acutely (96 h) and chronically (up to 8 wk) to 40 μg/L total dissolved PAH from Alaska North Slope crude oil [10]. Similarly, U_{crit} was reduced in juvenile mahi-mahi (*Coryphaena hippurus*) exposed for 24 h to 30 μg/L total PAH in Deepwater Horizon oil [8]. There are likely multiple mechanisms of oil-induced cardiotoxicity, owing in part

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to the complexity and instability of total PAH mixtures, including aryl hydrocarbon receptor (AhR)-dependent [11–13] and independent cellular pathways [11,14–16].

The present study tested the hypothesis that juvenile sockeye salmon exposed to sublethal and environmentally relevant concentrations of dissolved contaminants from dilbit would experience performance-impairing cardiotoxicity, as determined from molecular, histological, and whole-organism endpoints. The age of fish (yearling, late parr) and timing of experiments (April) were chosen to approximate when many parr in natural populations are nearing smoltification in preparation for seaward migration but are still largely confined to their nursery lakes [4]. Given the challenges of cleaning up dilbit spills and the resulting potential for extended environmental contamination [2], exposure durations of 1 wk and 4 wk at relatively low contaminant levels (parts per billion) were chosen to simulate what might occur following a pipeline failure near a nursery lake. If juvenile sockeye experience cardiotoxicity from dilbit exposure, then pipeline failures would impose a significant risk to these socioeconomically important fish.

MATERIALS AND METHODS

Fish

One-year-old sockeye salmon parr (*O. nerka*) were purchased from LSL Living Seafoods. To minimize stress and handling, fish were not weighed prior to distribution among 16 200-L fiberglass tanks ($n = 12$ per tank). Each tank was supplied with aerated flow-through dechlorinated city water (10 °C), and the photoperiod was 12:12-h light:dark. Fish were offered commercial salmon chow ad libitum once daily.

Dilbit exposure

Fish were acclimated in quadruplicate experimental tanks for 1 wk and then exposed continuously to 1 of 4 concentrations of the water-soluble fraction (WSF) of dilbit. The WSF of dilbit was generated as in Kennedy and Farrell [10] by continuously passing dechlorinated city water over ceramic beads thoroughly coated and soaked (~30 min) in summer blend dilbit from the Cold Lake region (Canada's second largest oil sands deposit), and collecting the water into 1 of 8 2000-L header tanks. The WSF of dilbit exposure water (free of droplets or emulsion) was pumped from the near bottom of header tanks into 2 replicate experimental tanks. A range of initial total PAH concentrations was achieved by varying the quantity of dilbit-soaked beads or by omitting the dilbit (control).

Water samples from each experimental tank were collected at 12 h following the initiation of exposures (0 d), and again at 12 d and 25 d. Samples were pooled by replicate experimental tanks prior to analysis (i.e., samples from 2 experimental tanks fed from the same header tank were combined at a given time point, for $n = 2$ samples analyzed per concentration per time point). Individual PAH and naphthenic acid concentrations were measured by Axys Analytical Services following standard procedures. Briefly, naphthenic acid from aqueous samples was collected on a Polar Organic Compound Integrative Sampler. Two deuterium-labeled carboxylic acids were used as internal standards, and 1-pyrenebutyric acid was used as a model carboxylic acid for quantification of naphthenic acid in the samples. A refined naphthenic acid blend was used as a control sample, and was prepared during the sample extraction step and carried through the entire analysis. Each extract was derivatized with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride. An aliquot of recovery standard ($^{13}C_3$-atrazine) was added, and the final volume was adjusted before analysis by liquid chromatography–tandem mass spectrometry with a reversed phase C18 column. The reporting limits for individual compounds ranged from 4.67 ng/L to 5.00 ng/L (average 4.85 ng/L), and the average percentage of recovery was 90.6%. Concentrations of target naphthenic acids were calculated as 1-pyrenebutyric acid equivalents, and are expressed as percentage of total naphthenic acid. All analyses were performed in duplicate. The concentrations of PAHs, alkylated PAHs, and alkanes were quantified from aqueous samples using gas chromatography–mass spectrometry. All samples were spiked with deuterated surrogate standards prior to extraction with dichloromethane and cleaned up with column chromatography on silica. Instrumental analysis was performed by low-resolution mass spectrometry using an RTX-5 capillary gas chromatography column operated in the electron impact ionization mode using multiple ion detection, acquiring at least 1 characteristic ion for each target analyte and surrogate standard. Reporting limits for individual compounds ranged from 0.13 ng/L to 1.13 ng/L (average 0.42 ng/L), and the average percentage of recovery was 100.3%. Concentrations of target PAHs were calculated using the isotope dilution method of quantification, and are expressed as percentage of total PAH. All analyses were performed in duplicate.

Swim trials

Prolonged swimming performance was assessed following either a 1-wk or 4-wk exposure to WSF of dilbit in a custom-built oval-shaped raceway [17]. Contaminant-free water in the raceway was constantly aerated and refreshed. On the day of a swim trial, 3 fish were carefully transferred to the swim tunnel, and acclimated for 45 min at a water velocity of 16 cm/s (0.8 body lengths/s) before undergoing a ramp U_{crit} test [18]. Briefly, water velocity was steadily increased over a 5-min interval to approximately 60% U_{crit} (estimated from a preliminary test of unexposed fish; actual ramp was $64 \pm 0.5\%$ of the measured U_{crit}), and then increased by 10 cm/s (0.6 body lengths/s) every 20 min to exhaustion. Exhaustion time was recorded when a fish no longer responded to mechanical stimulation and was used to calculate U_{crit} [18].

Tissue collection

Exhausted and nonexercised fish ($n = 3$ each) from the same experimental tank were euthanized on the same day by a 2-phenoxyethanol overdose ($n = 12$ per treatment). Mass and fork-length were recorded and used to calculate condition factor (K), where $K = [\text{mass (g)} \times 10^5]/[\text{fork length (mm)}^3]$. Blood was collected by free-flow from caudal vessels and clotted for 1 h at 10 °C; then serum was separated by centrifugation. The liver was removed, flash-frozen, and stored at –80 °C. The heart was removed and cleared of blood by passive perfusion with physiological saline. The bottom one-third of the ventricle was removed with a razor blade, transferred to RNAlater (Life Technologies), and stored at –20 °C for 1 mo. The top two-thirds of the ventricle was contracted in 1 M KCl before it was fixed overnight in 10% neutral buffered formalin and stored in 70% ethanol. Serial 6-μm transverse sections of paraffin-embedded ventricles were mounted on Superfrost Plus slides (Fisher Scientific).

Quantification of blood parameters

Hematocrit was measured with digital calipers. Serum glucose and lactate concentrations were determined spectrophotometrically by fitting absorbance values against standard

curves generated from known dilutions of glucose or lactate, respectively. Serum glucose was quantified in duplicate 200-μL reactions (0.2 M Tris, 5 mM nicotinamide adenine dinucleotide [NAD], 2 mM $MgSO_4$, 5 mM adenosine triphosphate, 0.08 U glucose-6 phosphate dehydrogenase; pH 7.4) by measuring the change in absorbance (340 nm) after incubating for 45 min at room temperature in the presence of 0.1 U hexokinase. Serum lactate was quantified in duplicate 200-μL reactions (0.2 M hydrazine sulfate, 0.5 mM NAD; pH 9.5) by measuring the change in absorbance (340 nm) after incubating for 30 min at room temperature in the presence of 5 U lactate dehydrogenase. All enzymes were purchased from Sigma-Aldrich.

Liver ethoxyresorufin O-deethylase assay

The induction of liver cytochrome P450 1 (CYP1) was quantified in a subset of frozen livers representing fish from all exposure concentrations at each time point ($n = 6$ fish) using an ethoxyresorufin O-deethylase (EROD) assay. Hepatic EROD activity in the microsomal fraction was determined following standard protocols [10] and is expressed relative to total protein, as determined by a Bradford assay.

Reverse-transcription quantitative polymerase chain reaction

Minimum information for publication of quantitative polymerase chain reaction experiments was considered in these experiments [19]. Ventricle pieces ($n = 12$ per concentration and time) were removed from RNAlater, snap-frozen on dry ice, and powdered with a mortar and pestle. Total RNA was extracted from the powdered tissue in Trizol (Life Technologies) exactly according to the manufacturer's instructions, and then checked for purity and yield using a NanoDrop (Thermo Scientific). The RNA integrity was verified by electrophoresis using a bleach–agarose gel [20]. Only samples with high purity and free from degradation were used to synthesis complementary DNA (cDNA; $n = 9$–11 per concentration and time). Then, 1 μg of total RNA was treated with DNaseI according to the manufacturer's instructions (Life Technologies) and requantified, and 500 ng DNA-free total RNA was reverse-transcribed to cDNA in 20-μL reactions using the High Capacity cDNA Synthesis Kit exactly according to the manufacturer's instructions (Life Technologies). Parallel reactions that omitted the Multiscribe reverse-transcribed enzyme (10% of samples, randomly chosen) served as non–reverse-transcribed controls. All cDNA and non-reverse-transcribed reactions were diluted 5-fold with molecular-grade water, aliquoted, and stored temporarily at –20 °C or longer at –80 °C.

Transcript abundances of *ahr*, *cyp1a*, and the reference gene *ribosomal protein L8* (*rpL8*) were measured in separate duplicate reactions using a Bio-Rad CFX96 and recommended cycling conditions. Each 12-μL reaction contained 1× Power SYBR Green (Life Technologies), 1.5 μL of each gene-specific primer (Table 1), and 3 μL of template (diluted cDNA, non-reverse-transcribed sample, or water). Each reaction plate included an internal control sample (average intra-assay coefficient of variation was 3.6%). All cDNA reactions produced single dissociation curves at the predicted amplicon melt temperature, whereas all reactions with non-reverse-transcribed samples or water as template failed to amplify any product. The quantification cycle of each reaction was determined by default settings and was used to calculate transcript abundances from 4-point calibration curves generated from serially diluted cDNA (Table 1). For each assay, all amplified samples had quantification cycle values in the middle of the calibration curve. Each sample was standardized to the abundance of *rpL8*, which remained constant across all treatments (mean quantification cycle 18.733 ± 0.046; $n = 164$ wells). For each gene of interest, data were normalized to the mean expression in control fish at 1 wk.

Histological staining and compact myocardium analyses

Ventricular sections of 4-wk exposed fish (0 μg/L, 3.5 μg/L, and 66. μg/L total PAH groups) were batch-stained for hematoxylin and eosin or picrosirius red (Electron Microscopy Sciences) following standard procedures. High-resolution (20×) brightfield image scans of stained sections were obtained on a Nikon Eclipse 90i microscope equipped with a 12-bit color digital camera (Q-Imaging) under identical acquisition settings, and assembled in ImageJ using a stitching plugin [21]. Each composite was converted to a binary image using the threshold function such that only stained components of interest (nuclei and myocyte cytoplasm for hematoxylin and eosin; collagen fibers for picrosirius red) were converted to black pixels, leaving all other components white. The proportion of black pixels in approximately 30% of the total compact myocardial area (~5% of total cross-sectional area) was quantified. All imaging and analyses were performed blind to treatment.

Statistics

A two-way analysis of variance (ANOVA) and Bonferroni post hoc test determined the effects of WSF of dilbit concentration and time on fish metrics (mass, fork length, K), U_{crit}, liver EROD activity, and ventricular gene expression. A three-way ANOVA and Holm–Sidak post hoc test determined the effects of WSF of dilbit concentration, time, and exercise on blood parameters (glucose, lactate, hematocrit). A one-way ANOVA and Student–Newman–Keuls post hoc test determined differences in the relative cellular fraction and collagen content in the compact myocardium. Any data that did not meet the

Table 1. Reverse-transcription quantitative polymerase chain reaction assay information

Gene	GenBank accession no.	Primer sequence[a] (5′–3′)	Concentration[b] (nM)	Amplicon size (bp)	Efficiency (%)	R^2
rpL8	FJ226384	F: ttggtaatgttctgcctgtg	200	130	103	0.994
		R: gggttgtgggagatgactg	200			
ahr	FJ226379	F: gctccagatgtggtcaagt	200	123	110	0.975
		R: gagtttgtccaggcgaga	200			
cyp1a	FJ226380	F: tcatcaacgacggcaaga	300	317	110	0.973
		R: gttcaccaagcccaacag	300			

[a]Primer sequences are from Veldhoen et al. [41].
[b]Final primer concentration in reaction.
bp = base pairs; *rpL8* = ribosomal protein L8; *ahr* = aryl hydrocarbon receptor; *cyp1a* = cytochrome P450 type 1a; F = forward primer; R = reverse primer.

assumption of normality was log-transformed prior to analysis. All data are expressed as mean ± standard error of the mean (SEM).

RESULTS

WSF of dilbit exposure

Water samples collected at 0 d, 12 d, and 25 d confirm the presence of PAH and naphthenic acid in experimental tanks supplied with WSF of dilbit, with initial total dissolved PAH values ranging from 0.002 μg/L (control), 3.5 μg/L, 16.4 μg/L, to 66.7 μg/L (average values from 2 composite samples per concentration). The initial total PAH concentrations in WSF of dilbit exposures are used hereafter to designate treatment groups. Total PAH and total naphthenic acid concentrations decreased by 50% to 70% between 0 d and 12 d, and then changed very little between 12 d and 25 d (Figure 1). Component breakdown for total PAH shows smaller and more volatile hydrocarbons (e.g., naphthalenes) predominating initially, with larger PAHs (e.g., phenanthrenes) becoming relatively more abundant with time (Supplemental Data, Table S1). The contribution of individual naphthenic acid was consistent over time (Supplemental Data, Table S2). The average mass and length of all fish ($n = 192$) was 101.6 ± 2.2 g and 20.8 ± 0.1 cm, respectively. Neither mass, length, nor *K* differed between treatments ($p > 0.05$; Supplemental Data, Table S3). No mortalities resulted from the WSF of dilbit exposure.

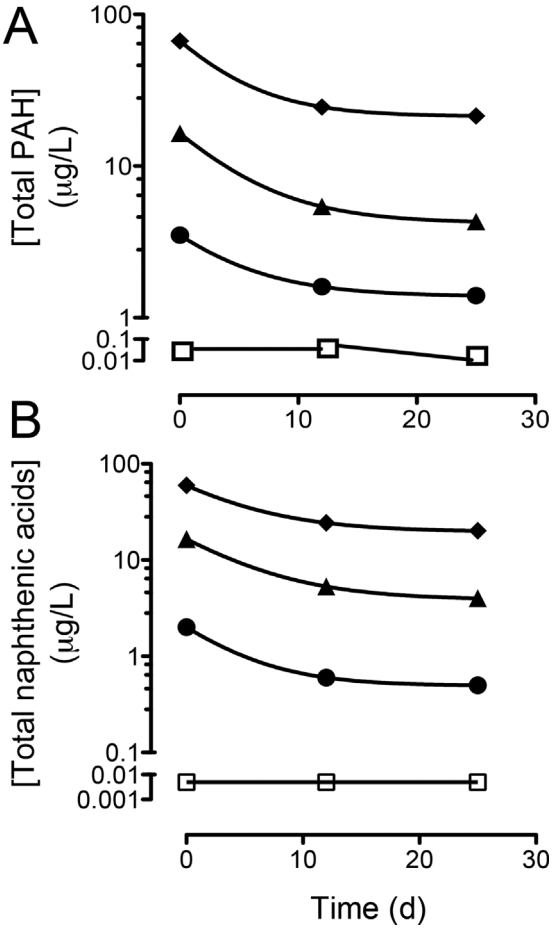

Figure 1. Concentrations of (**A**) total polycyclic aromatic hydrocarbons (PAHs) and (**B**) total naphthenic acids in experimental tanks during the 4-wk exposure period. Four exposure concentrations were generated by varying the amount of beads soaked in diluted bitumen (dilbit) held within generator columns, and replicated in 4 experimental tanks per concentration. Each data point is the average of 2 composite samples. Control (no dilbit, open squares), low (circles), medium (triangles), and high (diamonds) concentrations.

Swimming performance

Analysis of U_{crit} produced identical results whether expressed as absolute swimming speed (cm/s) or corrected to length (body length/s). Therefore, only length-corrected data are depicted in the present study (Figure 2). There was a significant interaction in the effects of WSF of dilbit concentration and time on U_{crit} ($p < 0.001$). Although there were no differences in U_{crit} among WSF of dilbit concentrations at 1 wk, U_{crit} at 4 wk in the 66.7 μg/L group was 10% lower than controls ($p < 0.001$), whereas the U_{crit} of fish in the 3.5 μg/L group was 7% higher than controls ($p < 0.01$). With the exception of the 66.7 μg/L group, U_{crit} values increased from 1 wk to 4 wk ($p < 0.001$). The blood parameters analyzed were primarily affected by exercise (Supplemental Data, Table S3). Plasma glucose increased with exercise in all treatments ($p < 0.05$), and there was an interaction in the effects of concentration and time ($p < 0.001$), with no differences between WSF of dilbit concentrations at 1 wk, but higher levels in WSF of dilbit-exposed fish at 4 wk (control = low < high < medium). The effects of concentration, time, and exercise interacted to affect plasma lactate concentrations ($p < 0.01$), with concentration-dependent increases with exercise at 1 wk but not at 4 wk. Hematocrit varied according to an interaction between time and exercise ($p < 0.001$), but was unaffected by WSF of dilbit concentration ($p > 0.05$); overall hematocrit was higher at 4 wk, but exercise decreased haematocrit only in 4-wk exposed fish.

Cellular biotransformation pathways

There was a significant interaction between time and concentration for all biotransformation endpoints quantified in the present study ($p < 0.05$). Overall, liver EROD activity

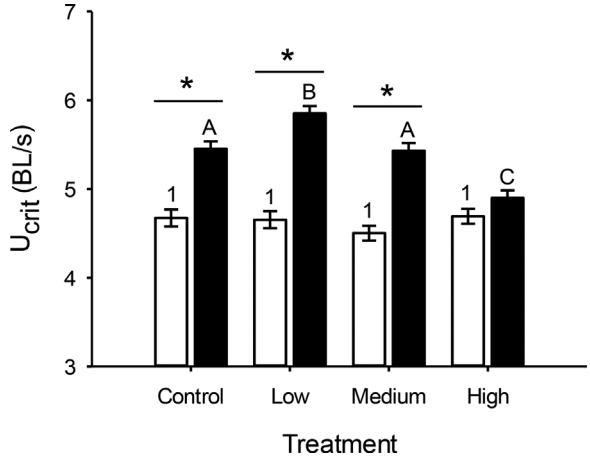

Figure 2. Critical swimming speeds (U_{crit}) of sockeye salmon parr exposed to various concentrations of the water-soluble fraction (WSF) of diluted bitumen (dilbit) for 1 wk (white bars) or 4 wk (black bars). A two-way ANOVA and Bonferroni multiple comparisons test were used to test for effects of WSF of dilbit and time, or their interaction ($p < 0.05$). A significant interaction between WSF of dilbit concentration and time existed. Within a time group, bars that do not share a common number (1 wk) or letter (4 wk) are significantly different. An asterisk indicates a significant difference between 1-wk and 4-wk values within a WSF of dilbit concentration. Data are means ± standard error of the mean for $n = 9$–12 fish.

was significantly elevated in WSF of dilbit-exposed fish relative to controls, with maximum induction occurring by 4 wk in the 3.5 μg/L group (Figure 3A). In the heart, transient changes in *ahr* transcript abundance occurred in the 2 higher exposure groups. Specifically, at 1 wk, *ahr* was over 1.5-fold greater in the 66.7 μg/L group relative to all others, and within the 16.4 μg/L WSF of dilbit group *ahr* was moderately higher at 4 wk than at 1 wk (Figure 3B; $n = 12$, $p < 0.05$). Transcript abundance of *cyp1a* in the hearts of the 66.7 μg/L group was over 2-fold greater than any other group at both 1 wk and 4 wk, and was also significantly elevated within the 16.4 μg/L group at 4 wk relative to 1 wk (Figure 3C; $n = 12$; $p < 0.05$).

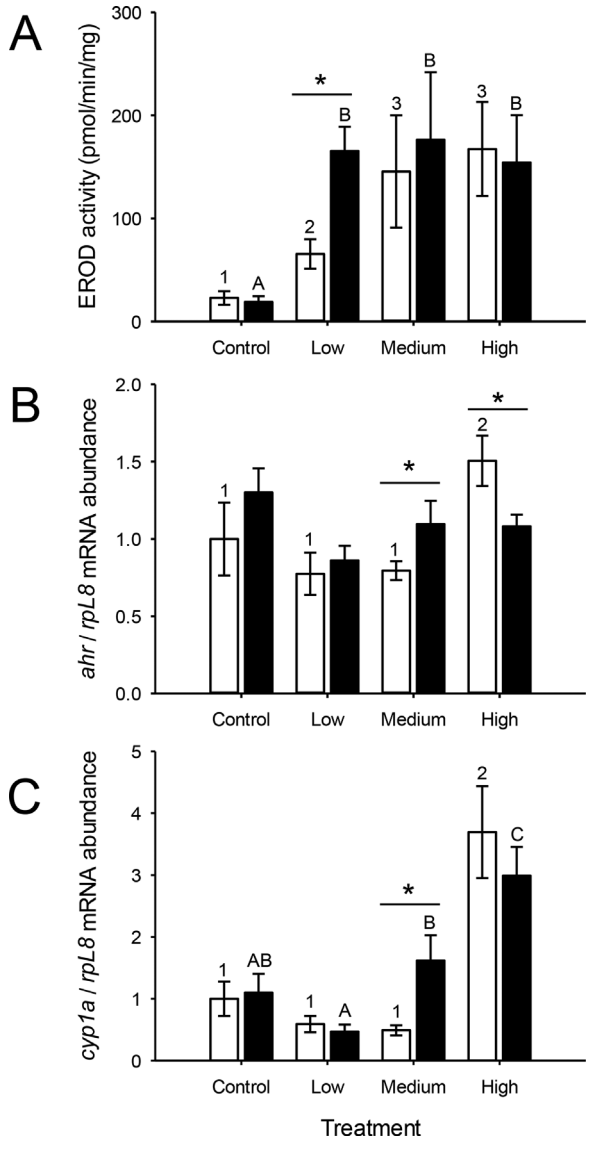

Figure 3. Activation of the phase 1 biotransformation pathway in fish exposed to various concentrations of the water-soluble fraction (WSF) of diluted bitumen (dilbit) for 1 wk (white bars) or 4 wk (black bars). (**A**) Liver ethoxyresorufin-O-deethylase (EROD) activity was quantified as a measure of cytochrome P450 1 (CYP1) activation. Transcript abundances of (**B**) aryl hydrocarbon receptor (*ahr*) and (**C**) *cyp1a* in the heart were measured by reverse-transcription quantitative polymerase chain reaction and standardized to the abundance of the reference gene ribosomal protein L8 (*rpL8*). To facilitate comparisons, data are shown normalized to the expression in control fish at 1 wk. A two-way analysis of variance and Bonferroni multiple comparisons test were used to test for effects of WSF of dilbit concentration and time, or their interaction ($p < 0.05$). A significant interaction between WSF of dilbit concentration and time existed for all variables. Within a time group, bars that do not share a common number (1 wk) or letter (4 wk) are significantly different. An asterisk indicates a significant difference between 1-wk and 4-wk values within a WSF of dilbit concentration. Data are means ± standard error of the mean for $n = 6$–12 fish.

Compact myocardium morphology

Changes to the integrity of the compact myocardium were measured on ventricle sections stained with either hematoxylin and eosin (Figure 4A–C) or picrosirius red (Figure 4E–G) by quantifying the relative proportion of cellular components or collagen content, respectively, on a binary representation of the color-thresholded image (Figure 4 insets). The cellularity in the compact myocardium of fish exposed to 3.5 μg/L total PAH in WSF of dilbit was significantly greater (90.8 ± 0.9%) than controls (88.3 ± 0.7%), whereas fish exposed to 66.7 μg/L had a significantly lower cellular fraction than both groups (86.8 ± 0.7%; $p < 0.05$, $n = 18$–20; Figure 4D). These differences reflected a significantly lower collagen content in the compact myocardium of fish in the 3.5 μg/L compared with the 66.7 μg/L group (4.3 ± 0.2% vs 5.3 ± 0.2%, respectively; $p < 0.01$, $n = 16$–21; Figure 4H), but neither group differed from controls (4.9 ± 0.3%).

DISCUSSION

The present study demonstrates for the first time that exposing juvenile freshwater fish to low, environmentally relevant (parts per billion range) dilbit concentrations causes performance-impairing cardiotoxicity, and that this response is sensitive to both the concentration and timing of exposure. Notably, while chronic exposure to WSF of dilbit at total PAH concentrations of 3.5 μg/L triggers compensatory mechanisms that are sufficient to maintain or modestly improve swimming performance, higher concentrations (66.7 μg/L) activate cellular biotransformation pathways in the heart, initiate a pathological remodeling response in the compact myocardium, and reduce swimming performance. These results are broadly relevant to fish species in freshwater ecosystems transected by dilbit transportation routes, but carry extra gravity for salmon populations in the Pacific Northwest of North America because impaired cardiovascular performance resulting from dilbit exposure could impede an obligatory life-stage transition by hindering the success of their seaward migration.

Cellular responses to WSF of dilbit

Phase 1 biotransformation is mainly a cellular detoxification pathway, and AhR-induced transcription of *cyp1a* and induction of liver EROD activity are known biomarkers of PAH exposure in fish [22,23]. Activation of this pathway can also directly contribute to in vivo toxicity, because reactive intermediates can be generated during CYP1A-mediated metabolism of some xenobiotics [24–26], and other AhR-induced genes may disrupt cell cycling and viability [23]. The sustained increase in liver EROD activity in all WSF of dilbit-exposed fish relative to controls clearly indicates that even very low concentrations of dissolved PAHs from dilbit are biologically available to fish tissues. In the heart, *ahr* and *cyp1a* show distinct time- and concentration-dependent responses. Notably, cardiac *cyp1a* is up-regulated at 1 wk and 4 wk in fish exposed to 66.7 μg/L total PAH in WSF of dilbit, and at 4 wk in the 16.7 μg/L group, supporting the bioactivity of PAHs in the heart itself. In line with these results, rainbow trout (*Oncorhynchus mykiss*) embryos exposed to retene (a high molecular weight alkyl PAH) showed extensive transcriptional changes in the heart

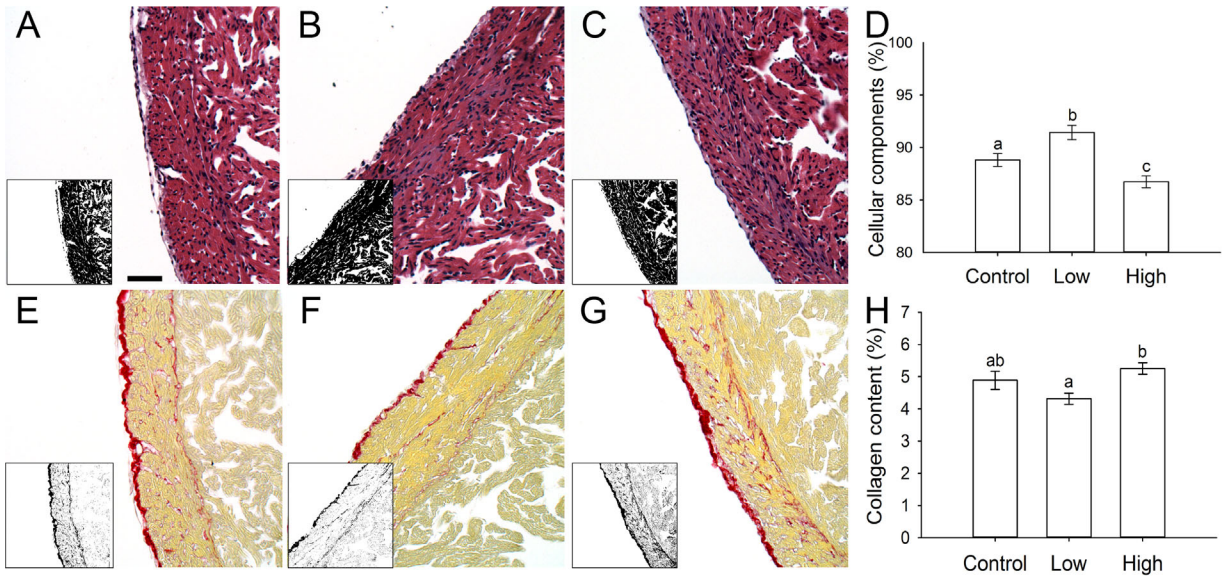

Figure 4. Representative brightfield photomicrographs of the compact myocardium in fish exposed to control (**A**, **E**), low (**B**, **F**), or high (**C**, **G**) concentrations of the water-soluble fraction of diluted bitumen for 4 wk (scale bar = 50 μm in A; applies to A–C, E–G). Ventricle sections were stained with either hematoxylin and eosin (**A**–**C**) or picrosirius red (**E**–**G**). Binary representations of color-thresholded images (insets) were used to quantify relative cellular fraction (hematoxylin and eosin; **D**) or collagen content (picrosirius red; **H**) in the compact myocardium ($n = 17$–22 fish per treatment). Data are means ± standard error of the mean. Significant differences were determined with a one-way analysis of variance and Student–Newman–Keuls post hoc test. Bars that do not share a common letter are significantly different ($p < 0.05$).

including up-regulation of *ahr* and *cyp* isoforms [27], and Japanese medaka (*Oryzias latipes*) embryos exposed to WSF of dilbit had increased whole-body *cyp1a* as well as increased expression of genes associated with oxidative stress and phase 2 biotransformation [28].

Cardiac remodeling response to WSF of dilbit

The compact myocardium is essential to supporting cardiac output and aerobic performance in athletic fish such as salmonids. Morphological plasticity (e.g., hypertrophy, extracellular matrix composition) in this layer occurs in response to thermal acclimation and is proposed to help maintain cardiac function during temperature-induced changes in muscle compliance and blood viscosity [29–33]. The reciprocal changes in the cellular and collagen fractions in the hearts of WSF of dilbit-exposed sockeye suggest that xenobiotics can also act as a remodeling stimulus in the teleost compact myocardium. These changes may be driven by a WSF of dilbit-induced reduction in blood oxygen carrying capacity [34–36], impaired cardiac function [11,16,37–39], and/or a direct PAH-induced transcriptional response in pathways regulating cell growth, proliferation, and structure [27,37,40]. Of particular interest are the concentration–dependent responses that were opposite in sockeye exposed to either 3.5 μg/L or 66.7 μg/L total PAH in WSF of dilbit. Increased myocytes/decreased collagen were associated with improved swimming performance in the 3.5 μg/L group, whereas decreased myocytes/increased collagen were associated with reduced swimming performance in the 66.7 μg/L group, suggesting both physiological and pathological elements to this remodeling response. The mechanisms underlying cardiac deformities [39] and altered heart shape [7,9] in fish exposed to crude oil as embryos likely include interference in developmental programming. In the present study, fish were exposed as juveniles, providing novel evidence of cardiac remodeling in response to crude oil exposure that is independent of ontogenic events.

Performance effects of WSF of dilbit

The present study is the first to document the effects of dilbit on swimming performance of any fish species. In general, performance impairment can reduce a fish's ability to capture prey and escape from predators. For Pacific salmon specifically, there are added concerns that performance impairment can reduce the success of their migration and transition into the oceanic phase of their life history. Interestingly, no differences in U_{crit} were observed in fish exposed for 1 wk to the concentrations used in the present study, and fish exposed to 3.5 μg/L total PAH in WSF of dilbit for 4 wk had slightly higher U_{crit} values compared to controls. Conversely, fish exposed to 66.7 μg/L total PAH in WSF of dilbit for 4 wk had a significant 10% reduction in U_{crit} relative to controls. This finding of concentration- and duration-specific exposure effects carries important implications, because it demonstrates that the amount and timing of dilbit release are major factors to consider when predicting the impact of a pipeline failure on Pacific salmon. Specifically, the lower the amount of spilled dilbit and the nearer the spill is to the onset of smoltification, the better the outcome for juvenile salmon.

Supplemental Data—The Supplemental Data are available on the Wiley Online Library at DOI: 10.1002/etc.3533.

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Data availability—Please contact the corresponding author with data requests (alderman@uoguelph.ca).

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