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Effects of diluted bitumen exposure and recovery on the seawater acclimation response of Atlantic salmon smolts

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ABSTRACT

Petrogenic chemicals are common and widespread contaminants in the aquatic environment. In Canada, increased extraction of bitumen from the oil sands and transport of the major crude oil export product, diluted bitumen (dilbit), amplifies the risk of a spill and contamination of Canadian waterways. Fish exposed to sublethal concentrations of crude oil can experience a variety of adverse physiological effects including osmoregulatory dysfunction. As regulation of water and ion balance is crucial during the seawater transition of anadromous fish, the hypothesis that dilbit impairs seawater acclimation in Atlantic salmon smolts (a fish at risk of exposure in Canada) was tested. Smolts were exposed for 24 d to the water-soluble fraction of dilbit in freshwater, and then transferred directly to seawater or allowed a 1 wk depuration period in uncontaminated freshwater prior to seawater transfer. The seawater acclimation response was quantified at 1 and 7 d post-transfer using established hematological, tissue, and molecular endpoints including gill Na^+/K^+-ATPase gene expression (*nka*). All smolts, irrespective of dilbit exposure, increased serum Na^+ concentrations and osmolality within 1 d of seawater transfer. The recovery of these parameters to freshwater values by 7 d post-transfer was likely driven by the increased expression and activity of Na^+/K^+-ATPase in the gill. Histopathological changes in the gill were not observed; however, CYP1A-like immunoreactivity was detected in the pillar cells of gill lamellae of fish exposed to 67.9 μg/L PAC. Concentration-specific changes in kidney expression of a transmembrane water channel, *aquaporin 3*, occurred during seawater acclimation, but were resolved with 1 wk of depuration and were not associated with histopathological changes. In conclusion, apart from a robust CYP response in the gill, dilbit exposure did not greatly impact common measures of seawater acclimation, suggesting that significant osmoregulatory dysfunction is unlikely to occur if Atlantic salmon smolts are exposed subchronically to dilbit.

1. Introduction

Crude oil is a major and ongoing contributor to contamination of the aquatic environment, and therefore poses an important risk to aquatic biota, including fish. Large historic marine spills, such as that from the Exxon Valdez in Prince William Sound, USA (1999), and the Deepwater Horizon disaster in the Gulf of Mexico, USA (2010), among many others, are the impetus for continued research into the adverse effects of crude oil exposure in fish. Indeed, environmentally-relevant, sublethal concentrations of crude oil cause an array of responses ranging from molecular changes and DNA damage, to impairments in individual performance and behavior, particularly in sensitive early life stages of fish (Dupuis and Ucan-Marin, 2015; Kennedy, 2015). Less well known are the effects of chronic crude oil exposure in juvenile and adult fish, and the recovery trajectory of fish once they are removed from the contaminated water (Claireaux et al., 2013; Mauduit et al., 2019). Such information is essential for a full assessment of the ecological risks of oil spills.

Canada ranks in the top five countries for contributions to the global oil market, with the majority (~90 %) of crude oil originating from the expansive oil sands region in the Western Canada Sedimentary Basin, and a smaller proportion (~10 %) from offshore drilling in eastern Canada (National Energy Board of Canada, 2019). Bitumen from the oil sands is a heavy type of crude that is mixed with lighter hydrocarbons

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to reduce viscosity, and the diluted bitumen (dilbit) is then transported thousands of kilometers across North America through a network of pipelines and railways. In an effort to keep pace with the rising rate of bitumen extraction, and to capitalize on overseas exports, calls to increase dilbit transport to Canadian tidewater have resulted in the approval (e.g. Trans Mountain Pipeline) and proposal (e.g. Energy East Pipeline) of pipeline expansion routes to the Pacific and Atlantic coasts, respectively. The largest dilbit spill to date resulted from a pipeline failure in 2010 that released 3.2 million liters of dilbit into the Kalamazoo River (Michigan, USA). As much as 30 % of the spilled oil remained unrecovered and entrained in river sediments despite extensive clean up and dredging efforts (Dew et al., 2015), highlighting that dilbit can sink in the aquatic environment. Thus there is a present and growing risk of accidental dilbit release and environmental contamination, including in the coastal watersheds that are critical habitat for the freshwater life stages of anadromous salmonids (Levy, 2009). Efforts to define the specific toxicity of dilbit are only recently initiated, and studies to date indicate that many of the adverse physiological effects of conventional crude oil exposure in embryonic and larval fish are also relevant for dilbit (Alderman et al., 2018; Alsaadi et al., 2018; Barron et al., 2018; Madison et al., 2017, 2015; McDonnell et al., 2019; Philibert et al., 2016). In juvenile sockeye salmon (*Oncorhynchus nerka*), dilbit exposure decreases swimming performance and induces cardiac fibrosis (Alderman et al., 2017b), and may increase post-exercise cellular damage in muscle, kidney, and other tissues (Alderman et al., 2017a). These changes could limit the successful migration of salmon from nursery lakes to the ocean.

The parr-smolt transformation, or smoltification, describes the suite of physiological, biochemical, and behavioural changes that occur in anadromous fish as they prepare for transition from a freshwater to a seawater habitat (Hoar, 1988). Salmon are particularly sensitive to anthropogenic stressors at this life history stage (Cohen, 2012; Moore et al., 2007), and declines in wild populations can be linked in part to poor survival of out-migrating smolts (Baldwin et al., 2009; Thorstad et al., 2012). In addition, considerable research has been devoted to building a mechanistic understanding of smoltification to enhance sea pen rearing of important aquaculture species like Atlantic salmon (*Salmo salar*) (Björnsson et al., 2011; Prunet et al., 1989). Moving from a freshwater to seawater environment is an extreme osmotic challenge that requires concerted molecular and cellular changes in osmoregulatory organs like the gill and kidney, in order to mitigate passive ion gain and water loss. For example, a shift in expression of isoforms for the ion pump Na^+/K^+-ATPase in the gill ensures active secretion of NaCl (Bystriansky et al., 2006). At the same time, the sharp reduction in glomerular filtration rate in seawater-acclimated salmon (Brown et al., 1978) is accompanied by changes in renal tubule expression of isoforms for the transmembrane water channels, aquaporins, which may facilitate water resorption and help reduce urine production (Engelund and Madsen, 2014; Tipsmark et al., 2010).

Fish exposed to some of the hydrocarbons found in crude oil can experience ion and osmotic disturbances (Brauner et al., 1999; Duarte et al., 2010; Englehardt et al., 1981; Kennedy and Farrell, 2005; Zbanyszek and Smith, 1984), resulting from a combination of direct and indirect effects on osmoregulation. For example, histopathological changes in the gill (Agamy, 2013; Englehardt et al., 1981; Medeiros et al., 2017) and renal tissue (DiMichelle and Taylor, 1978; Pal et al., 2011) have been observed in fish exposed to crude oil mixtures or to representative polycyclic aromatic compounds (PAC; a common constituent of crude oil). Also, activation of the aryl hydrocarbon receptor (AhR), for which certain PAC are also ligands, may disrupt interrenal cell cortisol production (Aluru and Vijayan, 2006, 2004), and this hormone facilitates seawater adaptation (McCormick, 1995) in addition to its role in the endocrine stress response (Wendelaar Bonga, 1997). While osmoregulatory dysfunction poses a long-term challenge for any animal, it is of particular concern during smoltification, where inadequate compensation for ion influx and water loss would not only increase the energy costs of osmoregulation but could also impair the function of other physiological systems or cause mortality. To date, no study has questioned how exposure to crude oil impacts seawater acclimation in salmonids. Therefore, the present study tested the hypothesis that dilbit exposure would impair the osmoregulatory ability of Atlantic salmon smolts.

2. Materials and methods

2.1. Fish

Hatchery-raised 8-month old pre-smolt Atlantic salmon (*S. salar*; average mass 61.3 ± 1.2 g and fork length 168.8 ± 1.1 mm; N = 272) were obtained from Marine Harvest Canada (Campbell River, BC) and evenly distributed among nine 200-L fiberglass tanks at the Alcan Aquatic Facility (Simon Fraser University, Burnaby, BC). Each tank was supplied with aerated flow-through dechlorinated municipal water at outside ambient temperature (10 °C). To induce smoltification, fish were maintained in constant light 24L:0D during an initial 4-wk acclimation period, and throughout the experiments described below. Fish were offered commercial salmon chow *ad libitum* once daily (Skretting, Vancouver, BC). Care and use of animals was approved by the Simon Fraser University animal care committee, as per the guidelines defined by the Canadian Council on Animal Care.

2.2. Dilbit exposure

After acclimating fish for 4 wk, fish were exposed to uncontaminated freshwater (control) or one of 2 concentrations of the water-soluble fraction of diluted bitumen (WSFd) for 24 d. Each concentration was replicated across triplicate experimental tanks, however fish were pooled across replicate tanks for all endpoint measures. WSFd were generated as previously described (Alderman et al., 2017b) by continuously passing dechlorinated municipal water over Siproax® ceramic beads (Aquatic Eco-Systems Inc., Apopka, FL) that were saturated with Cold Lake Summer Blend diluted bitumen (dilbit) and contained within polyvinyl chloride columns (15 cm diameter, 80 cm length). After passing over the beads, water was collected into one of 6 2000-L header tanks, where any remaining oil droplets collected on the water surface. WSFd exposure water was pumped from near the bottom of header tanks into 1 or 2 replicate experimental tanks containing fish, ensuring that only dissolved contaminants and not oil droplets were delivered to exposure tanks by continuous flow-through supply (~7.5 L/min). Initial total PAC concentrations in exposure water ranged from ~ 0–100 µg/L and were achieved by varying the number of dilbit-saturated ceramic beads or omitting the dilbit (Control) in each column. A subset of fish was removed from each experimental tank at the end of the 24 d exposure period for the seawater acclimation experiment described below. The water supply to experimental tanks was then returned to clean, uncontaminated flow-through water to initiate the 7 d depuration period.

Water samples were collected 12 h after initiating exposures on day 0, and again on days 10 and 21 of exposure. Water samples were pooled by replicate experimental tanks fed from the same header tank prior to analysis, resulting in 2 samples per concentration per time point. The abundances of 75 individual PAC were measured by SGS AXYS Analytical Services Ltd. (Sidney, BC, Canada) exactly as previously described (Alderman et al., 2017b).

2.3. Seawater acclimation

Seawater acclimation followed previously described methods (Bystriansky et al., 2006; Tipsmark et al., 2010). Briefly, at the end of the 3 wk exposure to WSFd, or after the 1 wk depuration period, a subset of smolts from each experimental group was either sampled directly (freshwater, FW; n = 6) or transferred to full-strength aerated

seawater (32 ‰ SW; static) for 1 or 7 d (n = 7 per concentration per time). Tank size, photoperiod, and water temperature were identical to acclimation conditions. Approximately one third of the SW in each tank was replaced daily by siphoning, with minimal disturbance to fish. Fish were not fed during the seawater acclimation experiment.

2.4. Tissue collection

Fish were euthanized in MS-222 after 0 d (FW), 1 d (SW1), or 7 d in SW (SW7). Each fish was blotted dry, and weight and fork length were recorded. The tail was severed and blood collected by free-flow into centrifuge tubes, then allowed to clot at room temperature (~17 °C) for 30 min before centrifuging and removing serum. The rostral 3 gill arches on the left side of each fish were removed and snap frozen on dry ice. The remaining left gill arch was removed, fixed overnight in 10 % neutral buffered formalin (NBF). A 1 cm segment was removed from the middle of the kidney and snap frozen on dry ice. A second 1 cm segment of the kidney was removed with adjacent skeletal muscle intact (for structural support) and fixed in 10 % NBF as above. Frozen tissue and serum were stored at −80 °C and fixed tissue was maintained at room temperature in 70 % ethanol.

2.5. Serum analysis

Serum Na^+ concentration was measured in duplicate by flame photometry (Jenway Corporation, Burlington, NJ, USA). Serum osmolality was measured in duplicate using a Vapro 5520 vapor pressure osmometer (Wescor Inc., Logan, UT, USA). Four samples were excluded from analysis due to a sampling error that resulted in visible erythrocyte lysis (all from SW1, 9.65 µg/L PAC, 24 d exposure).

2.6. Histopathology

Fixed gill and kidney tissue (n = 4–6 fish per treatment group, control and high concentrations only) were processed and embedded in paraffin following standard protocols, and serial 5 µm sections were collected onto SuperFrost Plus slides (Fisher Scientific, Toronto, ON, Canada). For gills, 10 paired lamellae positioned centrally on each of 2 adjacent filaments were analyzed for each fish (20 lamellae/fish). For kidneys, 10 tubules were chosen at random from a single photomicrograph and analyzed for each fish. A histopathology index (HI) was acquired by tallying the incidence and severity of lesions in FW smolts according to Bernet et al. (1999), using previously described abnormalities in crude oil-exposed fish gills (Medeiros et al., 2017) and kidneys (Pal et al., 2011). Briefly, each lesion type was given a severity factor between 1 (low) and 3 (high) corresponding to its likely impact on organ function. Using frequency histograms, the relative frequency of each lesion was used to assign a score value between 1 (rare; none or few occurrences) and 6 (frequent; 50 or more occurrences). In the case of kidney tubule degradation, the percentage of cells within a tubule showing signs of degradation was also factored into the frequency score value. For example, if 10 occurrences of degradation were counted on a section, a higher frequency score would be assigned if all of the affected cells were found on one tubule versus if they were divided amongst all 10 tubules. In this way, we were able to account for likely impact on tubule function without adjusting the severity factor. For each fish, the HI of the gill and kidney were calculated by multiplying the severity factor and frequency score of each lesion, and then taking the sum of all lesions. The effects of dilbit exposure and seawater acclimation on gill surface area were determined for FW, SW1, and SW7 smolts, by calculating the mean interlamellar cell mass (ILCM) height relative to mean lamellar length (Ong et al., 2007). All imaging and analyses were performed blind to treatment on a Nikon Eclipse 90i microscope equipped with a 12-bit colour digital camera (Q-Imaging) and NIS-Elements Advanced Research software package (v3.2.2).

2.7. Gill mucus cells

One gill section per fish (n = 4–6 fish per treatment group, control and high only) was stained with Alcian Blue (J.T. Baker Chemical Co., Phillipsburg, NJ) at pH 2.5 and Periodic Acid-Schiff (PAS; Electron Microscopy Sciences, Hatfield, PA) following standard procedures to differentiate between mucus cells containing neutral (magenta), acidic (blue), or a combination of mucins (purple). The number, type, and location (i.e. distal or proximal lamella, or interlamellar cell mass) of mucus cells were tallied on a total of 20 lamellae, as above.

2.8. Immunohistochemistry

Gill cells expressing Cyp1a were localized by immunohistochemistry as in Leguen et al. (2010) on one gill section per fish (n = 6 fish per treatment group, control and high only). Briefly, antigen retrieval was performed following standard deparaffinization/rehydration steps in sodium citrate buffer (pH 6.0, 90 °C) for 30 min, after which sections were cooled to room temperature before proceeding. Endogenous peroxidase activity was blocked in 3 % hydrogen peroxide, followed by a standard 30 min block in 1 % bovine serum albumin in phosphate buffered saline with 0.5 % Tween-20. Sections were incubated overnight at 4 °C in 1:500 mouse anti-fish CYP1A monoclonal antibody C10-7 (Biosense Laboratories AS, Thormøhlensgt, Bergen) in blocking buffer, and for 1 h at room temperature in 1:100 biotinylated goat anti-mouse IgG-B (Santa Cruz Biotechnology Inc., Dallas, TX). Signal amplification and visualization was performed using the Vectastain® ABC Kit and Vector® NovaRED peroxidase substrate kit (Vector Laboratories, Burlingame, CA) following manufacturer's instructions.

2.9. Na^+/K^+-ATPase (NKA) activity

Gill filaments (n = 6–7 fish per treatment) were homogenized with a motorized pestle in ice-cold buffer (150 mM sucrose, 10 mM EDTA, 50 mM imidazole, 0.5 % sodium deoxycholate; pH 7.5) and briefly centrifuged at low speed. The activity of NKA in the cleared homogenates was measured in triplicate assays containing 47.3 mM NaCl, 10.5 mM KCl, 2.6 mM $MgCl_2$, 3 U/ml lactate dehydrogenase (LDH), 3.75 U/ml pyruvate kinase (PK), 2.1 mM phosphoenolpyruvate, 2.6 mM ATP, and 0.38 mM NADH in 50 mM imidazole (pH 7.5) with or without the inclusion of 0.38 mM ouabain (McCormick, 1993). The assay couples the ouabain-sensitive hydrolysis of ATP with the oxidation of reduced NADH using PK and LDH. All enzymes and assay reagents were purchased from Sigma (St. Louis, MO, USA). NKA activity was calculated as the difference in ATP hydrolysis between ouabain-free and ouabain-inhibited reactions, normalized to the protein content of homogenates (Pierce BCA protein assay, Fisher), and expressed as µmol ADP/mg protein/h.

2.10. Semi-quantitative reverse transcriptase polymerase chain reaction (qRT-PCR)

A small piece of frozen gill filaments or kidney was homogenized using a Precellys Evolution (Bertin Technologies, Saint Quentin en Yvelines Cedex, France) in 500 µl Trizol Reagent (Life Technologies, Grand Island, NY, USA) with 1.4 mm zirconium oxide beads (Bertin) for two 25 s cycles at 5800 rpm. Cell debris was pelleted by centrifugation (5 min at 12 000 *g*), and total RNA was extracted from the supernatant as per manufacturer's instructions. Total RNA was checked for purity and quantified using a NanoDrop 2000, and then 1 µg total RNA was treated with DNase 1 and reverse transcribed to cDNA using the High Capacity cDNA Synthesis Kit all according to manufacturer's instructions (Life Technologies). Parallel reactions that omitted the Multiscribe RT enzyme were included during cDNA synthesis for 10 % of samples to serve as non-reverse transcribed (non-RT) controls. Transcript

Table 1
Comparison of serum Na^+ concentration and osmolality of control and dilbit-exposed smolts during seawater acclimation. Fish were transferred from freshwater (FW) to seawater for 1 d (SW1) or 7 d (SW7) after a 3 wk exposure and following 1 wk depuration. Values are mean ± s.e.m. Differences were determined by two-way ANOVA and Tukey's multiple comparisons test. Asterisks denote significant differences within the main effect of *seawater*, and pairwise differences are listed in the Statistics rows.

	3 wk exposure			1 wk depuration		
	FW	SW1	SW7	FW	SW1	SW7
Na^+ (mM)						
Control	188.1 ± 6.9	215.3 ± 7.1*	187.1 ± 8.8	188.1 ± 8.4	216.6 ± 8.3*	203.8 ± 7.7*
Low	189.8 ± 4.1	208.5 ± 10.4*	197.8 ± 6.9	188.5 ± 5.3	212.1 ± 5.8*	204.2 ± 3.9*
High	196.6 ± 4.7	214.4 ± 6.1*	210.4 ± 4.7	199.4 ± 4.0	206.0 ± 2.0*	211.7 ± 3.8*
Statistics	$P_{seawater}$ = 0.002 (FW = SW7) < SW1			$P_{seawater}$ = 0.0009 FW < (SW1 = SW7)		
	$P_{concentration}$ = 0.134			$P_{concentration}$ = 0.710		
	$P_{interaction}$ = 0.447			$P_{interaction}$ = 0.415		
Osmolality (mM)						
Control	302.8 ± 8.4	337.9 ± 6.9*	312.8 ± 4.3	306.3 ± 5.5	324.9 ± 4.3*	305.5 ± 2.4
Low	317.1 ± 2.4	335.7 ± 7.0*	311.3 ± 2.2	306.1 ± 3.8	324.9 ± 6.2*	310.9 ± 3.0
High	314.1 ± 3.5	326.9 ± 4.9*	318.8 ± 4.6	317.2 ± 2.0	321.0 ± 5.9*	311.3 ± 2.0
Statistics	$P_{seawater}$ < 0.0001 (FW = SW7) < SW1			$P_{seawater}$ < 0.0001 (FW = SW7) < SW1		
	$P_{concentration}$ = 0.778			$P_{concentration}$ = 0.505		
	$P_{interaction}$ = 0.120			$P_{interaction}$ = 0.393		

abundances of *cytochrome p450 1a* (*cyp1a*; (Olsvik et al., 2005)), *Na^+/K^+-ATPase isoform a1a* (*nka a1a*; (Bystriansky et al., 2006)), *nka a1b* (Bystriansky et al., 2006), *aquaporin 3a* (*aqp3a*; (Engelund and Madsen, 2014)), and the housekeeping genes *elongation factor 1α* (*ef1a*; (Engelund and Madsen, 2014)) and *β-actin* (Olsvik et al., 2005) were measured separately by qRT-PCR using a BioRad CFX96. Each duplicate 12 µl reaction contained 1x Power SYBR Green (Life Technologies), gene-specific primer pair (200 nM), and 1:40 vol:vol cDNA (or an equivalent volume of non-RT sample or molecular-grade water as negative controls). Recommended cycling conditions were followed, and a dissociation curve verified amplification of a single product, and all non-RT controls failed to amplify. Average threshold cycle values for each sample were used to calculate the transcript abundances from 5-point calibration curves generated for each primer set using serially diluted cDNA (Table 1). Abundance values were then standardized to the mean expression of the 2 reference genes, which was stable across experimental treatments.

2.11. Statistics

The ratio of *nka a1b:a1a* was calculated for each biological replicate to describe the relative expression of the seawater and freshwater *nka* isoforms in the gill during seawater acclimation. Differences in all parameters were determined in GraphPad PRISM v6.0 by 2-way ANOVA and Tukey's multiple comparisons test, using the main effects of *concentration* (control, low, high) and *seawater* (0, 1, or 7 d in seawater), and allowing for their interaction. Normality was verified using a Shapiro-Wilk test and data were ln-transformed if necessary, with the exception of mucus cell type distribution where a beta regression was used. All data are expressed as mean ± s.e.m. A significance level of 0.05 was used throughout.

3. Results

3.1. Dilbit exposure and depuration

The total PAC concentrations in experimental tanks (mean of n = 2 pooled samples per concentration) at the beginning of the exposure were 0.012 µg/L (control), 9.65 µg/L (low), and 67.9 µg/L (high). Total PAC in the control tanks remained low and stable throughout the 3-wk exposure (geometric mean 0.018 ± 0.003 µg/L), but declined ~80 % in the low and high concentration tanks (final concentrations 1.13 µg/L and 14.6 µg/L, respectively). As expected, these concentrations were sublethal, and no mortalities resulted from the dilbit exposure. Full details on water chemistry are available in Avey et al. (in review), and are consistent with our previous dilbit exposure studies (Alderman et al., 2018, 2017b).

3.2. Serum responses to dilbit exposure and seawater acclimation

Dilbit exposure did not impact serum Na^+ concentrations. All fish displayed a characteristic increase in serum Na^+ concentration during the first 24 h of seawater acclimation ($P_{seawater}$ = 0.002 and $P_{seawater}$ = 0.0009 at 3-wk exposure and 1-wk depuration, respectively), and a full (at 3-wk exposure) or partial (at 1-wk depuration) recovery to FW levels by 7 d in SW (Table 1). Serum osmolality showed a similar response to seawater acclimation that was also independent of dilbit exposure, with an increase after 24 h in seawater and a full or partial recovery by SW7 ($P_{seawater}$ < 0.0001at 3-wk exposure and 1-wk depuration; Table 1).

3.3. Gill responses to dilbit exposure and seawater acclimation

The gene expression of *nka* isoforms switched during seawater acclimation from primarily *nka a1a* in FW to *nka a1b* by 7 d in SW ($P_{seawater}$ < 0.0001) in smolts in the 3-wk exposure (Fig. 1A) and 1 wk depuration (Fig. 1B) treatments. At the end of the 3 wk dilbit exposure, smolts responded to seawater transfer by increasing NKA activity after 7 d of acclimation ($P_{seawater}$ < 0.0001), and this response was independent of dilbit concentration ($P_{concentration}$ = 0.29; Fig. 1C). After 1 wk depuration, there were no changes in NKA activity after seawater transfer ($P_{seawater}$ = 0.20) for any dilbit concentration ($P_{concentration}$ = 0.29; Fig. 1D).

There was a combined stimulatory effect of dilbit exposure and seawater acclimation on the expression of *cyp1a* in the gill at 3-wk exposure ($P_{interaction}$ = 0.009; Fig. 2A) and after 1-wk depuration ($P_{interaction}$ = 0.0005; Fig. 2B). In control fish, seawater acclimation consistently increased *cyp1a* mRNA abundance at SW1, and it remained elevated in the 3-wk exposure group but returned to FW levels by SW7 in the 1-wk depuration group. For fish exposed to 9.65 µg/L PAC for 3 wk, *cyp1a* abundance in the gill was at least 2.2-fold higher than control fish sampled in FW and SW1, and was stable throughout the seawater acclimation; but after 1-wk depuration *cyp1a* abundance was identical to control fish in FW and throughout the seawater acclimation. In contrast, at both experimental time points, fish exposed to 67.9 µg/L PAC had at least 2-fold higher gill *cyp1a* expression in FW compared to

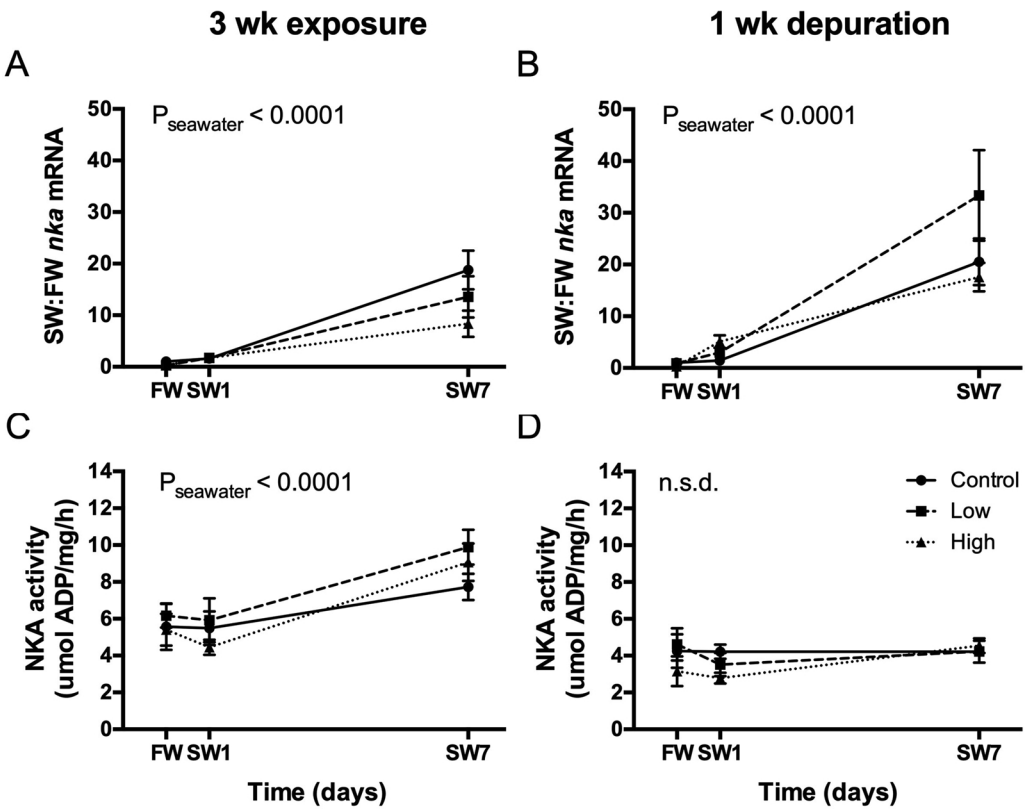

Fig. 1. Effect of dilbit exposure on gill Na^+/K^+-ATPase (NKA) during seawater acclimation. Following dilbit exposure and depuration, fish were sampled directly (FW) or transferred to seawater for 1 or 7 d (SW1 or SW7, respectively). Transcript abundances of *nka a1a* (FW isoform) and *nka a1b* (SW isoform) in the gills were quantified separately by qRT-PCR, normalized to the mean expression of *ef1a* and *b-actin*, and expressed as the proportion of SW:FW *nka* isoform for each fish at 3 wk exposure (A) and 1 wk depuration (B). NKA enzyme activity during SW acclimation was quantified spectrophotometrically in gill homogenates using an NADH-linked assay and standardized to total protein at 3 wk exposure (C) and 1 wk depuration (D). Data are mean ± s.e.m. Differences were determined by 2-way ANOVA and Tukey's multiple comparisons post-hoc test (n = 5–7 fish; $\alpha = 0.05$). n.s.d., no statistical differences.

control fish, and *cyp1a* mRNA abundance did not change for the duration of the seawater acclimation. Cyp1a-like immunoreactivity was absent in the gills of control fish in FW and throughout the seawater acclimation (n = 4 per time point; Fig. 2C). In contrast, $Cyp1a^+$ pillar cells and epithelial cells were abundant in the gills of fish exposed to 67.9 μg/L PAC when sampled directly from FW at the end of the 3-wk exposure (n = 4; Fig. 2D), and staining intensity was consistently greater at SW1 (n = 4; Fig. 2E). After 7 d in seawater, however, Cyp1a-like immunoreactivity was reduced, with weak staining evident only in pillar cells (Fig. 2F), and one fish did not have any $Cyp1a^+$ cells.

Differences in gill morphology were assessed blind to treatment for H&E stained sections of FW fish at 3 wk exposure and 1 wk depuration

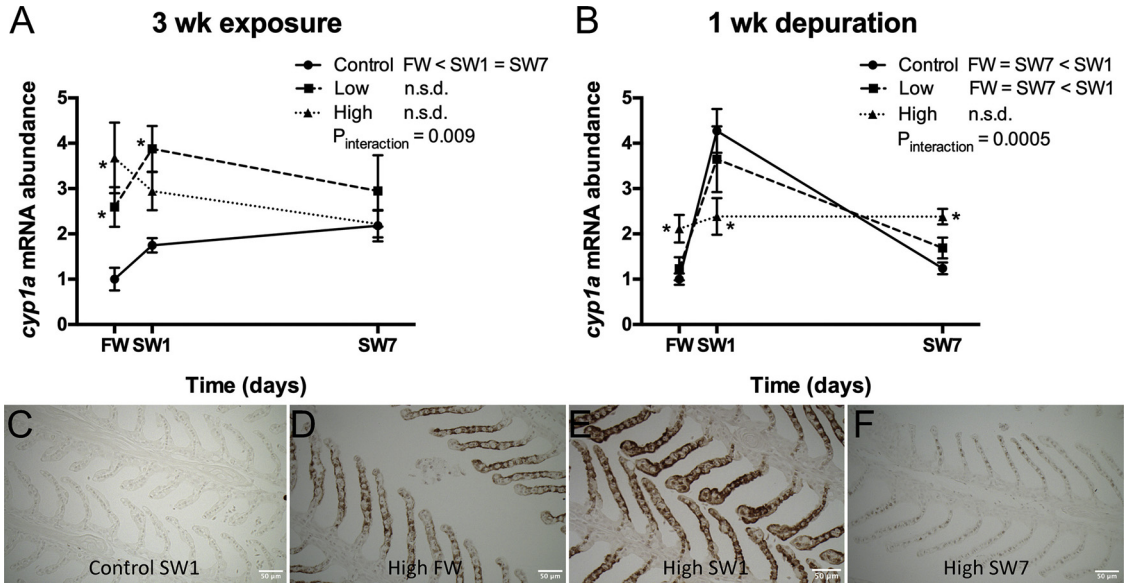

Fig. 2. Effect of dilbit exposure on gill Cyp1a expression during seawater acclimation. Following dilbit exposure and depuration, fish were sampled directly (FW) or transferred to seawater for 1 or 7 d (SW1 or SW7, respectively). The transcript abundance of *cytochrome p450 1a* (*cyp1a*) was quantified by qRT-PCR and normalized to the mean expression of *ef1a* and *b-actin* at 3 wk exposure (A) and 1 wk depuration (B). Data were analyzed by 2-way ANOVA and Tukey's post-hoc test (n = 5–7; $\alpha = 0.05$). For each concentration, significant differences in *cyp1a* abundance during seawater acclimation are listed in the figure legends. Asterisks denote differences between concentrations at each time point. n.s.d., no statistical differences. A qualitative assessment of Cyp1a expression in gills during seawater acclimation was made using immunohistochemistry on control fish (C) and fish exposed to 67.9 μg/L PAC (High; D–F) from the 3 wk exposure group. Cyp1a-like immunoreactivity is evident as a reddish-brown precipitate in pillar and epithelial cells of high exposed fish only.

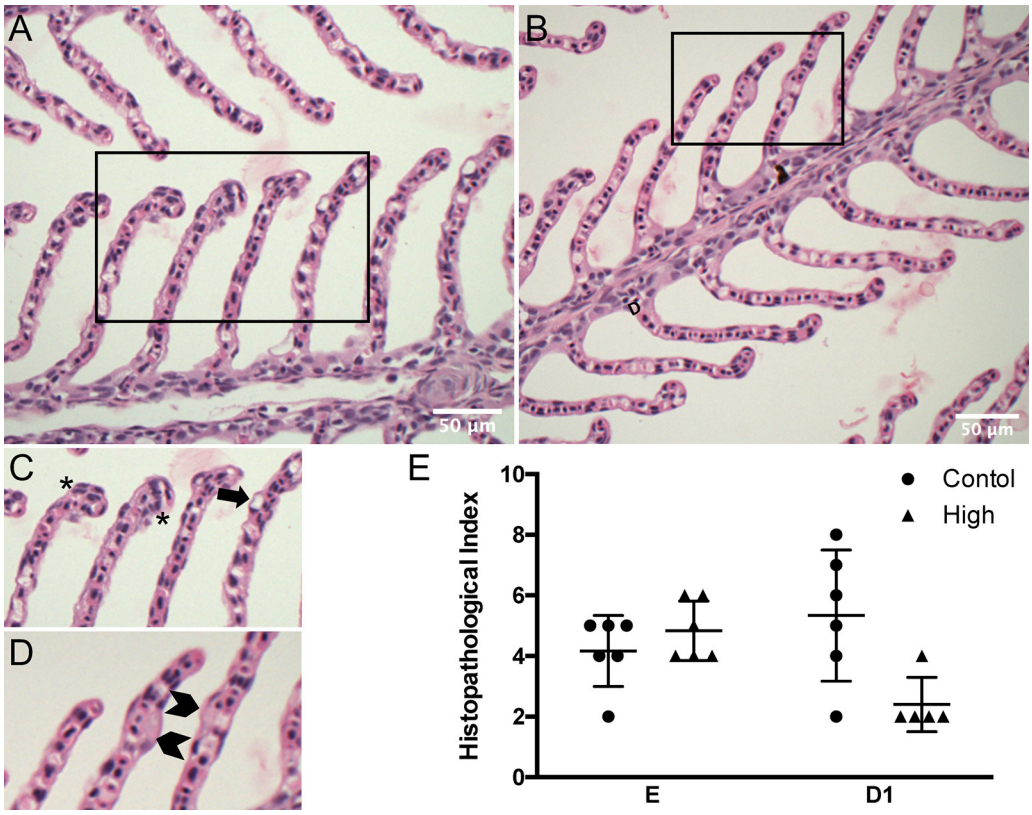

Fig. 3. Effect of dilbit exposure on gill histopathology. Representative H &E images of gill lamellae in a Control fish (A) and a fish exposed to 67.9 µg/L PAC (High; B) sampled immediately after the 3 wk exposure period. (C) Magnified image from the boxed outline in A, showing examples of hyperplasia (asterisks) and epithelial lifting (arrow). (D) Magnified image from the boxed outline in B, showing examples of hypertrophy (chevrons). (E) Lesions were tallied for 20 lamellae per section per fish and used to determine a histopathology index, with no differences between control and high-exposed fish at 3-wk exposure or after 1-wk depuration (E or D1, respectively; two-way ANOVA, n = 6 fish per treatment). Scale bar is 50 µm.

Table 2
Frequencies of histopathological changes in the gills and kidneys of smolts exposed to uncontaminated freshwater (Control) or dilbit at 67.9 µg/L PAC (High) for 3 wk and after 1 wk depuration. For each tissue, abnormalities are ranked from Stage 1–3 according to reversibility and severity. For each lesion, frequency scores were assigned as: 0 (absent), 0+ (present in few fish), + (present in most fish), + + (frequent).

		3 wk exposure		1 wk depuration	
	Stage	Control	High	Control	High
Gill					
Circulatory disturbances	1	0	0	0	0
Epithelial lifting	1	+	+	+	+
Lamellar fusion	1	0	0+	0	0+
Hypertrophy	1	+	+	+	+
Hyperplasia	2	+	+	+	0+
Necrosis	3	0	0	0	0
Kidney Tubules					
Tubule degradation	1	+	+	+	+
Hypereosinophilia	1	+	+	0+	+
Vacuolization	1	0	0+	0+	0+
Nuclear pyknosis	2	+	+	+	+
Necrosis	3	0	0	0	0

in control fish (Fig. 3A) and in fish exposed to 67.9 µg/L PAC (Fig. 3B). The most common abnormalities present on gill lamellae were hyperplasia (Fig. 3C), epithelial lifting (Fig. 3C), and hypertrophy, (Fig. 3D), although in all cases these were present infrequently (Table 2), and independently of dilbit exposure ($P_{concentration}$ = 0.16; n = 6; Fig. 3E). Interlamellar cell mass was consistent across all fish and did not change with dilbit exposure, depuration, or seawater exposure (n = 4–6; data not shown). Mucus cells were analyzed blind to treatment for Alcian blue-PAS stained sections of FW fish at 3 wk exposure and 1 wk depuration, and there were no differences in total mucus cell numbers, mucin type-specific cell numbers, or their distribution on and between gill lamellae (Supplemental Fig. S1).

3.4. Kidney responses to dilbit exposure and seawater acclimation

After 3-wk of dilbit exposure, the expression of *aqp3a* in the kidney was altered by the combined influence of dilbit exposure and seawater transfer ($P_{interaction}$ = 0.001; Fig. 4A). In control fish and fish exposed to 9.65 µg/L PAC, *aqp3a* mRNA abundance did not vary significantly during the 7d SW acclimation period. In contrast, fish exposed to 67.9 µg/L PAC had 2- to 3-fold higher *aqp3a* expression in FW than at either SW1 or SW7. Pairwise comparisons within each time point of the seawater acclimation revealed that the mRNA abundance of *aqp3a* was nearly 2-fold higher in fish exposed to 67.9 µg/L PAC when sampled in FW relative to controls, and was slightly but significantly higher in fish exposed to 9.65 µg/L PAC when sampled at SW1 relative to controls (Fig. 4A). These responses were abolished after 1-wk depuration in uncontaminated water, with *aqp3a* mRNA abundance similar throughout seawater acclimation ($P_{seawater}$ = 0.69) for all concentrations ($P_{concentration}$ = 0.62; Fig. 4B).

Differences in kidney morphology were assessed blind to treatment for H&E stained sections of FW fish at 3 wk exposure and 1 wk depuration. The most common abnormalities were tubule degradation, hypereosinophilia, and nuclear pyknosis (Fig. 5A), although in all cases these were present infrequently (Table 2), and independently of dilbit exposure ($P_{concentration}$ = 0.82; n = 6; Fig. 5B).

4. Discussion

This study shows that exposure to environmentally relevant concentrations of dilbit, a crude oil product, does not impact osmoregulation during seawater acclimation in Atlantic salmon smolts using well-defined hematological, tissue, and molecular endpoints. Smoltification is a critical life history stage for salmon, and represents a population bottleneck whereby successful outmigration of smolts from nursery lakes to the ocean is a primary determinant on future return of

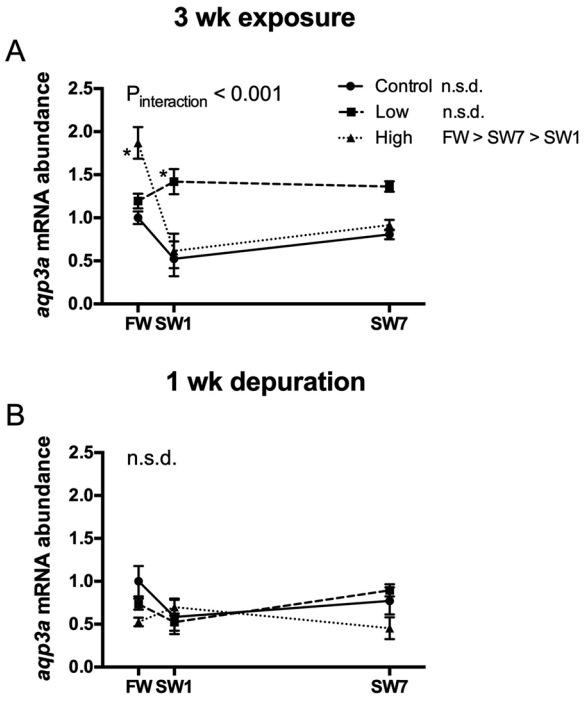

Fig. 4. Changes in kidney *aquaporin 3a* expression during seawater acclimation. Following dilbit exposure (A) and depuration (B), fish were sampled directly (freshwater, FW) or transferred to seawater for 1 or 7 d (SW1 or SW7, respectively). Transcript abundance of *aqp3a* in the kidney was quantified by qRT-PCR and normalized to the mean expression of *ef1a* and *b-actin*. Data were analyzed by 2-way ANOVA and Tukey's post-hoc test (n = 5–7; $\alpha = 0.05$). For each concentration, significant differences in *aqp3a* abundance during seawater acclimation are listed in the figure legends. Asterisks denote differences between concentrations at each time point. Data are mean ± s.e.m. n.s.d., no statistical differences.

spawning adults (Thorstad et al., 2012). Contamination of salmon freshwater and nearshore habitats by crude oil is a present and growing concern due to growth of Canada's oil industry. If a spill in salmon habitat were to occur, outmigration of smolts could be compromised by the reduction in swimming performance that is well characterized in fish exposed to conventional crude oil (e.g. Kennedy and Farrell, 2006; Mager et al., 2014; Nelson et al., 2017) and dilbit (Alderman et al., 2017b). There is also potential for crude oil exposure to impair other aspects of salmon physiology that are critical to smoltification and successful seawater transition, including behavior (Kawaguchi et al., 2012; Philibert et al., 2016), olfaction (Lari et al., 2015), and endocrine function (Kennedy and Farrell, 2005; Kennedy and Smyth, 2015; Truter et al., 2017), as well as the focus of the present study, osmoregulatory function (Brauner et al., 1999; Duarte et al., 2010; Englehardt et al., 1981; Kennedy and Farrell, 2005).

Seawater transfer induced a transient increase in serum Na^+ concentrations and osmolality in unexposed control fish. The recovery of these parameters to baseline by 7 d post seawater transfer were mediated in part by a switch in *nka* isoform expression and an increase in Na^+/K^+-ATPase activity in the gill. This seawater acclimation response is well-documented in salmonids (Bystriansky et al., 2006; Christensen et al., 2018). Atlantic salmon exposed to dilbit for 3 wk prior to seawater transfer showed a similar acclimation response as control fish. There was a trend towards higher serum Na^+ concentrations and osmolality in freshwater for both dilbit-exposed treatment groups relative to controls, and in the 67.9 µg/L PAC exposure group after 1 wk depuration, however these differences did not reach statistical significance. Given that neither gill *nka* gene expression nor Na^+/K^+-ATPase activity was altered by dilbit exposure, it is unlikely that this membrane transporter contributed to this modest change in serum Na^+ concentrations and osmolality. It is possible that the regulation of other ions was altered by dilbit, such as Mg^{2+} which is prevalent in seawater, or K^+ and Cl^- which both increased within 96 h of crude oil exposure in Pacific herring and remained elevated through 8 wk of continuous exposure (Kennedy and Farrell, 2005). Recently, Lin et al. (2019) reported that juvenile sockeye salmon exposed similarly to dilbit experienced reductions in plasma osmolality, Na^+ and Cl^-, as well as gill NKA activity, within 96 h of exposure to 34.7 µg/L and 124.5 µg/L PAC, and these reductions were sustained through 21 d of exposure. Taken together, the absence of a clear response in the Atlantic salmon used in the present study may be explained simply by species-level variation, or may in fact extend from genetic modifications towards increased resilience associated with successive generations in aquaculture. For example, variation in the seawater acclimation response was observed amongst several familial lines of Atlantic salmon broodstock (Mackie et al., 2005).

The gill is a high-surface area organ that directly interfaces with the environment and can be a site of contaminant uptake, particularly in freshwater when fish are not drinking. The gills of smolts exposed to dilbit showed near ubiquitous expression of Cyp1a in pillar and epithelial cells, while the gills of unexposed control fish were devoid of $Cyp1a^+$ cells. This response is consistent with AhR-mediated up-regulation of the Phase I biotransformation pathway (Collier et al., 2013), however we have previously observed tissue-specific variability in the *cyp1a* transcriptional response dynamics during sub-chronic dilbit exposures (S. Avey, personal communication). In the gill, up-regulation in Cyp1a protein was not matched by an increase in *cyp1a* mRNA abundance, indicating the transcriptional response to AhR occurred transiently and prior to the sampling time at 3 wk. Interestingly, seawater acclimation induced an early increase in *cyp1a* mRNA abundance in unexposed control fish which returned to baseline by 7 d. This transcriptional response to seawater did not result in the detection of Cyp1a protein in the gills using immunohistochemistry, which may have occurred transiently during the window between sampling periods.

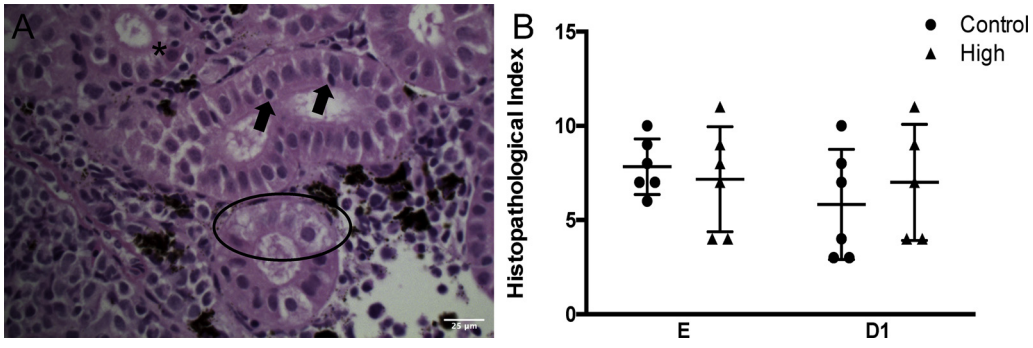

Fig. 5. Effect of dilbit exposure on kidney histopathology. (A) Representative H&E image of kidney tubules from a fish exposed to the highest dilbit concentration (67.9 µg/L PAC) for 3-wk, showing hypereosinophelia (asterisk), nuclear pyknosis (arrows), and tubule degradation (circle). (B) Lesions were tallied for 10 tubules per section per fish and used to determine a histopathology index, with no differences between Control and High fish at 3-wk exposure or after 1-wk depuration (E or D1, respectively; two-way ANOVA, n = 6 fish per treatment). Scale bar is 25 µm.

Others have suggested, based on similar findings, that Cyp1a plays a role in seawater acclimation (Leguen et al., 2010), although what that function may be is not clear. Nevertheless, our results indicate that gill Cyp1a is likely acting classically in chemical biotransformation during crude oil exposure, possibly helping to limit xenobiotic accumulation. As expected, Cyp1a-like immunoreactivity was reduced by 7 d in seawater, indicating that the induction of this protein reversed quickly once fish were removed to uncontaminated water.

Histopathological changes in the gill resulting from crude oil exposure have been described, most typically as hyperplasia and epithelial cell lifting which increase diffusion distances and may impair gill function (Kennedy and Farrell, 2005; McKeown and March, 1978; Medeiros et al., 2017). The extent of damage reported in these studies is variable and could be influenced by a number of experimental variables including oil source, duration and method of exposure, as well as species-specific sensitivities to the contaminants in crude oil. Contrary to previous studies, dilbit-exposed Atlantic salmon did not experience an increase in gill lesions. Other phenotypic responses in the gill were also examined, namely changes to interlamellar cell mass and mucus cell distribution. Previous studies have shown that salinity exposure (Blair et al., 2017, 2016) and air exposure (Ong et al., 2007) induce a rapid hyperplasia response in fish gills that packs the interlamellar space and greatly reduces gill surface area, but this did not occur following dilbit exposure in the present study. The number and type of mucous-secreting cells present on fish gills are influenced by abiotic factors including salinity and xenobiotic exposure (Blair et al., 2017; Dang et al., 2017; Paulino et al., 2012; Roberts and Powell, 2003; Solanki and Benjamin, 1982); however, we did not observe any effect of dilbit exposure on the number, type, or position of mucus cells on the gills of dilbit-exposed Atlantic salmon. Together, these results show a lack of tissue response at the level of the gill to the contaminants in dilbit.

The fish kidney plays an important role in maintaining osmotic balance and undergoes considerable changes in anadromous fish as it switches function from urine production in freshwater to water retention in seawater. Discrete expression and localization of aquaporins may play a key role in this process by facilitating the movement of water and select solutes, as occurs in the mammalian nephron (Nielsen et al., 2002). Indeed, seawater acclimation of euryhaline fish results in differential expression patterns of aquaporin isoforms in European eel (*Anguilla anguilla*) (Martinez et al., 2005) and in Atlantic salmon (Engelund and Madsen, 2014; Tipsmark et al., 2010), although the precise functions of each isoform are not certain. Here, the mRNA abundance of *aqp3* was elevated in the kidney of freshwater acclimated salmon following 3 wk exposure to 67.9 µg/L PAC, and after 1 d of seawater acclimation in salmon exposed to 9.65 µg/L PAC. Certainly, changes in gene expression could signify dysregulation of cell processes or a compensatory response to ameliorate an osmotic disturbance. However, since plasma osmolality was not greatly altered by dilbit exposure and no histopathological effects were found, the changes in *aqp3* expression are unlikely to contribute to higher-level physiological disturbances. By contrast, in juvenile sockeye salmon *cyp1a* mRNA abundance increased in renal tissue following dilbit exposure and this coincided with serum-level changes indicative of impaired kidney filtration, including increased serum protein levels and the appearance of a kidney-specific protein, dynein heavy chain 8, in the circulation (Alderman et al., 2017a). Moreover, osmoregulatory dysfunction was evident in juvenile sockeye within 24 h and throughout a 21 d dilbit exposure (Lin et al., 2019), again highlighting species-level variation in physiological responses to dilbit exposure.

Smoltification is a complex life history transition that requires the integration of regulators across biological levels of organization. While this study did not identify any major osmoregulatory dysfunction in dilbit-exposed smolts, other aspects of smoltification could be altered by crude oil exposure and warrant investigation. For example, smoltification is regulated by numerous endocrine factors (Björnsson et al., 2011), including prolactin, growth hormone, and cortisol (Kiilerich et al., 2011; Prunet et al., 1989). Crude oil dispersions, water-soluble fractions, individual PAH, and AhR agonists have all been shown to disrupt endocrine systems, including the cortisol stress axis (Aluru and Vijayan, 2006, 2004; Kennedy and Farrell, 2005; Stephens et al., 1997), thyroid axis (Stephens et al., 1997), and reproductive axis (Kennedy and Smyth, 2015). Future studies should consider endocrine endpoints in the context of smoltification and dilbit exposure in salmonids.

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CRediT authorship contribution statement

Sarah L. Alderman: Investigation, Formal analysis, Writing - original draft, Writing - review & editing, Visualization, Supervision, Project administration, Funding acquisition. **Christarin M. Dilkumar:** Investigation, Formal analysis. **Sean R. Avey:** Investigation. **Anthony P. Farrell:** Writing - review & editing, Funding acquisition. **Christopher J. Kennedy:** Resources, Writing - review & editing, Supervision, Project administration, Funding acquisition. **Todd E. Gillis:** Resources, Writing - review & editing, Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.aquatox.2020.105419.

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