The osmotic effect of hyper-saline hydraulic fracturing fluid on rainbow trout, *Oncorhynchus mykiss*


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Abstract

Flowback and produced water (FPW) is a complex, often brackish, solution formed during the process of hydraulic fracturing. Despite recent findings on the short-term toxicity of FPW on aquatic biota, longer-term impacts of FPW on fish have not yet been investigated and the mechanisms of chronic effects remain unknown. The aim of the present study was to observe the effect of a diluted FPW on ionoregulatory endpoints in the rainbow trout *Oncorhynchus mykiss*, following a 28-d sub-chronic exposure. A salinity-matched control solution (SW), recreating the salt content of the FPW, was used to differentiate the specific effect of the salts from the effects of the other FPW components (i.e. organics and metals). Overall, fish ionoregulation was not impacted by the chronic exposure. An accumulation of strontium (Sr) and bromide (Br) occurred in the plasma of the FPW-exposed fish only, however no change of plasma ions (Na, K, Cl, Ca, Mg) was observed in SW- or FPW-exposed fish. Similarly, exposures did not alter branchial activity of the osmoregulatory enzymes sodium/potassium ATPase and proton ATPase. Finally, FPW exposure resulted in modifications of gill morphology over time, with fish exposed to the fluid displaying shorter lamellae and increased interlamellar-cell mass. However, these effects were not distinct from morphological changes that also occurred in the gills of control groups.

Keywords: hydraulic fracturing, *Oncorhynchus mykiss*, salinity, osmotic stress

1. Introduction

Horizontal hydraulic fracturing (HF), an unconventional technique for hydrocarbon extraction, is a growing industrial practice, especially in North America (Stringfellow et al., 2014; Gagnon et al., 2015). Indeed, a 25% increase in gas and oil production by this method is expected in Canada by 2035 (Gagnon et al., 2015). Briefly, this process releases hydrocarbons from geologic
formations by injecting a highly-pressurized fluid into extraction wells resulting in rock fracture and eventual hydrocarbon release. The composition of the injected solution, known as fracturing fluid, is complex and specific to each well. However, some components are conserved, including corrosion inhibitors, biocides, and surfactants (King, 2012; Stringfellow et al., 2014). After injection, and once the pressure is released, a mixture of the fracturing fluid, hydrocarbons and other organic chemicals, flows back to the surface. This fluid that returns to the surface is termed flowback and produced water (FPW) and by volume represents 30 to 80% of the fluid initially injected (Alessi et al., 2017). Ultimately, the composition of FPW depends on the individual components of the injected fracturing fluid, residence time of the fluid in the formation and the composition of the geologic formation being fractured (Stringfellow et al., 2014; Kim et al., 2016).

Nevertheless, FPWs from a range of formations have been found to consist of a mixture of trace ions, metals, organic compounds, inorganic solids, and trace radionuclides (Entrekin et al., 2011; Jiang et al., 2014; Flynn et al., 2019).

Environmental concerns have accompanied the growth of the HF industry, particularly with respect to the large volumes of water extracted and water quality (Vidic et al., 2013; Kondash and Vengosh, 2015; Alessi et al., 2017). For example, it is estimated that in the last decade a total volume of 80 billion gallons of water has been used for HF oil and gas extraction in the U.S. (Vengosh et al., 2014). Furthermore, there are concerns regarding the effects of FPW spills into the aquatic environment. Whilst there exist fairly stringent regulations and guidelines for storage and transport of FPW, and the ratio of spills per wells drilled has decreased over the past 10 years, spills may still occur due to unforeseen challenges. These spills principally occur during transportation of the effluent or following a failure of the storage system (US EPA, 2015; Goss et al., 2015; Gagnon et al., 2015). As the practice of HF for extraction of shale oil and gas reserves
continues to grow, it is likely that threats of FPW contamination of waters will also increase (Teather and Parrott, 2006; US EPA, 2015). However, to date, there is a limited understanding of the long-term sub-chronic responses of aquatic organisms to FPW.

To assess the environmental impact of industrial fluids such as FPW, it is important to select appropriate model species that live in habitats that have a high risk of contamination. One such model is the rainbow trout (*Oncorhynchus mykiss*), a species native to North American freshwaters. This fish is widely employed in toxicological studies, in part due to its high sensitivity to environmental contaminants (Teather and Parrott, 2006). The rainbow trout is also a key test species in many regulatory guidelines for the protection of aquatic environments (e.g. Environment Canada, 1990).

The first studies investigating the impact of diluted FPW samples on aquatic biota have shown that FPW exposure can cause acute lethal and sub-lethal toxicity in freshwater organisms, and the organic fraction of the effluent is suggested to be the main driver of its toxicity. For example, studies have highlighted organic-associated effects on the metabolic responses of the rainbow trout, *Daphnia magna*, and the zebrafish (*Danio rerio*), as well as impacts on their development, fitness and swimming performance (He et al., 2017; 2018; Folkerts et al., 2017a,b; Blewett et al., 2017a,b; Blewett et al., 2018). It is also important to note that FPW is highly saline, with a salt content that is routinely greater than that of seawater, and it has been shown that this salinity is partly responsible for FPW toxicity. For example, Blewett et al. (2017b) showed that an acute (96 h) exposure to FPW caused changes in the gill morphology of freshwater rainbow trout, similar to those seen in seawater-acclimated fish. However, the consequences of longer-term FPW exposures to fish have yet to be investigated.
The present study examines the effects of a sub-lethal, longer-term (28-d) exposure of juvenile *O. mykiss* to FPW, with a specific focus on ionoregulatory endpoints (i.e. plasma ions, osmoregulatory enzyme activities, gill histology) that are susceptible to disturbance following an exposure to brackish FPW. Changes at the gill were of specific interest given the importance of the branchial epithelium in gas transport, ion and water exchange, the large branchial surface area, and the fact that the gills are in direct and constant contact with the environment (Evans et al., 2005). Consequently, the gills represent an important potential target for aquatic toxicants. This has been shown in previous work, wherein environmental stressors have been shown to induce morphological changes in the fish gill, including edema, lifting of the epithelium, lamellar fusion, hypertrophy and/or hyperplasia (Mallatt, 1985; Evans, 1987; Gilmour and Perry, 2018). Effects of environmental stressors on the gills of fish are not limited to morphological changes, but may also involve changes at the biochemical level. Sodium-potassium ATPase (NKA) and the proton pump (H⁺-ATPase) are key ionoregulatory enzymes located in branchial cells of fish (Dymowska et al., 2012), and which maintain plasma ion homeostasis. These enzymes may alter their activity either in response to osmotic imbalances caused by aquatic contaminants (Hollis et al., 1999; Bervoets and Blust, 2003; Nordlie, 2009), or because of direct effects of toxicants on these enzymes themselves (e.g. Dang et al., 2000).

The current study utilized a raw FPW sample, diluted to 3% of its initial strength based on the outcomes of an acute lethal exposure detailed herein. In addition, to separate the effects of the saline and non-saline components of FPW, exposures were also conducted with a salt water (SW) solution, which matched the salt content of the raw FPW sample, but with none of the metals or organic components. At this concentration, the salt content of exposure waters is below the limit where physiological changes are expected as a result of salinity (i.e. 3.4 parts per thousand (ppt),...
below the isosmotic point of 8-10 ppt) in this euryhaline species. Consequently, this allows the
delineation of effects of the other components (i.e. the metals and organics) of the FPW.

2. Material and methods

2.1. Animals

Juvenile rainbow trout (n = 96; 7.9 ± 0.5 g) were provided by the Sam Livingston Fish
Hatchery and Rearing Station (Calgary, Alberta, Canada) and were maintained in the aquatic
facility of the University of Alberta. Fish were held in a 120-L tank containing flow-through
moderately hard City of Edmonton dechlorinated water ([Na] = 14.6 ppm, [Ca] = 55.9 ppm, [Mg]
= 15.3 ppm, [K] = 2.5 ppm, titration alkalinity ≈ 119 mg/L as CaCO$_3$, pH ≈ 7.6, hardness ≈ 180
mg/L as CaCO$_3$, conductivity ≈ 385 μS/cm) at a temperature of 13 ± 1°C. Trout were acclimated
in these tanks for 3 months before the initiation of experiments and were fed daily with crushed
salmonid pellets (EWOS, Surrey, British Columbia, Canada) at 1.5% of their body mass. The
tanks were constantly aerated and kept under a 14h:10h light/dark photoperiod. All procedures
were conducted with the approval of the University of Alberta Animal Care Committee under
protocol AUP00001334.

2.2. Acute toxicity assay

A 3-h post well-opening FPW sample from a hydraulically fractured well in the Duvernay
Formation, Fox Creek, Alberta, Canada, was provided by Encana Corporation (December 2017).
This sample was collected at the well head, immediately after separation from the oil and gas
phases. The chemical analysis of this sample is detailed in Section 2.4.1, below.
The acute median lethal concentration (LC$_{50}$), representing the concentration at which 50% of exposed individuals are lethally affected, was determined for juvenile _O. mykiss_ exposed to the 3-h FPW sample for 96 h. After fasting for 24-h, fish were exposed to either control water (Edmonton City dechlorinated water) or increasing dilutions of the raw effluent sample, which undiluted was considered to be 100% FPW. Dilutions were made with City of Edmonton dechlorinated tap water. Six FPW concentrations and a control (no FPW present) were used to determine the LC$_{50}$: 0%, 2.5%, 5%, 10%, 15%, 20% and 25%. Four replicates were performed for each concentration. Each replicate consisted of six individual fish held in an 8-L tank containing 7 L of the appropriate treatment (i.e. control or FPW dilutions). All tanks were held at a temperature of 13 ± 1°C in a flow-through controlled temperature water bath, and a 50% water change was performed at 48-h. Survival was recorded at the end of the 96-h exposure, and an LC$_{50}$ value (with 95% confidence intervals) was calculated, using Toxicity Relationship Analysis Program (TRAP) version 1.30a (EPA, Washington, DC, USA). The calculated acute LC$_{50}$ was used to help guide the FPW concentration for the sub-chronic exposure.

2.3. **Sub-chronic exposure**

The exposure concentration for the sub-chronic test (3%) was chosen based on the acute LC$_{50}$ value (11.6% FPW), and thus represented a value close to the acute LC$_{10}$. Blewett and colleagues (2017b) previously showed that a 48-h acute exposure to an FPW sample diluted to 7.5% resulted in a salinity stress in _O. mykiss_. Thus, an exposure to 3% of the present effluent was considered sufficiently low as to not induce notable salinity effects, but sufficiently high such that any effects of the metal and organic composition could be delineated. In addition, while it is difficult to assess the average FPW volume spilled in real-world scenarios, and thus the exposure...
dose of such spills, it is noteworthy that many documented spills refer to high volumes of FPW (AER, 2018). Thus, 3% FPW likely represents an environmentally relevant concentration.

Juvenile rainbow trout were exposed to one of three solutions: 3% FPW (raw sample diluted to 3% of its initial strength), 3% SW (a solution made to match the salt content of the raw FPW, diluted to 3% of its initial strength) and a control (dechlorinated tap water). The dilutions of FPW and SW were conducted using dechlorinated tap water. The SW solution consisted of laboratory salts (NaCl, CaCl₂, MgCl₂, KCl; Sigma Aldrich, Oakville, Ontario, Canada) added to nanopure water to replicate the salt concentrations present in the raw FPW sample, which were determined as outlined in Section 2.4.1, below. For each condition six replicates were performed, with four juvenile rainbow trout per replicate. Each replicate contained 4-L of the appropriate solution, in previously acid-washed 8-L glass tanks. Trout were fed to satiation with crushed salmonid pellets every 2 to 3 days. Five hours after feeding, a 50% water change was conducted to clear tank of uneaten food and feces. All tanks were placed on a wet table supplied with flowing water, which maintained tank water at a temperature of 13 ± 1°C, and the exposure water quality was regularly sampled and monitored (see Section 2.4.2). A single fish from each replicate was sampled at 6-h, 48-h, 7-d and 28-d, following exposure initiation. At the time of sampling, fish were euthanized by cephalic concussion and subsequent spinal transection, before a blood sample was collected from the caudal vein. Following centrifugation (15000 g, 2 minutes), plasma was sampled and snap frozen in liquid nitrogen and stored at -80°C for later analysis of plasma ions and trace elements (see Section 2.4.2, below). In addition, gills (2nd gill arch, left side) were collected and either placed immediately in 4% paraformaldehyde (PFA) for histological analysis, or snap frozen in liquid nitrogen and stored at -80°C for biochemical analysis.
2.4. **Inductively coupled plasma-double mass spectrometry (ICP-MS/MS) analysis**

Inductively coupled plasma-double mass spectrometry (ICP-MS/MS) was conducted using an Agilent 8800 spectrometer, to analyze the metal content of the FPW sample, experimental waters, and plasma samples.

2.4.1. **FPW sample analysis**

Before chemical analysis, the raw FPW was filtered through a 0.2 µm nylon filter membrane and diluted using 18 MΩ ultrapure water: 850-fold (for sodium determination), 85-fold (for all other cationic elements, total sulfur and bromide), 2000-fold (for chloride), and 5-fold (for total carbon (TC) and total nitrogen (TN)). Diluted samples were then analyzed via one of three methods, detailed below.

Cations, bromide and total sulfur were quantified by ICP-MS/MS, operated with a microMist nebulizer, nickel/copper cones, and a 1550 W RF power and 18 W RF reflected power. After dilution, samples were acidified with 12 µL of 15.7 N trace metal grade nitric acid per 10 mL of sample. To overcome the high total dissolved solids of the FPW even after dilution, samples and standards were analyzed in high matrix mode in which aspirated samples were diluted with an 8 mL min\(^{-1}\) flow of argon. Additionally, a 0.5 ppm solution of indium was added to each sample using an inline internal standard addition system to account for instrumental drift. For a better mass resolution, MS/MS mode was utilized, and matrix interferences were reduced via the use of a collision gas reaction cell supplied with either helium, oxygen or hydrogen. Finally, a quality control was performed at the start, middle and end of each run by running a standard solution. The deviation was < 5% across all runs.
Chloride was measured via ion chromatography using a Dionex Ion chromatography DX 600 with a 4 mm analytical column (AS9-HC), guard column (AG9-HC), and a 4 mm ASRS Ultra suppressor. Due to the large dilution required for raw FPW analysis, bromide and sulfate were below the instrumental detection limit. A Shimadzu model TOC-V-CHS/CSN TOC analyzer was used to measure TC and TN.

2.4.2. Exposure water chemistry

Exposure water (50 mL) was sampled regularly over the course of the 28-d exposure, immediately before and after water changes. At least two replicate tanks were sampled at each water sample time-point, with the 28-d exposures sampled at least 3 times (i.e. 6 water samples). Water was stored at room temperature before analysis.

For elemental analysis, FPW samples were diluted 20-fold, SW was diluted 10-fold, and control water was analyzed undiluted, with the diluent varying depending on the preferred matrix for the target element (see below). For sodium analysis, samples and standards were prepared in a matrix of 2% HNO₃, and the sodium standards ranged from 0.1 to 100 ppm. For all other elements, samples and standards were also prepared in a matrix of 2% HNO₃, however 2,000 ppm NaCl was added to the standards to create a matrix similar to the diluted seawater and FPW samples. All FPW samples were diluted 1:2 for analysis of non-sodium elements, whereas SW and control water samples were analyzed undiluted. Standards covered a range of 0.0005 to 120 ppm in three tiers to accommodate varying concentration levels within the samples.

2.4.3. Plasma cations
Plasma ions and trace metals were analyzed following the protocol outlined in Gajek et al. (2013). Na, K, Ca, Mg, Li, Be, B, Al, Cr, Mn, Fe, Co, Ni, Cu, Zn, As, Br, Sr, Mo, Cd, Cs, Ba, Hg, Pb and U were measured. Samples were diluted 50 or 100 times in ultrapure water containing 2% NH₄OH w/v, 0.1% H₄EDTA w/v, 4% n-butanol w/v, and 0.1% triton X-100 w/v. Standards, covering a range of 0.0005 to 50 ppm in two tiers to accommodate varying concentration levels within the samples, were prepared in the same matrix as the samples.

2.5. Plasma chloride

Plasma chloride was measured via a Chloride Assay Kit according to the manufacturer’s protocol (Sigma Aldrich, St Louis, MO, USA). This assay is based on the competition of Hg²⁺ and Fe²⁺ for 2,4,6-Tris(2-pyridyl)-s-triazine (TPTZ). Briefly, plasma samples were initially diluted 24 times in nanopure water, and 10 μL of the diluted sample was plated in duplicate in a 96-well plate. An additional 5 times dilution was performed in the well as the sample volume was completed with nanopure water to reach a final volume of 50 μL, and 150 μL of chloride reagent was added to each well. The plate was then incubated at room temperature in the dark for 15 min before the absorbance of the samples was determined at 620 nm on a microplate reader (Versamax Molecular Devices) with the software Max Pro 5. To calculate the chloride concentration (in mM), a 10 mM chloride standard solution was used to generate a standard curve.

2.6. Sodium-Potassium ATPase (NKA) and H⁺-type ATPase activity

Enzyme activities were measured according to the protocols of McCormick (1993) and Lin and Randall (1993), with some modifications. Frozen gill tissues were homogenized in SEID-EGTA buffer (125 mM sucrose; 5 mM EGTA; 50 mM imidazole; 0.05 g 50 mL⁻¹ Na deoxycholate;
pH = 7.3) using a Fisherbrand handheld homogenizer. The homogenate was then centrifuged at 4°C for 3 minutes at 5000 g, and the supernatant was used to measure NKA and H⁺-ATPase activity.

Ten μL of supernatant was pipetted into each of 12 wells in a 96-well plate, representing triplicates of four different assay solutions. Three wells contained only the assay solution (2.8 mM phosphoenolpyruvate (PEP); 3.5 mM ATP; 0.22 mM NADH; 4 U mL⁻¹ lactate dehydrogenase (LDH); 5 U mL⁻¹ pyruvate kinase (PK); 189 mM NaCl; 10.5 mM MgCl₂·6H₂O; 42 mM KCl; 50 mM imidazole), while three other wells contained this assay solution in addition to 0.65 mM of ouabain (an NKA inhibitor). The remaining six wells were used to record H⁺-ATPase activity. All six of these wells contained the assay solution containing ouabain, described above, as well as 500 mM NaN₃. Finally, 100 mM N-ethylmaleimide (NEM; H⁺-ATPase inhibitor) was added to only three of these six wells. All enzymatic activity measurements were read at a wavelength of 340 nm on a microplate reader and normalized to the sample protein content measured via Bradford Reagent following the manufacturer’s protocol (Sigma Aldrich, St Louis, MO, USA). Enzyme activities are expressed in μmol ADP mg protein⁻¹ hour⁻¹.

2.7. Gill histology

Gill samples that were placed immediately in 4% PFA after sampling were fixed for 24-h at 4°C. Tissues then underwent a rinse with phosphate-buffered saline prior to being dehydrated with ethanol and embedded in paraffin wax. Embedded tissues were sectioned (7 μm) and stained using haematoxylin and eosin before being observed via light microscopy (Zeiss Scope A1). Digital images of 10 adjacent lamellae were captured using an optronic camera and analyzed using ImageJ software (National Institutes of Health). Lamellar width, lamellar length and interlamellar
cell mass (ILCM) were measured and the ratio of ILCM to lamellar length was calculated (see Ong et al., 2007). Each treatment (control, SW, FPW) at each time point (6-h, 48-h, 7-d and 28-d) was analyzed in triplicate.

2.8. Statistics

All data underwent tests of normality (Shapiro-Wilk) and equal variance (Brown-Forsythe). If data failed these tests, they were transformed until parametric assumptions were met. Differences in gill morphometrics, enzyme activities and plasma chloride and cation concentration were analyzed with a two-way ANOVA (time and treatment as the two factors), whereas the effects of FPW and SW on plasma trace element concentrations were tested via a one-way ANOVA (treatment only). A Tukey’s post hoc test was used to determine specific comparisons that differed, if the initial ANOVA highlighted significant differences. The exception to this was plasma copper (Cu) and strontium (Sr) levels, which were analyzed by a non-parametric Kruskal-Wallis test as they failed to meet parametric assumptions, even after transformation. SigmaPlot version 13.0 (Systat Software Inc., San Jose, CA, USA) was used to conduct all statistical analysis. Values are expressed as means ± SEM (standard error of the mean). For all analyses, an alpha level of 0.05 was used.

3. Results

3.1. Water chemistry

Analysis by ICP-MS/MS revealed a high concentration of salt ions in the raw FPW sample. Chloride (Cl) and sodium (Na) had the highest concentrations, 68,100 ppm and 47,500 ppm respectively. The next most abundant elements were calcium (Ca; 6,940 ppm), potassium (K;
1,600 ppm) and magnesium (Mg; 657 ppm). Of the trace elements analyzed in the FPW, strontium (Sr; 723 ppm) and bromide (Br; 259 ppm) were the most concentrated. The complete chemical analysis is available in Table 1.

3.2. Acute toxicity

The acute 96-h LC\textsubscript{50} value was determined to occur at a dilution of 11.6\% of the raw FPW sample (95\% C.I. 10.14 – 13.14\%) (Fig. 1). This value was used to help determine a sub-lethal exposure concentration for the 28-d sub-chronic exposure.

3.3. Sub-chronic exposure water chemistry

In terms of their ion content, the chemical composition of the two experimental treatments (3\% FPW and 3\% SW) were in close agreement (Table 2). In both solutions, representing the dilution of the raw sample to 3\% of its initial concentration, the salinity was equivalent to 10\% seawater salinity (~3.4 ppt). The sodium concentration of the FPW and SW treatments was 65 and 70 times higher, respectively, than the sodium level found in the control. Furthermore, as expected, the concentration of trace elements in 3\% SW was lower than in 3\% FPW.

3.4. Plasma ions and trace elements

Relative to the control group, neither FPW nor SW had a significant effect on plasma chloride concentrations in rainbow trout at any measured timepoint over a 28-d exposure duration (151.1 ± 1.1 mM average for all exposure groups; Fig. 2). Two-way ANOVA analysis revealed no significant differences with respect to time (p = 0.066), treatment group (p = 0.683), or interaction between these two factors (p = 0.983).
Even though some fluctuations were observed, the level of plasma salt cations (i.e. Na, K, Ca, Mg; Fig. 3) remained unchanged over the course of a 28-d sub-chronic exposure to 3% FPW and 3% SW. There was no effect of treatment on plasma ion levels (two-way ANOVA; p = 0.453 – 0.826). Among the four ions analyzed, only plasma Ca concentration from FPW-exposed fish displayed an effect of time (6-h versus 48-h samples; two-way ANOVA; p = 0.025; Fig. 3C). No difference between time points were highlighted for Na, K or Mg (two-way ANOVA; p = 0.176 – 0.464; Fig. 3A,B,D). No significant interaction term was observed in the analysis of the plasma levels of any of these salt cations (two-way ANOVA; p = 0.160 – 0.495).

Although the trace elements were analyzed throughout the 28-d of exposure, only the average concentration over time is presented. For plasma salt ion levels, the concentrations of most trace elements in plasma did not differ as a function of treatment group at Day 28 of the exposure (i.e. Fe, Cu, Zn, Li, Al, Ni, Co, Ba, Pb, As, Mo; Fig. 4) (one-way ANOVA; p-value range: 0.116 – 0.676). However, the plasma concentration of strontium (Sr) was ~2-fold higher in plasma of fish exposed to 3% FPW in comparison to fish in control water or SW (one-way ANOVA, p < 0.001; Fig. 4B). Although there was a trend of increasing boron (B; Fig. 4B), cadmium (Cd; Fig. 4D) and mercury (Hg; Fig. 4D) concentration in the plasma of fish exposed to FPW compared to the two other groups, it was not found to be statistically significant (one-way ANOVA, p = 0.072; 0.071 and 0.062 respectively). Finally, a significant ~2-fold increase of bromide (Br) plasma level (1.1 ± 0.2 mM) occurred in FPW-exposed fish relative to fish in control (0.6 ± 0.1 mM) and SW (0.5 ± 0.1 mM) treatments (one-way ANOVA, p < 0.001; Fig. 4A).

3.5. Enzyme activity
The activity of branchial NKA (Fig. 5A) remained constant throughout the course of the exposure, with no observable effect in response to the treatment or time (two-way ANOVA; \( p = 0.424 \) and \( p = 0.235 \) respectively; Fig. 5A). However, while the exposure treatment did not influence the \( \text{H}^+ \)-ATPase activity (two-way ANOVA; \( p = 0.910 \); Fig. 5B), it was dependent on time (two-way ANOVA, \( p = 0.043 \)). Despite an overall significant effect of time, the specific pairwise significant differences were unable to be identified by the post-hoc test. No significant interaction was observed in the analysis of NKA or \( \text{H}^+ \)-ATPase activity (two-way ANOVA; \( p = 0.533 \) and 0.308 respectively).

3.6. Gill morphometrics

The effects of FPW and SW exposure on gill morphology are shown in Figure 6, which provides a snapshot at 48-h of exposure. The lamellar width remained unchanged over the course of the 28-d exposure (effect of time: two-way ANOVA, \( p = 0.127 \); effect of treatment: two-way ANOVA, \( p = 0.424 \); Fig. 7A). In contrast, an effect of time was seen with respect to lamellar length (Fig. 7B) and ILCM (Fig. 7C) measurements (two-way ANOVA, \( p < 0.001 \) and \( p = 0.009 \) respectively). The lamellae became significantly shorter after 28-d in 3% FPW (89 ± 8.1 μm) compared to the fish gills exposed for 6-h (130.9 ± 10.4 μm). The branchial length was also reduced in the control group from 127.8 (± 13.1) μm at 6-h to 94.9 (± 9.9) μm after 28-d. Additionally, a rapid change in ILCM was seen in the FPW-exposed *O. mykiss*. The ILCM of the trout exposed for only 48-h to 3% FPW increased to a peak value of 20.7 (± 0.8) μm, representing a 2-fold increase relative to the 6-h FPW exposure, before stabilizing at 18.2 (± 2.6) μm after 28-d. An ILCM increase was also observed in the control fish. However, this increase occurred gradually over the course of the 28-d to reach a maximum value of 17.7 (± 0.2) μm. Despite
changes with respect to time, there was no effect of treatment for either lamellar length (two-way ANOVA, \( p = 0.724 \)) or ILCM (two-way ANOVA, \( p = 0.112 \)). There was also no significant interaction effect between treatment and time point for branchial width and length (two-way ANOVA; \( p = 0.176 \) and 0.812 respectively), but a significant interaction term was observed in the analysis of the ILCM (two-way ANOVA; \( p = 0.010 \)). The metric of ILCM:lamellar length was identical to the pattern obtained for the ILCM measurements alone (Fig. 7D).

4. Discussion

Sub-chronic exposure to low levels of FPW had little impact on ionoregulatory parameters in the rainbow trout. Overall, the gill morphology was altered during the 28-d exposure, but these changes were not attributed to either the FPW or SW treatment. Furthermore, the only changes in the plasma chemistry of FPW-exposed fish (i.e. differences in accumulation of Sr and Br), was likely due to the elevated concentration of these elements in the FPW treatment. Consequently, there is little evidence that exposure to 3% FPW causes significant changes in the ionoregulatory physiology of rainbow trout over a 28-d exposure.

4.1. Plasma ions and trace elements

When freshwater fish are transferred to seawater, there is an immediate increase in plasma ion levels, although this increase is rapidly corrected by regulatory mechanisms. However, if the fish remain in saline waters this will result in higher baseline ion concentrations relative to fresh water (Nordlie, 2009). In the current study, plasma chloride, sodium, potassium, magnesium and calcium concentrations (Figs. 2 and 3) were consistent with normal plasma ion levels for freshwater rainbow trout (Nordlie, 2009). Furthermore, they remained constant throughout the
sub-chronic exposure, and did not differ with respect to treatment. This suggests that the salinity
associated with 3% FPW/SW (i.e. 10% seawater) was either insufficient to trigger a change, or
that effective physiological responses were initiated to maintain homeostasis. With respect to this
latter explanation, there is considerable evidence showing that rainbow trout can rapidly adjust
plasma ion concentrations in response to an acute salinity change (e.g., Blair et al., 2016;
Bystriansky et al., 2006).

The lack of effect of FPW/SW exposure on plasma ions is therefore likely explained by
the level of exposure relative to the isosmotic point of rainbow trout. At 3% FPW, the salinity of
the exposure water is equivalent to ~10% SW (~ 3.4 ppt), which is below the point at which water
ion concentration exceeds fish ion concentration, between 8 and 10 ppt for rainbow trout (Morgan
and Iwama, 1991). Supporting this hypothesis, Morgan and Iwama (1991) showed that plasma Na
and Cl levels remained unchanged in rainbow trout exposed to a salinity close to their isosmotic
point (i.e. 9 ppt), whereas freshwater fish exposed to hyperosmotic water (i.e. 18 ppt), exhibited
significant fluctuations in plasma ions. Hence, our results suggest that plasma salt ion regulation
in rainbow trout would not be impacted by the salinity found in 3% FPW. This outcome is also
supported by the results of ionoregulatory enzyme activity assays, discussed below in Section 4.2.
Importantly, the lack of difference between the SW and FPW group indicates that the organic and
metal components of the FPW did not exert effects on plasma ion homeostasis after 28-d, even
though this sub-chronic exposure concentration represents a value similar to the acute LC$_{10}$.

Patterns of trace element plasma accumulation over a 28-d exposure to 3% FPW showed
that for most of these elements there were no significant changes relative to the control group,
despite often elevated elemental concentrations in the exposure waters (Fig. 4). The exceptions to
this general pattern were Br and Sr, which were significantly elevated in the plasma of FPW-
exposed fish. Currently, little literature exists on the effect of Sr on fish. However, Sr is known to mimic Ca, and thus is thought to exert toxic effects through this mechanism (Chowdhury and Blust, 2011). The presence of Sr in the blood, as noted in the current study, is likely a reflection of Sr transport from the site of uptake (i.e. the gills) to calcified tissues such as bone, where it is accumulated. Sr reportedly has a relatively low toxic effect to rainbow trout, with a 28-d LC$_{50}$ of 0.2 ppm for rainbow trout eggs exposed from fertilization through 4-d post hatch (Birge, 1978; Chowdhury and Blust, 2011). The concentration of Sr associated with the 3% FPW-exposure in this study was ~ 100 times the reported LC$_{50}$ (20.9 ± 0.6 ppm; Table 1). The reasons for the lack of toxic effect observed in the current study are unknown but may relate to either the stage of exposure (eggs in previous studies versus juvenile rainbow trout in the current work), or the complexity of the exposure media mitigating the toxicity. Strontium did accumulate in the plasma of 3% FPW-exposed fish, likely gaining entry through Ca uptake mechanisms. Indeed, there was a slight decrease in plasma Ca concentration between the fish exposed to FPW for 6-h and 7-d (Fig. 3C), an effect that is consistent with documentation of Sr inhibition of Ca uptake in fish and a resulting decline in plasma Ca levels (Chowdhury and Blust, 2011). However, this effect was not significantly different from the SW group lacking Sr. It is therefore possible that Sr accumulation is limited by the elevated Ca concentrations in FPW, which will outcompete Sr (Chowdhury et al., 2000; Chowdhury and Blust, 2011), and thus limit any toxic impacts associated with enhanced concentrations of this element in FPW. Clearly, these noted differences in Sr toxicity, plus the elevated Sr concentrations measured in FPW, demonstrate the need to further investigate the mechanism by which mitigation of Sr toxicity occurs in rainbow trout chronically exposed to FPW.
The impact of Br on fish has mostly been investigated in the form of sodium bromide (NaBr). Stormer and colleagues (1996) exposed *O. mykiss* to 1 mM (79.9 ppm) NaBr, a concentration approximately 10 times higher than our 3% FPW exposure (8.0 ± 1.6 ppm). In their study, plasma Br reached concentrations of 51 mM after 14-d. However, in the present study, a 28-d exposure to 3% FPW resulted in plasma Br concentrations of only 1.1 (± 0.2) mM (Fig. 4A). These differences could reflect the presence of other components in the FPW, but more likely are simply the result of the lower concentrations in our study being significantly below the Michaelis constant of the transporter involved, resulting in negligible transport. It is unlikely that the Br accumulation observed in the plasma of FPW-exposed fish had significant toxicological consequence, as this element is considered to have a low toxicity towards freshwater aquatic organisms (Alexander et al., 1981; Canton et al., 1983; Wester et al., 1988). For example, the LC$_{50}$ value for fathead minnows exposed to NaBr is 16 g/L (Alexander et al., 1981). However, the effect of this element does vary depending on the species studied, and because of this variability, early research suggested a water quality criterion for Br of 1 ppm (Canton et al., 1983). Hence, even though Br toxicity to fish is low, an exposure to 3% FPW still represents an exposure concentration 8 times higher than the suggested current criterion for Br.

### 4.2. Ionoregulatory enzyme activity

NKA and H$^+$-ATPase are two key osmoregulatory enzymes, whose activities can reflect perturbations of ionic homeostasis. NKA and H$^+$-ATPase activities are usually increased in freshwater fish following an exposure to seawater. For instance, Bystriansky and colleagues (2006) showed that NKA activity increased significantly in salmonids, including rainbow trout, following 10-d of exposure to seawater. However, the activities of these enzymes did not change at any of
the timepoints (i.e. 6-h, 48-h, 7-d and 28-d) in either SW or FPW in the current study (Fig. 5), an outcome consistent with a lack of change in plasma major ions.

The lack of effect in 3% FPW suggests that the metal content of this treatment is insufficient to cause effects on NKA activity, even though dissolved metals are frequently associated with inhibition (e.g. Cd, Hg, Ag, Cu, Zn; reviewed by Evans, 1987; Wendelaar Bonga and Lock, 1991; Morgan et al., 1997). For example, the metals in our exposure waters known to exhibit the strongest inhibitory actions against NKA (e.g. Cu, Ag), were below the threshold at which such effects may be expected (Wendelaar Bonga and Lock, 1991). Overall, the data showing a lack of change in NKA and H⁺-ATPase support our hypothesis that a 3% FPW-exposure does not cause an osmotic stress to *O. mykiss*, be it the salt content or the metal/organic content, and that this freshwater species is capable of coping with the salinity associated with these exposure regimes.

Our outcomes are consistent with studies that have exposed euryhaline fish species to increases in salinity. For example, Urbina and Glover (2015) exposed inanga (a euryhaline galaxiid fish) to elevated salinities for 7-d and measured NKA activity. While these authors showed a trend of NKA activity decrease around the isosmotic point, they showed no significant change in NKA activity at 10% SW, the same salinity and outcome noted in the current work.

### 4.3. Gill morphometry

The gills of fish are highly responsive to waterborne toxicants. For example, an increase in the interlamellar cell mass (ILCM) is a common protective mechanism against pollutants in the water, as it acts to decrease the effective branchial surface area in contact with the environment, thus decreasing the potential uptake of toxicants, and subsequent toxicological impact (Mallatt,
Histological analysis revealed no change of the branchial tissues in the current study in FPW exposures relative to SW and control fish. Throughout the course of the 28-d exposure, however, there were changes with respect to exposure time, with a decrease of the lamellar length and an increase of the ILCM being observed, leading to an overall increase of the ratio of ILCM to lamellar length (Fig. 7B-D). This relative lack of change with respect to treatment is opposed to observations made by Blewett et al. (2017b). These authors showed that fish displayed wider lamellae and a reduced ILCM after a 48-h exposure to 7.5% FPW or SW, whereas the lamellar length remained unchanged. These gill morphological changes were attributed principally to the salt components of this FPW and were proposed to be an indicator of hyperosmotic stress. The different outcomes between these two studies are likely due to the different concentrations, and thus salinities, of the raw and exposure samples tested (7.5%/13.5 ppt versus 3%/3.4 ppt herein).

In a field-based study, changes in gill morphology in response to FPW have been observed previously. Papoulias and Valesco (2013) sampled two freshwater fish species (creek chub and green sunfish) chronically exposed to FPW for a month, after a spill occurred in the environment. In those fish, they observed branchial lesions and hyperplasia (i.e. cells filling the interlamellar spaces) attributed to the presence of metals (i.e. Al and Fe) in the FPW. Unfortunately the specific water chemistry of this spill is unknown, and so the FPW concentrations cannot be easily compared to the current study. It seems, however, most likely that the lack of significant changes in gill morphology in rainbow trout in the present work relates to a relatively lower FPW exposure concentration than that observed in this field-based study. However, it is also possible that factors, such as species ionoregulatory capacity will play a role, especially should exposures occur to high FPW dilutions. For example, Papoulias and Valesco (2013) studied two exclusively freshwater
fish, which are less likely to have refined mechanisms for limiting morphological changes associated with FPW salinity. Supporting this, Blair et al. (2016) investigated the salinity tolerance of the Arctic grayling, *Thymallus arcticus*, and of the rainbow trout, two freshwater salmonid species. Each were acutely exposed to 50% seawater (17 ppt) for 96-h, in an attempt to replicate exposure of these species to the salinity content of an FPW spill. These authors measured ILCM:lamellae length and showed that *O. mykiss*, the more euryhaline species, was able to physiologically compensate for this high salinity, whereas *T. arcticus*, a more stenohaline species, was not. The study of Blair and colleagues therefore, showed that responses of fish to salinity associated with FPW exposure can be dependent on the species being examined, even though the current study suggests that FPW concentration is likely to be the most significant factor.

5. Conclusion

The exposure scenario in this study was chosen to mimic an environmental spill of low-level of FPW over a long period of time (i.e. 3% FPW over 28-d), a constancy of exposure similar to that which may be experienced in a pipeline leak into a small slow-flowing river, pond or lake. This study is the first to investigate the specific impact of FPW salinity following sub-chronic exposure to environmentally relevant, albeit low levels of the effluent. This exposure had little significant measurable impact on juvenile rainbow trout. Gills were the principal tissue affected by a sub-chronic exposure, as seen by the signs of hyperplasia when fish were exposed to FPW over the course of 28-d. However, these changes were not significantly different relative to the control groups. Changes over time in the FPW-exposed fish gills were likely driven by the metals contained in the effluent, but more investigations would be needed to determine the mechanism of effect.
Our results, which showed no significant difference between a 3% FPW treatment and a 3% salt-matched control, suggest that the salt concentration associated with 3% FPW was not sufficiently high to induce a salinity stress. However, even though most salmonids have been showed to be capable of acclimation to a wide range of salinity, the impact of such exposures rely heavily on the fish species exposed (Bystriansky et al., 2006; Blair et al. 2016). Thus, it is difficult to determine whether the outcomes of the current work apply to other fish species that may be exposed to an FPW spill. It is also worth noting that this study focused on ionoregulatory endpoints, and thus excluded other potential mechanisms (e.g. oxidative stress, endocrine disruption), which could be altered by exposure to FPW spills.

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Table 1. Water chemistry of the raw 3-h FPW sample measured by ICP-MS/MS; where TDS = total dissolved solids; TN = total nitrogen; TC = total carbon.

<table>
<thead>
<tr>
<th>Element</th>
<th>Concentration (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TDS</td>
<td>150000</td>
</tr>
<tr>
<td>Na</td>
<td>47500</td>
</tr>
<tr>
<td>K</td>
<td>1600</td>
</tr>
<tr>
<td>Ca</td>
<td>6940</td>
</tr>
<tr>
<td>Mg</td>
<td>657</td>
</tr>
<tr>
<td>Cl</td>
<td>68100</td>
</tr>
<tr>
<td>Sr</td>
<td>723</td>
</tr>
<tr>
<td>S</td>
<td>100</td>
</tr>
<tr>
<td>Br</td>
<td>259</td>
</tr>
<tr>
<td>Ba</td>
<td>22.6</td>
</tr>
<tr>
<td>B</td>
<td>74.8</td>
</tr>
<tr>
<td>Li</td>
<td>35.3</td>
</tr>
<tr>
<td>Mn</td>
<td>5.71</td>
</tr>
<tr>
<td>Fe</td>
<td>167</td>
</tr>
<tr>
<td>TC</td>
<td>302</td>
</tr>
<tr>
<td>TN</td>
<td>272</td>
</tr>
</tbody>
</table>
Table 2. Water chemistry of exposure waters (control and 3% FPW and SW) measured by ICP-MS/MS. Reported values represent means ± SEM. BDL = below detection limit. N = 6 (water sampled over the course of 28-d). Elements below detection limit in all treatments are not shown in this table.

<table>
<thead>
<tr>
<th>Ion concentration (ppm)</th>
<th>Detection limit range</th>
<th>Control</th>
<th>3% SW</th>
<th>3% FPW</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na</td>
<td>0.65 - 21.7</td>
<td>20.4 ± 1.7</td>
<td>1411.4 ± 166.8</td>
<td>1330.6 ± 100.1</td>
</tr>
<tr>
<td>K</td>
<td>1.00 - 2.10</td>
<td>3.3 ± 0.2</td>
<td>51.5 ± 4.4</td>
<td>50.6 ± 2.8</td>
</tr>
<tr>
<td>Mg</td>
<td>0.044 - 2.1</td>
<td>14.0 ± 0.2</td>
<td>35.3 ± 2.9</td>
<td>33.1 ± 0.9</td>
</tr>
<tr>
<td>Ca</td>
<td>0.88 - 2.02</td>
<td>38.4 ± 1.5</td>
<td>247.4 ± 24.5</td>
<td>259.7 ± 16.9</td>
</tr>
<tr>
<td>B</td>
<td>0.00049 - 0.12</td>
<td>1.0 ± 0.8</td>
<td>0.1 ± 0.05</td>
<td>2.1 ± 0.1</td>
</tr>
<tr>
<td>Ba</td>
<td>0.00052 - 0.0315</td>
<td>0.05 ± 0.01</td>
<td>0.1 ± 0.01</td>
<td>0.4 ± 0.03</td>
</tr>
<tr>
<td>Sr</td>
<td>0.050 - 0.30</td>
<td>0.4 ± 0.2</td>
<td>0.6 ± 0.2</td>
<td>20.9 ± 0.6</td>
</tr>
<tr>
<td>S</td>
<td>0.052 - 1.06</td>
<td>17.2 ± 1.0</td>
<td>18.9 ± 2.2</td>
<td>19.9 ± 1.3</td>
</tr>
<tr>
<td>Mn</td>
<td>0.00046 - 0.012</td>
<td>0.001 ± 0.0002</td>
<td>0.01 ± 0.002</td>
<td>0.2 ± 0.01</td>
</tr>
<tr>
<td>Fe</td>
<td>0.0097 - 0.106</td>
<td>BDL</td>
<td>BDL</td>
<td>0.6 ± 0.2</td>
</tr>
<tr>
<td>Br</td>
<td>0.050 - 0.35</td>
<td>1.0 ± 0.5</td>
<td>2.1 ± 0.7</td>
<td>8.0 ± 1.6</td>
</tr>
</tbody>
</table>
Figure 1. Percentage of 96-h survival of juvenile *Oncorhynchus mykiss* exposed to increasing concentration of raw sample of FPW. Error bars represent the mean (± SEM) of 3 to 4 replicates.
Figure 2. Chloride concentration in plasma sampled from juvenile rainbow trout exposed to control water, 3% SW and 3% FPW within the course of a 28-d sub-chronic exposure. Bars represent the mean (± SEM) of 3 to 4 replicates.
Figure 3. Salt cations in plasma sampled from juvenile rainbow trout exposed to control water, 3% SW and 3% FPW over the course of a 28-d sub-chronic exposure: A) sodium, B) potassium, C) calcium, and D) magnesium. Bars represent the mean (± SEM) of 3 to 4 replicates. Different lower-case letters represent difference across timepoints within the same treatment, whereas upper-case letters denote differences within the same timepoints (two-way ANOVA, post hoc Tukey’s test), at alpha = 0.05. No letter indicates no significant differences with respect to either time or treatment.
Figure 4. Trace elements concentrations in plasma sampled from juvenile rainbow trout exposed to control water, 3% SW and 3% FPW after a 28-d sub-chronic exposure: A) Fe, Cu, Zn, Br; B) Li, B, Al, Ni, Sr; C) Co, Ba, Pb; D) As, Mo, Cd, Hg. Bars represent the mean (± SEM) of 4 replicates. Asterisks represent significant differences between treatments (one-way ANOVA, post hoc Tukey’s test; or Kruskal-Wallis test), at alpha = 0.05.
Figure 5. Branchial activity of Sodium-Potassium ATPase (NKA) (A) and H⁺-ATPase (B) of juvenile rainbow trout exposed to control water, 3% SW and 3% FPW within the course of a 28-d sub-chronic exposure. Bars represent the mean (± SEM) of 5 to 6 replicates.
Figure 6. Micrographs of gill tissues stained with haematoxylin and eosin sampled from juvenile rainbow trout exposed to control water (A), 3% SW (B) and 3% FPW (C) for 48-h.
Figure 7. Effect of a 28-d sub-chronic exposure to control water, 3% SW and 3% FPW on the gill morphology of juvenile rainbow trout. A) Lamellar width, B) lamellar length, C) interlamellar cell mass (ILCM), D) and ratio of ILCM to lamellar length were measured at 6-h, 48-h, 7-d and 28-d of exposure. Bars represent the mean (± SEM) of 3 replicates. Different lower-case letters represent difference across timepoints within the same treatment, whereas upper-case letters denote differences within the same timepoints (two-way ANOVA, post hoc Tukey’s test), at alpha = 0.05. No letter indicates no significant differences with respect to either time or treatment.