The impact of hydraulic fracturing fluid on two key aquatic species: the water flea, *Daphnia magna,* and the rainbow trout, *Oncorhynchus mykiss.*

by

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Abstract

Wastewater produced during hydraulic fracturing activities (termed flowback and produced water; FPW), is a complex solution containing metals, organics and high concentrations of salts. The number of reported FPW spills affecting aquatic and/or terrestrial environments has increased with the rise of hydraulic fracturing activities (especially in North America), leading to concerns regarding the effects of such spills on aquatic biota.

This thesis investigates the effect of FPW on two key freshwater species, *Daphnia magna* and *Oncorhynchus mykiss*. Using a salinity-matched control (SW) which replicated the major anionic and cation salt concentrations found in the raw FPW sample, specific salinity-induced effects were differentiated from other FPW component effects (i.e. metals and organics).

First, the impact of FPW on the phototactic behaviour of the freshwater water flea *D. magna* was investigated. Overall, effects on daphnid phototactic responses were dependent on the FPW dose and the pre-exposure history of the animal. Naïve *Daphnia* acutely exposed to increasing FPW concentrations displayed an erratic behaviour and faster swimming speeds in response to light stimuli, defined as an accentuated positive phototactic behaviour (i.e. aversion response). A similar response was observed in SW exposed organisms, suggesting that the dose-dependence response was likely driven by the salinity of the FPW. Interestingly, pre-exposure of *Daphnia* to low concentrations of FPW and SW reduced positive phototactic behaviours compared to naïve, acutely exposed *Daphnia*, indicating acclimation to these treatments may occur. Despite the clear effect of salinity on organism behaviour, differential phototactic responses between FPW and SW exposed *Daphnia* were observed. FPW and SW pre-exposures resulted in a diminution of the positive phototaxis of the organisms (i.e. decrease of their swimming speed) and a loss of aversion response. However, only *Daphnia* pre-exposed to SW showed signs of possible

acclimation in their behavioural responses. These results indicate that salt and non-salt components of FPW differentially affect the phototactic behaviour of *D. magna* through distinct mechanisms of action.

Second, I investigated the effects of a 28-d exposure to low FPW concentrations on the ionoregulatory physiology of *O. mykiss*. My results suggest that low, sub-chronic exposures to 3% FPW and SW (~3.4 ppt; below the isosmotic point of *O. mykiss*) do not result in ionoregulatory disturbance in fish. Following these sub-chronic exposures, concentrations of plasma ions (Na^+, K^+, Cl^-, Ca^{2+}, Mg^{2+}) were unchanged regardless of treatment condition, and branchial activities of Na^+-K^+ ATPase and H^+-type ATPase (two key osmoregulatory enzymes) similarly did not change between treatment groups. However, sub-chronic FPW exposure did cause accumulation of certain trace elements (i.e. Br and Sr) in fish plasma and modified the fish gill morphology over time. Branchial remodeling following FPW exposure was observed as a function of time, but these effects were not distinct from changes that also occurred in control groups.

This work contributes to the understanding of FPW toxicity on freshwater aquatic species that inhabit environments where hydraulic fracturing practices occur and where spills of FPW are most likely. By improving our knowledge on how certain behavioural and physiological traits of organisms may be affected following exposure to FPW, this study provides important insights for FPW risk assessment development, biomonitoring of FPW spills, and minimization of post-spill environmental effects.

Preface

This thesis is an original work by Perrine L. M. Delompré and was conducted under the supervision of Dr. Chris N. Glover, of Athabasca University, and Dr. Greg G. Goss, of the University of Alberta; with the assistance of Dr. Tamzin A. Blewett (University of Alberta). PLMD was principal researcher for all experimental work, and in association with TAB, CNG, GGG, designed the described experiments. No part of this thesis has been previously published. The contributions of all co-authors for each chapter are described below:

Chapter 2: Shedding light on hydraulic fracturing fluid toxicity: the effect of flowback and produced water on phototactic behaviour in *Daphnia magna*.

PLMD was responsible for the data collection, data analyses, and manuscript composition. CNG assisted with the data collection and contributed to the manuscript edits. All co-authors contributed to editing the final manuscript.

Chapter 3: The osmotic effect of hyper-saline hydraulic fracturing fluid on the rainbow trout, *Oncorhynchus mykiss*.

PLMD was responsible for the data collection, data analyses, and manuscript composition. CNG and TAB assisted with the data collection. TAB, CNG and GGG contributed to editing of the final manuscript. ICP-MS analyses were performed by Shannon Flynn and Katherine N. Snihur, , in the laboratory of Daniel S. Alessi.

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List of Abbreviations

µE	micro Einstein
µg	microgram
µS	micro Siemens
ADP	adenosine diphosphate
AER	Alberta Energy Regulator
Ag	silver
Al	aluminum
As	arsenic
ATP	adenosine triphosphate
B	boron
Ba	barium
Be	beryllium
Br	bromide
Ca/Ca^{2+}	calcium
$CaCl_2$	calcium chloride
$CaCl_2.2H_2O$	calcium chloride dihydrate

$CaCO_3$	calcium carbonate
Cd	cadmium
Cl/Cl^-	chloride
cm	centimeter
Co	cobalt
Cr	chromium
Cs	caesium
Cu	copper
EGTA	ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid
Fe/Fe^{2+}	iron
FPW	flowback and produced water
H^+-ATPase	proton pump
H_4EDTA	ethylenediaminetetraacetic acid
HF	hydraulic fracturing
Hg/Hg^{2+}	mercury
$HgCl_2$	mercuric chloride
HNO_3	nitric acid

ICP-MS/MS	inductively coupled plasma-double mass spectrometry
ILCM	interlamellar cell mass
K/K^+	potassium
KCl	potassium chloride
kPa	kilopascal
LC_{10}	concentration causing mortality in 10% of the exposed population
LC_{50}	median lethal concentration
LDH	lactate dehydrogenase
Li	lithium
mg	milligram
Mg/Mg^{2+}	magnesium
$MgCl_2$	magnesium chloride
$MgCl_2.6H_2O$	magnesium chloride hexahydrate
$MgSO_4.7H_2O$	magnesium sulfate heptahydrate
mL	milliliter
mmol/mM	millimolar
Mn	manganese

Mo	molybdenum
Na/Na^+	sodium
NaBr	sodium bromide
NaCl	sodium chloride
NADH	nicotinamide adenine dinucleotide
$NaHCO_3$	sodium bicarbonate
NaN_3	sodium azide
NEM	n-ethylmaleimide
NH_4OH	ammonium hydroxide
Ni	nickel
NKA	sodium-potassium ATPase
nm	nanometer
NOM	natural organic matter
OECD	Organization for Economic Cooperation and Development
Pb	lead
PEP	phosphoenolpyruvate
PFA	paraformaldehyde

PK	pyruvate kinase
ppm	part per million
ppt	part per thousand
rpm	revolutions per minute
SEID	sucrose, EDTA (ethylenediaminetetraacetic acid), imidazole, deoxycholic acid
SW	sea-water matched control
TC	total carbon
TDS	total dissolved solids
TN	total nitrogen
TOC	total organic carbon
TPTZ	2,4,6-Tris(2-pyridyl)-s-triazine
U	uranium
US EPA	United States Environmental Protection Agency
UV	ultraviolet
w/v	weight/volume
Zn	zinc

Chapter 1 - Introduction

Canadian freshwaters are threatened by multiple factors, including anthropogenic processes. A novel recent human stress is the growing practice of hydraulic fracturing, which presents high risks of contamination of freshwaters (Vengosh et al., 2014; see below). Because surface freshwaters (e.g. lakes, rivers) and groundwaters are a critical natural resource, representing 7.6% of the surface area of Canada (Schindler, 2001), it is important to protect these ecosystems. However, the specific impacts of hydraulic fracturing on freshwater ecosystems, and the biota that reside within, is still poorly understood.

Hydraulic fracturing

Hydraulic fracturing (HF) is a process by which hydrocarbons (i.e., gas or oil) are extracted from deep sedimentary deposits. This occurs through the injection of a highly pressurized liquid (69,000 kPa; Stringfellow et al., 2014), which fractures rock, thereby increasing porosity, and hydrocarbon flow. This process started in the 1940s and has been rapidly and extensively developed over the past 70 years (Vidic et al., 2009). In recent years, the use of HF has intensified through its adoption as a mechanism to extract shale gas from low production reservoirs (Vidic et al., 2009). This increase has been largely driven by increased demand for gas, which has made these less profitable sources more viable, and thus HF has presented as an alternative to the conventional drilling of gas and oil (review by Owen et al., 2010). The fracturing technique allows access to unconventional resources trapped in rocks of low permeability, often with higher oil and gas yield than typical drilling techniques, but at a lower cost (Vidic et al., 2009). Because of its economic benefits, HF is becoming more widespread in North America. It has been estimated that

by 2022, ~50% of oil and gas production in the US will be extracted via this process (Stringfellow et al., 2014), while in Canada hydrocarbon extraction via HF is expected to increase 25% by 2035 (Gagnon et al., 2015).

However, as HF grows as an industrial process, issues arise regarding its potential environmental impact (Vidic et al., 2009; Entrekin et al., 2011; Rivard et al., 2014). The fluids injected as part of HF are highly complex chemical mixtures, making risk assessment difficult. Indeed, until recently HF companies were not legally required to provide a detailed record of the chemical constituents that comprised their HF fluids, and often these were kept as trade secrets (Rivard et al., 2014). However, since 2012 many jurisdictions have passed legislation requiring disclosure of HF fluid additives, although the exact chemical formulations of the constituents do not need to be disclosed and exemptions exist for proprietary trade secrets. Nevertheless, the baseline composition is likely common to all the fluids, being a combination of water, sand, and lubricant (Vidic et al., 2009). In addition, measures must be taken to prevent rock fractures from closing once pressure is released, and the injected fluid flows back to the well head. Therefore sand, ceramic beads, and inert solids are added as "proppants" in the injected fluid to provide structural support once the pressure is removed (Vidic et al., 2009; Stringfellow et al., 2014). In addition to proppants, the mixture contains additives to control the liquid viscosity, surface tension, and to prevent bacterial development in the well which may lead to issues with extraction.

Flowback and produced water (FPW)

After pressurization and fracture, the well is opened, the pressure is released, and fluid returns to the surface. This fluid is termed Flowback and Produced Water (FPW) as the differentiation between flowback (i.e. the injected water return) and produced (i.e. fluid containing

oil and gas) waters is based more on well operational definitions rather than chemically-based characterization. Generally, the fluid returning immediately to the surface within the first few days is considered flowback whereas the fluid containing the newly extracted hydrocarbons remains longer in the formation and is termed produced water (Stringfellow et al., 2014). Consequently, FPW contains the original chemicals injected into the well, the hydrocarbons extracted during the process, and additional components derived from the geologic formation, and is, therefore, a very chemically complex mixture. Additional components in flowback water include transformation products from the originally-injected chemicals, trace metals, naturally occurring radioactive materials, and petrogenic organics (Entrekin et al., 2011; Stringfellow et al., 2014). Furthermore, salts accumulate in the FPW during the extraction resulting in a hyper-saline solution that can reach salinities up to 8-10 times that of seawater. The salinity level of the FPW is determined by the salt content of the formation itself and the time of flowback, such that early flowback has lower salinity and the salinity increases with flowback time (Alessi et al., 2017). Each well, therefore, produces a unique FPW whose components are influenced by the site-specific rock formations and fracturing fluids used (Stringfellow et al., 2014; Alessi et al., 2017).

The FPW produced during the extraction process is often the biggest environmental concern. Indeed, the volumes of FPW produced during this process vary, but they are always considerable. Single wells are reported to use between 25,000 m^3 and up to 1 million m^3 of water (Goss et al., 2015). It is estimated that FPW surface return represents 10 to 80% of the volume injected into the well initially (Vengosh et al., 2014; Alessi et al., 2017), and thus this effluent represents a large volume of a potentially toxic mixture where the natural gas or oil must be separated before the wastewater is either reused or transported to storage sites via pipelines or trucks.

Considering the large volumes of FPW produced, and that well sites are often located at a distance from storage/disposal sites, there is a significant risk of FPW spills during transportation. For example, 205 spills of FPW occurred in Alberta between January 2017 and July 2018 according to the Alberta Energy Regulator website (AER, 2018). Most of these spills have been documented following a failure of transportation or storage of the effluent (US EPA, 2015; Goss et al., 2015; Gagnon et al., 2015). As more HF is scheduled to occur, more FPW will be generated, and this increases the probability of more spills. However, prior to the last few years, little was known about the possible toxicity of FPW to aquatic organisms.

Toxicity of FPW to aquatic biota

Owing to its complex chemistry, including metals, organics, and salts, a spill of HF fluid has the potential to significantly impact the organisms living in receiving environments. Most HF occurs inland, so aquatic settings of highest risk are freshwater streams and lakes. Some recent research has started to elucidate the effects of FPW on freshwater organisms.

One of the first studies to specifically examine the toxicity of FPW to aquatic animals was conducted by Blewett and colleagues (2017a). These authors chronically exposed the freshwater crustacean *Daphnia magna* to dilutions of the raw FPW collected from the well-head over 21 d. It was found that FPW impaired the survival (LC_{50} of 0.19% and 0.75% for neonates and adults respectively, where this percentage value represents the dilution of the raw sample collected at the well-head) and the reproduction (0.004% FPW) of the organisms. Overall, daphnids exposed to the wastewater presented a delayed time to first brood and produced fewer neonates. Furthermore, a chronic exposure to concentrations as low as 0.004% FPW induced a perturbation of metabolism, with FPW-exposed daphnids displaying a significantly lower oxygen consumption. Changes in

gene expression were also identified, with transcripts implicated in detoxification mechanisms and the moulting process significantly altered following chronic exposure (0.004% FPW) (Blewett et al., 2017a). Using an activated charcoal control solution (removal of the organics from the FPW sample) and a salinity-matched control solution (containing only the matching salt concentrations of the raw FPW sample), the authors were able to attribute these toxic impacts principally to the organics contained in FPW, but a distinct effect of higher salt concentrations was also observed.

The effect of FPW on fish has also been investigated recently. Data from studies conducted on model freshwater fish species (i.e. rainbow trout *Oncorhynchus mykiss*; zebrafish *Danio rerio*) have established that FPW affects fish survival and swimming performance (Folkerts et al., 2017b), induces developmental toxicity (He et al., 2018; Folkerts et al., 2017a; b), and impairs their metabolism (He et al., 2017b; Blewett et al., 2017b). Furthermore, in *O. mykiss*, evidence of oxidative stress and endocrine disruption was elucidated after a 48-h acute exposure to 7.5% FPW, an effect attributed mostly to the organic components of the fluid (He et al., 2017b; Blewett et al., 2017b). On the other hand, FPW salinity affected the gill morphology of the rainbow trout. Indeed, branchial remodeling was observed in fish acutely exposed to FPW (Blewett et al., 2017b).

Thus, due to its complex chemical composition, FPW has the potential to affect different aquatic species at many biological levels (i.e. molecular, biochemical, physiological, behavioural and morphological). This thesis will focus on the impact of FPW exposure on two freshwater species, the water flea *Daphnia magna* and the rainbow trout *Oncorhynchus mykiss*, both of which are model organisms used in toxicological studies and regulatory compliance.

The water flea, *Daphnia magna*

Daphnia magna is a freshwater cladoceran extensively used as a model species in toxicity testing and water quality assessment, in part because of their generally high sensitivity to aquatic contaminants (US EPA 2002; van Leeuwen and Vermeire, 2007). They also present a broad worldwide distribution, being found in the freshwaters of all continents, which makes outcomes of studies using this species widely applicable to freshwater settings globally (US EPA, 2002; Adamowicz et al., 2009). This crustacean species is also easily cultured in laboratories and is characterized by the short time they take to reach reproductive maturity (production of offspring within ~10-12 d), making them especially amenable to chronic toxicity studies (Baudo, 1987**)**. In addition, *Daphnia* are a key component of the aquatic ecosystem, playing an important role in freshwater food webs, where they are the preferred prey of secondary consumers (e.g. predatory invertebrates and planktivorous fish) (Tabor et al., 1996; Miner et al., 2012).

Daphnia behaviour as a toxicologically-relevant endpoint

Another key attribute of *Daphnia* is their amenability to behavioural toxicity assessment. Behavioural endpoints are principally used in toxicology for three reasons (Warner et al., 1966). First, they are considered integrated endpoints, representing the sum of physiological and biochemical perturbations which manifest at the whole animal level. They are also non-destructive, which is of benefit from the perspective of animal ethics in vertebrate species. Finally, behavioural endpoints have the potential to be used as early warning signals in toxicity studies due to their higher sensitivity (10-1000 times) relative to lethality tests (Hellou, 2011).

A key behavioural trait in *Daphnia* is their response to light (i.e. phototaxis). *Daphnia* undergo diurnal vertical migration, a behaviour that results in a downwards movement in the water

column during the day and a movement towards the water surface during the night (Ringelberg, 1964). This behaviour is thought to be driven, in part, by the search for food and the avoidance of predators (Clarke, 1932; De Meester, 1993). However, the vertical migration of aquatic organisms is a complex behaviour governed by many other environmental factors, including carbon dioxide concentration, water temperature, the polarization of the light, and light quality (visible or ultraviolet (UV) light) (reviewed by Ringelberg, 1964).

Daphnia can undergo two different phototactic behaviour. Positive phototaxis (i.e. movement towards the light source) generally occurs in response to visible light, while negative phototaxis (i.e. movement away from the light source) is the usual response to UV light (Ringelberg, 1964; Gerritsen, 1982; Storz and Paul, 1998; Glaholt et al., 2016). Importantly, *Daphnia* phototaxis has been shown to be perturbed in presence of specific toxicants in water. For example, several studies investigating the impact of different common environmental chemicals (e.g. metals, pesticides, organics) have shown a disruption of positive phototactic behaviour in daphnids (Di Delupis and Rotondo, 1988; Michels et al., 1999; Martins et al., 2007). Thus, the phototactic behaviour of *D. magna* has been proposed as an endpoint to examine water quality in sub-lethal exposure studies.

This first component of my thesis will focus on the impact of FPW on the phototactic behaviour of *D. magna*. Given that phototaxis is implicated in the organisms' feeding behaviour and avoidance of predation, an impairment of *Daphnia* phototactic behaviour might negatively affect the survival of the individuals, as well as affecting population status. This study will be the first to examine the behaviour of aquatic organisms in response to FPW exposure.

The rainbow trout, *Oncorhynchus mykiss*

Rainbow trout, *Oncorhynchus mykiss*, is a salmonid fish species that originates from Pacific Coast freshwaters of North America, but which now has a global distribution in cold freshwaters (MacCrimmon, 1971). In 1919, rainbow trout were first introduced to Alberta, although the Athabasca rainbow trout is a subgroup which is considered native to the province (MacCrimmon, 1971). Today, in addition to their natural occurrence in freshwaters worldwide, rainbow trout are extensively cultured, both for direct harvest for food, and as a sportfish. Consequently, this species is of significant economic and environmental importance.

Oncorhynchus mykiss is a euryhaline fish able to tolerate a wide salinity range. This capacity exists even in those populations which are resident year-round in freshwaters and which do not undergo a migration between freshwater and seawater, as well as in migratory species (i.e. steelhead trout). Because of their salinity tolerance, and ease of maintenance in laboratory culture, rainbow trout have become an important model for the study of ionoregulation in fish. Furthermore, *O. mykiss* are known to be sensitive to water contaminants (Teather and Parrott, 2006). These factors, coupled with their worldwide distribution, has led to the development of the rainbow trout as an important model species for toxicity studies. For example, rainbow trout has been used for decades for toxicity testing, and is a key component used in the establishment of regulations and guidelines for the protection of aquatic ecosystems in Canada (Environment Canada, 1990). Effects of aquatic contaminants on trout are often of direct relevance to freshwaters as effects on this species could result in ecological and economic impacts. As a consequence of their status as a model toxicological species, extensive knowledge of rainbow trout sensitivity to aquatic contaminants has been accumulated over the last several decades (Environment Canada, 1990).

In the current work, rainbow trout was chosen as a model owing to its presence in the cold freshwaters largely associated with HF in Alberta and British Columbia. Furthermore, it has a well-studied biochemistry and physiology associated with ionoregulation, a potential target of toxicity owing to the high salt content of FPW. This thesis will investigate the impact of FPW salinity on *O. mykiss* following a sub-chronic exposure to the wastewater, the first study of sub-chronic effects of FPW in fish. Specifically, gill morphology, branchial osmoregulatory enzymes, and plasma ion levels will be analyzed to determine the effect of FPW salts on the rainbow trout.

Importance of the gills

In rainbow trout, as in all teleost fish, the gills perform a number of critical biological roles (Evans et al., 2005). They are a multifunctional tissue facilitating gas exchange as well as ion and water fluxes through the lamellar epithelium, but are also responsible for nitrogenous waste excretion. Briefly, the gill consists of four branchial arches, themselves composed of many filaments (Fig. 1-1). Each of these branchial filaments is subdivided into primary lamellae, on top of which sit secondary lamellae, the site of exchange between blood and water (Hughes and Morgan, 1973; Evans, 1987). The lamellar epithelium is composed of pavement cells and cells rich in mitochondria, previously called chloride cells but now termed ionocytes, which are the principal site of ion exchange (Karnaky, 1986; Evans et al., 2005; Dymowska et al., 2012). The ionocytes are of great importance in ionoregulation and it has been shown that the proliferation of these cells enables a rainbow trout to cope with osmotically challenging scenarios (Evans, 1987; Richards et al., 2003). Such situations include migration from freshwater to seawater, or exposure to a toxicant that disrupts ionic homeostasis.

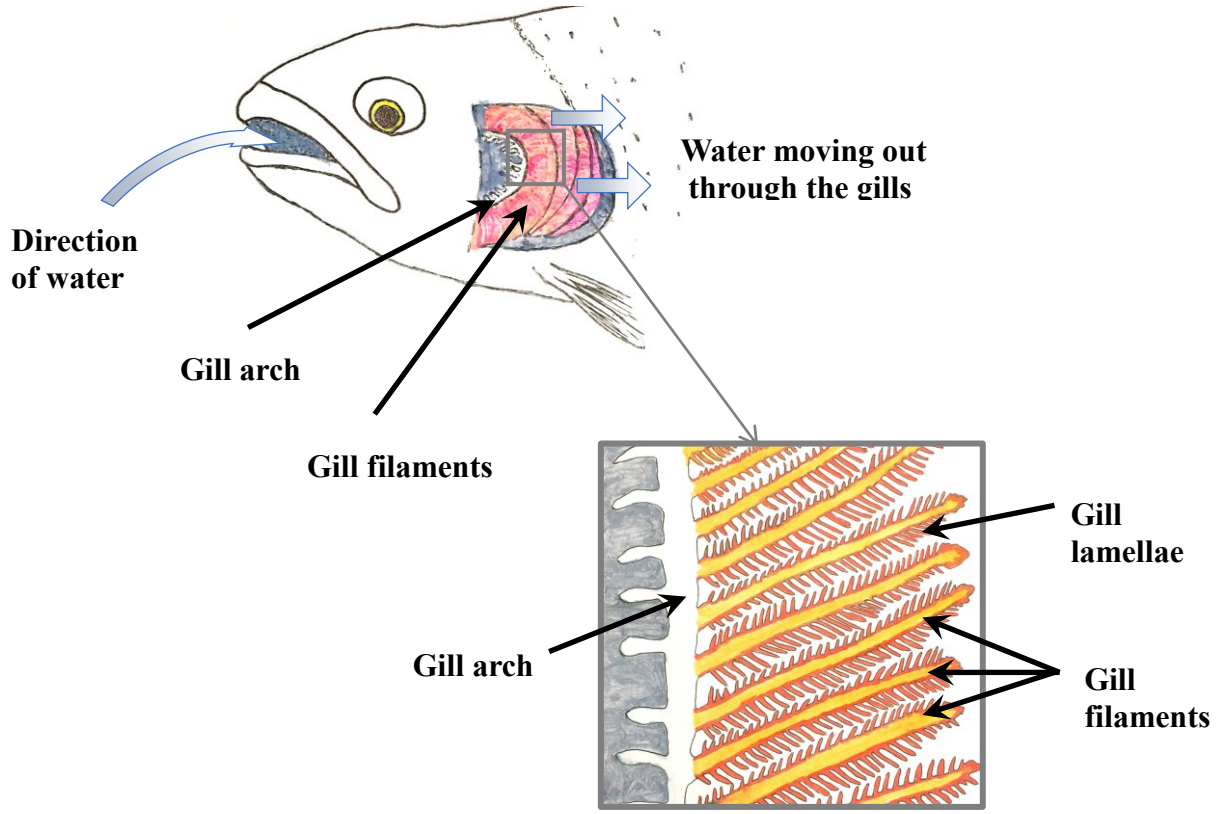

Figure 1-1. Diagram of the gill structure of teleost fish (thanks to Dr. Edyta Jasinska for providing this figure).

As gills are in direct contact with the environment, they are a primary target for an environmental chemical. For example, the presence of irritants often causes branchial lesions in fish such as edema, lifting of the epithelium, lamellar fusion, hypertrophy or hyperplasia. Because of such structural damage of the gills, the branchial physiological functions (e.g. exchange of water and ions; respiratory functions) can be negatively impacted, thus disrupting fish osmoregulation (Wendelaar Bonga and Lock, 1991). However, gills have been shown to display a high plasticity in response to environmental stressors, and so morphological changes may be induced which act to minimize the harmful effects of stressor exposure (Mallatt, 1985; Evans, 1987; Gilmour and Perry, 2018).

A structural change often observed following the exposure of the gill to stressors is the modification of the interlamellar epithelium, which can proliferate or diminish, leading to changes in interlamellar cell mass (ILCM). An increase in ILCM, occurring through hyperplasia, has been largely associated with the presence of metals (reviewed by Evans, 1987). This hyperplasia acts to decrease the branchial surface area exposed to the environment. This reduces the available surface area for uptake of aquatic contaminants and may also increase the diffusion distance for some toxicants. Consequently, this morphological change has been defined as a protective mechanism (Mallatt, 1985). Conversely, a decrease of the ILCM has also been documented in the literature. For example, hypoxia (i.e. low environmental oxygen) is known to induce this morphological change. Sollid and colleagues (2003) showed that hypoxic Crucian carp (*Carassius carassius*) undergo ILCM reduction in order to facilitate oxygen uptake by increasing the surface area of the gill in contact with the environment. A decrease of ILCM has been attributed to an inhibition of cell proliferation, in addition to the apoptosis of cells in the inter-lamellar tissue.

Because of its large surface area in contact with a potentially contaminated environment, the importance of its many functional roles, and due to its high plasticity in response to stressor exposure, the gill is an important toxicological tissue. In the current thesis, the changes in gill structure (histology) and function (see below) were investigated in response to FPW exposure in order to ascertain whether FPW impacted branchial function, specifically as it relates to ionoregulation.

Ionoregulatory enzymes: NKA and H^+-ATPase

As mentioned previously, rainbow trout are euryhaline and can acclimate to a wide salinity range. In freshwater, fish must actively absorb environmental Na^+ across the branchial epithelium, to counterbalance the salts lost via diffusion from the more concentrated animal to the more dilute environment. These fish also produce a dilute urine to eliminate diffusive water gain. These water and ion fluxes are reversed in seawater, such that fish tend to lose water and gain salts. Thus, to compensate for dehydration and salt influx, fish must drink water as well as actively excrete salt ions across the gills and produce small volumes of concentrated urine (McCormick and Saunders, 1987; Avella and Bornancin, 1989; McCormick, 1995; Evans et al., 2005).

Two enzymes, sodium-potassium ATPase (NKA) and the proton pump (H^+-ATPase) play an important role in homeostasis of osmoregulation. For example, transfer of euryhaline fish such as rainbow trout from freshwater to seawater is associated with an increase of the gene expression and activity of NKA (McCormick and Saunders, 1987; McCormick, 1995; Marshall, 2002; Richards et al., 2003). This enzyme is, however, important in both freshwater and seawater ionocytes, providing the electrochemical energy required for either net uptake of ions and acid-base regulation in freshwater, or excretion of ions and acid-base regulation in seawater. As such,

it has been used as a biomarker for ionoregulatory disturbance in a number of toxicological studies (Blair et al., 2016). For a full review of ionoregulatory models of the fish gill, please see Dymowska et al. (2012).

H^+-ATPase also makes an important contribution to cellular ion regulation in the gill of freshwater fish, including rainbow trout. This apically-located transporter is thought to be coupled with a sodium conductive channel which, in concert, drive Na^+ uptake from the environment (Dymowska et al., 2014), although current models also suggest roles for a Na^+/H^+ exchanger/Rh protein metabolon in regulating Na^+ uptake in FW fishes (Wright and Wood, 2012).

An exposure to a hyper-saline FPW solution has the potential to perturb the ionoregulatory system of freshwater aquatic species, including *O. mykiss*. Thus, studying enzymes implicated in osmoregulation is of significant importance in understanding how organisms are able to cope with the osmotic stress of FPW exposure.

Plasma ions

The roles of NKA and H^+-ATPase are to maintain plasma ion homeostasis. This needs to occur even in the presence of environmental stressors that may induce osmotic imbalances (Bervoets and Blust, 2003; Nordlie, 2009). Imbalances in ion homeostasis will result in a higher metabolic cost in fish, which could lead to a long-term impact on their physiology (e.g. decrease of growth or reproduction) (Wendelaar Bonga and Lock, 1991). Many toxicological studies have investigated the influence of the chemical composition of the environment, such as a change of salinity or the presence of metals, on the plasma ion levels of freshwater organisms.

The effect of an increase in environmental salinity on plasma ion levels in fish has been well described in the literature. The instantaneous effect of transferring a fish from freshwater to

seawater is an immediate increase of their plasma ion concentrations, triggering compensatory responses (increases in NKA and H^+-ATPase activity), that restore plasma ion homeostasis. However, the new steady state level may differ, with a higher plasma osmolality noted in seawater fish, than in freshwater, likely a mechanism for reducing diffusive fluxes, and thus the costs of plasma ion maintenance (Eddy, 1982; Nordlie, 2009).

Overall, exposure to a hyper-saline FPW effluent is likely to disturb the plasma ion levels in fish, setting off a cascade of negative effects on proper cellular function. Consequently, examining plasma concentrations is a useful sub-lethal endpoint to monitor following FPW exposure.

Plasma trace elements

While plasma ions can provide insight into the effects of the high salinity of FPW on fish health, analysis of plasma composition can also inform the toxicological importance of the trace element components of FPW. Depending on the element considered (e.g. an essential or non-essential metal) there is usually a positive relationship between the concentrations of trace elements in fish tissues and the levels found in the environment. For example, Bervoets and Blust (2003) studied a freshwater sentinel fish species, the gudgeon (*Gobio gobio*), and showed that an increase of metal levels in the plasma of the fish could be seen following an increase of the metals in the water.

It is known that metals and other trace elements can be taken up and accumulated in fish tissues, principally in gills, liver, kidney, muscles or plasma (Hollis et al., 1999; Jezierska and Witeska, 2006; Agah et al., 2009). For example, it has been reported that fish exposed to cadmium display an accumulation of this element in their plasma (Hollis et al., 1999). Thus, monitoring

accumulation of trace elements in fish plasma can be a useful tool to monitor the impacts of FPW exposure.

Thesis aims

When FPW spills occur, they can result in the release of potentially large volumes of hyper-saline water to freshwater environments. Such events have the potential to induce severe osmotic stress and induce toxic effects in aquatic organisms living in receiving waters. The goal of this thesis is to investigate the specific effect of FPW salinity on two freshwater model species, *Daphnia magna*, and the rainbow trout. My first objective is to examine the impact of FPW on the phototactic behaviour of naïve and pre-exposed *D. magna*. Based on the known toxicity of this effluent on *Daphnia* and on the high sensitivity of the behavioural endpoint to different toxicants (described above), *FPW exposure is hypothesized to alter the phototactic behaviour of the freshwater water flea.* This is the first-time behavioural endpoints are being used to evaluate the impact of FPW exposure. My second objective is to examine the impact of a sub-chronic low-level FPW exposure and a parallel associated low salinity exposure as a paired control, on *O. mykiss* ionoregulatory physiology and gill morphology. Given that the euryhaline rainbow trout species is known to be able to cope with high salinity range, *I hypothesize that such exposure will not induce an osmotic stress in O. mykiss, thus revealing any potential impacts of other constituent contaminants.* To date, the impact of a long-term (28 day) FPW exposure has never been investigated in fish.

Chapter 2 - Shedding light on hydraulic fracturing fluid toxicity: the effect of flowback and produced water on phototactic behaviour in *Daphnia magna*

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Introduction

Freshwater cladocerans, such as *Daphnia magna*, are important ecological and toxicological model species. Widely distributed in worldwide freshwaters (Adamowicz et al., 2009), *D. magna* have a number of important ecological functions. In particular, they are a critical component of freshwater food webs, through their consumption of phytoplankton, and their role as prey to higher trophic levels (Miner et al., 2012). The daphnids are also commonly used toxicological test species, often displaying sensitivities to aquatic contaminants that are among the highest of all freshwater organisms (Baudo, 1987). Consequently, species such as *D. magna* have a significant influence on the establishment of water quality guideline values (van Leeuwen and Vermeire, 2007), and thus establishing the sensitivity of daphnids is a key initial step for assessing the risk of emerging toxicants to aquatic ecosystems.

One such emerging threat to Canadian freshwaters is the effluent generated by hydraulic fracturing. Hydraulic fracturing is a process that facilitates access to hydrocarbons trapped in geologic formations of low permeability, thus allowing the recovery of gas and oil (Stringfellow et al., 2014). Briefly, a highly pressurized proprietary combination of water and chemical additives such as surfactants, scale inhibitors, friction reducers, and biocides, is injected into the ground to fracture oil and gas-containing shale, enabling the release of hydrocarbons (King, 2012; Stringfellow et al., 2014). The water that returns to the surface is termed Flowback and Produced Water (FPW), which is a complex solution containing the extractable hydrocarbons, but also high concentration of salts, trace metals and organic chemicals (Entrekin et al., 2011; Jiang et al., 2014, He et al., 2017a). Owing to the considerable growth in hydraulic fracturing, the increased production of FPW, and thus the enhanced risk of FPW spills, there is significant concerns regarding the toxicological potential of hydraulic fracturing and its effluents. Indeed, more than

100 large volume FPW spills have been recorded in the Canadian province of Alberta alone, occurring mostly during the transportation of the fluid for storage and/or disposal (Vengosh et al., 2014; Goss et al., 2015). The specific composition of FPW depends on the characteristics of the geologic formation, the time spent in contact with the well, and the composition of the initial injectate (Maule et al., 2013; Stringfellow et al., 2014). Hence, each well produces a unique FPW, a fact that complicates the assessment of the risk associated with FPW spills (Alessi et al., 2017).

Only recently have the first studies appeared that have characterized the toxicity of FPW to freshwater biota (He et al., 2017b; 2018; Folkerts et al. 2017; Blewett et al. 2017b). For example, FPW has a toxic effect on *Daphnia magna* survival and reproduction. The 48-h median lethal concentration (LC_{50}) for neonates was determined to occur when the raw FPW sample was diluted to 0.19% of its original strength, while over a 21-day chronic exposure, an FPW dilution of 0.04% had significant effects on *Daphnia* reproduction (Blewett et al. 2017a). A notable finding of the studies on FPW toxicity performed to date is that the salt component of the effluent appears to have a major influence on the toxic responses (Blewett et al., 2017a; b). In the raw FPW samples, salt contents can be as high as 8-fold greater than the salinity of seawater, and thus even significant dilution of a spill into freshwater, may still result in an environmentally-elevated salinity. From a physiological perspective, elevated salinity represents a significant challenge to freshwater organisms. Under normal conditions, these animals constantly lose salt from their more concentrated bodies, where it is used to maintain optimal ionic balance for a myriad of cellular functions. To maintain ionic homeostasis epithelial ion transporters are used to recover lost ions. The presence of high salt can disrupt this dynamic steady state, leading rapidly to toxicity (Blewett et al., 2017a).

To date, however, there is no understanding of behavioural responses of aquatic biota to hydraulic fracturing wastewaters. Behavioural endpoints in toxicology have significant value. They are integrative measures, representing the combined effects of molecular, biochemical and physiological changes. Furthermore, behaviour is highly sensitive, with data indicating that behavioural endpoints may be 10 to 1000 times more sensitive than measures of lethal toxicity (Hellou, 2011). Importantly, changes in an organism's behaviour can have significant real-world relevance, with disruption in behaviour potentially leading to impairments in migratory patterns, reproduction, acquisition of food, and escape from predation. As a consequence, accounting for behavioural responses of animals to toxicants can contribute significantly to water quality assessments (Di Delupis and Rotondo, 1988; Michels et al., 1999; Martins et al., 2007).

In *Daphnia magna*, phototactic behaviour has been a widely used endpoint for assessing the toxicity of aquatic contaminants (Wolf et al., 1998; Martins et al., 2007). For example, trace metals such as cadmium (Baillieul and Blust, 1999; Michels et al., 2000) and copper (Gerhardt, 1995; Michels et al., 1999; Untersteiner et al., 2003) have been shown to affect responses of *Daphnia* to light. In general, *Daphnia* move towards a light source, and therefore display positive phototaxis. This response plays a critical role in daphnid biology (Ringelberg, 1964; Gerritsen, 1982; Glaholt et al., 2016), particularly with respect to the vertical migration of the animals within the water column, a factor of importance for feeding and avoiding predation (De Meester, 1993). Consequently, the aim of the current study was to investigate the effect of FPW on the behaviour of *D. magna*. Initially, we characterized the behavioural responses of *Daphnia magna* to light in a custom-built chamber, before utilizing this chamber to investigate the effects of FPW, and its salinity component, on *Daphnia* phototaxis.

Material and methods

Animals

Daphnia magna were initially obtained from Aquatic Research Organisms, Inc. (Hampton, New Hampshire, USA), and maintained in culture in the Department of Biological Sciences at the University of Alberta. *Daphnia* were subjected to a 12h:12h light/dark photoperiod at 20 ± 1°C and cultured in 8-L glass aquaria following Organization for Economic Cooperation and Development (OECD) guidelines (OECD, 2008), where the water chemistry was as follows: 2 mM $CaCl_2.2H_2O$; 0.5 mM $MgSO_4.7H_2O$; 0.77 mM $NaHCO_3$; 0.08 mM KCl. Water was changed every 2 days and animals were fed daily to satiation with Roti-Rich liquid invertebrate food (VWR, Edmonton, Alberta, Canada).

Experimental apparatus and test protocol

Phototaxis in *D. magna* was assessed using a custom-built apparatus, diagrammed in Figure 2-1. This apparatus consisted of three clear polycarbonate tubes (length 34 cm; diameter 3 cm), that fitted snugly within shorter (29 cm) black polycarbonate sleeves (Fig. 2-1A). The sleeves were secured into a housing chamber (cardboard shoe box). The clear inner tube could be placed inside the sleeve, and slid back and forth, such that one end protruded from the sleeve by 5 cm (Fig. 2-1B). Holes were drilled in the center of the sleeve and inner tube, allowing for the introduction of the daphnid, and the entire apparatus was placed on top of a light box. Before being slid into the sleeve, inner tubes were filled with test waters (~175 mL), with bungs at each end. An experiment was started with the introduction of an individual daphnid into each of the three *Daphnia* ports, before the inner tubes were pushed through the sleeve such that an end protruded, and the light box was switched on. This light source generated light in the visible spectrum only,

with no UV light. This resulted in the illumination of one end of the inner tube. The time taken for a daphnid to appear in the illuminated end of the tube and remain in this zone for at least 5 consecutive seconds was deemed a positive phototactic response and recorded. Any individual failing to display this response after 5 minutes was given a score of 300 seconds. Five minutes was considered an appropriate response time as > 90% of the adults (fed and fasted) responded within this time frame. This test approach is consistent with previous studies (Ringelberg, 1964). The speed of the animals was then calculated (i.e. [time needed to reach the light]/[distance traveled in the tube]). Between each replicate, the tubes were cleaned with 5% ethanol solution and rinsed with dechlorinated City of Edmonton facility water. The simultaneous measurement of three individuals allowed for each test condition to be matched with a control to account for any replicate-to-replicate variation. The placement of the control and test tubes were randomized to eliminate any bias associated with a specific location within the housing chamber.

Establishing the effects of extrinsic factors on phototactic response

To establish the influence of factors extrinsic to the water chemistry in the test chambers, the effects of fed state (24 h fasted *versus* food present in breeding colony at time of removal for testing), time of day (hourly test of naïve individuals between 9:30 AM to 4:30 PM), and life-stage (neonates <24-h old *versus* adults 12-15 days old) were examined. These tests were conducted with individual daphnids (n value range from 6 to 48), in OECD water, using the test chambers as described above. Subsequently, all following assays were conducted with adult daphnids (12-15 days old), collected from exposure chambers with food present, and during daylight hours.

Effects of FPW and salinity on phototactic response

A raw sample of FPW was obtained from Encana corporation, an oilfield operator serving as an industrial collaborator. This sample has been chemically-characterized in previously published works from our laboratory (Blewett et al., 2017a). A salinity-matched control (saline water; SW) was also generated, which had a salt profile matched to that of the raw FPW sample, but which lacked all other components of the effluent (see Blewett et al., 2017a).

Experiment 1- Effect of dose: The effect of FPW on *Daphnia* phototaxis was tested in a dose-dependent manner. Naïve animals (i.e. those that had never been exposed to FPW) were transferred directly from holding colonies maintained in OECD water, into the test apparatus, which contained either 0% (OECD water without FPW), 1%, 5%, 10%, 20% or 40% of the raw FPW sample diluted in OECD water (control, n = 27; FPW, all n = 9).

An identical study was conducted in waters that matched the salt content of the FPW, but which lacked any other constituents. Individual fed adult daphnids were tested for their phototaxis response in either 0% (OECD water without addition from the SW stock solution), 1%, 5%, 10%, or 20% of the SW stock solution, diluted in OECD water (n = 6-7).

As the FPW and SW dose-response data were generated at different times with different set of controls, an additional study was conducted to simultaneously compare the responses of SW-exposed and FPW-exposed daphnids. These studies were conducted at dilutions of 1% or 10% (relative to the raw FPW and stock SW solutions), tested simultaneously for direct comparison (i.e. one 'run' of the test apparatus was conducted concurrently with control, FPW and SW, randomized between the three testing chambers). There were 6 replicates of each treatment.

Experiment 2- Effect of pre-exposure: In this study six different pre-exposure conditions were examined: 24-h exposure to 1.5% FPW or SW, 96-h exposure to 0.75% FPW or SW, and 96-

h exposure to 0.25% FPW or SW. For 96-h exposures, a water change was conducted at 48-h, and in all exposures daphnids were fed daily. Control daphnids (OECD water alone) were held under identical conditions. Following the exposure, all pre-exposed and control animals were tested in either OECD water (i.e. control conditions) or a 10% solution of the pre-exposure solution (i.e. FPW pre-exposed animals (and controls) were tested in 10% FPW, while SW pre-exposed animals (and controls) were tested in 10% SW). The aims of these experiments were to: ascertain the behavioural effects of longer exposures to FPW or SW; determine whether behavioural effects induced by FPW and SW relied on the presence of these solutions in the test chamber; and examine the possibility of acclimation of phototaxis behaviour to prolonged exposure. To place these pre-exposure concentrations in context, the 48-h LC_{50} for adult daphnids exposed to FPW is 0.75%, while for SW-matched solution the 48-h LC_{50} is 2.6% (Blewett et al., 2017a).

Experiment 3- Effect of colour: The raw FPW sample was coloured orange-brown, and even when diluted added a noticeable hue to the test waters. In order to determine whether the clarity of the water was a factor influencing phototaxis, *Daphnia* behaviour was examined in OECD water containing 10 mg L^{-1} Suwannee River natural organic matter (NOM; International Humic Substances Society, St. Paul, MN, USA). The powdered NOM was reconstituted in OECD water, and phototaxis was determined relative to a concurrently run control (n = 7). The light intensity passing through the test chamber filled with control OECD water, 20% FPW or NOM, was measured with a light meter (AccuPAR LP-80, Meter Environment, Pullman, WA, USA).

Statistics

All statistical analyses were conducted using SigmaPlot version 13.0 (Systat Software Inc., San Jose, CA, USA). All data were subjected to tests of normality (Kolmogorov-Smirnov) and

homogeneity of variance (Levene's). Data that failed these tests were transformed until parametric assumptions were met, or a non-parametric test was performed (two cases: pre-exposure to 0.25 and 0.75% FPW tested in OECD water analyzed by Kruskal-Wallis test). The effect of fed state and time of day were assessed via a two-way ANOVA, whereas the effect of SW or FPW dose and pre-exposure on phototactic behaviour was tested with a one-way ANOVA, both employing a Tukey's post hoc test where significance was identified. The effect of the colour was analyzed using a paired t-test. Significance was accepted at $\alpha = 0.05$, and throughout values are reported as means ± standard error of the mean.

Results

The feeding state of the adults significantly influenced their phototactic behaviour (two-way ANOVA, $p = 0.032$; Fig. 2-2.A), but there was no effect of the time at which the test was conducted (two-way ANOVA, $p = 0.850$), nor was there a significant interaction between time of test and fed state (two-way ANOVA, $p = 0.239$). A post-hoc analysis showed that fasted organisms presenting a higher speed than fed organisms, albeit only at a single tested timepoint (1:45 PM). In contrast to adults, there was no effect of feeding status observed in neonates (two-way ANOVA, $p = 0.187$). There was, however, a significant effect of time of testing (two-way ANOVA, $p = 0.002$; Fig. 2-2.B), with both fasted and fed neonates presenting a slower phototactic response at 11:45 AM, relative to their speed of response at 9:45AM (in the case of fed neonates) or at 10:45 AM and 2:45 PM (in the case of fasted neonates). Pooling all data, independent of time of day, an effect of life-stage and fed state could be seen (Fig. 2-2.C; two-way ANOVA, $p < 0.001$ and $p = 0.017$ respectively). While the phototactic behaviour of the neonates did not differ between feeding conditions (fed: 0.16 cm s^{-1}; fasted: 0.20 cm s^{-1}), fasted adults had a more positive phototactic

behaviour than fed adults (0.51 vs. 0.38 cm s^{-1}). Furthermore, adults were always faster than the neonates, irrespective of fed state. Therefore, all subsequent tests were conducted on adult daphnids fed 45-60 min prior to experimentation.

Increasing the dose of FPW in the behavioural test chamber resulted in an increase in the speed of the *Daphnia* to reach the illuminated zone of the test apparatus (one-way ANOVA, $p < 0.001$; Fig. 2-3). Indeed, the organisms exposed to 40% FPW displayed response speeds that were 3-fold faster than in OECD water alone. Similarly, when *Daphnia* were exposed to SW solutions representing the salt content of the FPW, a similar dose-dependence was observed, with *Daphnia* speed increasing to a peak value of 1.1 (± 0.3) cm s^{-1} at a 10% SW dilution, representing a 5.4-fold increase relative to the control (one-way ANOVA, $p < 0.001$; Fig. 2-4). In both treatments (SW and FPW), the organisms exposed to the highest exposure concentrations (20 and 40%) presented a clear erratic behaviour (visual observation). Given that the FPW and salt dose-response curves were determined at different times, we confirmed the similarity of the stimulation in positive phototaxis by testing 1% and 10% solutions of FPW and SW simultaneously with OECD water controls (Fig. 2-5). At both dilutions, the responses to SW and the FPW were statistically indistinguishable and matched the response obtained in these solutions during the dose-response study.

The similar responses induced by FPW (coloured) and SW (uncoloured) suggested that the colouration of test waters by FPW did not have an impact on phototactic response. Regardless, we conducted a series of tests in the presence/absence of NOM in the test chamber solutions to eliminate this as a variable. As predicted, there was no statistically significant effect of 10 mg L^{-1} Suwannee River NOM on *Daphnia* phototaxis (t-test, $p = 0.519$; Fig. 2-6). The measured light

intensity through the different test solutions was 10, 10 and 7 µE for OECD water, NOM, and 20% FPW, respectively.

To determine whether *Daphnia* could acclimate to test solutions, three pre-exposure regimes were examined for both FPW and SW solutions (24-h exposure to 1.5% FPW or SW, 96-h exposure to 0.75% FPW or SW, and 96-h exposure to 0.25% FPW or SW). In each of these conditions, phototaxis was then examined in either the presence, or absence, of a 10% solution (10% FPW for FPW pre-exposed animals, and 10% SW, for salt pre-exposed animals) to determine whether effects on phototaxis were related directly to the presence of the contaminant, or were due to downstream effects in terms of detection of, or response to, the test chemistry (Fig. 2-7 and 2-8). The only statistically significant effect of pre-exposure tested in control chemistry (OECD water) was a significant decrease in the speed of *D. magna* pre-exposed to 1.5% FPW and SW for 24 h relative to the pre-exposed control (one-way ANOVA, $p = 0.004$; Fig. 2-7.A). This response was distinct from the stimulation of swimming speed observed in the dose-response study performed in naïve *Daphnia* (Fig. 2-3). All other FPW pre-exposure regimes and test conditions resulted in responses that were statistically unchanged relative to the concurrently-tested control animals (one-way ANOVA, p value range: 0.089-0.398; Fig. 2-7.B-C).

However, the only statistically-significant effect of pre-exposures subsequently tested in 10% FPW/SW was in daphnids maintained for 24-h at 1.5% SW, then tested in 10% SW. In contrast to the significant increase in speed observed in naïve animals (Fig. 2-4), these daphnids displayed a significant decrease in speed (one-way ANOVA, $p = 0.003$; Fig. 2-8.A). Notably, no significant effects of the lower pre-exposure concentrations over 96 h were observed (one-way ANOVA, p-value range: 0.184-0.941; Fig. 2-8.B-C), but for those animals tested in 10% SW, this

represented a different outcome from naïve animals, which showed a stimulated speed of phototactic response in this test condition (as seen in Fig. 2-4).

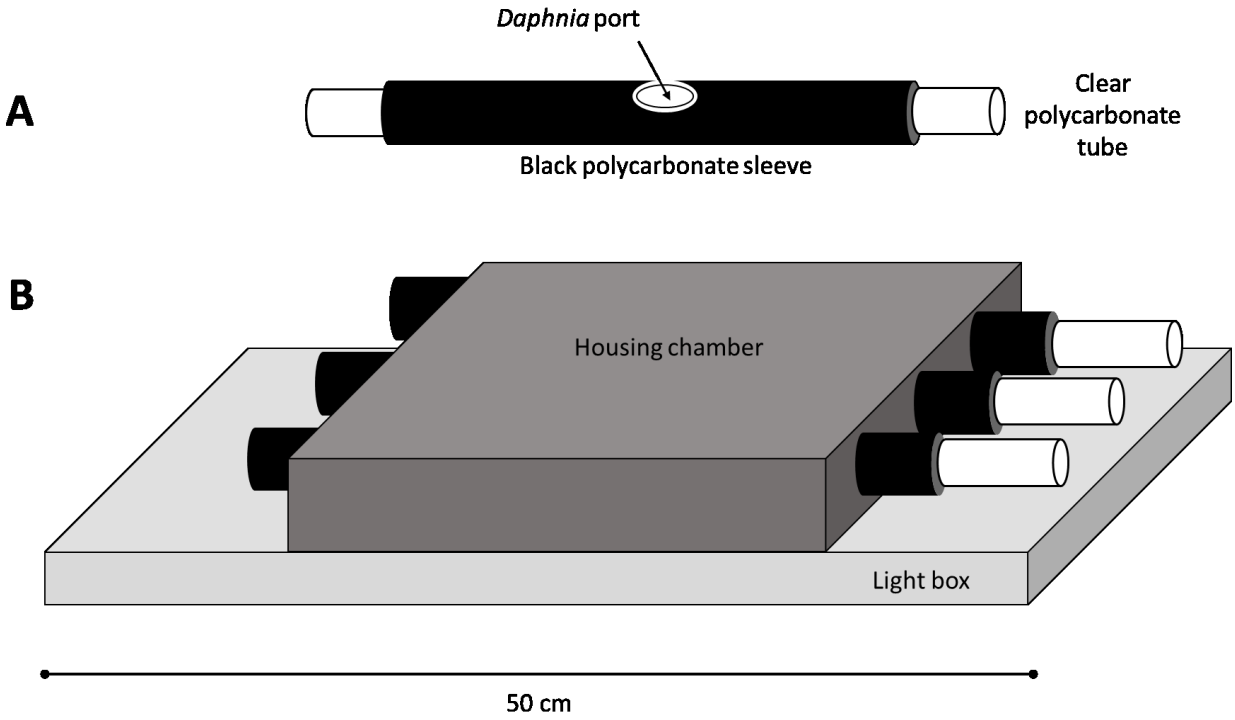
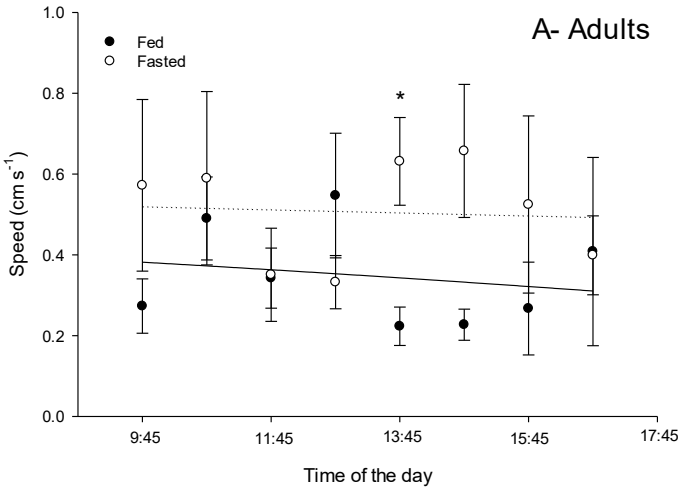
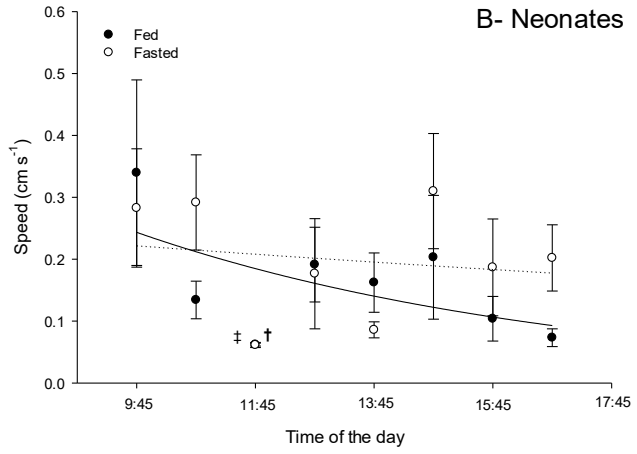


Figure 2-1. Diagram of test apparatus, showing individual test chamber (A), and an overview of the holding chamber in position for behavioural testing (B).


A- Adults
Fed
Fasted
Speed (cm s-1)
1.0
0.8
0.6
0.4
0.2
0.0
*
9:45
11:45
13:45
15:45
17:45
Time of the day


B- Neonates
Fed
Fasted
Speed (cm s-1)
0.6
0.5
0.4
0.3
0.2
0.1
0.0
‡ ♌ †
9:45
11:45
13:45
15:45
17:45
Time of the day

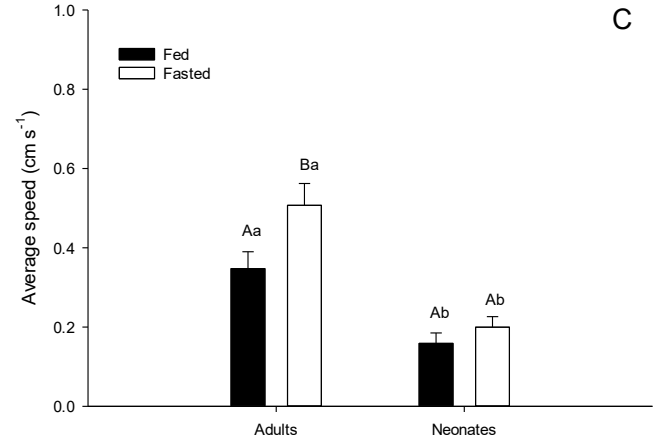

C
Fed
Fasted
Average speed (cm s-1)
1.0
0.8
0.6
0.4
0.2
0.0
Aa
Ba
Ab
Ab
Adults
Neonates

Figure 2-2. (Previous page) Phototactic behaviour of fed (filled circles) and fasted (open circles) adult (A) or neonate (B) *Daphnia magna* as a function of time of day when tested in OECD water (no FPW). Plotted points represent the mean (± SEM) of 6 replicates. An asterisk denotes a significant different between a fed and fasted group within a tested time. A single dagger represents a fed group that differs significantly to other fed groups tested at 9:45 AM, while a double dagger represents a fasted group that differs significantly to other fasted groups tested at 10:45AM and 2:45 PM. Pooled data from Panels A and B independent of time of day are exhibited in Panel C. Plotted points represent the mean (± SEM) of 48 replicates. Plotted points sharing uppercase letters are not significantly different with respect to fed state within a life-stage, whereas points sharing lowercase letters are not significantly different with respect to life-stage within a feeding status, as determined by two-way ANOVA, followed by Tukey's post hoc test.

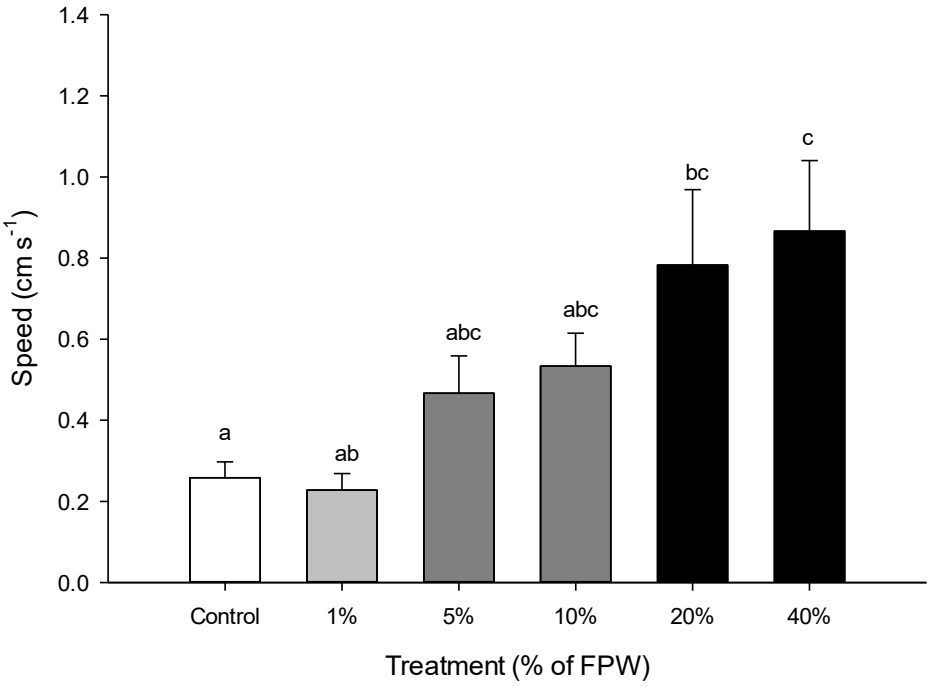


Figure 2-3. Effect of increasing FPW dilutions on the phototactic behaviour of fed adult *Daphnia magna*. Bars represent the mean (± SEM) of 9 (all FPW solutions) or 27 (control) replicates. Bars sharing letters are not statistically significantly different (one-way ANOVA, post hoc Tukey's test), at alpha = 0.05.

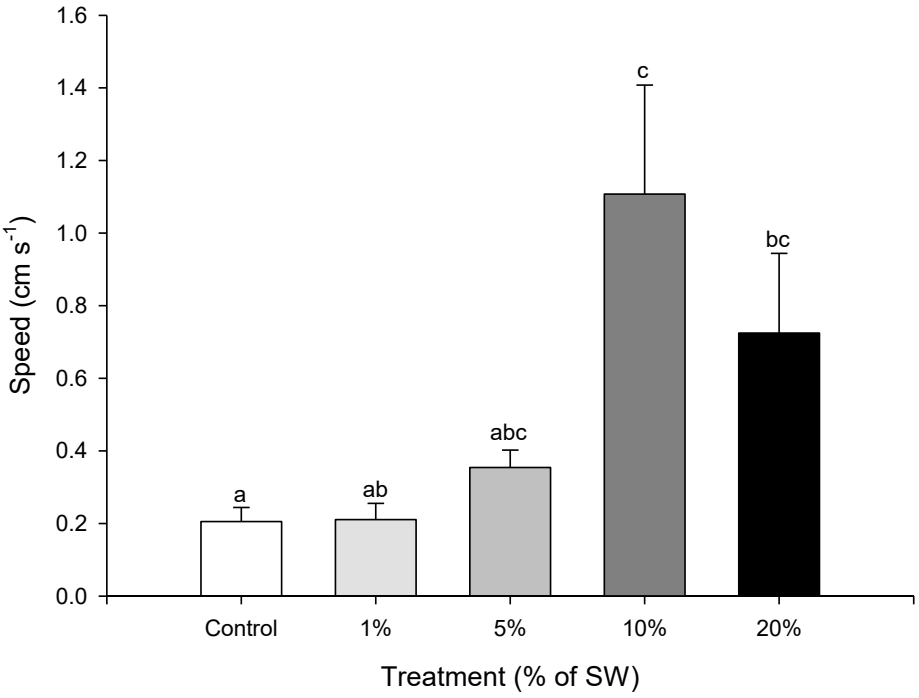
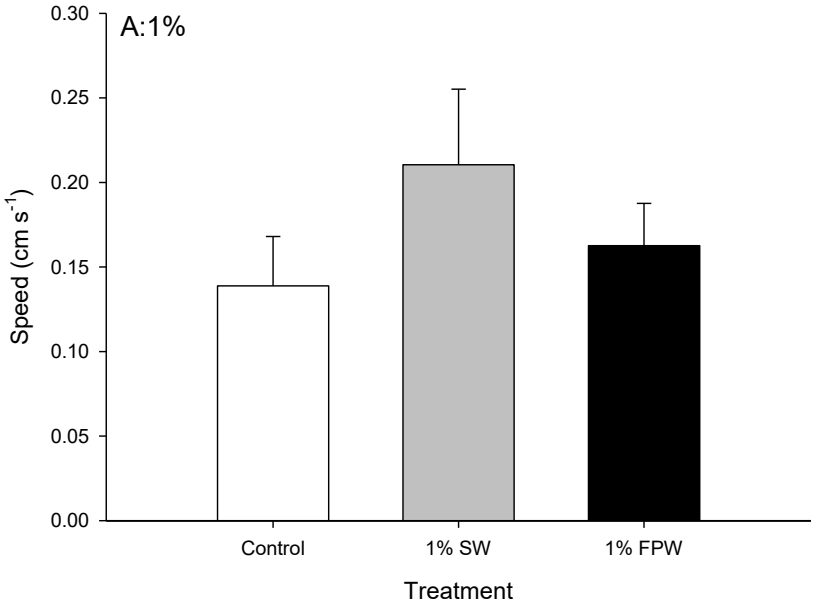


Figure 2-4. Effect of increasing SW dilutions on the phototactic behaviour of fed adult *Daphnia magna*. Bars represent the mean (± SEM) of 6-7 (all SW solutions) or 19 (control) replicates. Bars sharing letters are not statistically significantly different (one-way ANOVA, post hoc Tukey's test), at alpha = 0.05.


A:1%
Speed (cm s-1)
0.30
0.25
0.20
0.15
0.10
0.05
0.00
Control
1% SW
1% FPW
Treatment

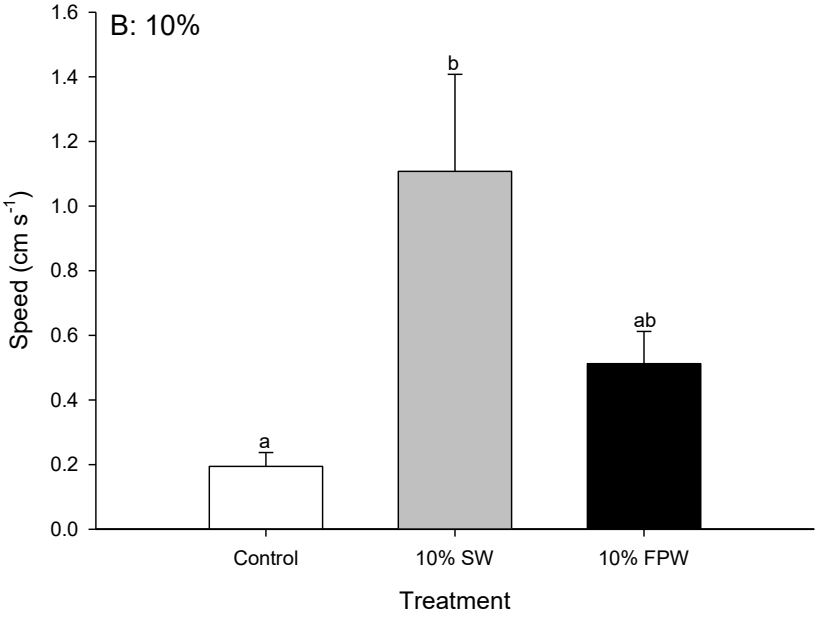

B: 10%
Speed (cm s-1)
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
a
b
ab
Control
10% SW
10% FPW
Treatment

Figure 2-5. (Previous page) Effect of a simultaneous assay of 1% (A) or 10% (B) SW or FPW solutions on the phototactic behaviour of fed adult *Daphnia magna*. Bars represent the mean (± SEM) of 8 replicates. Bars sharing letters are not statistically significantly different (one-way ANOVA, post hoc Tukey's test), at alpha = 0.05.

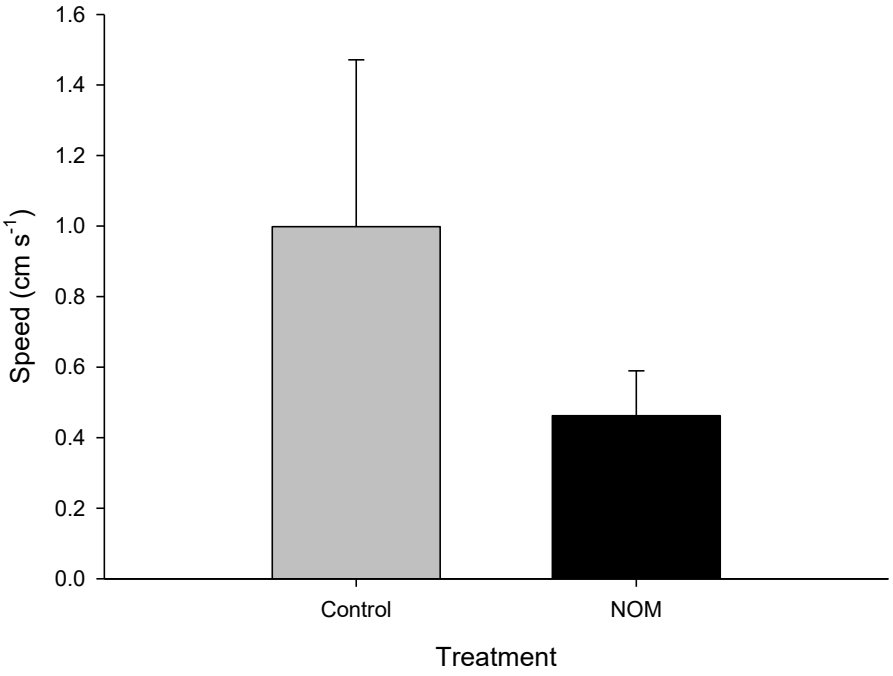
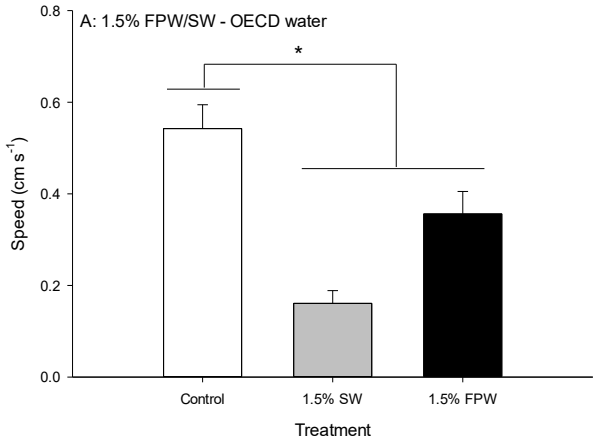
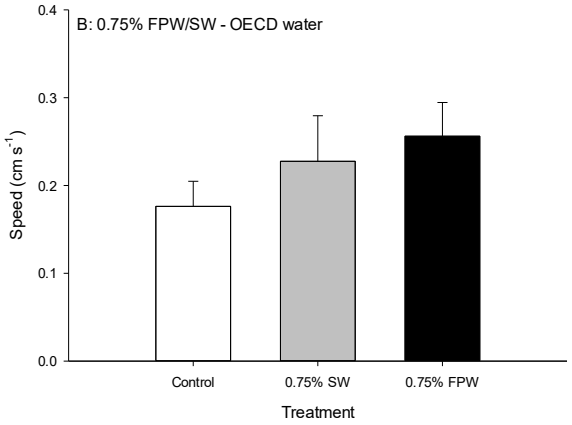


Figure 2-6. Effect of colour (10 mg L-1 Suwannee River NOM) on the phototactic behaviour of fed adult *Daphnia magna*. Bars represent the mean (± SEM) of 7 replicates.


A: 1.5% FPW/SW - OECD water
*
Speed (cm s-1)
0.8
0.6
0.4
0.2
0.0
Control
1.5% SW
1.5% FPW
Treatment


B: 0.75% FPW/SW - OECD water
Speed (cm s-1)
0.4
0.3
0.2
0.1
0.0
Control
0.75% SW
0.75% FPW
Treatment

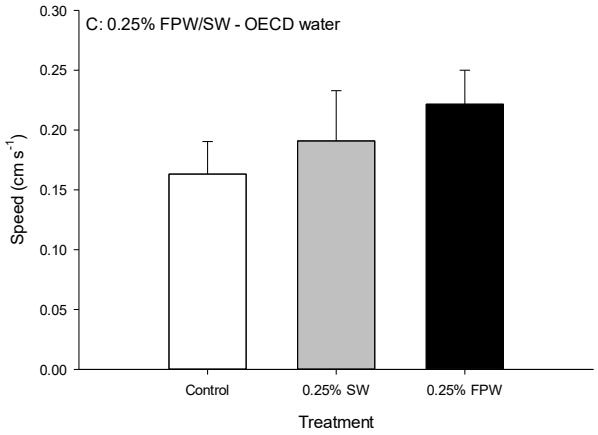
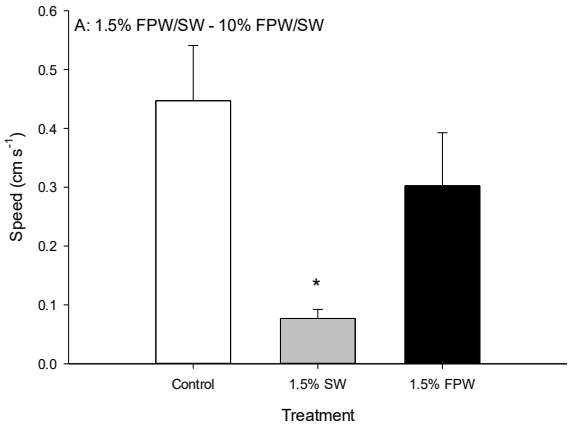
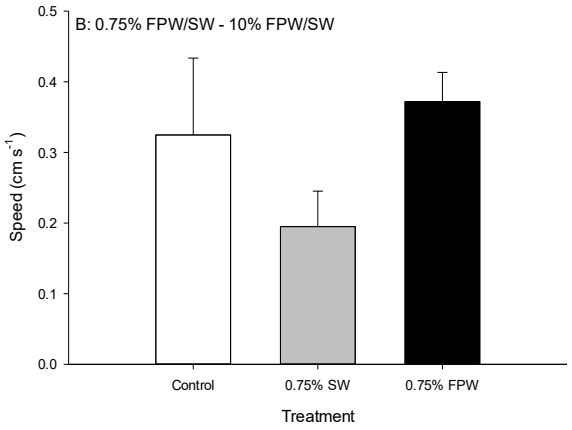

C: 0.25% FPW/SW - OECD water
Speed (cm s-1)
0.30
0.25
0.20
0.15
0.10
0.05
0.00
Control
0.25% SW
0.25% FPW
Treatment

Figure 2-7. (Previous page) Effect of pre-exposure to FPW and SW (A: 1.5% FPW/SW, 24 h; B: 0.75% FPW/SW, 96 h; C: 0.25% FPW/SW, 96 h) on subsequent tests of the phototactic behaviour of fed adult *Daphnia magna* conducted in OECD water. Bars represent the mean (± SEM) of 25-76 (FPW) or 6-7 replicates (SW). Asterisks indicate statistically significantly differences between control and exposed groups (one-way ANOVA), at alpha = 0.05.


A: 1.5% FPW/SW - 10% FPW/SW
Speed (cm s-1)
0.6
0.5
0.4
0.3
0.2
0.1
0.0
*
Control
1.5% SW
1.5% FPW
Treatment


B: 0.75% FPW/SW - 10% FPW/SW
Speed (cm s-1)
0.5
0.4
0.3
0.2
0.1
0.0
Control
0.75% SW
0.75% FPW
Treatment

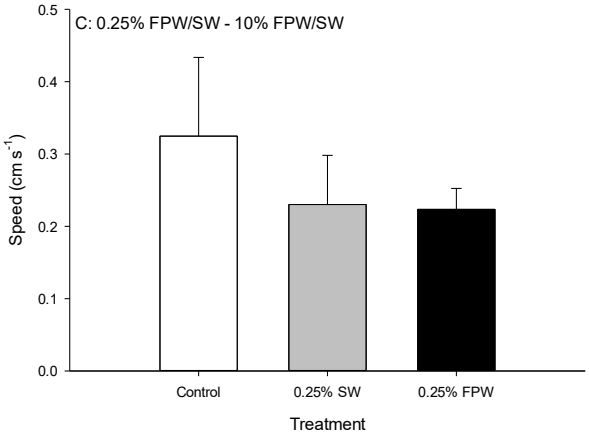

C: 0.25% FPW/SW - 10% FPW/SW
Speed (cm s-1)
0.5
0.4
0.3
0.2
0.1
0.0
Control
0.25% SW
0.25% FPW
Treatment

Figure 2-8. (Previous page) Effect of pre-exposure to FPW and SW (A: 1.5% FPW/SW, 24 h; B: 0.75% FPW/SW, 96 h; C: 0.25% FPW/SW, 96 h) on subsequent tests of the phototactic behaviour of fed adult *Daphnia magna* conducted in 10% FPW or SW. Bars represent the mean (± SEM) of 6-7 replicates. Asterisks indicate statistically significantly differences between SW treatment and control and FPW groups (one-way ANOVA), at alpha = 0.05.

Discussion

The present study is the first to investigate the effects of FPW on the behaviour of the water flea *Daphnia magna*. These data show that FPW induces perturbations in the positive phototactic response (i.e. movement toward a light source) of the exposed organisms, and that these effects appear to be principally driven by the salt content of these wastewaters. There were also notable differences in the nature of the effect depending on the exposure history, with naïve animals displaying enhanced phototactic behaviour, but animals exposed to 1.5% solutions for 24-h, displaying diminished positive phototaxis.

Effects of extrinsic factors on *D. magna* phototaxis

In our test conditions, *Daphnia magna* were shown to display positive phototactic behaviour, moving rapidly towards the illuminated end of the test chamber. In adults, this behaviour was consistent, independent of the time of the day a test was conducted (9:45 AM – 4:45 PM; Fig. 2-2). Although previous observations have shown that *Daphnia* phototactic behaviour fluctuates between day and night, our finding is consistent with these studies which also observed that within hours of daylight there is little variation in phototactic response (Cushing, 1951; Ringelberg, 1964; Ringelberg and Servaas, 1971). It is important to note that while adult behaviour was consistent across tested times, there was some significant variation in the speed of phototactic response of neonates with time of test (Fig. 2-2.B). This is consistent with previous observations, which have noted the responses of neonates are more variable than those of adults (Whitman and Miller, 1982).

In contrast, there was a significant effect of fed state on phototaxis in adult *Daphnia*, with fasted organisms responding more rapidly to light than fed organisms (Fig. 2-2.C). The more rapid

response of fasting animals can be attributed to a stronger drive to move towards the light, a stimulus associated with feeding. For example, an early study by Clarke (1932), showed *D. magna* were less responsive to a light stimulus in the presence of higher food densities, relative to conditions where food was less available. This finding was later corroborated in a review by Folt and Burns (1999), which emphasized that in conditions of low food concentration, *D. magna* increase their swimming speed in an attempt to actively seek out nutrients.

The current study also showed that differences in swimming speeds occur between adult and neonate daphnids. Previous research has shown that adult *D. magna* swim velocity averages 0.55 cm s^{-1} (Buchanan and Goldberg, 1981), in line with the range of values in our study (0.35 and 0.51 cm s^{-1} for fed and fasted animals, respectively; Fig. 2-2.C). Differences in swimming speeds between neonate and adult *Daphnia* is a function of body size, with larger animals capable of producing a more powerful swimming stroke than smaller individuals (Dodson and Ramcharan, 1991).

Effect of FPW and SW on phototaxis

Exposing naïve animals to FPW or SW resulted in a clear dose-dependent increase in positive phototactic response (i.e. swimming speed towards the illuminated zone of the test chamber; Fig. 2-3 and 2-4), as well as an erratic behaviour when the organisms were exposed to the highest concentrations of SW and FPW, characteristic of a startle response (Drummond and Russom, 1990). The magnitude and threshold of effects of SW, a water chemistry which mimicked the salt content of FPW but without metal and organic constituents, were very similar to those seen in the FPW itself. This suggests that the effect of FPW on *Daphnia* phototactic behaviour is mediated by salinity. An effect of FPW driven by its salt content is consistent with previous

research. For example, salinity was shown to play a role in the effects of FPW on survival and fitness in *Daphnia* (Blewett et al., 2017a).

An increase in speed in response to a stimulus, as observed in our study, is a phenomenon that has been observed previously in aquatic behavioural ecotoxicology and has been characterized as a hyperactive response. Hyperactivity is suggested to be a reversible adaptive response enacted in order to escape the negative stimulus, and thereby reduce potential negative effects resulting from exposure (Evans, 1994; Wolf et al., 1998; Gerhardt, 2007). Consequently, organisms tend to move away from a potential toxicant. This has been principally defined as "avoidance" (Gerhardt, 2007), and is a commonly observed response of aquatic biota, such as fish and invertebrates, to a wide variety of chemical contaminants (reviewed by Tierney, 2016; Lefcort et al., 2004; Wiklund et al., 2006). Importantly, however, the response of adult *D. magna* in the current study cannot be classified as avoidance. This is in part because of the nature of the test chamber, which does not offer a choice between a contaminated zone and a contaminant-free zone, and thus does not allow the test animal to avoid the exposure. Furthermore, the response of *D. magna* in our study became statistically significant only at 20% dilutions of FPW and 10% dilutions of SW, orders of magnitude higher than acute lethal concentrations (adult 48-h LC_{50} for FPW is 0.75%; adult 48 h LC_{50} for SW is 2.6%; Blewett et al., 2017a). This contrasts with true avoidance responses which are generally displayed at low concentrations, below the threshold for toxicity (Tierney, 2016). Consequently, the responses of daphnids to FPW and SW in our system are best characterized as aversion behaviour, a definition that is consistent with the responses of some aquatic invertebrates to predator chemical cues (Kats and Dill, 1998).

Our data do, however, indicate an ability of *D. magna* to sense FPW and SW, and to respond in a manner that might allow them to escape exposure in a natural setting. The ability of

Daphnia to detect changes in water chemistry is likely mediated by chemosensory systems, such as olfaction (Roozen and Lürling, 2001). Consistent with our observation that aversion responses are mediated by the salt content of FPW, studies on fish have demonstrated that they are behaviourally responsive to salts such as calcium (Dew and Pyle, 2014). At a dilution of 10%, test solutions for FPW and SW contain a calcium concentration of 21 mmol L^{-1}, 2-fold the concentration of calcium in seawater. It is, therefore, possible that a similar mechanism for detecting dissolved salt content drives *Daphnia* behaviour. However, there are few studies that have examined the effects of ions on *Daphnia* behaviour. An exception is research by Baillieul and colleagues (1998), which showed that increasing salinity (up to 8 parts per thousand; ~25% seawater) resulted in significantly slower swimming velocities in *D. magna*. These findings contrast to our significantly increased velocity values. However, in this previous work, the effects of salinity on behaviour were first examined only after two days of exposure and are therefore not directly comparable to our dose-dependence studies that examined the immediate response of naïve animals. Intriguingly, as discussed below, pre-exposure to salts in our study did slow swimming speeds, consistent with the outcomes of Baillieul et al. (1998).

Effect of FPW and SW pre-exposure and their presence in test chambers

Pre-exposure to 1.5% FPW for 24 h, and subsequent testing in the absence of an FPW stimulus, significantly impaired the positive phototaxis response in *Daphnia*. This decrease in swimming speed was not apparent when this same pre-exposure group was tested in 10% FPW. The most likely explanation for this effect is that the pre-exposure to FPW impairs the ability of the animal to either detect, or respond to, the light stimulus. However, in the presence of FPW in the test chamber, this inhibition of response is eliminated. This indicates that FPW is still capable

of inducing an increase in swimming speed, albeit from a baseline behavioural response that is lower than that of a naïve animal (i.e. loss of startle response; as seen in Fig. 2-3). This hypoactivity of the FPW pre-exposed animals is likely a reflection of an impaired capacity to respond. Exposure to toxicants modifies energy balance, via the diversion of energetic resources to homeostasis and damage repair (Knops et al., 2001; Sokolova et al., 2012). Consequently, 24-h exposure to 1.5% FPW, but not a 96-h exposure to 0.75 or 0.25% FPW, appears to reduce basal locomotory ability, an effect observed previously for toxicant-exposed *Daphnia* (Wolf et al., 1998). It is also possible that components of the FPW might cause more specific effects. For instance, cadmium exposure is suspected to induce a "neurological failure", leading to a decreased beat frequency of the second antennae which drive the swimming stroke in *D. magna* (Baillieul and Blust, 1999). Similarly, copper which also impairs *Daphnia* phototaxis, appears to do so by disrupting neurotransmitter function (Untersteiner et al., 2003).

Underlining the importance of salinity in driving the effect of FPW on the behavioural response of *Daphnia* to light, effects of salt pre-exposure were similar to those induced by FPW, albeit with subtle differences. In all animals that had been pre-exposed to SW, the testing of phototaxis in the presence of 10% SW either significantly decreased (24-h, 1.5%) or had no effect (96-h, 0.75 and 0.25%) on the speed of response. This was distinct from the enhanced swimming speed seen in naïve animals (Fig. 2-4). Similarly to FPW, daphnids that were exposed to 1.5% SW for 24 h and tested in OECD water displayed a statistically significant decrease in speed relative to control animals. Thiseffect on positive phototaxis following SW pre-exposure likely indicates an effect of the salt components of FPW, in addition to the non-salt components of FPW (i.e. metals, as discussed above), causing the inhibition of the basal phototaxis response seen in Fig. 2-7.A.

A specific effect of FPW salinity was, furthermore, observed when the pre-exposed animals were tested in 10% SW, with a significant decline in swimming speed relative to the control. This pattern was distinct from that in FPW pre-exposed animals tested in 10% FPW which exhibited no significant difference in response relative to control animals tested in 10% FPW. Our data therefore suggest that *Daphnia* are likely able to acclimate to the salinity component of FPW, such that there is an elimination of the aversion and startle responses (Fig. 2-8.A). Acclimation of daphnid swim behaviour has been observed previously. In a study examining the swimming speed responses of *Daphnia* to two volatile organic carbon toxicants, Watson and colleagues (2007) showed that initial exposure induced a stimulation in response, but that continued or subsequent exposures induced no effect. This pattern was attributed to behavioural acclimation of *Daphnia* to the toxicant. In our study, we observed a similar effect in response to SW, albeit an inhibition in phototaxis was observed after acclimation, rather than a null effect. However, this might be a particular response to salts, as a decline in *Daphnia* swimming speed has been reported following 48-h of salt exposure to this species, and attributed to an unknown, but specific, physiological impairment (Baillieul et al., 1998).

This work by Baillieul and colleagues also provides further evidence of the ability of *Daphnia* to acclimate to salinity. In their study, two days of exposure to enhanced environmental salinity, resulted in a significantly impaired *Daphnia* swimming speed (Baillieul et al., 1998). As the exposure continued over 8 days, swimming speeds in salinity-exposed daphnids remained lower than controls. However, because salinity impeded growth, which in turn influences swimming behaviour, correction of swimming speed for *Daphnia* size, resulted in no significant difference in size-specific swimming speeds, therefore suggesting that acclimation to salinity had occurred. It is important to note that these observations refer specifically to locomotory responses,

which are only one component of the positive phototactic response. To date, little is known about the effects of salts on sensory reception and processing in *Daphnia*, but this could also be a mechanism by which changes in phototaxis are mediated.

Effect of water colour

Hydraulic fracturing effluents often present a brown or orange colouration (personal observation). Consequently, if a spill occurs into clear freshwater, the colouration of the water could change the light intensity, and thus affect phototactic behaviour. To eliminate the possibility that the effects of FPW on *Daphnia* phototaxis were a consequence of water colour, behaviour was examined in the presence of NOM which results in a tea colouration similar in nature to that presented at high FPW dilutions. Our data showed that there was no impact of NOM on the response of *D. magna* to light (Fig. 2-5). However, although the NOM coloured the water, it did not affect light intensity, which was unchanged relative to the OECD water control, and higher than that recorded for 20% FPW (10 vs. 7 μE). Thus, our data do not allow us to definitively state that the effects of FPW on *Daphnia* behaviour are independent of the reduced clarity of the exposure water associated with FPW treatments. However, it is worth noting that previous studies do not note any significant change in *Daphnia* phototaxis with light intensity (Storz and Paul, 1998), which indicates that it is unlikely that the effect of FPW is mediated by changes in water clarity.

Conclusion

Our data show that the effects of FPW on daphnid behaviour depend significantly on the nature of the exposure. Naïve animals will respond by an aversion response, but only at relatively

high concentrations (10-20% dilution of the raw sample). However, at concentrations as low as 1.5% of the raw sample, a 24-h exposure is sufficient to induce lethargy, be it the result of impaired chemosensation or locomotory capacity. Consequently, in natural settings, the impact of an FPW spill will ultimately depend on the nutrient status of the water body (fed *versus* fasted animals; Fig. 2-2.C), the age distribution of the zooplankton fauna (Fig. 2-2.C), as well as on exposure history. Overall, the inability of *D. magna* to react appropriately to a spill of FPW, by detecting and escaping it, could have significant repercussions on the survival of the organisms. Importantly, the presence of FPW and the disruption of phototaxis will impair an animal's ability to perform a diel vertical migration, a critical component of both feeding and predation avoidance in this species (De Meester, 1993).

Chapter 3 - The osmotic effect of hyper-saline hydraulic fracturing fluid on the rainbow trout, *Oncorhynchus mykiss*

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Introduction

Horizontal hydraulic fracturing, 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 extracted via this method is expected in Canada by 2035 (Gagnon et al., 2015). Briefly, this process releases hydrocarbons from the geologic formation by injection of a highly-pressurized fluid into extraction wells. The composition of the injected fluid, 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 various chemicals formed during the process flows back to the surface. This wastewater returning 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 composition of the injected fracturing fluid, the time spent in the formation and the type of rock present at the drilling site (Stringfellow et al., 2014). Nevertheless, some components are common to all FPW, including salts, metals, organics and some radionuclides (Entrekin et al., 2011; Jiang et al., 2014).

The growth of the HF industry is, however, associated with environmental issues, particularly with respect to water abstraction and water quality. Of specific concern is the large volumes of water required for extraction using HF. 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. In fact, there have been numerous documented spills over the last decade, which have principally occurred 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 HF grows in magnitude, it is likely that threats of FPW contamination of waters will also grow (Teather and Parrott, 2006; US EPA, 2015). However, to date, there is a limited understanding of the toxicity of FPW to aquatic organisms.

To assess the environmental impact of industrial effluents such as FPW, it is important to select appropriate model species living in the habitats presenting a high risk of contamination. One such model is the rainbow trout (*Oncorhynchus mykiss*), a species native to North American freshwaters. This fish species is widely employed in toxicological studies, in part because it is highly sensitive to contaminants (Teather and Parrott, 2006), and because of this, and its relevance to North American waters, the rainbow trout is a key test species in many regulatory guidelines for the protection of aquatic environments (Environment Canada, 1990).

Recently, the first studies investigating the impact of FPW on aquatic biota have emerged. This research has shown that FPW exposure causes 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 evidence of an organic-associated effect on the metabolic responses of the rainbow trout and the zebrafish (*Danio rerio*), as well as on their development, fitness and swimming performance (He et al., 2017b; 2018; Folkerts et al., 2017a; b; Blewett et al., 2017a). However, some of these studies have also described an important effect of the salt component of FPW on toxicity. This effluent is highly saline, with overall salt content up to 8 times that of seawater. For example, Blewett et al. (2017b) showed that an acute exposure to FPW caused changes in the gill morphology of rainbow trout, similar to those seen in seawater-acclimated fish. However, the consequences of sub-chronic FPW exposure to fish have yet to be investigated.

The present study examines the long-term (28 day) exposure of juvenile *O. mykiss* to FPW, with a specific focus on ionoregulatory endpoints (e.g. plasma ions, osmoregulatory enzyme activities, gill histology; see section 1-5). To separate the effects of FPW exposure to saline and non-saline components, exposures were also conducted with a salt water (SW) solution, which closely matched the salt content of the raw FPW sample, but with none of the metals or organic components. This study utilized a raw FPW sample, diluted to 3% of its initial strength, based on the outcomes of an acute lethal exposure detailed herein. At this concentration, the salt content of exposure waters is below that at which physiological changes are expected as a result of salinity (i.e. 3.4 ppt, below the isosmotic point of 8-10 ppt), in this euryhaline species. Consequently, this approach allowed us to uncover any specific effects of the non-salt components (i.e. the metals and organics) of the FPW.

Material and methods

Animals

The Sam Livingston Fish Hatchery and Rearing Station (Calgary, Alberta, Canada) provided the juvenile rainbow trout which were maintained in the aquatic facility of the University of Alberta. Fish were held in a 120L tank containing flow-through moderately hard City of Edmonton dechlorinated water ($[Na^+]$ = 14.6 ppm, $[Ca^{2+}]$ = 55.9 ppm, $[Mg^{2+}]$ = 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 10 to 13˚C. Trout were acclimated in these tanks for 3 months before starting the experiment and were fed daily with crushed salmonid pellets (EWOS, Surrey, British Columbia, Canada) to 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.

FPW sample analysis

A 3-hour FPW sample from a hydraulically fractured well in the Duvernay Formation, Fox Creek, Alberta, Canada, was provided by Encana Corporation (December 2017). This is a sample representative of FPW returning to the surface three hours after the pressure in the well was released and the fluid began flowing back to the well head. This sample was collected at the well head, before separation of the oil and gas phases.

Before chemical analysis, the raw FPW was filtered through a 0.2 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 using inductively coupled plasma-double mass spectrometry (ICP-MS/MS) with an Agilent 8800 spectrometer, 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 6 µL of 15.7 N trace metal grade nitric acid per 10 mL of sample. To overcome the high TDS (total dissolved solids) of the FPW that persisted even after dilution, samples and standards were analyzed in high matrix mode in which aspirated samples were diluted with a flow 8 mL min^{-1} 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 high dilution required for 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.

Acute toxicity assay

The acute median lethal concentration, representing the concentration at which 50% mortality occurs (LC_{50}), 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 Edmonton City dechlorinated water. Six FPW concentrations were used to determine the LC_{50}: 2.5%, 5%, 10%, 15%, 20% and 25%. Three to four replicates were performed for each concentration. A single 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 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 (and 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.

Sub-chronic exposure

The exposure concentration for the sub-chronic test (3%) was chosen based on the acute LC_{50} value (11.6% FPW), thus representing 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. It is also important to note that given the availability of raw FPW sample and the total volume of exposure water needed over the course of the 28 d, 3% was the maximal concentration that allowed completion of the study using a single raw sample source.

A total of 96 juvenile rainbow trout (7.9 ± 0.5 g) 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 (Edmonton City dechlorinated water). The dilutions of FPW and SW were conducted using dechlorinated Edmonton City water. The SW solution consisted of laboratory salts (NaCl, $CaCl_2$, $MgCl_2$, KCl; Sigma Aldrich, Oakville, Ontario, Canada) added to nanopure water to replicate the salt concentrations present in the raw FPW sample (Table 3-1; see Blewett et al., 2017a). For each condition six replicates were performed, with four juvenile rainbow trout per replicate. Each replicate contained 4-L of the appropriate solution, in an 8-L acid-washed glass tank. Trout were fed to satiation with crushed salmonid pellets (EWOS, Surrey, British Columbia, Canada) every 2 to 3 days, and thereafter, a 50% water change was conducted which involved siphoning 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. 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 (12000 rpm, 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. Gills (2^{nd} gill arch, left side) were also collected for histological analysis (placed immediately in 4% paraformaldehyde (PFA)), and for biochemical analysis (snap frozen in liquid nitrogen, then stored at -80°C).

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). These analyses were performed using ICP-MS/MS. For sodium analysis, samples and standards were prepared in a matrix of 2% HNO_3, 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% HNO_3, 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.

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 Hg^{2+} and Fe^{2+} for 2,4,6-Tris(2-pyridyl)-s-triazine (TPTZ). The binding of Hg^{2+} to TPTZ displays no colour. However, in presence of chloride, Hg^{2+} precipitates in the form of $HgCl_2$ thus freeing TPTZ which now can bind to Fe^{2+}. The Fe-TPTZ complex exhibits a blue colour, the absorbance of which is proportional to the chloride concentration in solution. 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.

Plasma cations

Plasma ions and trace metals were analyzed by ICP-MS/MS following the protocol outlined in Gajek et al. (2013). Na^+, K^+, Ca^{2+}, Mg^{2+}, 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% NH_4OH w/v, 0.1% H_4EDTA 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.

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

Enzyme activities were measured according to the protocols of McCormick (1993) and Lin and Randall (1991), 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 loaded 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 $MgCl_2.6H_2O$; 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 NaN_3. 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.

Both of these assays work on the principle that ATP is hydrolyzed by the ATPases. This hydrolysis, sensitive to the presence of ouabain and NEM, is paired with the oxidation of NADH via the two enzymes PK and LDH. The difference between the ATP hydrolysis in the samples treated with and without inhibitor (i.e. ouabain or NEM) represents the gill enzymatic activity (i.e. NKA or H^+-ATPase). All enzyme activities were 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}.

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.

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, whereas the effects of FPW and SW on plasma trace element concentrations were tested via a one-way ANOVA. 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.

Results

Water chemistry

Analysis by ICP-MS/MS revealed a high concentration of salt ions in the raw FPW sample. Chloride (Cl) and sodium (Na) were the most concentrated, reaching 68 115 ppm and 47 484 ppm respectively; values 4-fold greater than the salinity of full-strength seawater. The calcium (Ca) concentration reached 6 936 ppm, whereas potassium (K; 1 598 ppm) and magnesium (Mg; 657 ppm) were the major ions present at the lowest concentrations. Of the trace elements measured in the raw sample of FPW analyzed, strontium (Sr; 723 ppm) and bromide (Br; 259 ppm) were the most concentrated. The complete chemical analysis is available in Table 3-1.

Acute toxicity

The acute 96-h LC_{50} value was determined to occur at a dilution representing 11.6% of the raw FPW sample (95% C.I. 10.14 – 13.14%) (Fig. 3-1). This value was used to help determine an exposure concentration for the 28-d sub-chronic exposure.

Sub-chronic exposure water chemistry

The exposure water chemistry is presented in Table 3-2. In terms of their ion content, the chemical composition of the two experimental treatments (3% FPW and 3% SW) were in close agreement. In both of these 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). Furthermore, as expected, the concentration of trace elements in 3% SW was lower than in 3% FPW.

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. 3-2). 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 seen, the level of plasma salt cations (i.e. Na^+, K^+, Ca^{2+}, Mg^{2+}; Fig. 3-3) 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^{2+} concentration from FPW-exposed fish displayed an effect of time (6-h *versus* 48-h samples; two-way ANOVA; $p = 0.025$; Fig. 3-3.C). No difference between time points were highlighted for Na^+, K^+ or Mg^{2+} (two-way ANOVA; $p = 0.176 – 0.464$; Fig. 3-3.A-B-D). No 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 days 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. 3-4) (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. 3-4.B). Although there was a trend of increasing boron (B; Fig. 3-4.B), cadmium (Cd; Fig. 3-4.D) and mercury (Hg; Fig. 3-4.D) concentration in the plasma of fish exposed to FPW compared to the two other groups, it was not 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. 3-4.A).

Enzyme activity

The activity of branchial NKA (Fig. 3-5.A) 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. 3-5.A). However, while the exposure treatment did not influence the H^+-ATPase activity (two-way ANOVA; $p = 0.910$; Fig. 3-5.B), 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.

Gill morphometrics

The effects of FPW and SW exposure on gill morphology are shown in Figure 3-6, 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. 3-7.A). In contrast, an effect of time was seen with respect to lamellar length (Fig. 3-7.B) and ILCM (Fig. 3-7.C) measurements (two-way ANOVA, $p < 0.001$ and $p = 0.009$ respectively). The lamellae became significantly shorter after 28 days in 3% FPW (89 ± 8.1 μm) compared to the fish gills exposed for 6 hours (130.9 ± 10.4 μm). The branchial length was

also reduced in the control group from 127.8 (± 13.1) μm at 6 hours to 94.9 (± 9.9) μm after 28 days. Additionally, a rapid change in ILCM was seen in the FPW-exposed *O. mykiss*. The ILCM of the trout exposed for only 48 hours 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 days 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. 3-7.D).

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

Element	Concentration (ppm)
TDS	150162
Na	47484
K	1598
Ca	6936
Mg	657
Cl	68115
Sr	723
S	100
Br	259
Ba	23
B	75
Li	35
Mn	6
Fe	167
TC	302
TN	272

Table 3-2. 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 days). Elements below detection limit in all treatments are not shown in this table.

Ion concentration (ppm)	Detection limit range	Control	3% SW	3% FPW
Na	0.653 - 21.7	20.4 ± 1.7	1411.4 ± 166.8	1330.6 ± 100.1
K	1.002 - 2.10	3.3 ± 0.2	51.5 ± 4.4	50.6 ± 2.8
Mg	0.0436 - 2.1	14.0 ± 0.2	35.3 ± 2.9	33.1 ± 0.9
Ca	0.88 - 2.02	38.4 ± 1.5	247.4 ± 24.5	259.7 ± 16.9
B	0.000485 - 0.12	1.0 ± 0.8	0.1 ± 0.05	2.1 ± 0.1
Ba	0.00052 - 0.0315	0.05 ± 0.01	0.1 ± 0.01	0.4 ± 0.03
Sr	0.0496 - 0.30	0.4 ± 0.2	0.6 ± 0.2	20.9 ± 0.6
S	0.0518 - 1.06	17.2 ± 1.0	18.9 ± 2.2	19.9 ± 1.3
Mn	0.000459 - 0.012	0.001 ± 0.0002	0.01 ± 0.002	0.2 ± 0.01
Fe	0.0097 - 0.106	BDL	BDL	0.6 ± 0.2
Br	0.0496 - 0.35	1.0 ± 0.5	2.1 ± 0.7	8.0 ± 1.6

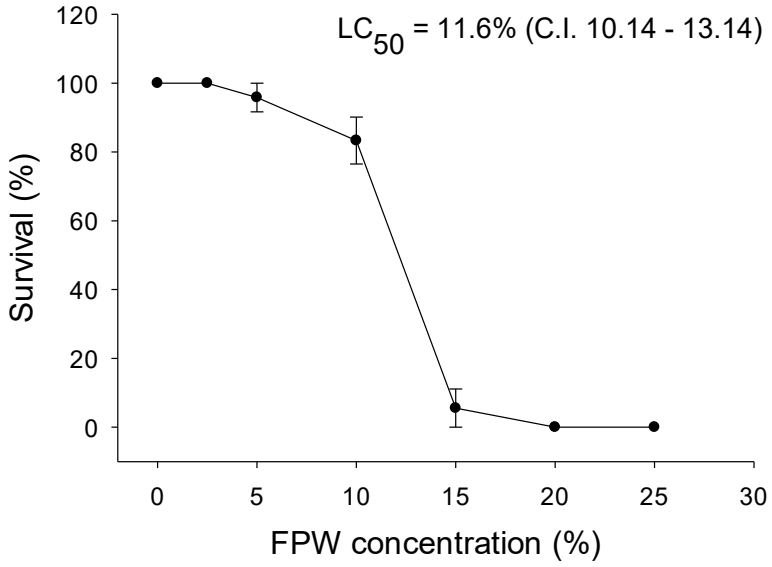

Figure 3-1. Percentage of 96-h survival of juvenile *Oncorhynchus mykiss* exposed to increasing concentration of raw sample of FPW. Error bars represent the mean (± SEM) of 3 to 4 replicates.

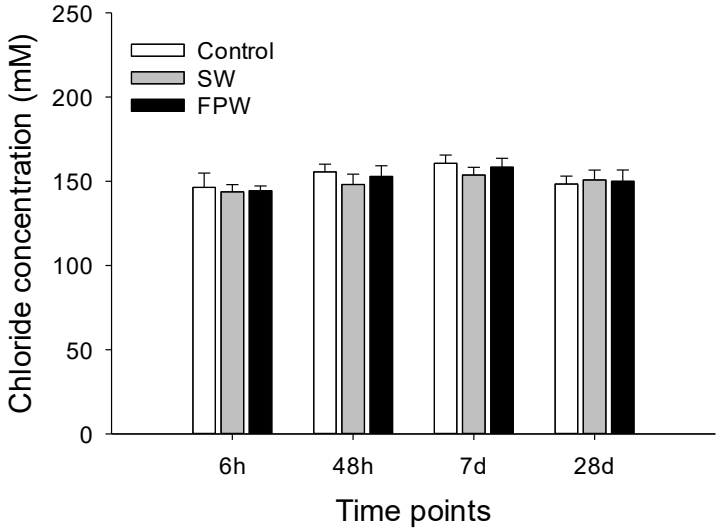

Figure 3-2. Chloride concentration in plasma sampled from juvenile rainbow trout exposed to control water, 3% SW and 3% FPW over the course of a 28-d exposure.

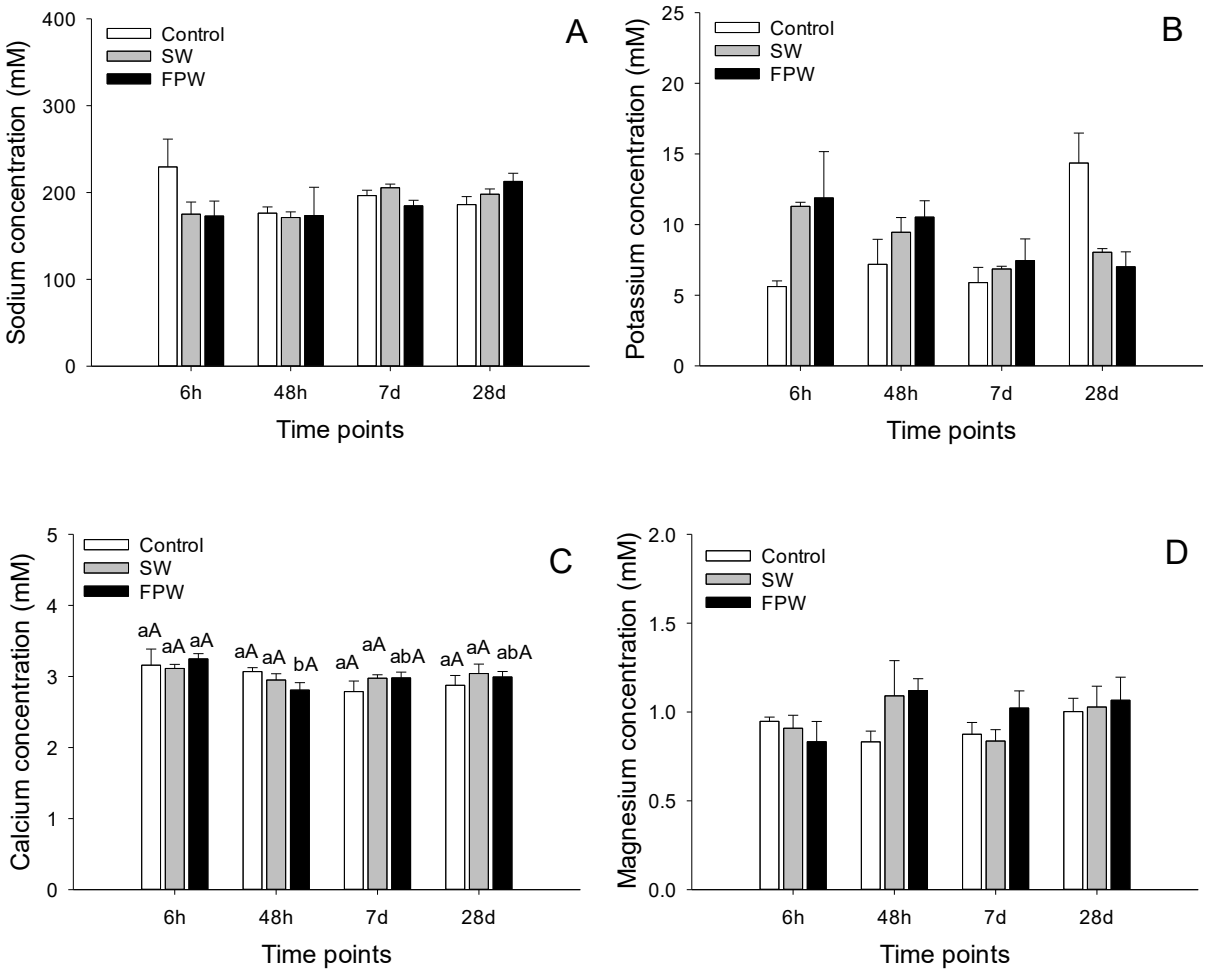

Figure 3-3. Salt cations in plasma sampled from juvenile rainbow trout exposed to control water, 3% SW and 3% FPW over the course of a 28-d exposure: sodium (A), potassium (B), calcium (C) and magnesium (D). Bars represent the mean (± SEM) of 3 to 4 replicates. Different lower-case letters represent difference between 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.

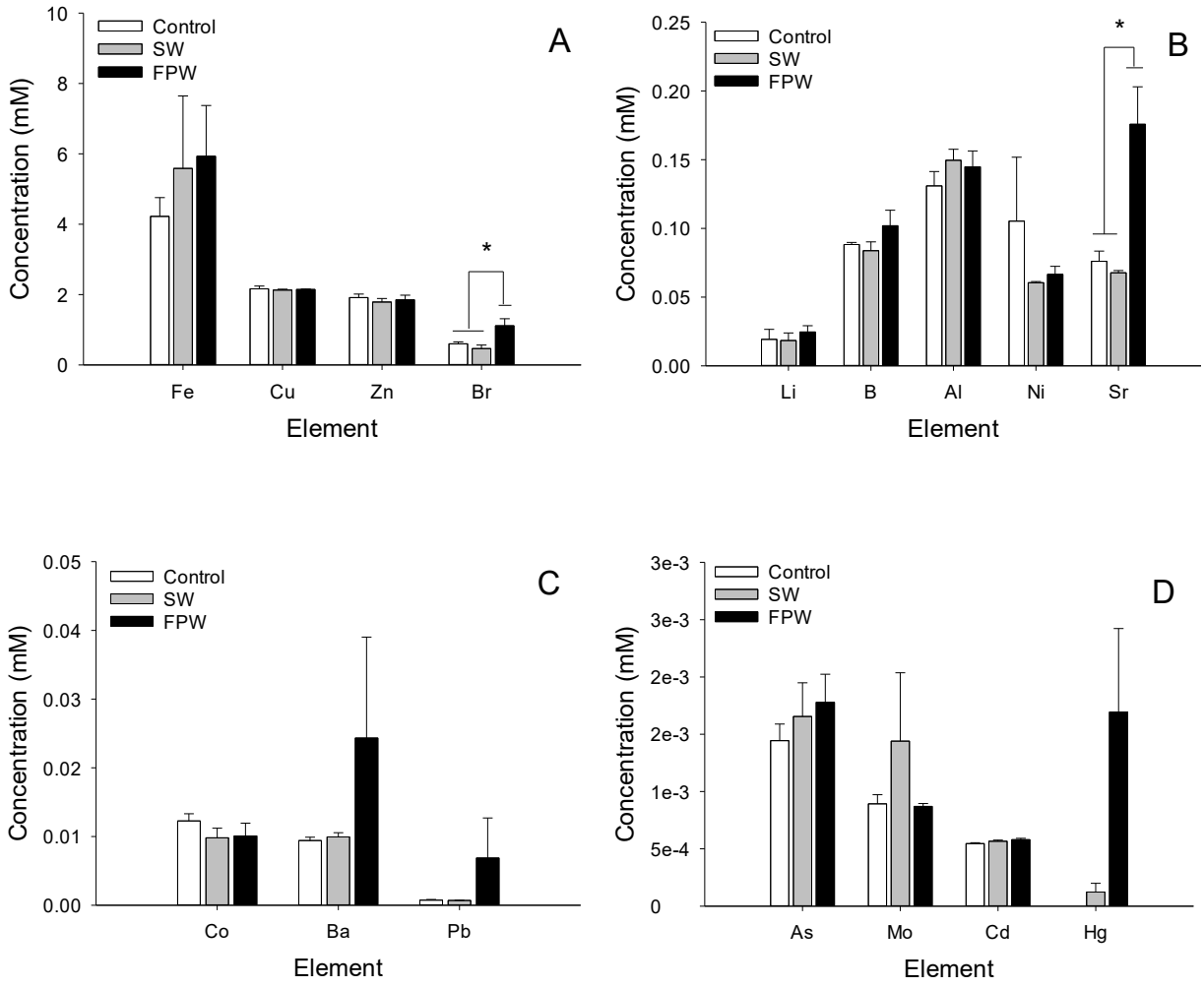

Figure 3-4. Trace elements concentrations (Fe, Cu, Zn, Br (A); Li, B, Al, Ni, Sr (B); Co, Ba, Pb (C); As, Mo, Cd, Hg (D)) in plasma sampled from juvenile rainbow trout exposed to control water, 3% SW and 3% FPW after a 28-d exposure. Bars represent the mean (± SEM) of 4 replicates. Asterisks represent significant differences between treatments (one-way ANOVA, post hoc Tukey's test; or Kruskal-Wallis test), at alpha = 0.05.

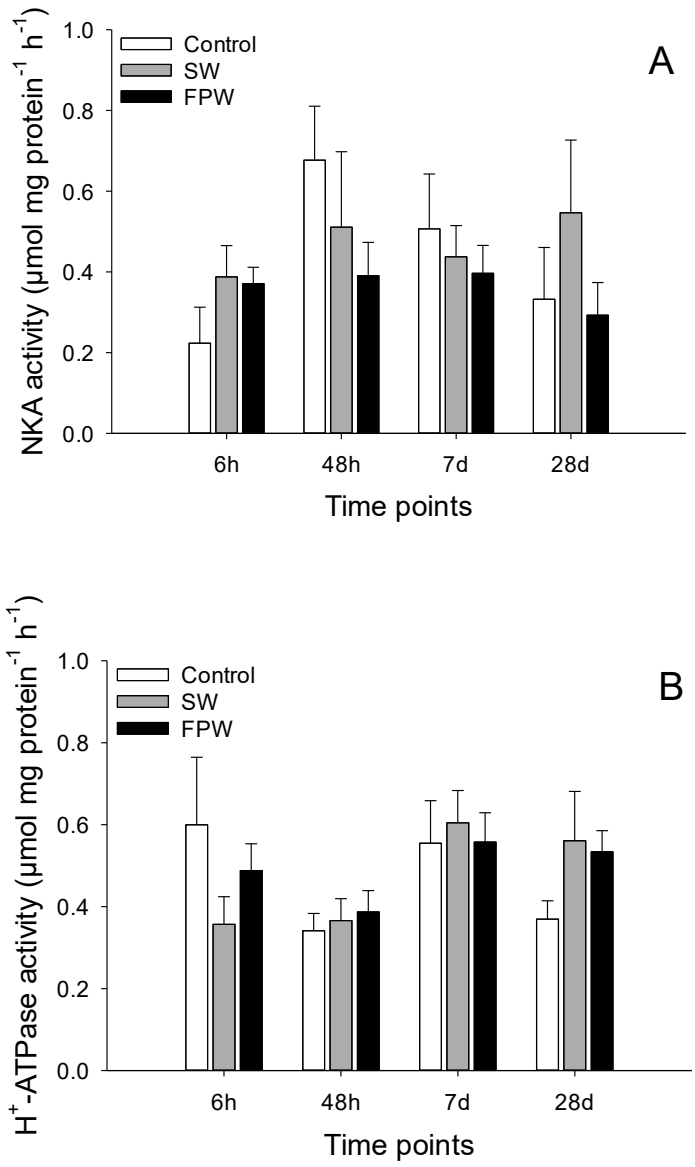

Figure 3-5. Branchial activity of Sodium-Potassium ATPase (NKA) (A) and H^+-ATPase (B) of juvenile rainbow trout exposed to control water, 3% SW and 3% FPW over the course of a 28-d exposure. Bars represent the mean (± SEM) of 5 to 6 replicates.

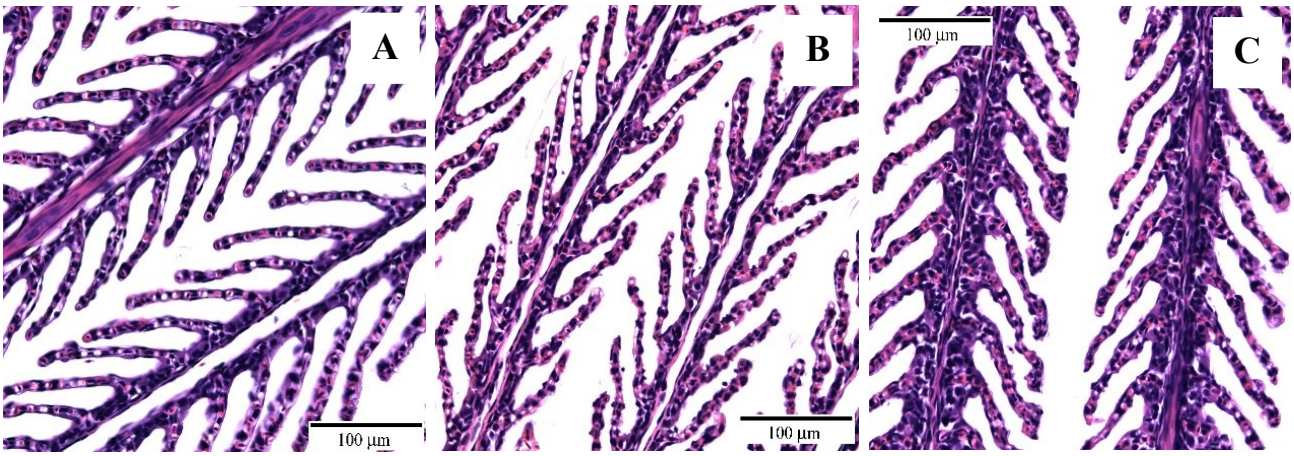

Figure 3-6. Micrographs of gill tissues stained with Hematoxylin and Eosin sampled from juvenile rainbow trout exposed to control water (A), 3% SW (B) and 3% FPW (C) for 48 hours.

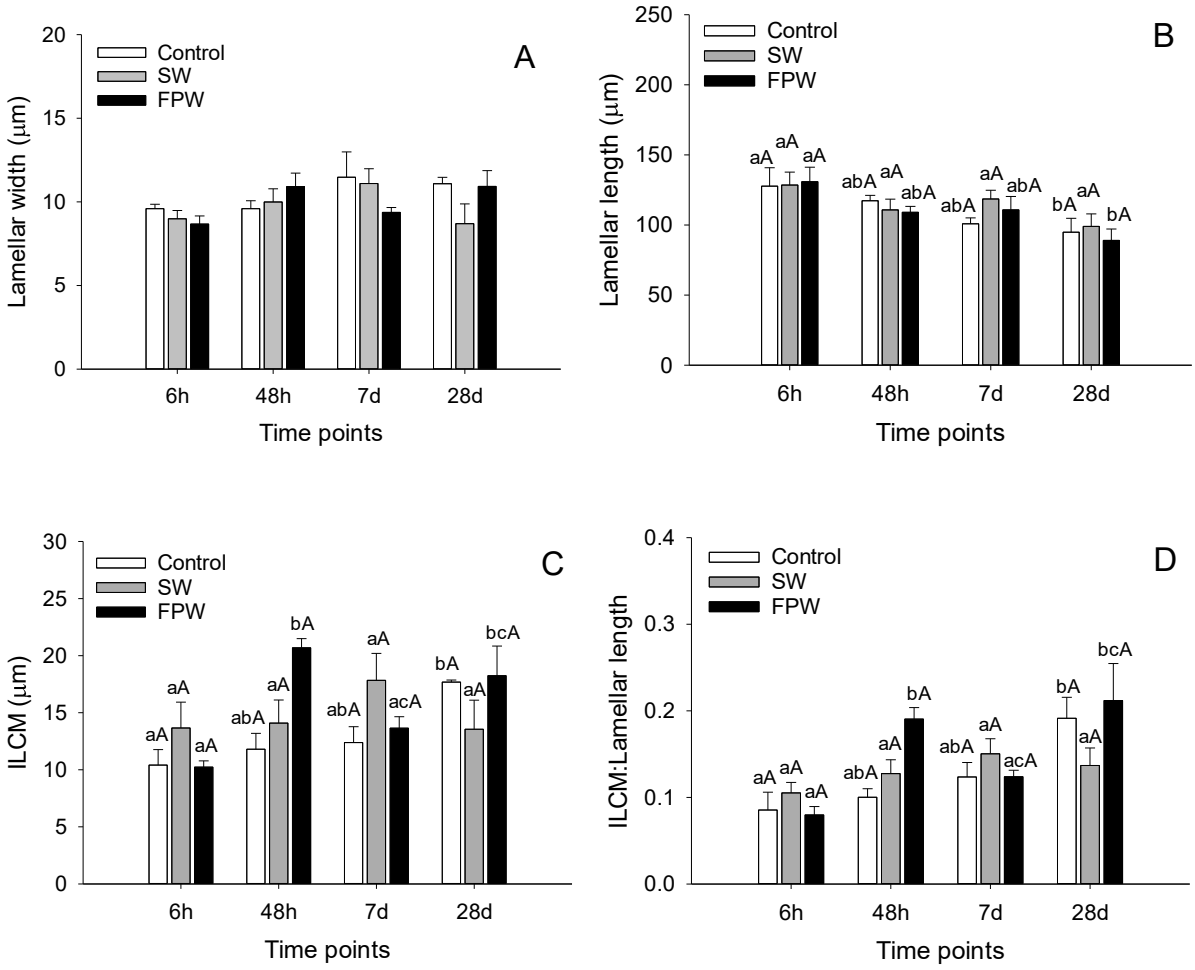
A
Control
SW
FPW
Lamellar width (μm)
0
5
10
15
20
6h
48h
7d
28d
Time points
B
Lamellar length (μm)
0
50
100
150
200
250
aA aA aA
abA aA abA
abA aA abA
bA aA bA
C
ILCM (μm)
aA aA aA
abA aA bA
abA aA acA
bA aA bcA
D
ILCM:Lamellar length
0.0
0.1
0.2
0.3
0.4

Figure 3-7. (Previous page) Effect of a 28-d exposure to control water, 3% SW and 3% FPW on the gill morphology of juvenile rainbow trout. Lamellar width (A), lamellar length (B), interlamellar cell mass (ILCM) (C) and ratio of ILCM to lamellar length (D) 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 between 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

Discussion

Sub-chronic exposure to low levels of FPW had little impact on ionoregulatory parameters in the rainbow trout. Overall, the gill morphology was modified over the course of the 28 days of exposure, but there were no changes that could be 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), can be attributed to the 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.

Plasma ions and trace elements

When freshwater fish are transferred to seawater, osmotic stress commonly results in an immediate increase in plasma ion levels, although this increase is rapidly corrected by regulatory mechanisms. If the fish remain in saline waters a higher baseline ion concentration relative to freshwater is usually the result (Nordlie, 2009). In the current study plasma salt ion concentrations (Fig. 3-2 and 3-3) 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 literature evidence showing that rainbow trout can rapidly adjust plasma ion concentrations in response to acute salinity change. For example, even though an increase of Na^+ and Cl^- in the plasma occurs in rainbow trout following a 24 h exposure to 50% seawater, the fish were able to efficiently reduce the concentrations of these ions back to levels close to freshwater

control groups by 96 h (Blair et al., 2016). Similarly, *O. mykiss* are able to reduce their plasma Na^+ and Cl^- concentrations back to levels not significantly different from baseline concentrations even after 30 days of exposure to 100% seawater (Bystriansky et al., 2006). However, our study provides no evidence that the salinity of either the 3% FPW and 3% SW exposures were sufficient to induce even an initial disturbance in plasma ion homeostasis. Even at 6 h, a timepoint at which plasma measurements may be expected to reflect ion dysregulation induced by salinity exposure owing to delayed onset of ion regulatory mechanisms, the levels of Na^+, K^+, Cl^-, Ca^{2+}, and Mg^{2+} remained unchanged in treatment groups relative to the time-matched control.

This lack of effect of FPW/SW exposure on plasma ions is 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, which is determined to occur between 8 and 10 ppt (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 isotonic point (i.e. 9 ppt), whereas freshwater fish exposed to hyperosmotic water (i.e. 18 ppt), exhibited significant fluctuations in plasma ions. Hence, our present results suggest that plasma salt ion regulation in rainbow trout would not be impacted by the salinity found in 3% FPW. 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, even though this sub-chronic exposure concentration represents a value similar to the acute LC_{10}.

Patterns of trace element 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. 3-4). The exceptions to this

general pattern were for bromide (Br) and strontium (Sr). Little literature exists on the effect of Sr on fish. However, Sr is known to be a Ca mimic, and thus is thought to exert toxic effects through this mechanism (Chowdhury and Blust, 2011). This mimicry also explains the accumulation of Sr in the fish body. 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 high toxicity to rainbow trout, with a 28-d LC_{50} of 0.2 mg/L for rainbow trout eggs exposed from fertilization through 4 days 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 mg/L; Table 3-2). The reasons for this discrepancy are unknown but likely related to either the stage of exposure (eggs *versus* juvenile rainbow trout), or the complexity of the exposure media mitigating the toxicity. Given that Sr is known to interfere with Ca homeostasis, Sr did accumulate in the plasma of 3% FPW-exposed fish. There was a slight decrease in Ca plasma concentration between the fish exposed to FPW for 6 h and 7 d (Fig. 3-3.C), 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 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.

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 mg/L) NaBr a

concentration ~ 10 times higher than our 3% FPW exposure (8.0 ± 1.6 mg/L). In their study, after 14 days, plasma bromide reached concentrations of 51 mM. 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. 3-4.A). 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-Menten affinity constant of the transporter involved, resulting in negligible transport. It is unlikely that the Br accumulation observed in the plasma of FPW-exposed fish has significant toxicological consequences, as this element has been associated with a low toxicity to freshwater aquatic organisms in the literature (Alexander et al., 1981; Canton et al., 1983; Wester et al., 1998). 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 mg/L (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.

Ionoregulatory enzyme activity

As mentioned in the general introduction (Section 1-5), 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 days of exposure to seawater. However, in the current work, 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. 3-5), an outcome consistent with a lack of change in plasma major ions.

The lack of effect in the 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 instance, an exposure to 25 μg Cd/L for 35 days or to 10 μg Ag/L for 48 hours (Wendelaar Bonga and Lock, 1991; and Morgan et al., 1997 respectively) induced a decreased NKA activity in freshwater teleost fish. It is, however, possible that an increase in NKA, in response to salinity, was counteracted by an inhibitory effect of the metals present in the FPW. Arguing against this, is that there was no effect of NKA activity seen in the SW control, which was relatively free of trace elements. Furthermore, although elevated, the concentrations of the metals known to exhibit the strongest inhibitory actions against NKA (e.g. Cu, Ag) were still 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) held inanga (a euryhaline galaxiid fish) to elevated salinities for 7 days 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.

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, 1985; Evans, 1987). Histological analysis of the branchial tissues in the current study showed that FPW impacted the gill morphology of the rainbow trout over time. Throughout the course of the 28-d exposure, a decrease of the lamellar length and an increase of the ILCM could be observed, leading to an overall increase of the ratio ILCM to lamellar length (Fig. 3-7.B-D). However, those changes were never statistically different from the control and SW-matched control treatment groups. These results are opposed to observations made by Blewett et al. (2017). In this previous research, rainbow trout were exposed to a 10-day FPW sample originating from the same formation as the effluent tested in the present study (i.e. Fox Creek, Duvernay formation). 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 wastewater and proposed to be an indicator of a hyperosmotic stress. The different outcomes between these two studies are likely due to the different salinities of the raw and exposure samples. Blewett et al. (2017) exposed fish to a 10-day FPW sample whereas a 3-hour sample with a lower salinity was used in our study. For the former research, FPW was diluted to 7.5% of its original concentration, corresponding to a salinity of 13.5 ppt, while a 3% dilution (~ 3.4 ppt) was used in the current research.

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 unknown concentrations of FPW for a month, after a spill occurred in the environment. In those fish, they observed branchial lesions and hyperplasia attributed to the presence of metals (i.e. Al and Fe) in the wastewater. These data were consistent with other studies that have shown gill morphological changes, such as hyperplasia (i.e. cells filling the interlamellar spaces) and lesions of lamellae (review by Evans, 1987), in response to metal exposure (e.g. Al, Fe, Hg, Cd) (Mueller et al., 1991; Pratap and Wendelaar Bonga, 1993). There are also studies suggesting that Br (at least in its methylated form) can cause morphological changes in the gill. After a 4-d exposure to methylbromide concentrations as low as 1 mg/L (i.e. significantly lower than the 8 mg/L total Br concentration measured in 3% FPW), hyperplasia and swelling was noted in medaka (*Oryzias latipes*) and guppy (*Poecilia reticulata*) gills (Wester et al., 1988). Given that we did not measure the speciation of Br in our FPW, it is possible that brominated FPW components are responsible for the temporal changes in gill morphology that were observed here for rainbow trout. However, these effects were insufficient to cause morphological differences relative to control fish.

It is possible that differences in responses to FPW depend on the species being examined. Indeed, in their field study Papoulias and Valesco (2013) studied two exclusively freshwater fish, whereas an euryhaline species was used in the current work. An euryhaline species may simply have more refined mechanisms for limiting morphological changes associated with FPW salinity. Supporting this, Blair and colleagues (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 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. They performed gill histological analysis and measured the ILCM:lamellae length. Rainbow trout gill morphology was not impacted by salinity, whereas the Arctic grayling demonstrated an increased ILCM:lamellae length following exposure This study, therefore, showed that effects of fish to salinity associated with FPW exposure can also be dependent on the species being examined.

One potential explanation as to why there was a lack of effect of FPW and SW on gill morphology in rainbow trout is that there were changes in gill morphology occurring throughout the exposure in the control fish (Fig. 3-7.B-D). This may be explained by a difference in feeding behaviour. Although feeding was not quantified during the course of the 28 days of exposure, it was observed that control fish fed more than SW or FPW-exposed fish. This may have resulted in higher ammonia levels in the water, potentially resulting in the observed changes in gill morphology over time in this group (Sinha et al., 2014). Such an effect may have rendered the time-dependent changes in FPW gill morphology non-significant relative to the control fish gills.

Conclusion

The exposure scenario in this study was chosen to mimic an environmental spill of low-level of the FPW over a long period of time (i.e. 3% FPW over 28 days), a constancy of exposure similar to that which may be experienced in a pipeline leak into a small river, pond or lake. While, it is difficult to assess the average FPW volume spilled in real-world scenarios, and 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 of the wastewater.

This study is the first to investigate the specific impact of FPW salinity following sub-chronic exposure to 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 days. 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. For example, a paired feeding study coupled with measurement of ammonia in exposure waters could be insightful, owing to the suggestion in this study that feeding rates, which can alter gill morphology (Alix et al., 2017), may have differed between exposure treatments. 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.

Chapter 4: General conclusion

General summary

Within the past few years, the first studies have been published that seek to understand the effect of the hydraulic fracturing wastewater on aquatic biota, and the mechanism by which this toxicity is mediated. The goal of this thesis was to investigate the impacts of FPW on two key freshwater model organisms. Within this thesis, I have established the effect of FPW on the behaviour of the water flea *Daphnia magna*. I have also revealed the effects of a sub-chronic FPW exposure on ionoregulatory endpoints in the rainbow trout *Oncorhynchus mykiss*. This general conclusion will first summarize the main findings of my thesis, then I will introduce some future directions for my research.

Daphnia magna and phototaxis

In my thesis, I analyzed the phototactic behaviour of *Daphnia magna* in control OECD water and in presence of FPW and SW, either after exposure of naïve organisms or following a 24 and 96-h pre-exposure. I observed a disruption of behaviour in both cases.

The first part of this study consisted of an acute exposure of naïve individuals to high concentrations of FPW and SW, with measurement of behavioural responses immediately after the initiation of the exposure. These data revealed an aversion response to FPW and SW in adult *D. magna*, as seen by the increase in swimming speed of the organisms exposed to increasing concentration of solutions (up to 40% FPW and 20% SW). As the response was very similar in terms of its threshold and magnitude for both FPW and SW, it was likely driven by the salt component of the exposures. More specifically, Ca^{2+} may be involved in this response, as this is known to impair the chemosensory system of fish (Dew and Pyle, 2014). A direct exposure to

increasing FPW levels mimics the onset of a large spill, for example from a leaking pipeline or from a truck during FPW transportation.

The second component of the *Daphnia* behavioural study aimed to investigate the consequence of a pre-exposure to low-levels of FPW and SW, with subsequent testing of the behavioural response in control conditions and sought to determine whether daphnids could acclimate to such pre-exposure. *Daphnia* displayed a loss of aversion response following a 24 and 96-h pre-exposure to FPW and SW. The comparison between FPW and SW treatments allowed for the distinction between the specific effect of FPW salinity and the potential effect of the non-salt components present in FPW. More particularly, adult daphnids exposed to 1.5% FPW for 24 h presented a significant decrease of their swimming speed when tested in OECD water compared to control organisms acclimated in control water. This finding suggests that adult *D. magna* undergo an inhibition of their basal phototactic response, rather than an incapacity to detect the toxicants (i.e. chemosensory defect). This effect could be mediated by FPW such as Cd and Cu, which are known to disrupt *D. magna* neural function, leading to an impairment of their phototaxis comparable to the one observed in the present study (Baillieul and Blust, 1999; Untersteiner et al., 2003). However, that this effect was also observed in SW exposures, it suggests that the effect is more likely to be mediated by a salt component of the effluent.

Furthermore, I tested the phototactic behaviour of pre-exposed daphnids in presence of 10% FPW or SW in the test chamber. Compared with the pre-exposed organisms tested in OECD water, this experimental series was used to determine if acclimation of *D. magna* to the effluent could occur. In 10% FPW, *D. magna* pre-exposed to 1.5% FPW for 24 h did not display the hypoactivity response noted when pre-exposed animals were tested in the absence of FPW in the observation chamber. This null effect indicates that pre-exposed daphnids tested in FPW are still

able to detect and react to the presence of this effluent, displaying a swimming speed greater than that of pre-exposed daphnids tested in OECD water only. This result supports the hypothesis formulated previously: FPW impairs the basal phototactic behaviour of *D. magna*. Moreover, I also defined a specific effect of the organisms to FPW salt content. Indeed, when tested in 10% SW, adult *D. magna* pre-exposed to 1.5% SW for 24 h displayed a significant inhibition of phototaxis and a complete loss of aversion response, a sign of an acclimation to the salts (Baillieul et al., 1998). These data provide evidence that salt and non-salt components (i.e. organics and metals) of FPW may present two different mechanisms of action on the phototactic behaviour of *D. magna*. Further investigations are necessary to fully assess of the mechanisms of the organic and metal (e.g. Cd and Cu) components of FPW on *Daphnia* behaviour.

Rainbow trout and ionoregulatory physiology

The second part of my thesis examined the sub-chronic impact of FPW on a freshwater fish, the rainbow trout *Oncorhynchus mykiss*. The goal of this chapter was to reproduce a scenario of an environmental spill into a low-flow freshwater, subsequent to a leak from a pipeline. This study focused on the potential ionoregulatory disruption caused by FPW. To delineate between effects associated with salinity, and those associated with metals and organic constituents, exposure occurred to a salinity below the isosmotic point (8 – 10 ppt), where changes related to salinity would not be expected. Furthermore, studies were conducted with a SW control, which matched the salt content of the FPW, but without the metal and organic components. Overall, I found that *O. mykiss* displayed few significant changes related to either SW or FPW, during an exposure representing a dilution of an FPW sample to 3% of its raw concentration over 28 days.

Following the sub-chronic exposure, plasma analysis revealed little change in ion and trace element concentrations. Only Sr and Br were accumulated in the plasma of FPW-exposed fish at concentrations greater than those of the time-matched controls. Furthermore, the activities of the two osmoregulatory enzymes studied (NKA and H^+-ATPase) remained unchanged throughout the 28 days of exposure. Therefore, a salinity of ~ 3.4 ppt was suggested to be insufficient to disrupt fish ion homeostasis and thus trigger changes in plasma ions or enzyme activity, physiological modifications commonly observed to result from an osmotic stress (Morgan and Iwama, 1991; Urbina and Glover, 2015).

Branchial remodeling was observed throughout the 28-d exposure in fish exposed to 3% FPW (i.e. decrease of lamellar length and increase of ILCM). However, these observations were never significantly different from the control and SW-matched control. This lack of significant effect may have been due in part to changes that also occurred over time in the control fish. It therefore remains possible that trace element components of the FPW could alter gill morphology. Notably, the methylated form of Br, an element that accumulated significantly in rainbow trout plasma in the current work, has been shown to induce modification of gill morphometry (Wester et al., 1988). Nonetheless, few studies exist on the impact of these trace elements on fish, and more investigations are necessary to assess the risk associated with such exposure and determine the mechanism of toxicity of these elements.

This study showed that a low FPW salinity level does not result in an osmotic stress on the rainbow trout *O. mykiss*. This will help guide future studies that can minimize the influence of the salinity component of FPW as a factor driving toxicity and examine the sub-chronic effects of metals and organics in whole FPW effluents on other physiological systems and toxic endpoints (e.g. oxidative stress, endocrine disruption).

Future directions

My thesis establishes, for the first time, that FPW exposure induces changes in the phototactic behaviour of *Daphnia magna*. These changes were in part attributed to FPW salinity, however, an additional impact of non-salt components (i.e. metals and organics) could be observed following pre-exposure to the effluent. Further investigations could involve the study of the specific effect of metal and organic components present in FPW to better understand the mechanisms of action of the fluid on *Daphnia* phototaxis.

The study of a 28-d sub-chronic exposure to FPW and SW on the rainbow trout, *Oncorhynchus mykiss,* revealed that low salinity levels did not disturb the ionoregulation of the fish. However, a significant accumulation of Sr (20.9 ± 0.6 mg/L) was seen in the plasma of FPW-exposed fish at concentrations that are known to be lethal (Birge, 1978). Nevertheless, no mortality was observed throughout the exposure. It is possible that the complexity of the FPW mixture played a role in mitigating the Sr toxicity towards the fish, but this is an interesting avenue for investigation.

Furthermore, I determined that no osmoregulatory disturbance was observed in rainbow trout following a long-term exposure to 3% FPW. However, previous research has shown evidence that acute exposure to FPW causes endocrine disruption and oxidative stress in fish; effects driven by the organics and metals contained in the wastewater (He et al., 2017b; Blewett et al., 2017a). Thus, future studies are required to examine these endpoints following a sub-chronic FPW exposure.

Finally, one key aspect of the current work was that there were changes in the gill morphology over time in control groups, which may have obscured significant changes related to

FPW exposure. I hypothesized that this could be a consequence of feeding behaviour, in that control fish appeared to have higher feeding rates, which may have led to higher waste excretion, which in turn could have altered gill morphology (Sinha et al., 2014; Alix et al., 2017). Studies that are monitoring the feeding behaviour of control, SW and FPW-exposed fish during a long-term exposure will be important to determine whether this is the case and to determine whether feeding behaviour is an endpoint that is sensitive to FPW exposure.

Aquatic organisms are tightly linked to each other by their position in the food web and if one species is perturbed by environmental stress (e.g. presence of toxicants), the whole ecosystem might be impacted. For example, rainbow trout largely feed on zooplankton, including *Daphnia*, and the abundance of these prey has been correlated to the growth of the fish (Tabor et al., 1996). Organisms occupying lower trophic levels are smaller in size and are usually associated with a higher sensitivity to contaminants. I have observed a disturbance of *D. magna* phototactic behaviour due to FPW salinity. This effect could lead to a perturbation of their vertical migration and consequently alter their position in the water column. On the other hand, rainbow trout did not display negative effects following a sub-chronic exposure to low FPW salinity level. Hence, it would be interesting to study the global consequences of FPW on the food web. Even though fish might not be impacted by the presence of low levels of FPW in a given water, even over sub-chronic exposure periods, they might be indirectly affected by a decrease or redistribution of prey populations.

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