After the spill: The mechanisms behind recovery and exposure to flowback and produced waters in *Daphnia magna*

by

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A thesis submitted in partial fulfillment of the requirements for the degree of

Master of Science

in

Physiology, Cell and Developmental Biology

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Abstract

Hydraulic fracturing for unconventional oil and gas extraction produces a complex wastewater known as flowback and produced water (FPW). FPW effluents contain inorganic constituents (e.g., Na, Ca, Mg, Zn, Cu, Cl) and organic constituents (e.g., surfactants, proppants, polycyclic aromatic hydrocarbons). These FPW effluents are often transported away from their well of origin for reuse or disposal, creating the possibility of large FPW spills. The possibility of FPW spills entering freshwater environments is of particular concern, with the potential to cause long term damage to exposed organisms. Previous research has shown that FPW can cause impairments in reproduction and molting in *Daphnia magna*, a freshwater invertebrate, after a chronic sublethal exposure to FPW. However, recovery from FPW in this species has not been extensively studied and a species unable to return to pre-stressor conditions can have drastic impacts on a population and the ecological scale.

Thus, this thesis investigated recovery within a single generation after an acute 48-hour FPW spill and in multiple generations after a chronic 21-day spill. Recovery was evaluated by endpoints including time to first brood, neonate output, and molt production returning to control pre-exposure levels. It was hypothesized that recovery can occur in *Daphnia magna* but full recovery from initial FPW exposure would require more than a single generation.

The first objective of my thesis was to understand if a 19-day period in clean water resulted in sufficient recovery from an acute 48-h FPW spill by pairing physiological endpoints with molecular analyses. We found reproduction and molting significantly impaired, with a mean value of 12.4 ± 4.6 neonates and 2.5 ± 0.6 molts produced per daphnid after exposure to 0.75% FPW, compared to the control daphnids that produced 66.2 ± 4.9 neonates and 7.5 ± 0.3 molts per replicate. Systems-level quantitative proteomic analyses demonstrated extensive perturbation of

metabolism and protein transport in both 0.25% and 0.75% FPW treatments after a 48-h FPW exposure. Collectively, our data indicate that even an acute 48-h exposure renders recovery of *Daphnia magna* impossible in the first generation.

The second objective of my thesis was to investigate if recovery from a chronic 21-day exposure to FPW was possible in four generations of *Daphnia magna*. Four generations were studied as the F_3 generation was the first unexposed germ line to the F_0 FPW exposure. To understand recovery, F_0 *Daphnia magna* were exposed to 0.1% FPW for 21-day and then placed in clean water for the F_1-F_3 generations (recovery group). This treatment group was compared to a chronic FPW exposure group where all four generations were exposed to 0.1% FPW. Overall, time to first brood was delayed initially in the FPW exposed F_0 (recovery and chronic). The time to first brood was 12.3 ± 0.5 days for the chronic group and 12.0 ± 0.4 days for the recovery group, ~2.5 days later than the control group at 9.6 ± 0.2 days, but no differences between neonate and molt production were found. No differences were found in the F_1 and F_2 generations between any of the three treatments. Meanwhile, the chronic F_3 generation had a time to first brood of 11.7 ± 0.7 days compared to the control at 9.8 ± 0.3 days.

This thesis gives insight into the potential recovery from FPW exposure in the key indicator species, *Daphnia magna.* After as little as 48 hours of FPW exposure, *Daphnia* were unable to recover, likely due to latent mortality. This emphasizes the importance of preventing spills, rather than reactive cleanup measures to best protect freshwater ecosystems. Notably, after a chronic exposure to FPW, *Daphnia* were able to recover by the F_1 generation, but will face delayed maturation time if the spill is not remediated by the F_3 generation. This indicates that spill remediation should be completed as soon as possible to prevent future organisms from having an adverse response to continued FPW exposure, after an initial spill.

Preface

This thesis is an original work by Ivy Luu. Chapter 2 of this thesis is in submission to Environmental Science and Technology as I. Luu, A. Boyd, D. Mehta, S. P. Myers, K. R. Shivakumar, K. Snihur, D. S. Alessi, M. C. Rodriguez, H. Veilleux, R. G. Uhrig, and T. A. Blewett, "Persisting effects following an acute exposure to flowback and produced waters in *Daphnia magna*." I. Luu was responsible for data collection, analysis, and manuscript composition. A. Boyd and S. P. Myers assisted in the data collection. K. R. Shivakumar and K. Snihur from D. S. Alessi's laboratory had conducted the ICP-MS analyses. D. Mehta and M. C. Rodriguez from R. G. Uhrig's laboratory and H. Veilleux assisted in the data analysis for the proteomics section. T. A. Blewett was the supervisory author and was involved with concept formation and manuscript composition. All co-authors contributed to editing the manuscript.

Chapter 3 of this thesis, "A multigenerational study on the recovery of Daphnia magna exposed to flowback and produced water" has not been previously published. I. Luu was responsible for data collection, analysis, manuscript composition and editing. H. Lowes helped with data collection and editing. K. Snihur in the laboratory of D. S. Alessi had conducted the ICP-MS analyses. T. A. Blewett was the supervisory author, contributed the concept, and edited/co-wrote the MS.

Acknowledgements

Firstly, I would like to thank my supervisor Dr. Tamzin Blewett for being the most supportive supervisor a person could ask for. I really appreciate her endless dedication and compassion to her students. Tamzin's support has pushed me to secure funding, present at a handful of conferences, travel to Belgium and learn new skills, expand my network, flourish my CV, and further ignite my passion for science. I think my growth under her supervision has been exponential. Not only have I become a better scientist, I think I have learned to be a better functioning human being.

Secondly, I would like to thank my family for their continuous support, with special mention to my mother, Phung Chanh Luu, who has brought me into this world and to my husband, Alexander James Baker, for keeping me in it. Special thanks to my cousin Kevin Lien for not only checking on my mental health while I devolved into madness writing this thesis, but also for going above and beyond with making sure this thesis is more coherent.

Thirdly, I am grateful to the friends I have made on this academic journey who have kept me as sane as I could be doing a MSc during a 2 year pandemic. Thank you Anika Cyr, Christina Lummer, Abigail Robinson, Christiana Frost, Robert Griffin, Hannah Lowes, Sienna Overduin, Aaron Boyd, Connor Stewart, Arash Shahriari, Troy Locke, Tad Plesowicz, Lauresha Kepuska, Mike Barteski, and Ryan Lewis.

Of course, I have been supported by my friends, mentors, and peers outside of the University of Alberta. But I would like to give honourable mentions to my friends Joy Roasa and Norman Tran for their tremendous feedback on how to improve my writing and helping me edit parts of my thesis with exceptional turnaround time.

Lastly, during my MSc, I have been lucky enough to receive scholarships and awards to help keep me from being hungry and homeless. I would like to acknowledge that my research was funded by the Alexander Graham Bell scholarship from NSERC and the Alberta Graduate Excellence Scholarship. Thank you.

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List of Abbreviations

µg/L	micrograms per litre
µL	microliters
ACN	acetonitrile
AER	Alberta Energy Regulator
AGC	automatic gain control
Al	aluminum
ARO	Aquatic Research Organisms
ATP	adenosine triphosphate
Ba	barium
BCA	bicinchoninic acid
Bi	bismuth
BLAST	Basic Local Alignment Search Tool
Br	bromine
Ca	calcium
$CaCl_2 \bullet 2\ H_2O$	calcium chloride dihydrate
CAPP	Canadian Association of Petroleum Producers
CCM	complex chemical mixture
Cl	chloride
Cu	copper
DDA	data dependent acquisition
DNA	deoxyribonucleic acid

DTT	dithiothreitol
EROD	ethoxyresorufin-O-deethylase
EtOH	ethanol
F_0	parental generation
F_1	first filial generation
F_2	second filial generation
F_3	third filial generation
FA	formic acid
FDR	false discovery rate
Fe	iron
FPW	flowback and produced water
Ge	germanium
GST	glutathione S-transferase
GO	gene ontology
GOBP	gene ontology biological processes
HCD	higher-energy C-trap dissociation
HEPES-KOH	(4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) – potassium hydroxide
HNO_3	nitric acid
IA	iodoacetamide
ICP-MS/MS	inductively coupled plasma mass spectrometry/mass spectrometry
In	indium
K	potassium

KCl	potassium chloride
KEGG	Kyoto Encyclopedia of Genes and Genomes
L	litres
LC50	concentration inducing 50% mortality in the population
LC MS/MS	liquid chromatography mass spectrometry/mass spectrometry
Lu	lutetium
Mg	magnesium
Mg/L	milligrams per litre
$MgSO_4 \cdot 7\ H_2O$	magnesium sulfate septahydrate
MS	mass spectrometry
m/z	mass to charge
n	sample size
N_2	nitrogen
Na	sodium
NaCl	sodium chloride
$NaHCO_3$	sodium bicarbonate
NPRI	National Pollutant Release Inventory
OECD	Organization for Economic Cooperation and Development
PAH	polycyclic aromatic hydrocarbons
pCi/L	picocuries per litre
PFAS	perfluoroalkyl and polyfluoroalkyl substances
PSM	peptide-spectrum match
ROS	reactive oxygen species

S	sulphur
Sc	scandium
SDS	sodium dodecyl-sulfate
SEM	standard error of the mean
SMPD	sphingomyelin phosphodiesterase-like protein
Sr	strontium
TFA	trifluoroacetic acid
TDS	total dissolved solids
U	uranium
UOG	unconventional oil and gas
U.S.A.	United States of America
U.S. EIA	United States Energy Information Administration
U.S. EPA	United States Environmental Protection Agency
VOC	volatile organic compound
v/v	volume per volume
w/v	weight per volume
WIPI2	WD repeat domain phosphoinositide-interacting protein 2
YCT	yeast/cerophyll/trout chow
Zn	zinc
$ZnSO_4$	zinc sulfate

Chapter 1 – Introduction

Human processes have led to anthropogenic effluents entering aquatic environments. Anthropogenic pollutants have the potential to affect the health of individuals and whole ecosystems, ultimately leading to biodiversity loss (Groh et al., 2022). Pollutants, such as oil and gas effluents can enter the environment intentionally (e.g., as anti-dusting or for agricultural use) (Tasker et al., 2018) or unintentionally via sewage effluents (Annevelink et al., 2016) and spill releases during transport and clean up (Barron et al., 2020). Common pollutants of concern entering freshwater ecosystems include, road tire wear, polycyclic aromatic hydrocarbons (PAHs) from diesel range organics, forever chemicals like perfluoroalkyl and polyfluoroalkyl substances (PFAS), metal contaminants from mining effluents (Groh et al., 2022), and anthropogenic salinization from hydraulic fracturing (Folkerts et al., 2020a), among many others. In the natural aquatic environment, chemical effluents are rarely found as singular isolated chemicals and in watershed areas, most anthropogenic effluents are considered to be complex chemical mixtures (CCMs). CCMs have the potential to interact in an additive, synergistic, or antagonistic manner with respect to organism health (Warne and Hawker, 1995). Among CCMs present in the environment, flowback and produced water (FPW) from hydraulic fracturing practices are considered to be contaminants of concern (Acharya et al., 2020).

Hydraulic Fracturing/FPW

Hydraulic fracturing for oil and gas exploration is a billion-dollar industry in Canada (CAPP, 2022), occurring primarily within the western provinces of British Columbia and Alberta (Figure 1-1). Hydraulic fracturing for crude oil is considered to be an unconventional practice,

with conventional crude oil extraction specifically referring to oil found in accessible pools within a porous, permeable rock formation (Soeder, 2021). Unconventional oil and gas exploration requires more effort to recover oil and gas, as it is trapped within low-permeability rock formations that require energy intensive processes to extract (Soeder, 2021). Therefore, unconventional or hydraulic fracturing practices will be the focus of this thesis. Unconventional methods are considered to be a relatively new technology with the first reported use in 1947 in Kansas, U.S.A. (Veatch Jr et al., 1989). The hydraulic fracturing process starts by injecting water and extraction chemicals under high pressure (e.g., ~69,000 kPa) and temperature (i.e., above 132^{o}C within the well environment) both vertically and horizontally along the medium to extract oil and gas from subterranean formations of low permeability (e.g., shale, coal beds, tight sands; Figure 1-2) (Stringfellow et al., 2014). After injection, the well head is closed and the pressurized fluid is "shut" into the well for a period to target specific areas horizontally along the formation (Folkerts et al., 2020a). Once pressure is released from the fracture, the fluid within the formation flows back to the surface (Soeder, 2021). This formation fluid includes both the initial chemical composition of the injected flowback fluid in addition to various other chemical constituents from the well-bore environment (Stringfellow et al., 2014). After this period, oil and gas are separated from the mixture based on density, and the effluent left behind is termed "flowback fluid" and "produced water" (Lockhart et al., 1987).

During production of a well, the distinction between these two fluids is unclear, and usually refers to the time spent within the formation. However, more generally, flowback fluid is characteristic of a shorter shut-in time and resembles a similar chemical composition to the injection fluid, while produced water is more characteristic of a longer shut-in time (> 30 days) (Stringfellow and Camarillo, 2019). Even though flowback fluid and produced water (FPW) are

characteristically different, it is difficult to distinguish between them because mixing occurs within the formation. Well shut-in times vary based on operators and locations, and the injection fluid mixture is considered proprietary by some hydraulic fracturing practitioners.

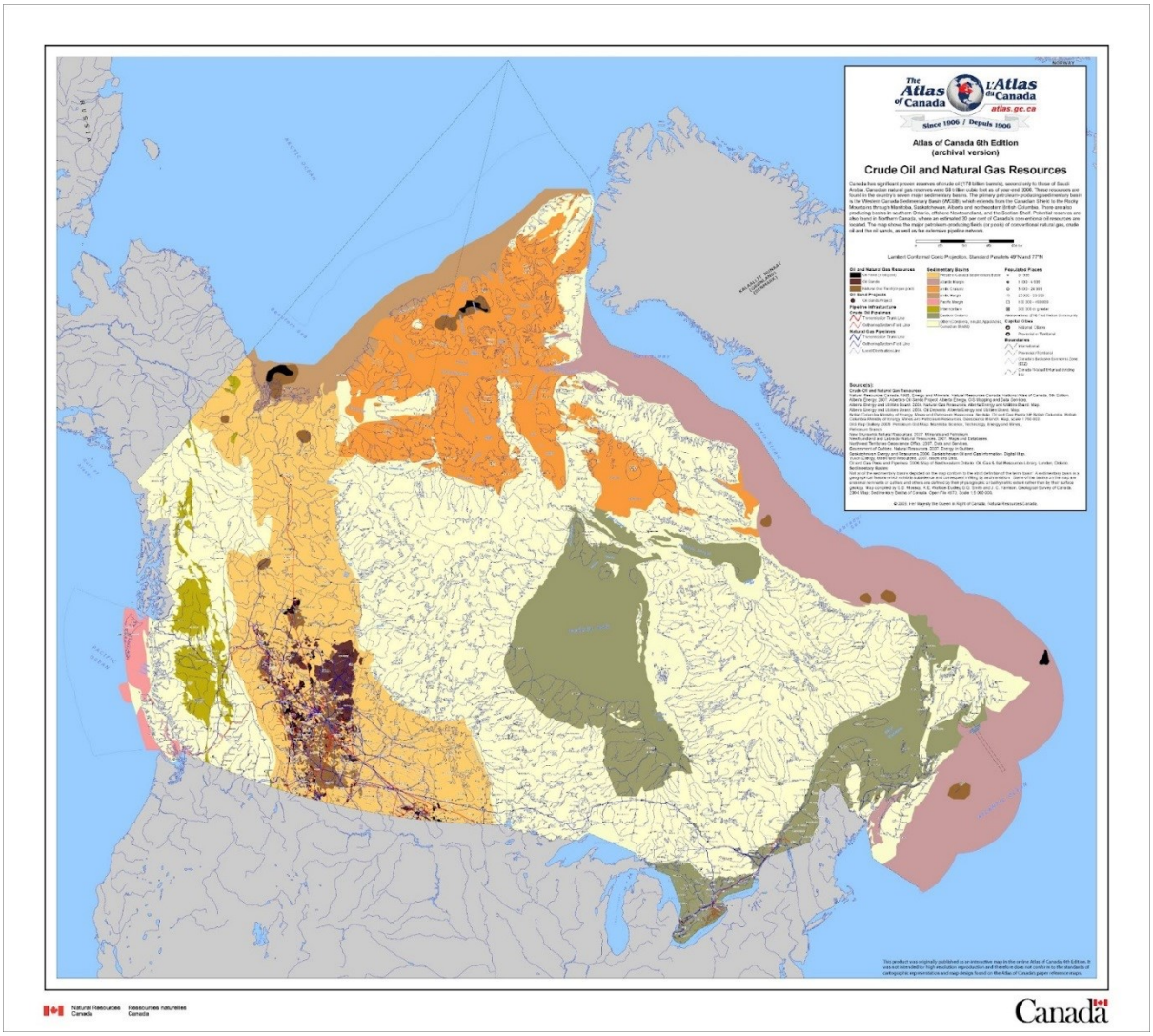

Figure 1-1. Crude Oil and Natural Gas Resources map (obtained from Natural Resources Canada, 2021). The areas shown in black, maroon, and brown indicate oil and natural gas resources.

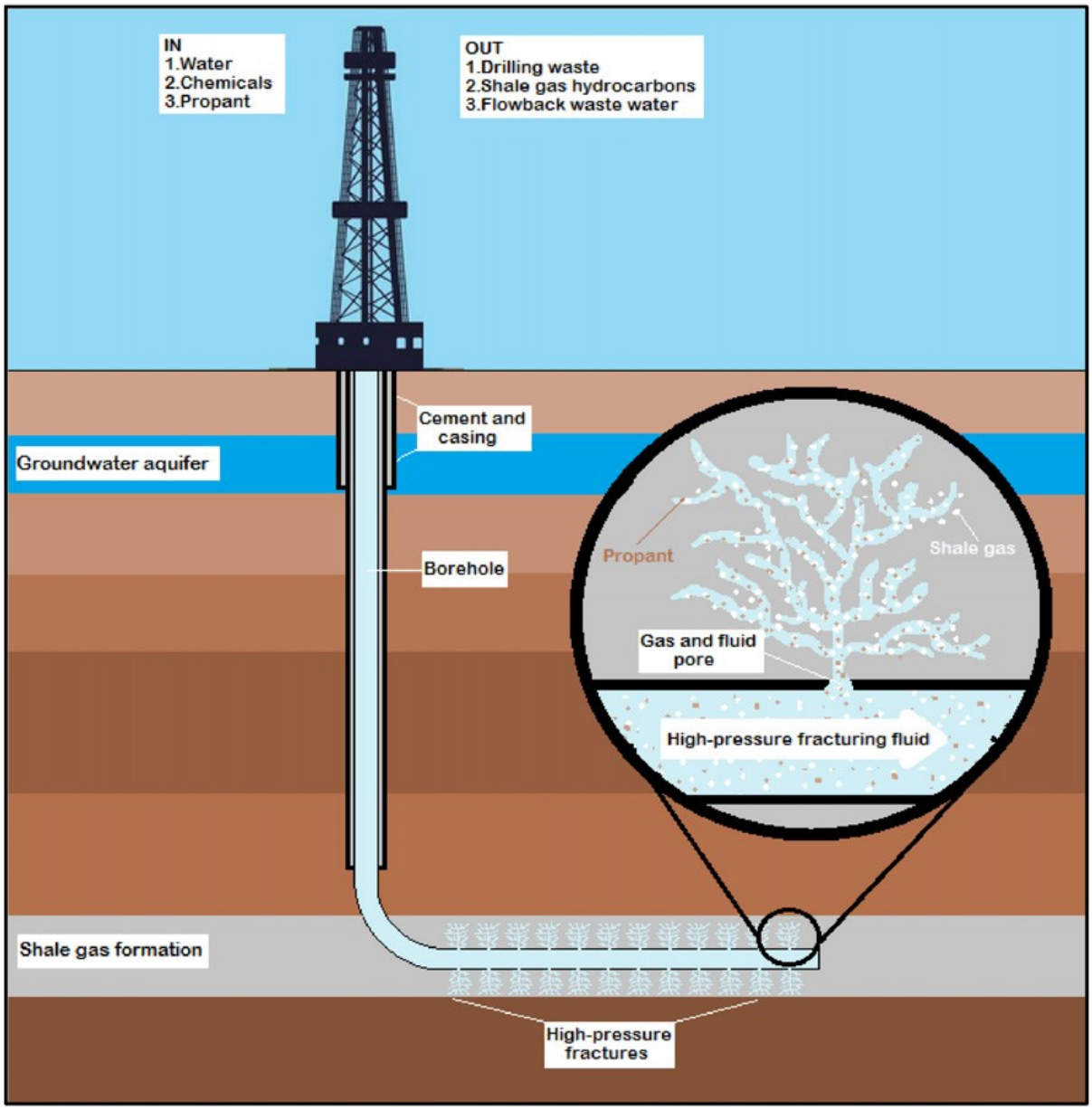

Figure 1-2. Schematic of horizontal hydraulic fracturing (obtained from Annevelink et al., 2016).

FPW effluents consist of a wide range of organics such as surfactants (e.g., ethoxylated surfactants), volatile organic compounds (e.g. benzene), and polycyclic aromatic hydrocarbons (e.g. phenanthrene) (Acharya et al., 2020; Lester et al., 2015; Thurman et al., 2014). FPW also constitutes of inorganics, such as metals (e.g., strontium, barium, lead), naturally occurring

radioisotopes (e.g., radium, uranium), and salts (e.g., calcium, magnesium, sodium, chloride) (Acharya et al., 2020; Folkerts et al., 2020a; Rosenblum et al., 2017b, 2017a; Tasker et al., 2018). These components are derived from the injection fluid, from the formation, and from the process of hydraulic fracturing itself. All these different compounds can induce toxicity depending on their concentration which will be discussed below.

A common organic surfactant found in FPW are neutral ethoxylated surfactants (Thurman et al., 2014). Ethoxylated surfactants with non-polar properties are more likely to be adsorbed into lipid membranes (Patoczka and Pulliam, 1990). Shorter chain ethylene oxides induce greater toxicity, where 0.71% of one ethoxylated surfactant (i.e., tridecyl alcohol ethoxylate) had induced mortality in half the mysid population after an acute 48-hour exposure (LC50) (Patoczka and Pulliam, 1990). These compounds can bind to cellular membranes causing membrane leakiness (Ivanković and Hrenović, 2010). Organics also include volatile organic compounds (VOCs), such as benzene and its substituted constituents, which have been known to affect estrogen pathways of mammals (Mihaich and Borgert, 2018). In aquatic organisms, phenolic compounds have been found to inhibit ATP production by uncoupling mitochondrial oxidative phosphorylation (Buikema et al., 1979). Other common organics found in FPW include polycyclic aromatic hydrocarbons (PAHs), such as naphthalene, a highly toxic and carcinogenic substance that induces oxidative stress in benthic fauna (Jesus et al., 2022). PAHs in FPW are never found as single constituents, but rather as mixtures with other PAHs of low and high molecular weights. In one study, common PAHs found in FPW (i.e., phenanthrene, fluorene, and pyrene) were found at concentrations of 0.145 μg/L, 0.545 μg/L, and 0.0373 μg/L respectively (He et al., 2017a). The concentration of PAHs in FPW could exceed the median environmental levels (e.g., 0.04 μg/L) by up to 14-fold, as seen with fluorene concentrations (Kolpin et al.,

2002; Olmstead and LeBlanc, 2005). PAHs may deter growth through several different mechanisms, such as non-polar narcosis and the generation of reactive oxygen species (ROS) through the aryl hydrocarbon receptor pathway (Arfsten et al., 1996; Barata et al., 2005). Non-polar narcosis occurs when a non-polar chemical enters an organism's lipid membrane and disrupts cellular function, causing immobility, and eventually leading to death (Barata et al., 2005; Di Toro et al., 2000). Lipid membrane perturbations will affect cell signaling of receptors on the membrane, membrane fluidity, and membrane function. Non-polar narcosis is generally a mechanism of action of lower-molecular weight PAHs (Barata et al., 2005), while both higher and lower-molecular weight PAHs may donate electrons to oxygen, forming ROS that can generate DNA adducts (Wilk et al., 2013).

In addition to PAHs, metals are also present in FPW effluents. Common metals found in FPW include barium, strontium and iron, which can be found in the mg/L range in raw undiluted FPW (Folkerts et al., 2020a). Metals may complex with organics such as PAHs whereby increasing the toxicity of FPW or decreasing it depending on the biotic and abiotic factors present (Folkerts et al., 2020a). Metal mixtures (e.g., zinc, mercury and cadmium) have been shown to induce additive toxicity on *D. magna* in comparison to single metal exposures (Biesinger et al., 1986). In freshwater fish, zinc alters gill ionoregulatory function, acid-base balance, gill morphology, and oxygen transport (Hogstrand, 2012). In general, metals cause lethality either through ionoregulatory dysfunction, respiratory issues, or via ROS production, all of which can decrease growth, reproduction, or survival (Wood, 2012). Some metals can also be radioisotopes, emitting radiation to cause malignant tumours in organisms (Brues, 1949). For example, one study found radium levels in FPW to be at 1,230 pCi/L, exceeding the drinking

water standards of 5 pCi/L by 246-fold (Tasker et al., 2018). If consumed, radium can pose adverse effects on human health since it is a known carcinogen (Tasker et al., 2018).

Lastly, FPW is dominated by inorganic ions (primarily salt) from the formation itself. Present in these mixtures are high concentrations of sodium (Na), chloride (Cl), potassium, magnesium, and calcium (He et al., 2017a). Salts such as Na, require a much higher concentration than metals to induce 50% mortality (e.g., 1480 mg/L Na) and 50% reproductive impairments (e.g., 1020 mg/L Na) in the freshwater organism *D. magna* (Biesinger and Christensen, 1972). However, FPW has elevated salt concentrations reaching upwards of 70,000 mg/L Na and 136,000 mg/L Cl (He et al., 2017a), inducing mortality by ~47-fold and sub-lethal reproductive impairment by ~69-fold. When compared to a freshwater environment (Cl < 20 mg/L) (Hintz and Relyea, 2019), Cl in FPW can be up to 6800-fold higher. Increased concentrations of Na and Cl have been found to immobilize, suppress feeding, inhibit growth and reproduction in zooplankton (Evans and Frick, 2001). In fish, salts cause swelling of the interlamellar cell mass decreasing gill surface area, and ultimately lowering oxygen uptake (Folkerts et al., 2020a). Salts also induce stress-related hormones (e.g., cortisol), changes in electrolyte equilibrium, and increased energy metabolism (Folkerts et al., 2020a).

Considering that these chemicals have the potential to induce toxicity in organisms, the persistence of these chemicals within the environment may pose a distinct problem. Salts and metals do not degrade overtime in a similar manner to organics, and will require complexation with dissolved organic matter or dilution to decrease their concentrations in the environment (Hintz and Relyea, 2019; Skeaff et al., 2002). Alternatively, some organics may be degraded by microorganisms (Xu et al., 2018) which transform into different products through reactions with light and oxygen. Although these methods can decrease organic concentrations, not all organics

can be degraded, and many can even increase in toxicity after transformation reactions (Buhler and Williams, 1988). VOCs such as benzene, toluene, ethylbenzene, and xylene, have low boiling points and many will "gas off" before entering aquatic environments (Folkerts et al., 2020a). Alternatively, PAHs are larger, have higher molecular weights, and are generally more stable; resulting in persistence within aquatic environments (Folkerts et al., 2020a). Lower molecular weight PAHs are more commonly dissolved in water (Szopińska et al., 2019), while higher molecular weight PAHs have a higher hydrophobicity and are, therefore, more readily taken up by organisms through their hydrophobic outer lipid bilayer (Hylland, 2006).

Over the course of a well's lifetime, oil and gas will be separated from FPW effluents and FPW may be reused again for further injection or transported to a disposal well (Alessi et al., 2017). Wells in Canada use about 200 – 4600 L per well (Johnson and Johnson, 2012). In British Columbia, FPW can be reused to reduce water consumption, but must go through a treatment process (e.g., wastewater treatment plant using activated sludge) before entering back into the cycle of use (Alessi et al., 2017). While in Alberta, this is not a requirement so FPW ends up in the ground where it will remain for the lifespan of the well (Carter et al., 2013). Furthermore, in the United States, FPW is either reused after the initial injection or in some states sold as an alternative end-product for deicing or dedusting on roads (Robbins et al., 2022; Tasker et al., 2018). Both methods may result in accidental and intentional FPW release into the environment.

During projection, the volume of FPW produced for a single well can reach up to 3 to 4-fold more than the initial volumes used during injection (Goss et al., 2015). It is estimated that in the United States between 2000 to 2015, the total volume of FPW produced was approximately 803 billion liters (Kondash and Vengosh, 2015). With such high volumes of FPW being produced and the need to transport it for disposal or recycling, this may result in a higher

likelihood of FPW entering the environment. One pathway of accidental FPW release is through spills during transport to the final well destination. In recent years, between 2019 - 2020 in Canada, 165 spills of FPW were reported (data collected from the AER incidents compliance dashboard, 2021) while in the United States, it was estimated that ~28.6 million liters of FPW were spilled over 11 states between 2006 – 2012 (U.S. Environmental Protection Agency, 2015). In 2013, one of the largest Canadian spills occurred in Zama City, Alberta where 9.5 million litres of FPW entered the environment (Vanderklippe, 2013) and in the United States, the largest spill was ~4.9 million litres of FPW in 2008 (U.S. Environmental Protection Agency, 2015).

In the United States, reporting requirements for FPW spills vary from state to state. For example, in Colorado, a spill must be reported if it exceeds ~160 L (Patterson et al., 2017). If discovered, FPW spills in Alberta, Canada need to be reported only if they are released offsite into a water body or may cause adverse effects (Figure 1-3) (AER, 2022). Within 24 hours of discovery, the spill is reported to the Alberta Energy Regulator, who then dispenses a team to clean up the spill. Usually, the physical removal of contaminated material is employed to remediate a spill. This may be not completely effective as spills may seep or spread so the spill is not easily contained for removal, leaving residual FPW in the environment. Unfortunately, depending on the size of the spill and the spill components, the time to contain a spill is highly variable. Thus, spills can enter freshwater environments and can possibly remain for prolonged lengths of time without remediation.

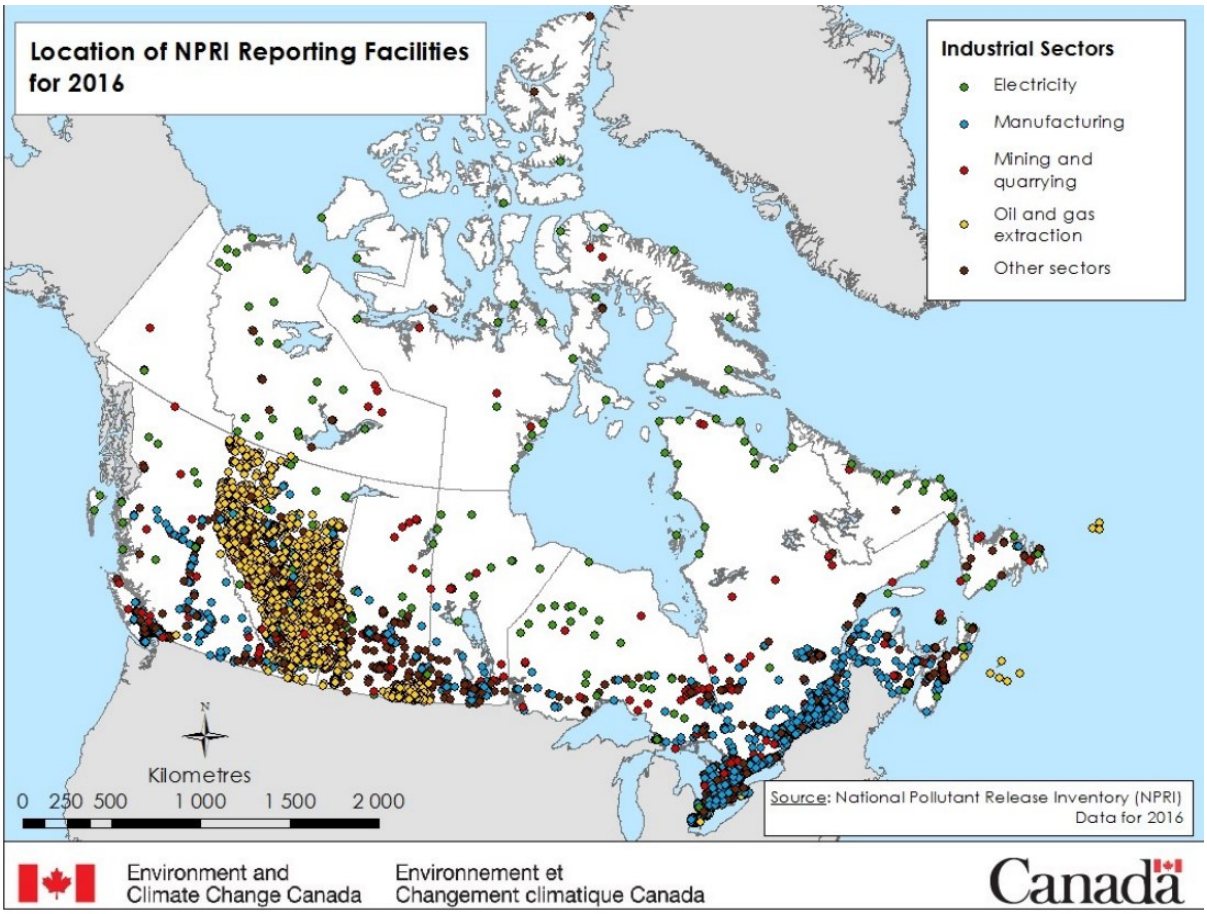

Figure 1-3. Map of Canadian facilities, by industry, that report to the NPRI (National Pollutant Release Inventory) in 2016 (obtained from Environment Canada, 2016). Facilities that report to the NPRI are those that meet the certain criteria when releases of any of the listed pollutants occur.

Daphnia magna

Daphnia magna are small invertebrates found ubiquitously in freshwater environments (Hebert, 1978) and are a representative species of Albertan lakes and rivers (Patalas et al., 1994). In these freshwater ecosystems, *D. magna* are key organisms that act as a prey food resource for many other freshwater species (Miner et al., 2012). *D. magna* are commonly used as a model organism in laboratory experiments since they are small and hardy with relatively simple upkeep,

making them easy to culture and maintain (Seda and Petrusek, 2011). Small organisms like *D. magna* also have short lifespans (~60 days) and reach sexual maturity around 12 days at 18^{o}C (MacArthur and Baillie, 1929). Earlier sexual maturity makes *D. magna* useful for chronic multigenerational studies because they can produce the next generation within a brief time span (e.g., months) in comparison with some fish species that take years to reach maturity.

Previous work has shown that *D. magna* are sensitive to FPW effluents (Blewett et al., 2017a; Boyd et al., 2021; Folkerts et al., 2019; Tasker et al., 2018). After a 48 hour exposure to the Duvernay FPW sample, the LC50 was 0.19% FPW in *D. magna* neonates, while the LC50 for the adult population was 0.75% FPW (Blewett et al., 2017a). After a chronic 21-day exposure to sublethal concentrations of FPW (e.g., 0.004% FPW), brood size was reduced, time to maturation was delayed, and changes in gene expression were observed (Blewett et al., 2017a). These concentrations are the lowest reported for *D. magna,* but FPW concentrations that cause negative impacts on *D. magna* can be highly variable (Folkerts et al., 2019; Boyd et al., 2021). As FPW spills can enter freshwater environments, issues with *D. magna* fitness and survivability can serve as a proxy for the health of the ecosystem.

Recovery

In toxicity studies, recovery is one where an organism experiences an adverse outcome with respect to an anthropogenic stressor but returns to pre-exposure homeostasis after the anthropogenic is removed (Pandher et al., 2012). This can be measured through multiple levels of biological organization, from cellular to whole organism recovery. However, it is important to note that recovery can be tough to characterize in such a simplistic manner as different levels of biological organization will have different timescales and magnitudes of recovery. For example, recovery on the biochemical level with enzyme activity returning to basal conditions will be

different compared with organismal recovery with physiological competency being achieved. Partial recovery or complete recovery may indicate a toxicant is biotransformed into a lesser toxicant, or that latent mortality is not an issue. For the purpose of this thesis, organismal recovery can be defined as the ability for an organism to return to a comparable basal state prior to toxicant exposure. This state can be determined relatively using a control group of *D. magna* to monitor the physiological endpoints of growth and reproduction. Organismal recovery is related and central to ecological recovery of a freshwater system. If the effects of FPW are irreversible and damage is permanent in *D. magna*, this can lead to potential *Daphnia* population collapse. A population collapse for a key prey species can be devastating to higher trophic levels that feed on *D. magna* (Miner et al., 2012). Therefore, recovery in individual *D. magna* to FPW may be crucial to prevent an ecosystem crash. We have defined toxicological whole-organism recovery in the context of this thesis as: *D. magna* are considered recovered when they return to pre-stressor reproductive units (e.g., return to control levels of neonate outputs).

Other freshwater organisms (e.g., rainbow trout, *Oncorhynchus mykiss*) are also affected by FPW exposure (Blewett et al., 2017b; He et al., 2017b). Previous research has shown that FPW exposure in trout causes increases in oxidative stress, where hepatic detoxification and antioxidant activity are increased (Blewett et al., 2017b; He et al., 2017b). It appears that after FPW exposure ends (approx. 3 weeks), ethoxyresorufin-O-deethylase (EROD) activity - a measure of xenobiotic detoxification - returns to control levels (Weinrauch et al., 2021). This shows the possibility that organisms have the potential to recover after FPW exposure, but it is not known whether this translates to whole organism recovery or the time it might require to recover. Furthermore, Weinrauch et al. (2021) assessed a suite of molecular endpoints for recovery after 48-hour exposure to FPW (e.g., gene expression and metabolism). Molecular or

cellular recovery can indicate key pathways of recovery and elucidate if whole organism recovery is possible, where subcellular changes can cause a cascade of changes within an organism leading to recovery. A common method of quantifying cellular recovery is assessing gene expression or targeted omics approaches. Gene expression is rapid and in constant flux, posing as a snapshot of the cell at a given moment (Weinreb et al., 2018). However, quantifying protein abundance is slower and gives a more realistic understanding of an organism's basal state compared with gene expression (Greenbaum et al., 2003). Thus, evaluating cellular recovery using protein abundance to measure functional proteins would be more reliable than gene expression, which may not always be translated to a protein.

Proteomics

Proteomics is the study of all proteins and their interactions within a cell at a given moment (Ong et al., 2003). One gene can code for different isoforms of multiple proteins after post-translational modifications are made (Cho, 2007). This makes gene expression more variable compared with protein abundance. In proteomics, one protein can be linked back to one gene and has a unique function which is specific to the protein (Cho, 2007). Multiple proteins combined on a systems-wide level have functions that give rise to a specific phenotype of an organism. Key proteins that are responsible for a given phenotype can be determined using quantitative proteomics. Quantitative proteomics is the measure of protein abundance and, specifically, relative quantitative proteomics compares this expression to a control group (Elliott et al., 2009). In terms of this thesis, quantitative proteomics was used to determine the differential protein abundance of FPW exposed daphnids compared with daphnids never exposed to FPW, further discussed in chapter 2. Proteomics can then explain the possible whole organism

responses seen in FPW exposed daphnids. By pairing molecular endpoints to physiological endpoints, it gives a holistic understanding of recovery to FPW spills on *D. magna*.

Thesis Aims

FPW spills are common and have the potential to be environmentally devastating. *Daphnia magna* were chosen to be studied as they are prey for many higher trophic organisms, thus their population size can serve as a surrogate for whole ecosystem level changes. The goal of this thesis was to investigate whether *D. magna* can recover from an FPW spill once the contaminant is removed. The first aim was to determine the potential for recovery in one generation of *D. magna* after an acute 48-h FPW spill. *I hypothesized that recovery will not be possible within one generation* due to the likelihood that latent mortality will occur after acute neonatal exposure to FPW. Protein abundance of differentially expressed proteins was investigated to understand the potential mechanisms that were seen on the whole organism scale. The second aim expanded on the first to determine the potential for recovery in multiple generations of *D. magna* after a chronic 21-d FPW spill exposed to the first generation. *I hypothesized that recovery will be possible by the F_3 generation* as this is the first generation unexposed to FPW, since parental (F_0) FPW exposure extends to the germline (F_2). Physiological endpoints related to reproduction were examined, such as time to first brood, neonate production, and number of molts.

Chapter 2 - Persisting effects following an acute exposure to flowback and produced waters in *Daphnia magna*

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1.0 Introduction

Unconventional oil and gas (UOG) operations, which include extraction techniques such as hydraulic fracturing, accounted for 51% of the total oil recovery in the United States in 2015 (U.S. Energy Information Administration, 2016). These approaches have facilitated the exploitation of oil and gas reserves previously considered uneconomical; However, the growth of UOG has led to increased concerns regarding its environmental footprint (Burton et al., 2014; Folkerts et al., 2020a). Potential environmental impacts of UOG include an increase in seismic activity during drilling processes (Atkinson et al., 2016; Davies et al., 2013), discharge of volatile gases during production (Annevelink et al., 2016; Vinciguerra et al., 2015), and the potential for surface water contamination through accidental releases (Burton et al., 2014). The accidental release of flowback and produced water (FPW), a complex mixture containing oil and gas constituents as well as waste materials, is of particular concern (Folkerts et al., 2020a). For example, a recent survey found that FPW was the most common material associated with accidental releases from UOG activities (U.S. Environmental Protection Agency, 2015). FPW is a complex saline mixture containing diesel range organics, volatile organic compounds (VOCs), radioisotopes, geogenic derived ions and metals, and transformation products from the resulting well-bore environment (Folkerts et al., 2020a; He et al., 2017a; Lester et al., 2015). The specific composition of a given FPW effluent is unique and dependent on the geology of the formation, the shut-in time of the well, and the initial fracturing fluid composition (Folkerts et al., 2020a).

On average, hydraulic fracturing wells located in the Canadian Montney formation (Alberta/British Columbia Area) use approximately 200 – 4600 L of water per injection, ranging between 800 – 13,000 L for a single well (Johnson and Johnson, 2012). In addition, an estimated further 1×10^7 – 1×10^8 L of water may be used to maintain flowback per well, which also

contributes to the wastewater burden (Alessi et al., 2017). Ultimately, FPW needs to be transported for reuse or for disposal via deep well injection, and it is during these processes that accidental releases occur (Folkerts et al., 2020a). Between 2019 and 2020, there were approximately 165 FPW spills into freshwater environments in Alberta, Canada (data collected from the AER incidents compliance dashboard, 2021) and in the United States approximately 2-16% of wells reported a spill every year, with the largest encompassing 3,756,000 L (Patterson et al., 2017). Critically, such releases often occur in close proximity (~300 m) to bodies of freshwater (Entrekin et al., 2011).

Knowledge of how FPW affects organismal biology is a critical component in the development of regulatory practices and policies that will protect freshwater systems against the impacts of FPW spills (Jones et al., 2015). Effects of FPW on freshwater organisms have been well characterized in both vertebrate and invertebrate species. These include developmental deformities in zebrafish (*Danio rerio*) embryos (Folkerts et al., 2017a; He et al., 2018), impacts on fish cardiac and respiratory function (Folkerts et al., 2020b, 2017a, 2017b), an increase in oxidative stress and altered gill morphology in rainbow trout (*Oncorhynchus mykiss*) (Blewett et al., 2017b), depletion of energy stores in trout (Weinrauch et al., 2021), and a number of effects in *Daphnia magna* (Blewett et al., 2018, 2017a; Boyd et al., 2021; Folkerts et al., 2019).

D. magna can be used to assess the health of aquatic communities and are considered to be a useful model for toxicological studies due to their wide distribution, sensitivity to various environmental contaminants, and ease of use in the laboratory (Altshuler et al., 2011). Furthermore, *D. magna* are also important ecologically as they play a pivotal role in the success of a freshwater ecosystem, by providing food for many other aquatic species (Miner et al., 2012). If *D. magna* populations are drastically reduced due to contaminant exposure, populations of

higher trophic level aquatic organisms that prey on *D. magna* may also decline. Previous work has shown that the 48-h LC50 for *D. magna* neonates is variable but ranged from 0.19% - 1.58% for FPW (Blewett et al., 2017a; Folkerts et al., 2019). 21-day chronic toxicity also varies in *D. magna* exposed to FPW, where 2% of one sample was lethal, but for the three other samples survival was significantly higher (Boyd et al., 2021). At lower concentrations (0.004% FPW), reproduction and time to maturation decreased in *D. magna*. Indeed, at this concentration neonate production was reduced by ~2.5-fold compared to control values. In the wild, daphnids experiencing this exposure might see a drastic decrease in populations. As such, FPW spills could have an important impact on both daphnids and the secondary consumers that are dependent on *D. magna* as a food source (Miner et al., 2012).

To date, the capacity of organisms to recover after an FPW spill event has not been thoroughly investigated. Organismal recovery can be defined as an organism's ability to return to physiological competency (i.e., basal state) after a stress event or scenario (Wu et al., 2005), however, recovery can be characterized at different levels of biological organization. For example, at an ecological level, recovery may be recognized as the return of population density or composition to pre-exposure conditions. Yet, ecological recovery may occur at a different rate than recovery at molecular, biochemical, physiological, and behavioural levels (Adams et al., 2002). To our knowledge, only one study has investigated the potential for an organism to recover from an FPW exposure (Weinrauch et al., 2021). This study showed that after an acute 48-h exposure to FPW (2.5% and 7.5% of raw FPW), liver glucose and amino acid metabolism in rainbow trout (*Oncorhynchus mykiss*) were increased, but that normal function had returned after a 3-week recovery period in clean water. This research suggests that some FPW effects can be reversible, and that organismal recovery can occur in the absence of the initial stressor.

The aim of the current study was to investigate organismal recovery in *D. magna* after a 48-h acute exposure to low concentrations of FPW (dilutions of 0.25% and 0.75% of full-strength FPW). To assess reproductive endpoints, we recorded neonate production, molting and time to maturation. We complemented these measurements with systems-level quantitative proteomic analyses to assess changes in whole animal protein profiles. Collectively, this study provides a critical physiological and molecular understanding of acute FPW exposures, and the potential for organismal and population level recovery after such events.

2.0 Materials and Methods

2.1 *Daphnia culturing*

Daphnia magna were obtained from a research colony (Aquatic Research Organisms (ARO), New Hampshire) in September 2019, and housed in the Department of Biological Sciences at the University of Alberta. The colony was maintained following the Organization for Economic Cooperation and Development guidelines (OECD) for *Daphnia* (OECD, 2004) with minor adjustments. Briefly, *Daphnia* colony water (OECD water) was made using dechlorinated Edmonton tap water with the following recipe: 2 mM $CaCl_2 \cdot 2\ H_2O$, 0.5 mM $MgSO_4 \cdot 7\ H_2O$, 0.77 mM $NaHCO_3$, 0.08 mM KCl (OECD, 2004; see Table 2-1). The colony was maintained in 2 L glass beakers containing 1 L of OECD water. Both the colony and experiments were held at a 12:12 h light/dark photoperiod and at a temperature of 20 ± 1°C. Water was changed every 2-3 days, and *Daphnia* were fed a daily mixture of 5 mL yeast/cerophyll/trout chow (YCT) mix and 5 mL of concentrated algae (sp. *Raphidocelis subcapitata*; ARO, New Hampshire) and supplemented once weekly with 100 µL of Roti-Rich™ (VWR, Edmonton, Alberta, Canada).

2.*2 FPW Information and Chemical Analysis*

FPW was obtained from a hydraulically fractured well (Well ID: 02-12-81-18 W6 Pad – termed Well O) in the Montney formation (Dawson Creek, British Columbia, Canada) on June 2, 2019. This FPW sample was obtained from the two-hour flowback period/2-h post initiation of well flowback and was termed 100% FPW. For the duration of the study and for storage, the FPW sample was stored in the dark at room temperature, 20 ± 2°C. FPW samples were then diluted to 0.25% or 0.75% using OECD water (See Section 2.3, Table 2-1). Prior to experimentation, all glassware was soaked for ≥ 24 h in 5% nitric acid (HNO_3, Sigma Aldrich), rinsed, and followed subsequently by a 10% ethanol (EtOH) wash, and then rinsed in distilled water. Experimental water samples (40 mL) were taken for inorganic chemical characterization. Random collection of the samples occurred over the 21-day experiment for the control (n=16) and the 0.75% treatment only (n=4 for day 0-2 and n=10 for day 3-21). FPW samples were held at 4ºC until ICP-MS/MS analysis, they were then diluted 40x for trace elements and 400x for sodium (Na) and chloride (Cl). Finally, samples and standards were prepared in a matrix of 2% HNO_3. Standards covered a range of 0.0005-120 mg/L in three tiers to accommodate varying concentrations within the samples. Standards for trace elements analysis were matrix matched with 2000 mg/L NaCl. The ICP-MS/MS measurements were made using various collision/reaction gases. Supplemental Table 2-1 indicates the measured masses in Q1 and Q2 and the used collision or reaction gases to eliminate isobaric interferences. An internal standard mix of Sc, Ge, In, Lu, and Bi was used to account to instrumentation drift for each analysis across all collision/reaction gas cells.

2.3 *Recovery Experiment*

Less than 24-hour old *D. magna* neonates were placed individually into 20 mL glass scintillation vials in three separate treatments: control with OECD water only (n=25), 0.25% FPW

(n=20) and 0.75% FPW (n=25) where exposures lasted for 48-h. After the 48-h exposure period, neonates were moved to new 20 mL (pre-cleaned, see above) vials containing OECD water only for the rest of the experiment – 19 days, considered the recovery phase. Every 2 days, 80% of the water was replaced with fresh OECD water, and *D. magna* were fed 100 µL of algae and 100 µL of YCT daily. *D. magna* survival, molting, and neonate production were recorded daily. Any offspring produced were removed from the vials each day.

2.4 *Proteomics*

2.41 *Sample Preparation and Digestion*

Snap-frozen *D. magna* (n=32) were ground to a fine powder under liquid N_2 using a mortar and pestle. Any dead *D. magna* neonates were removed prior to freezing. Ground samples were then extracted using a 50 µL 1:2 (w/v) extraction solution of 50 mM HEPES-KOH pH 8.0, 50 mM NaCl, 4% (w/v) sodium dodecyl-sulfate (SDS), vortexed and placed in a 95ºC tabletop incubator (Eppendorf) shaking at 1100 RPM for 15 mins followed by an additional 15 mins of shaking at room temperature. *D. magna* homogenates were then spun at 20,000 x g for 5 mins to clarify extractions, with the supernatant retained in a fresh 1.5 mL microcentrifuge tube (Eppendorf). Protein concentrations were measured by a bicinchoninic acid (BCA) assay (Pierce™; Thermo Scientific). Samples were then reduced with 10 mM dithiothreitol (DTT; BioShop) at 95ºC for 5 mins, cooled, then alkylated with 30 mM iodoacetamide (IA) for 30 min in the dark without shaking at room temperature. Subsequently, an additional 10 mM DTT was added to each sample, quickly vortexed and incubated for 10 mins without shaking at room temperature.

Total proteome peptide pools were generated using a KingFisher Duo automated sample preparation device and trypsin digestion (Promega) as outlined by Leutert et al., 2019 without

deviation. Generated tryptic peptides were dried in a speedvac, then dissolved in 3% (v/v) acetonitrile (ACN) / 0.1% (v/v) trifluoroacetic acid (TFA), desalted using ZipTip C18 pipette tips (ZTC18S960; Millipore) as previously described (Uhrig et al., 2019), dried by speedvac and dissolved in 3.0% (v/v) ACN / 0.1% (v/v) formic acid (FA) prior to MS analysis.

2.42 *LC MS/MS Analysis*

Total proteome peptide samples were analyzed using a LUMOS Orbitrap mass spectrometer (Thermo) in data dependent acquisition (DDA) mode. Dissolved samples were injected using an Easy-nLC 1200 system (Thermo) and separated on 50 cm ES803a Easy-Spray PepMap C18 Column (Thermo). The column was equilibrated with 100% solvent A (0.1% FA) in water). Peptides were eluted using the following gradient of solvent B (0.1% FA in 80% ACN): 5% to 46% B, 0 – 110 min; 22% - 35% B, 110 – 120 min; 35 - 95% B, 120 – 125 min at a flow rate of 300 nL/min at 50^{o}C. Full scan mass spectra (300 - 1700 m/z) were acquired with a resolution of 120,000 at 200 m/z after accumulation to a target value of 2.0e5. DDA MS/MS were recorded in a linear ion trap using quadrupole isolation in a window of 1.6 m/z. Selected ions were HCD fragmented with 35% fragmentation energy. The ion trap was run in rapid scan mode with a 2.0e4 AGC target and a maximum injection time of 100 ms. Precursor ions with a charge state of +2 - +7 and a signal intensity of at least 5.0 x 10^3 were selected for fragmentation. All precursor signals selected for MS/MS were dynamically excluded for 30s. All raw data has been deposited to ProteomeExchange through the PRoteomics IDEntification database (PRIDE; https://www.ebi.ac.uk/pride/) and can be found using dataset identification number PXD021962.

2.43 *Label-free Quantification and Analysis*

Raw data were processed using MaxQuant software version 1.6.14 (http://www.maxquant.org/) (Cox and Mann, 2008; Tyanova et al., 2016a). MS/MS spectra were searched with the Andromeda search engine against a decoyed (reversed) version of the *D. magna* protein database obtained from UniProt (https://www.uniprot.org/). Trypsin specificity was set to one missed cleavage and a protein and PSM false discovery rate of 1%. Minimal peptide length was set to seven and match between runs option enabled. Fixed modifications included carbamidomethylation of cysteine residues, while variable modifications included methionine oxidation. MaxQuant outputs were then imported into Perseus version 1.6.10.43 (Tyanova et al., 2016b) for downstream data analyses. Here, reverse hits and contaminants were removed, the data $\log_2$-transformed, followed by a data sub-selection criterion of n=3 of 4 replicates in at least one sample type. Missing values were replaced using the normal distribution imputation method.

The 26,600 proteins of the *Daphnia magna* proteome were annotated using default settings within OmicsBox 1.411 (BioBam Informatics, 2019). Specifically, 99.6% of the sequences had successful matches to proteins from the non-redundant protein sequence BLAST database (nr v5) using BLASTP (accessed December 2020). Using Goa 2020.10, Gene Ontology (GO) terms were mapped to 67.6% of *Daphnia magna* sequences, of which 92.0% were annotated (Gotz et al., 2008). In addition, InterProScan, version 5.51-83.0 (Blum et al., 2021), was performed, retrieving domain/motif information and corresponding GO terms for 91.2% of the protein sequences. GO terms from InterPro were merged to the annotation, resulting in 68,399 validated GO terms. To gain a broad overview of the functions associated with these protein sequences, a GO-Slim analysis was applied to the annotation. REVIGO (accessed May 2021) (Supek et al., 2011) was then used to summarize related GO terms. Similarly, a combined pathway analysis was conducted within OmicsBox 2.0.036 (BioBam Informatics, 2019) to determine enrichment of KEGG and Reactome

pathways among the same differentially expressed protein set, using default settings and an FDR < 0.05 (Fabregat et al., 2018).

2.5 *Statistics*

The normality of the physiological data was assessed using the Shapiro-Wilk Normality test while data was tested for equal variance using Levene's test. Data was not normal, and all transformations attempted to normalize the data had failed. Thus, a one-way ANOVA on ranks was performed, followed by Dunn's post hoc test (SigmaPlot version 11.1; Systat Software, San Jose, CA). All data was analyzed and graphed on SigmaPlot version 11.1 (Systat Software, California, U.S.A). Data have been expressed as means ± standard error of the mean (SEM). Significance for all tests was accepted at $\alpha = 0.05$.

Significantly changing, differentially abundant proteins were identified via ANOVA and a post-hoc t-test corrected for multiple comparisons in Perseus (Benjamini-Hochberg FDR *p-value ≤ 0.05; q-value ≤ 0.05*). For GO enrichment, a Fisher's Exact Test in OmicsBox with a false discovery rate (FDR) of 5%, enrichment of Gene Ontology Biological Processes (GOBPs) was performed for the 443 shared differentially expressed proteins (LogFC ≥ 1 or ≤ -1) between the two treatments (0.25% FPW and 0.75% FPW) relative to control conditions.

3.0 Results

3.1 *Water Chemistry*

Inorganic analyses are reported in Table 2-1. Overall, stock concentration of FPW was dominated by Na (7130 ± 30 mg/L), Mg (324 ± 11 mg/L), Cl (12600 ± 1070 mg/L), Ca (1380 ± 30 mg/L), and K (374 ± 71 mg/L) with bromine (Br) being the dominant metal present at 26.3 ±

0.6 mg/L. The 0.75% dilution was comparable to the stock solution, but with some notable absences (Table 2-1). For example, bromine was not detected in the 0.75% dilution. Both OECD control, and the day 3-21 OECD recovery treatment were consistent with similar ionic and metal compositions.

3.2 *Recovery Experiment*

Mortality was observed in *D. magna* exposed to FPW, with most deaths occurring by day 5 (Figure 2-1). Remaining *Daphnia* survived for the duration of the 21-day experiment. Half of the 0.25% FPW exposed daphnids survived the acute 48-h exposure and 19-day recovery period, while in the 0.75% FPW exposed daphnids only 32% survived (Figure 2-1). After 48 hours of exposure to FPW and 19 days in clean water recovery (21-day total), *Daphnia* exposed to the FPW treatments showed reduced neonate production compared to the control treatment (Figure 2-2A). The average number of neonates produced per *Daphnia* was 66.2 ± 4.9 in the control treatment, 33.4 ± 7.8 in 0.25% FPW treatment and had a 5-fold decline of 12.4 ± 4.6 in 0.75% FPW treatment (Figure 2-2A, $p < 0.001$). Overall total brood size was decreased in our exposure treatments compared to control neonates (Figure 2-2B).

Daphnia exposed to 0.75 % FPW for 48 hours and then subsequent clean water displayed a significantly delayed time to first brood in comparison to *Daphnia* in the control treatment (Figure 2-3A, $p = 0.03$). FPW treated *Daphnia* displayed a delayed maturation by 3 days in comparison to control treatment. In both FPW treatments (0.25% and 0.75%), fewer molts occurred compared to control *Daphnia* (Figure 2-3B, $p = < 0.001$). Molting was reduced by 3-fold in the 0.75% FPW group compared to control treatment (Figure 2-3B).

3.3 *Proteomic Analysis*

Comparative quantitative proteomic analysis of *D. magna* exposed to 0.25% and 0.75% concentrations of FPW found close PCA clustering of 0.25% and 0.75% treated replicates compared to control replicates, suggesting each treatment has a similar overall impact on the *D. magna* proteome (Figure 2-4A). A total of 443 proteins of the 2566 quantified proteins in the study were found to change significantly ($Log_2FC \geq 1.0$; *q-value* ≤ 0.05) compared to control samples in both 0.25% and 0.75% treatments, while a total of 27 and 62 proteins were found to change significantly in only 0.25% and 0.75% treatments; respectively (Figures 2-4B and 2-4C). Scatterplot analysis shows that protein-level changes in the two treatments clustered around a 45-degree line, indicating that proteins that changed in both treatments did so in a similar manner. Only a single protein, WD repeat domain phosphoinositide-interacting protein 2 (WIPI2) changed in opposing directions in either treatment, demonstrating a -1.0 Log_2FC in the 0.25% treatment and a 3.04 Log_2FC in the 0.75% treatment. The protein with the highest decreased abundance, sphingomyelin phosphodiesterase-like protein (SMPD), decreased 6 Log_2FC in both FPW treatments (Figure 2-4C). Lastly, analysis of the ratios in abundance of either treatment relative to the control was undertaken (Figure 2-4D). A ratio of 1.0 indicates that those proteins exhibited identical changes in abundance in either the 0.25% or 0.75% treatment relative to controls. The distribution demonstrates that 77% of the proteins exhibiting a significant change in abundance ($Log_2FC \geq 1.0$; q-value ≤ 0.05) were changing almost identically in either treatment. The slight right skew in the frequency distribution indicates that protein-levels were mildly sensitive to treatment condition, with the 0.75% eliciting a slightly larger change in protein abundance relative to the 0.25% treatment.

Proteins in both FPW treatments relative to controls resulted in significant enrichment (FDR < 0.05) of 46 GOBP terms, which were further sorted into 16 groups using REVIGO (Table 2-2) (Supek et al., 2011). Six of the seven terms related to metabolic function (metabolic process, organic substance metabolic process, catabolic process, carbohydrate metabolic process, lipid metabolic process, and small molecule metabolic process; Table 2-2) were enriched from proteins that were, for the most part, decreased expression relative to controls. All other terms were enriched for proteins that were primarily increased in the two FPW treatments relative to control and were involved in transport, cell organisation, organic substance biosynthesis, and transcription (Table 2-2). Among the proteins enriched for metabolic GOBP terms, were several chitinase orthologs: probable chitinase 10 (decreased) and chitinase 15 and chitinase 3-like protein 1 (increased; Table S2-7). Also, enriched proteins related to drug metabolism glutathione S-transferase and glutathione S-transferase 1-like, decreased in comparison to the control (Table S2-7).

4.0 Discussion

The current study shows that recovery within a generation did not occur after an acute 48-h exposure to FPW. Recovery in an organism can be defined as the biological sum of molecular, biochemical and physiological functions returning to post-stressor homeostasis. Reproduction was used as a surrogate for organismal recovery in this context as it is highly energy dependent and is thought to reflect the increased cost of toxicant exposure (Blewett et al., 2017a). At the end of the 21-day experiment, reproduction, molting, and time to maturation were all shown to be reduced in both FPW exposures (0.25% and 0.75%) compared to control animals. Protein level changes were measured at 48-h post-exposure and suggested decreased metabolic performance regardless of

FPW exposure concentration. These metabolic depressions relate to the physiological responses seen after the 21-day experiment.

4.1 *Water Chemistry*

In the 0.75% treatment, the inorganic analysis resulted in metals that were below the detectable limit, and that Na and Cl values were 3-4 times the concentrations of that of the OECD control water (Table 2-1). The Na and Cl concentrations of this sample were 6 – 8-fold and 6.5 – 8.5-fold less concentrated respectively, compared to previous FPW samples from the Duvernay formation in Alberta, Canada (Folkerts et al., 2019; Blewett et al., 2017a). Comparatively to FPW samples from the same well pad on the Montney formation, this sample was approximately 1 – 2-fold less concentrated in Na and Cl concentrations (Boyd et al., 2021). Na and Cl were well below LC50 measurements previously recorded in *D. magna* (NaCl 4,745 mg/L) (Arambašić et al., 1995). LC50 measurements for a variety of metals in *D. magna* (e.g. magnesium 322 mg/L, iron 9.6 mg/L, copper 0.060 mg/L) were also higher than the concentrations found within the 0.75% treatment (Biesinger and Christensen, 1972; Cui et al., 2018), suggesting that the salts or metals of FPW were not the major contributors to the toxicity of the sample. However, the possibility of additive or synergistic toxicity with the combined effects of salt, metals, and organics cannot be discounted (Blewett et al., 2017a). It should be noted that the 0.75% dilution was not linear from the concentrated stock solution. This could be due to organic and anionic complexation with both metal and salt ions (Kozelka and Bruland, 1998; Saito and Moffett, 2001). Furthermore, loss of both metals and salts due to adsorption to the side of the glass vial would influence the potential metal and ion concentrations (Eichholz et al., 1965; Struempler, 1973). Chemical concentrations may also vary due to the surfactants in FPW, such as ethoxylates and polyethylene glycols

(Thurman et al., 2014), causing FPW to adsorb to surfaces (e.g. pipettes, containers holding FPW) (Lunkenheimer and Wantke, 1981).

4.2 *Recovery Experiment*

Survival was investigated over the course of the 21-day time period (Figure 2-1). Mortality was similar to what was reported previously in Boyd et al., 2021 with the same FPW effluent and concentration (20% survival at 0.75% FPW). In this experiment, the percentage of surviving adult daphnia was 50% and 32% in the 0.25 % FPW and 0.75% FPW respectively, at the end of the test period. The low survivorship of *D. magna* in the FPW concentrations demonstrates latent mortality, due to the organism's inability to recover from an anthropogenic stressor even when exposure to the contaminant was discontinued (Zhao and Newman, 2004). Latent mortality is commonly seen in a variety of aquatic invertebrates in response to a contaminant (e.g., weathered crude oil in *Anchoa mitchilli* (Munnelly et al., 2021), FPW in *Lumbriculus variegatus* (Mehler et al., 2020), copper and zinc in *Strongylocentrotus purpuratus* (Colvin et al., 2021)

Surviving *D. magna* from FPW treatments had decreased reproductive outputs, a reduction in molting and a delayed time to maturation (Figures 2-2 and 2-3). These results are similar to previous work in *Daphnia* exposed to FPW for 21 days (Blewett et al., 2017a; Boyd et al., 2021). Neonate *Daphnia* are more sensitive than their adult counterparts, due to their high mass-specific metabolic rate and higher surface area to volume ratio, increasing the potential contact points for transport across the epithelium (Yu and Wang, 2002). Previously, Muyssen and Janssen (2007) showed that neonate *Daphnia* had lower survival, neonate production, and first brood size, to metal stressors than 7-day adult *Daphnia*, and Blewett et al. (2017a) also showed that LC50s were vastly lower in neonates (0.19%) than in adults (7-day old) *Daphnia* (0.75% FPW).

It is difficult to determine the specific mechanism of action of FPW due FPW being a complex chemical mixture. These include salt forming ions (e.g. Na, Ca, and Cl), trace metals (e.g. Fe, Zn, Sr, and Ba), and organic compounds (e.g., polycyclic aromatic hydrocarbons (PAHs), volatile organic compounds (VOCs) (Acharya et al., 2020; Colborn et al., 2011; Folkerts et al., 2020; Goss et al., 2015; He et al., 2017a). These compounds may contribute to additive, synergistic, or antagonistic toxicity, but we did not test this in the current study. This is further complicated by the fact that new materials are made under the conditions in the well-bore environment and every well seemingly has different mixture compounds and concentrations (Folkerts et al., 2020a). This study did not measure the organic constituents within our FPW sample due to its complexity, but previous studies have determined that common organic contaminants found in FPW include PAHs (e.g. fluorene, phenanthrene, naphthalene, pyrene (He et al., 2017a)). PAHs elicit bioactivation where cytochrome p450 enzymes form intermediate PAH epoxides which potentially can interact with DNA, creating a downstream effect of toxicity (Buhler and Williams, 1988). The toxicity from various PAHs can affect the time to first brood and number of broods in *D. magna*, ultimately affecting their reproductive fitness and survival (Feldmannová et al., 2006; Holst and Giesy, 1989; Parkhurst et al., 1981). The chronic effects of decreased reproduction and molting seen in this study may largely be attributed by the organic fraction in FPW, with an increased likelihood of organic toxicity caused by PAHs. This is because the inorganic analyses between the OECD control and FPW were not too different from one another. A complex mixture of organic compounds, like that is present in FPW, can have increased toxicity compared to its individual components (Deneer et al., 1988). In Blewett et al. (2017a), the components of FPW were isolated to see the effect of organics, which displayed higher toxicity compared to the salt and combined salt and metal fractions. Biotransformation of these organic

compounds within *D. magna* can lead to increased toxicity (Buhler and Williams, 1988). Despite the organic fraction likely being the primary driver of toxicity, these effects can be exacerbated by the salts and metals present in FPW. *D. magna* has been shown to have impaired reproduction after exposure to individual metals (Biesinger and Christensen, 1972; De Schamphelaere et al., 2007) and to the salt, NaCl (Ghazy et al., 2009; Martínez-Jerónimo and Martínez-Jerónimo, 2007). Since FPW consists of organic compounds, metals, and salts forming elements, all of which influence reproduction, it can be inferred that these groups of chemicals can act in conjunction to cause even more toxic reproductive effects in *D. magna*. These effects are likely seen on the protein level, where proteins linked to reproduction may give insight in determining the mechanisms behind FPW toxicity.

4.2 *Proteomic Analysis*

Overall, *D. magna* exposed to 0.25% and 0.75% FPW concentrations exhibited similar protein abundance changes that were vastly different from the control (Figure 2-4). The proteins that decreased within the FPW exposed treatments were those related to metabolism, meanwhile the proteins that increased were those related to cellular transport, cellular organization, and transcriptional processes (Table 2-2). Oftentimes in response to a stressor (≥ 96 h), metabolism is increased to mobilize energy stores (Becker et al., 2018; Sancho et al., 2009), reducing lipid content and lipid reserves (Vandenbrouck et al., 2010), while transcriptional processes are decreased (Becker et al., 2018). However, this was not the case in this experiment.

It appears that in more acute stress scenarios (8 h), there is an induction of stress genes and a downregulation of metabolic genes, which reverses once daphnids are acclimated to the stressor (Becker et al., 2018). In general metals and parent PAHs, common chemicals found in FPW, downregulate energy-related metabolic pathways (Campos et al., 2021). Lipid metabolism, a

GOBP term associated with downregulated proteins following FPW exposure, plays a role in *D. magna* reproduction and molting. Lipids are metabolized and stored as lipid droplets that are used for egg and molt formation (Tessier and Goulden, 1982), which in turn is linked to reproduction as egg production occurs after the adult molts (Ebert, 2005). Molting and neonate production were decreased after 48-h FPW exposure (Figures 2-2 and 2-3), following a reduction in lipid metabolism (Table 2-2). Proteins involved in molting, such as chitinases 3 and 15 (Qi et al., 2018) were found to increase in both FPW treatments (0.25% and 0.75%; Table S2-7). Alternatively, probable chitinase 10 was decreased (Table S2-7), which causes an excess in chitin production in *Drosophila* (Dong et al., 2020). Here, up-regulation of molting proteins and excess chitin production after neonatal FPW exposure may represent a method to remove FPW damaged cells and produce new cells to avoid mechanical senescence caused by stress, as seen in decapods (Vogt, 2012). Exposure to FPW altered proteins involved in lipid metabolism in *D. magna*, which may directly influence reduced molting and reproduction. Alternatively, pathways associated with upregulated proteins were primarily involved in translation (Table S2-6). Protein translation is energetically expensive but essential for cell survival. Protein translation or translation initiation may increase in response to cellular stress to produce conserved stress proteins (Heckmann et al., 2008; Jia et al., 2018), contrasting the downregulation of essential pathways like detoxification (Table S2-6).

Detoxification pathways of xenobiotic compounds relate to phase I and II metabolism, with glutathione metabolism as an example of phase II metabolism (Table S2-6). Phase I metabolism converts the xenobiotic into a more polar molecule, while phase II metabolism conjugates the compound to increase solubility (Buhler and Williams, 1988). Phase I and glutathione metabolism can ultimately lead to the excretion of the contaminant from the cells (Baldwin and LeBlanc,

1994). Glutathione s-transferase, a protein involved with glutathione metabolism, decreased in both the 0.25% and 0.75% FPW treatments (Table S2-7). Decreased glutathione-s-transferase abundance suggests *D. magna* may have a limited ability to biotransform and then excrete contaminants found in FPW. Exposure to high concentrations of PAHs, commonly found in FPW, have been shown to impair molting and reproduction in *D. magna* (Vandenbrouck et al., 2010). In conjunction with metals, PAHs can induce reactive oxygen species production (Xie et al., 2006), which can cause damage to many cellular macromolecules, such as nucleic acids, lipids, and proteins (Kurutas, 2016). Ultimately, cell death will occur when enough cellular damage has been done. FPW exposed cells were likely undergoing cell death and were unable to mobilize energy to remove these apoptotic cells (Yin and Heit, 2021), leading to increased mortality, reduced fecundity and molting in FPW treatments (Figures 2-1 to 2-3).

Proteome profiles of *D. magna* neonates exposed to FPW at 0.25% and 0.75% concentrations were very similar (Figures 2-4A and 2-4B). In both FPW treatments, similar changes in protein abundance were observed in comparison to control *Daphnia*. Sphingomyelin phosphodiesterase-like protein (SMPD) was the most heavily downregulated (-6 Log_2FC) protein in the two FPW treatments (Figure 2-4C). SMPD is a sphingomyelinase which converts sphingomyelin to ceramide to increase cellular ceramide concentration (Sawai and Hannun, 1999). Apoptosis has been linked to ceramide production (Obeid et al., 1993), thus a decreased abundance of SMPD leads to decreased ceramide production, preventing apoptosis. A deficiency of SMPD causes accumulation of sphingomyelin, a commonality in Niemann-Pick disease, which induces dysfunction in the central nervous system and the rapid onset of death (Bienias et al., 2016). Increased sphingomyelin levels are associated with a delayed time to first brood and decreased

neonate production, as seen in a study where *D. magna* neonates were exposed to the toxicant, triclosan (Sengupta et al., 2017).

Changes in protein abundance were similar between the two FPW treatments, with the only exception ($Log_2FC \geq 1.0$) being WD repeat domain phosphoinositide-interacting protein (WIPI2), whose abundance decreased in the 0.25% and increased in 0.75% FPW treatment (Figure 2-4C). In particular, WIPI2 in the aquatic invertebrate *Hydra*, is related to autophagosome formation, a process that transports cellular debris for degradation (Tomczyk et al., 2020). Daphnids increase autophagy to degrade damaged or abnormal cytoplasmic contents caused by toxicant exposure as their cells undergo apoptosis (Bacchetta et al., 2017). In this present study, WIPI2 was increased by 3-fold in the higher FPW treatment indicating that cell death was occurring at the 0.75% concentration, matching the increased mortality, impaired reproduction and decreased molts seen. Interestingly, at lower concentrations, WIPI2 is decreased 1-fold compared to the control (Table S2-7). A decrease in WIPI2 could mean little to no autophagy is occurring and in light of the 0.75% treatment there may be a threshold for triggering autophagy. In a similar study exposing contaminants to *D. magna,* only high concentrations of zinc sulfate ($ZnSO_4$) had elicited autophagy vacuoles (Bacchetta et al., 2016). The concentration of FPW at which WIPI2 function may switch is a topic to be further explored. This information provides insight into a potential mechanism of action of a complex chemical mixture at different sub-lethal concentrations.

4.3 *Implications and Conclusions*

Overall, these data show that recovery was not possible within the first generation exposed to FPW. Even after an acute exposure to FPW, detrimental protein changes in *D. magna* occurred (Figure 2-4). Furthermore, the data are supported by extensive global proteome changes, including those related to reproduction and molting; processes that when impaired ultimately lead to

mortality (Figures 2-1 to 2-3). Importantly, these results indicate that if a spill were to occur and be cleaned up within 48 hours, the impact on aquatic organisms will be devastating. *D. magna* populations can be decimated from a FPW spill, disrupting the ecosystems that they reside in. Although recovery was not possible in the first generation, future directions could investigate the potential for recovery within multiple generations after parental exposure. Like this study, a holistic approach could be taken so that not only molecular endpoints are assessed, but also physiological endpoints. Another future direction would be to study the acute and chronic toxicities of many more FPW samples to look for correlations between chemical constituents and their effects.

Table 2-1. Elemental analysis (mg/L) of experimental solutions over the 21-day period. The FPW sample came from a hydraulically fractured well (Well ID: 02-12-81-18 W6, Well O) with a 2-hour shut-in time. Water samples were obtained around the same time over the course of 21 days, with *D. magna* exposed to FPW for the initial 48 hours and the subsequent 19 days in clean OECD water. Water samples were randomized and averaged. Sample sizes were the following: Control (n=16), FPW Stock (n=1), FPW Day 0-2 (n=4), FPW Day 3-21 (n=10). Less than values indicate concentrations below detection limits. Data was indicated as ± SEM.

	Element	Control (OECD)	FPW		
			Stock	Day 0-2 (0.75%)	Day 3-21 (OECD)
Concentration (mg/L)	Li	< 0.165	< 0.165	< 0.165	< 0.165
	B	< 0.0377	12.1 ± 0.21	0.0946 ± 0.0009	< 0.0377
	Na	29.3 ± 0.3	7130 ± 30	97.5 ± 1.0	28.4 ± 0.3
	Mg	25.7 ± 0.8	324 ± 11	27.5 ± 0.9	26.3 ± 0.8
	Al	0.0243 ± 0.0009	< 0.00089	0.0066 ± 0.0005	0.0237 ± 0.0009
	Si	2.25 ± 0.06	17.0 ± 0.8	2.36 ± 0.08	2.25 ± 0.04
	Cl	194 ± 10	12600 ± 1070	393 ± 12	209 ± 9
	K	4.65 ± 1.76	374 ± 71	7.84 ± 1.76	4.79 ± 1.76
	P	0.286 ± 0.036	< 0.00052	0.185 ± 0.022	0.282 ± 0.039
	S	48.8 ± 1.7	23.1 ± 1.6	57.2 ± 0.7	56.2 ± 1.7
	Ca	103 ± 1	1380 ± 30	125 ± 1	111 ± 1
	Cr	< 0.0936	< 0.0936	< 0.0936	< 0.0936
	Mn	< 0.0880	3.57 ± 0.09	< 0.0880	< 0.0880
	Fe	0.0552 ± 0.0009	13.51 ± 0.16	0.125 ± 0.002	0.0503 ± 0.0011
	Co	0.0710 ± 0.0083	2.88 ± 0.21	< 0.0707	< 0.0707
	Ni	< 0.127	< 0.127	< 0.127	< 0.127
	Cu	< 0.0571	< 0.0571	< 0.0571	< 0.0571
	Zn	0.0350	1.60 ± 0.10	< 0.0334	< 0.0334
	Br	< 0.331	26.3 ± 0.6	< 0.331	< 0.331
	Sr	0.435 ± 0.15	136 ± 6	1.511 ± 0.059	0.430 ± 0.15
	As	0.0009 ± 0.0009	0.0341 ± 0.0001	0.0011 ± 0.0013	0.0009 ± 0.0012
	Mo	0.00346 ± 0.00051	0.201 ± 0.029	0.00367 ± 0.00070	0.00333 ± 0.00050
	Cd	< 0.00216	0.0873 ± 0.0175	< 0.00216	< 0.00216
	Ba	0.0653 ± 0.0033	2.71 ± 0.15	0.0813 ± 0.0029	0.0654 ± 0.0036
	Pb	< 0.0026	< 0.0026	< 0.0026	< 0.0026
	U	0.0029 ± 0.0001	0.106 ± 0.011	0.0029 ± 0.0001	0.0029 ± 0.0001

Table 2-2. Summary of enriched gene ontology (GO) biological processes (BP) related to FPW exposed *Daphnia magna*. The main summarized GOBP term ("Description") was assigned to a general function, indicated by colour. Differential protein abundance for each protein (log2FC > 1; Fisher's Exact Test, FDR < 0.05) that either increased or decreased among all terms per "Description" was totaled. See supplemental tables 2-5 for the breakdown of the terms for each "Description".

GO IDs	General Function	Description	#Terms	#Proteins	Abundance Change - 0.25% FPW	Abundance Change - 0.75% FPW
GO:0071705	Transport	nitrogen compound transport	4	33	-18 48	-19 53
GO:0016192		vesicle-mediated transport	1	18	-9 28	-9 29
GO:0015031		protein transport	3	16	-10 24	-10 27
GO:0071840	Cell organization	cellular component organization or biogenesis	12	60	-27 94	-29 104
GO:0051179		localization	1	34	-19 48	-20 53
GO:0033036		macromolecule localization	1	16	-10 24	-10 27
GO:0061024		membrane organisation	1	12	-6 15	-6 16
GO:1901576	Metabolism	organic substance biosynthetic process	11	15	-1 28	-1 30
GO:0044281		small molecule metabolic process	1	28	-33 10	-36 11
GO:0006629		lipid metabolic process	1	28	-50 8	-55 7
GO:0008152		metabolic process	1	163	-190 146	-219 162
GO:0071704		organic substance metabolic process	2	119	-151 106	-175 116
GO:0009056		catabolic process	1	48	-77 17	-88 22
GO:0005975		carbohydrate metabolic process	1	37	-74 6	-81 6
GO:0010467	Transcription	gene expression	1	30	-12 53	-15 58
GO:0006397		mRNA processing	4	13	-5 28	-6 32

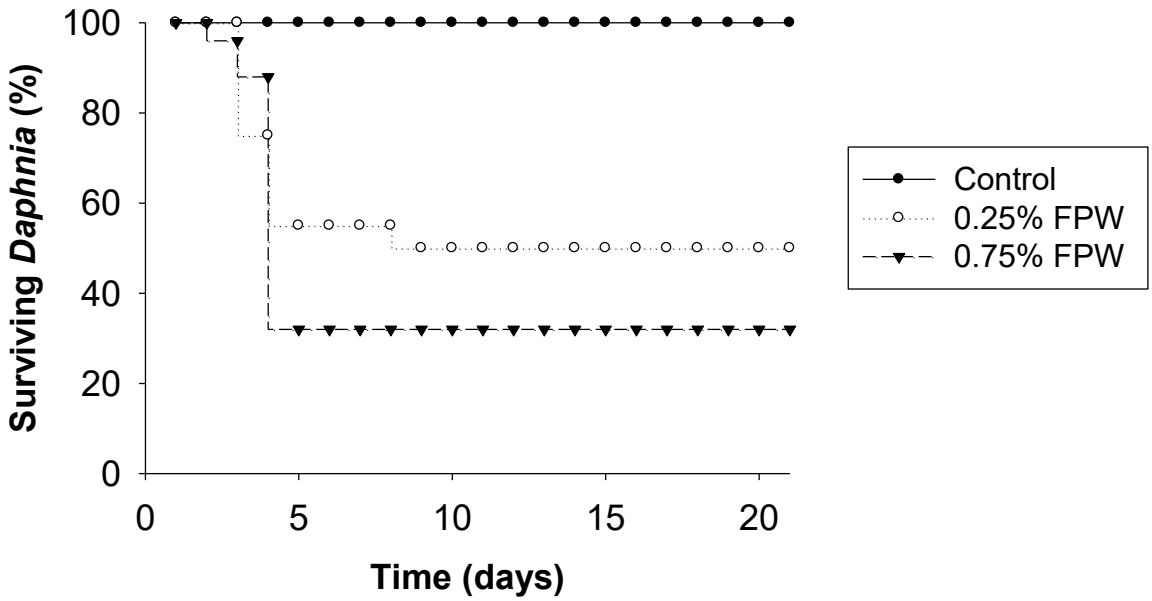

Figure 2-1. Survivorship curve of < 24-h *D. magna* exposed to FPW for 2 days and then placed in clean water for 19 days, with 25 individuals in the control and 0.75% FPW treatments and 20 individuals in the 0.25% FPW treatment.

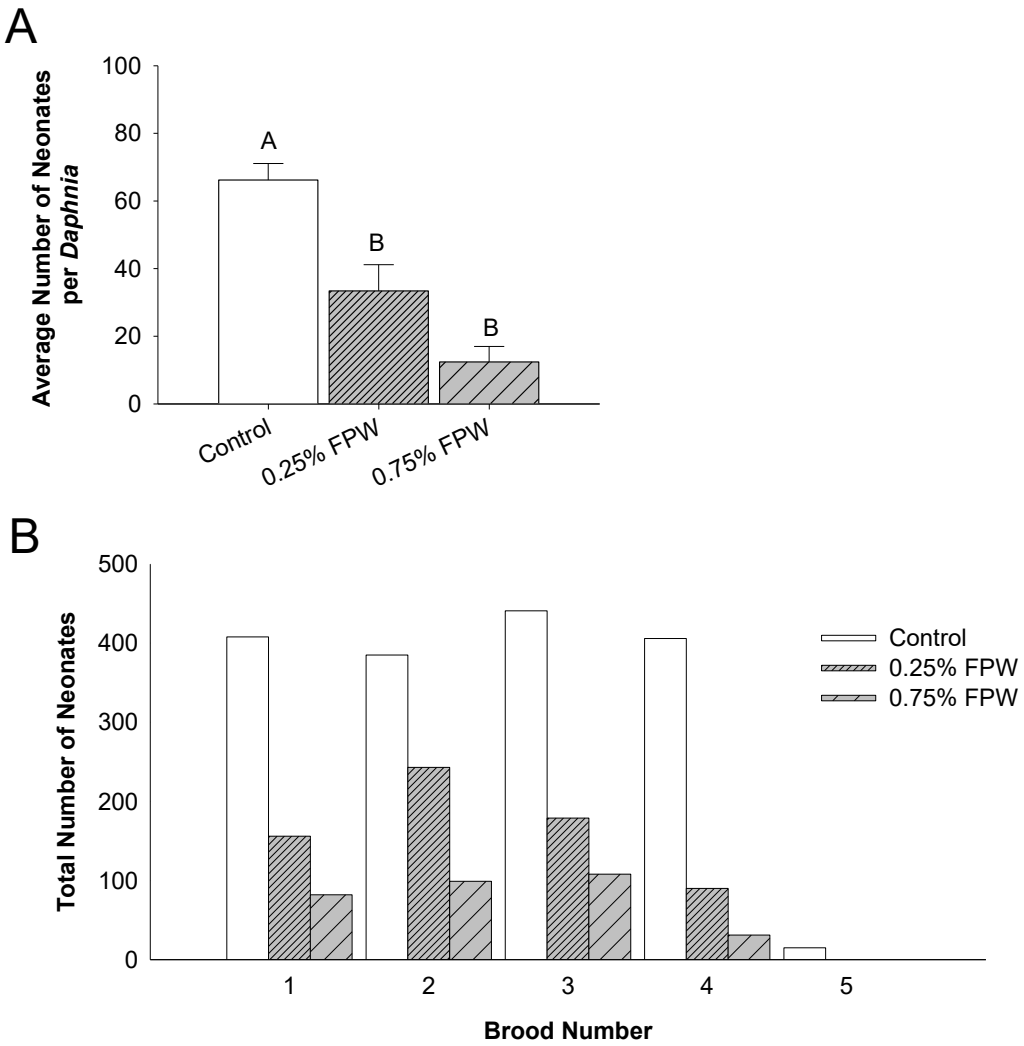

Figure 2-2. The brood characteristics of *Daphnia* exposed to FPW for 2 days, then clean water for 19 days for the average number of neonates produced per **(A)** all *Daphnia* and **(B)** the total number of neonates per brood. Bars represent the mean ± SEM. Significance was denoted with different letters (Kruskal-Wallis, $p < 0.05$).

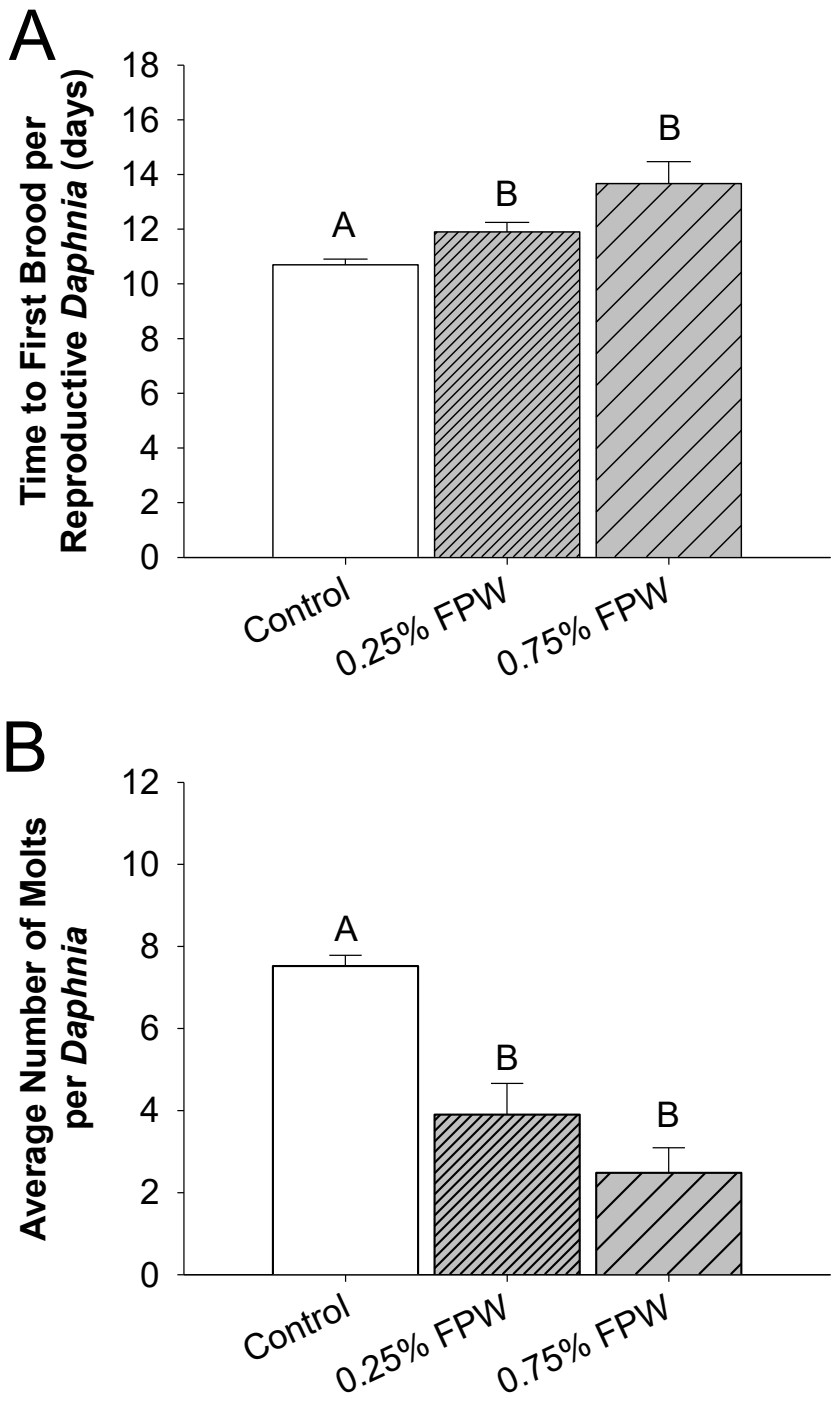

Figure 2-3. Less than 24-h old *Daphnia* were exposed to FPW for 2 days, then clean water for 19 days and scored for **(A)** the time to first brood for each reproducing daphnid and **(B)** the average number of molts for all *Daphnia*. Bars represent the mean ± SEM. Significance was denoted with different letters (Kruskal-Wallis, $p < 0.05$).

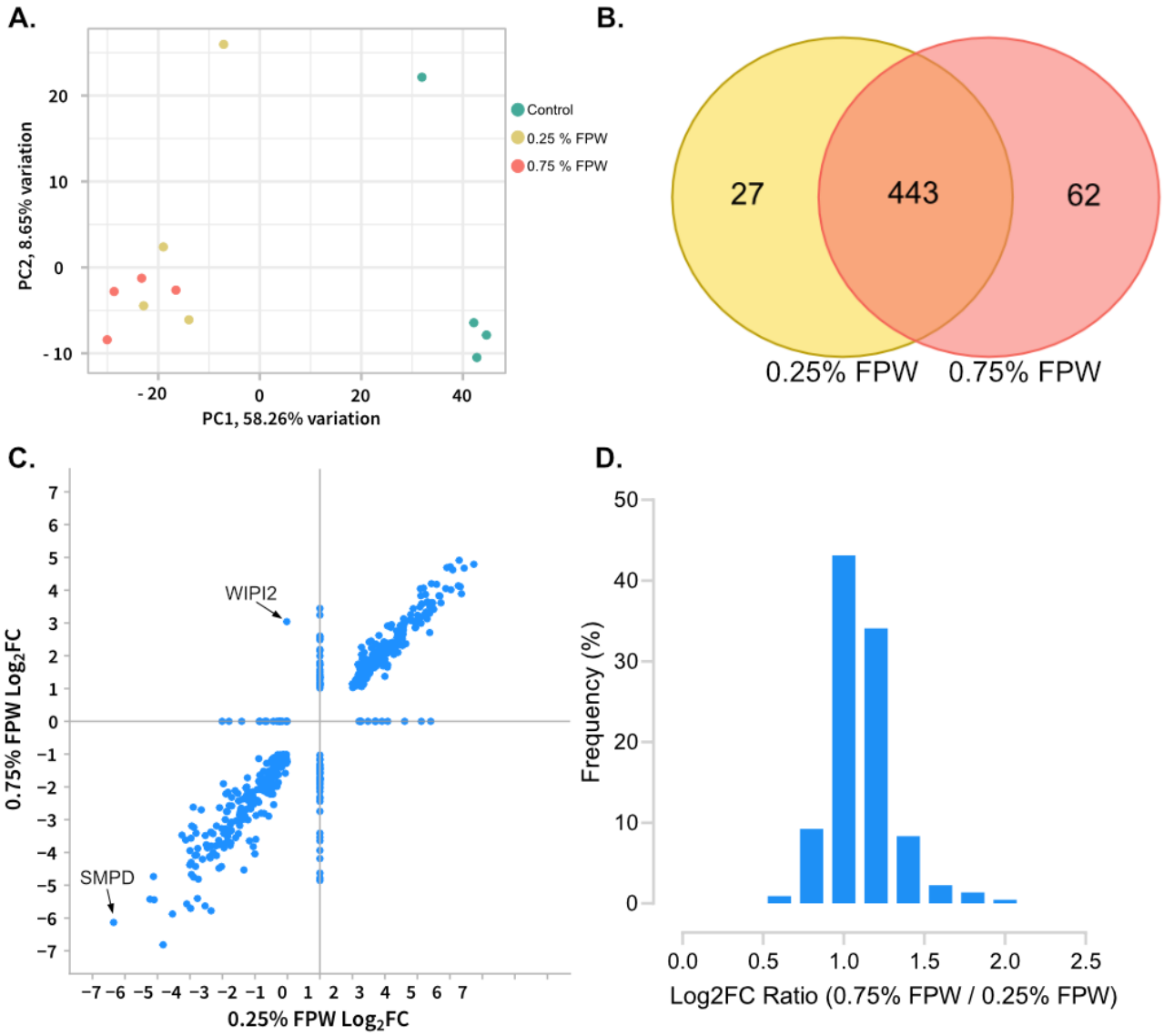

Figure 2-4. Quantitative proteomics of *D. magna* cells. **(A)** Principal component analysis of quantified proteins in control and treated samples. **(B)** Venn diagram of significantly changing proteins (q-value <0.05; Log2FC >1) after 0.25% and 0.75% treatments. **(C)** Scatter plot showing changes in abundance of individual proteins upon 0.25% (X-axis) and 0.75% (Y-axis) treatment. **(D)** Frequency distribution of the ratios in protein changes (Log2FC) upon 0.75% vs 0.25% treatment.

Chapter 3 – A multigenerational study on the recovery of *Daphnia magna* exposed to flowback and produced water

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1.0 Introduction

Hydraulic fracturing is an unconventional method used to extract oil and gas from subterranean formations of low permeability (Lester et al., 2015). This method of extraction requires large volumes of water mixed with proppants and other chemicals to facilitate higher yields of oil and gas retrieval (Kim et al., 2016). Fluid is injected into the ground at high temperatures and pressures to fracture shale, coal beds, and tight sands, releasing the oil and gas constituents trapped within (Stringfellow et al., 2014). To retrieve the oil and gas, the high-pressure injection creates a pressure buildup within the well such that when the well is reopened, the pressure difference forces the fluid in the formation to flow back to the surface (Soeder, 2021). The fluid that returns to the surface post-pressure release contains hydrocarbons, other organic and inorganic constituents from both the formation and the initial injection fluid (Stringfellow et al., 2014). The hydrocarbons are separated from the rest of the fluid (a.k.a., wastewater) according to density (Lockhart et al., 1987). The hydrocarbons are collected for oil and gas usage, meanwhile the wastewater is transported for disposal (Alessi et al., 2017). This wastewater is termed flowback and produced water (FPW) (Stringfellow et al., 2014). During transport of FPW spills can occur and potentially contaminate water bodies (Entrekin et al., 2011; Folkerts et al., 2020a).

FPW consists of three major chemical groupings: inorganics (e.g., Na, Cl, Cu, Zn), organic (e.g., PAHs, surfactants), and geogenic chemicals made from the wellbore environment (Folkerts et al., 2020a). Firstly, the injection fluid may contribute to a small portion of the salts found in FPW (Rosenblum et al., 2017a; Stringfellow et al., 2014) but the majority of metal and salt content is from the formation producing a highly saline FPW (e.g., total dissolved solids > 200,000 mg/L) (Acharya et al., 2020; Rosenblum et al., 2017a). In contrast, freshwaters have a total dissolved solid content of < 1000 mg/L, so FPW can be > 200-fold more saline (Vengosh et al., 2014). FPW

is dominated primarily with sodium (Na) and chloride (Cl), which can reach concentrations of 95,500 mg/L and 207,000 mg/L (Folkerts et al., 2020a). With increases in human activity, elevated salt concentrations are more commonly found in water bodies. Although more common, salts may still have adverse effects when they enter into a freshwater source. Anthropogenic salinization in freshwater ecosystems from a FPW spill can cause ionoregulatory distress, reactive oxygen species (ROS) production, or mortality (Folkerts et al., 2020a). Ionoregulatory distress has been shown in rainbow trout (*Oncorhynchus mykiss*) where interlamellar cell mass decreased, affecting oxygen uptake (Blewett et al., 2017b). Salts also affected antioxidant capacity in the gill and liver of rainbow trout which was attributed to an overproduction of ROS causing damage in those organs (Blewett et al., 2017b; He et al., 2017b).

Metals derived from the geological formation are usually found in the mg/L levels and are toxic to aquatic biota at these concentrations (Folkerts et al., 2020a). However, metals and ions present in FPW are subject to complexation with other chemicals and competition with other ions and metals leaving them potentially less bioavailable to the organism (Norwood et al., 2003). Some metals are essential for all organisms in low concentrations, but FPW can have elevated levels of essential metals such as iron (Fe), copper (Cu), and zinc (Zn) which can potentially cause toxicity (Folkerts et al., 2020a). High concentrations of essential and non-essential metals can cause toxicity due to their interactions with ions and other metals which can lead to osmoregulatory distress and dysfunction in cellular activity (Grosell, 2012), induction of ROS (Lin and Spallholz, 1993), and inhibition of energy production (Tseng, 2004).

Organic chemicals originate primarily from the injection fluid, but hydrocarbons from the formation can contribute to the total organic load and vary between wells (Folkerts et al., 2020a). Although organics are prevalent in FPW, the specific organic makeup will vary between wells.

Many organic compounds are formed under the high heat (e.g., 200^{o}C) and high pressure (e.g., 69,000 kPa) well-bore environment (Kahrilas et al., 2016). A multitude of organic constituents occur in FPW, including classes of compounds with known toxic effects, such as polycyclic aromatic hydrocarbons (PAHs), volatile organic compounds (VOCs), and ethoxylated surfactants (Lester et al., 2015; Rosenblum et al., 2017b; Thurman et al., 2014). Some organic chemicals can cause endocrine disruption (Kassotis et al., 2018), reproductive impairments (Feldmannová et al., 2006), increased pericardial edemas and lowered heart rates (McGruer et al., 2021), growth and developmental inhibition (Cong et al., 2021), and ROS formation (Xie et al., 2006). However, organic compounds may complex in the aquatic environment reducing their bioavailability (Moeckel et al., 2014). They can also be subjected to microbial degradation with further degradation occurring over time via photolysis or oxidation (Shemer and Linden, 2007; Xu et al., 2018). Once ingested the potential for toxicity might also be reduced due to biotransformation pathways (Buhler and Williams, 1988). Conversely, there is the possibility that biotransformation or abiotic transformations may increase toxicity in aquatic systems (Buhler and Williams, 1988; Marrot, 2018). Moreover, complexation with metals or salt ions may induce synergic toxicity (i.e., non-additive toxicity) (Deneer et al., 1988; Xie et al., 2006) and some compounds are so complex that microorganisms are unable to degrade them (Xu et al., 2018). As a result, organic compounds in FPW may also persist and bioaccumulate in the aquatic environment.

Once released, many of the individual components of FPW may persist in the environment long term (e.g., hydrocarbons persists for >25 years) (Fingas, 2000; Wang et al., 1998) potentially interacting with aquatic organisms over a chronic timescale. Thus, it is crucial to monitor the effects of FPW over multiple generations to understand the impacts after remediation of a spill. *Daphnia magna* is a freshwater invertebrate shown to be highly sensitive to FPW (Blewett et al.,

2017a; Folkerts et al., 2019). *D. magna* start developing eggs in their brood chamber around 5 days at 20°C and produce a brood every 3-4 days (Ebert, 2005), with multiple neonates per brood. In general, multigenerational studies use third brood neonates as they are considered to be the hardiest against environmental perturbations and have more consistent results than earlier or later broods (Lampert, 1993; Lyu et al., 2016). Multigenerational studies for *D. magna* require monitoring for at least four generations if the parental (F_0) generation was exposed to the initial stressor. This is because germ line exposure ends at the fourth generation. The first filial generation (F_1) is exposed to the stressor as embryos and the second filial generation (F_2) is exposed as germ cells during F_0 exposure (Jeremias et al., 2018). The fourth generation, also known as the third filial generation (F_3) is considered to be the first generation unexposed to the stressor (Jeremias et al., 2018).

Studying multiple generations of *D. magna* can elucidate the degree with which the toxicity might persist within a generation and across a generation once the toxicant is removed. Whether or not *D. magna* can survive and exhibit reproductive fitness after FPW exposure over multiple generations is, in part, a measure of recovery. Recovery can be defined as the reversibility of a toxicant's effects after its removal, and a return to "pre" stress conditions (Pandher et al., 2012). In this study, recovery of three subsequent *D. magna* generations in clean water was tested after parental exposure to FPW. A treatment group exposing all four generations to FPW was also monitored to compare if there were differences by the F_3 generation after continuous exposure. It was hypothesized that recovery to FPW exposure would be possible by the F_3 generation, the first generation unexposed to FPW. Neonates were followed to the F_3 generation – the first generation beyond the germ cell line.

2.0 Materials and Methods

2.1 *Colony Maintenance*

The colony for *Daphnia magna* was originally acquired from Aquatic Research Organisms (ARO, New Hampshire, U.S.A.) and housed in the Aquatics Facility of the Department of Biological Sciences at the University of Alberta, since 2019. Colony health was maintained following the *Daphnia magna* care guidelines from the Organization of Economic Cooperation and Development (OECD, 2004) with a few modifications. Briefly, daphnids were held in 1 L of OECD water made from dechlorinated Edmonton tap water with the following salt concentrations: 2 mM $CaCl_2$ • 2 H_2O, 0.5 mM $MgSO_4$ • 7 H_2O, 0.77 mM $NaHCO_3$, 0.08 mM KCl (OECD, 2004) (Table 3-1). Water changes occurred every 48 to 72 hours, and temperature and light were kept consistent at 20 ± 1ºC and 12 h/12 h light/dark photoperiod, respectively. The colony was fed 3 mL yeast/cerophyll/trout chow (YCT) and 3 mL of concentrated algae daily (ARO, New Hampshire, U.S.A.) and a weekly supplement of 100 µL of Roti-Rich™ (VWR, Alberta, Canada).

2.2 *FPW Information and Analysis*

All glassware was soaked for at least 24 h in 5% HNO_3, 10% EtOH, and then rinsed in distilled water prior to experimentation. We obtained the FPW sample from the Montney formation on June 2, 2019 (Well ID: 02-12-81-18 W6 Pad/Well O). The sample was collected during a 2-hour post-injection flowback period and was considered 100% FPW. The FPW sample was stored in an airtight opaque container at 20 ± 2°C until experimentation. Water samples for inorganic chemical characterization were collected once a week throughout the experiment for the stock solutions (OECD water and 0.1% FPW) and averaged. The measured inorganic chemical characterization is listed in Table 3-1.

2.3 *Physiological Experiment*

D. magna neonates (< 24 h) were placed individually in glass scintillation vials (20 mL) containing 20 mL of exposure medium: control, recovery, and chronic (n=20). See Figure 3-1 for a schematic on the experimental design. All control generations (F_0-F_3) were placed in clean uncontaminated OECD water (see Section 2.1). The recovery treatment had the parental generation (F_0) exposed to 0.1% FPW and all subsequent generations (F_1-F_3) exposed to clean uncontaminated OECD water. The chronic treatment had all generations (F_0-F_3) exposed to 0.1% FPW. To make the final 0.1% FPW stock, 100% FPW was mixed to ensure a uniform concentration, and then subsequently diluted with OECD water to yield a final concentration of 0.1% FPW. Exposures were followed over the course of 21 days, and every 2 days, *D. magna* was replaced and exposures were refreshed, whether that be OECD water or 0.1% FPW. *D. magna* were fed 100 µL of algae and 100 µL of YCT daily, regardless of water change. *D. magna* survival, molts, and offspring production were observed and recorded daily at the same time to ensure consistency. Any offspring produced were discarded daily, except for third brood offspring. Three representatives (< 24 h) from the third brood of each individual were used to start the subsequent generation and were maintained in the appropriate medium as seen in the experimental design (Figure 3-1). On day 7, two of the three individuals were chosen as representatives to follow for the F_1 generation due to the many deaths that occurred in the F_0 generation. For the F_2 and F_3 generations, only one of the three individuals were chosen. Individuals were chosen if they were female and given preference if they had eggs. If the selection criteria were met for multiple females, then the individual was randomly chosen (based on a number generator) to be the representative for the next generation.

2.4 *Water Chemistry Analysis*

Water samples (n=30) for each stock solution were analyzed on an inductively coupled plasma-mass spectrometer/mass spectrometer (ICP-MS/MS) where the samples were diluted 40x in 2% nitric acid (HNO_3). Standards were also prepared in 2% HNO_3 and used as a measure to verify sample concentrations were correct. Scandium, lutetium, bismuth, germanium, and indium were used as an internal standard mixture for the various ICP-MS/MS measurements, which used the collision gases helium, oxygen, and hydrogen.

2.5 *Molecular Epigenetic Exposures*

D. magna neonates (< 24 h) were placed in groups of 25 into 600 mL glass beakers. Each beaker contained 500 mL of either OECD water (all control and F1-F3 recovery) or 0.1% FPW (F0 recovery and all chronic) with 3 replicates for each treatment. For each water change, the beaker would be rinsed with distilled water and dried with a paper towel before adding 500 mL of either OECD water or 0.1% FPW depending on experimental treatment. Any dead *D. magna* were removed during water changes. *D. magna* were fed daily 2.5 mL of algae and 2.5 mL of YCT daily consistent with the physiological experiment above. On the first day that neonates were produced between days 17-19 (generally time for third brood), 25 neonates, if available, were selected to start the next generation. At the end of the 21 days for each generation, the ratio of males to females was recorded, then adult female *D. magna* were filtered, had their eggs removed, frozen in liquid nitrogen, and stored at -80^{o}C until processing.

Processing the samples for epigenetic analysis will require DNA extraction using the Epicentre MasterPure Complete DNA and RNA Purification Kit (Mandel Scientific Company, Guelph, Canada). Three snap-frozen adult female *D. magna* (n=3) from the F_0 control, F_0 chronic, and F_3 recovery treatments will undergo DNA extraction. After extraction, samples will be sent to

a sequencing center (Genome Quebec, Montreal, Canada) for whole genome bisulfite sequencing using the NEBNext Enzymatic Methyl-seq library preparation kit (New England Biolabs, Whitby, Canada). Epigenetic analyses are currently underway, but will not be discussed further in this thesis as they are not completed yet.

2.6 *Statistics*

All data had failed the Shapiro-Wilk Normality and no transformations made could normalize the data. Data that did not pass the equal variance test was transformed using log10. Normality likely failed due to a lack of variability in the small number of female surviving daphnids used for the analyses. A Two-Way ANOVA was performed using the Holm-Sidak method on SigmaPlot version 11.1 (Systat Software, California, U.S.A). SigmaPlot version 11.1 was used to graph all data where data was expressed as means ± standard error of the mean (SEM; Systat Software, California, U.S.A). Significance was accepted at $\alpha = 0.05$ and p values were denoted.

3.0 Results

3.1 *Water Chemistry*

Stock solutions for the two treatments (control and 0.1% FPW) have their elemental concentrations reported in Table 3-1. Between the two treatments, more than half of the analyzed elements were below detection limits (Table 3-1). In 0.1% FPW, there was a slightly elevated strontium (Sr) concentration of 0.464 ± 0.006 mg/L compared to the control at 0.346 ± 0.004 mg/L. The other nine detectable elements [i.e., sodium (Na), magnesium (Mg), aluminum (Al), sulphur (S), calcium (Ca), barium (Ba), and uranium (U)] had similar concentrations between the two

treatments. The 0.1% FPW did not follow a linear dilution from the measured 100% stock (Boyd et al., 2021).

3.2 *Physiological Experiment*

The F_0 generation had a total of 6, 7, and 9 males out of 20 daphnids in the control, recovery, and chronic treatments, respectively (Table 3-2). Upon F_0 FPW exposure in the recovery and chronic treatments, there were 6 and 7 deaths respectively (Table 3-2), decreasing the sample number for the F_1 generation. The F_0 control group had 3 deaths compared with the FPW exposure groups (F_0 recovery and F_0 chronic; Table 3-2). The total number of *D. magna* in the filial generations do not correlate with the number of leftover daphnids after removing the dead and the males (Table 3-2).

For the time to first brood, there was a statistically significant interaction between generation and treatment (Figure 3-2, $p < 0.001$). In the F_0 generation, treatments exposed to FPW (recovery and chronic) but not control had their time to first brood delayed by ~2.5 days (Figure 3-2B, $p < 0.001$). The time to first brood was not different between the treatments in the F_1 and F_2 generations whether FPW exposure continued (i.e., chronic) or not (i.e., recovery; Figure 3-2). The first generation unexposed to parental FPW exposure (F_3 recovery) had a time to first brood of 9.8 ± 0.1 days, which was not significantly different compared to the control group at 9.8 ± 0.3 days (Figure 3-2B, $p = 0.901$). In the F_3 chronic treatment, the time to first brood was 11.7 ± 0.7 days, 2 days later than the F_3 control group (Figure 3-2B, $p < 0.001$).

In the control treatments, there was a noticeable decrease in the time to first brood for the F_1 generation compared with the F_2 generation (Figure 3-2A, $p = 0.003$). This was not seen when the dead daphnids were removed from the data (Figure 3-2B, $p = 0.009$). The F_0 recovery group

was significantly different compared with all the subsequent generations (F_1, F_2, $F_{3)}$ (Figure 3-2B, $p < 0.001$). The chronic F_0 time to first brood was later than the F_1 and F_2 generations, but the same as the F_3 generation (Figure 3-2).

There was a statistically significant difference between generations for the average number of neonates produced per total and surviving reproductive daphnid (Figure 3-3, $p < 0.002$), and a statistically significant difference between treatments for the average neonates produced for all daphnids (Figure 3-3, $p = 0.019$). The average number of neonates produced per *Daphnia* was lower in the F_0 generation compared to the filial generations (F_1, F_2, F_3) (Figure 3-3A). In the F_0 generation, there was also a decrease in the number of neonates produced in the chronic FPW treatment compared to the control group (Figure 3-3, $p = 0.004$). When accounting for only surviving reproductive *Daphnia*, the neonate production was not significantly different in any of the treatments or generations (Figure 3-3B, $p = 0.010 – 0.821$).

The average number of molts was statistically different between generations for all daphnids (Figure 3-4A, $p < 0.001$), while for the surviving reproductive daphnids, there was a statistically different average number of molts between the treatments (Figure 3-4B, $p = 0.025$). The number of molts also did not differ between any of the treatments when accounting for all daphnids (Figure 3-4A, $p = 0.035 – 0.847$). But, there was a difference in the recovery treatment between the F_0 with the F_1 and F_2 (Figure 3-4A, $p = 0.010$ and 0.003, respectively). The number of molts was lower in the F_2 chronic treatment at 8.0 ± 0.5 molts compared to the F_2 recovery treatment at 9.1 ± 0.2 molts (Figure 3-4B, $p = 0.012$).

3.3 *Molecular Exposure Summary*

As stated prior, the molecular experiment is of interest and is underway. Although the epigenetic analysis could not be completed, the group exposures gave valuable information regarding the survivorship and sex ratios of *D. magna* in this experiment that reflected the results seen in the physiological experiment. Like the physiological experiment, all treatments had a high number of males in the F_0. The males accounted for at least a third of the total daphnids from the F_0 generation for all treatments (Table 3-3). Males in future generations (F_1-F_3) were not as common, with a maximum of 11 males produced in the F_3 recovery group (Table 3-3). In the F_0 FPW exposed treatments (recovery and chronic), the dead accounted for another third of the total daphnids (Table 3-3). Contrasting the individual exposures, there was a higher percentage of deaths in the F_1 generation for the recovery treatment at 41% and chronic treatment at 51% (Table 3-3). In the F_2 and F_3 generations, only the chronic treatment had deaths exceeding 20% (Table 3-3).

4.0 Discussion

Recovery - in terms of this thesis - can be measured via reproductive fitness in *D. magna*, where reproduction has returned to a state prior to FPW exposure. Reproduction was used as a measure of recovery because *Daphnia* allocate 60% of their total energy to reproduction under ideal conditions (McCauley et al., 1990). Only the time to first brood was delayed in *D. magna* exposed to FPW in the F_0 generation. In subsequent generations (F_1 and F_2), whether or not FPW was present did not affect the time to first brood. The time to first brood was the same as the control in the F_3 recovery treatment whereas the time to first brood was delayed in the F_3 chronic treatment. Thus, at 0.1% of this FPW sample, the time to maturity was only delayed after the F_3 – the

generation first unexposed to F_0 FPW exposure. At higher concentrations of this FPW sample, reproductive outputs may be impaired in addition to maturation time, as seen in previous studies. Testing a larger concentration range and various sources of FPW could give a more robust conclusion to the findings from this study.

4.1 *Water Chemistry Analysis*

Inorganic measurements were taken to determine the salt and metal concentrations in the stock OECD and 0.1% FPW solutions. Concentrations were mostly below detection limits for many elements at the 0.1% dilution (Table 3-1). The 0.1% dilution was not linear from the 100% stock solution (See Boyd et al., 2021). This sample had a lower concentration of salts and metals compared with other FPW samples with similar shut-in times (Folkerts et al., 2020b, 2019; Weinrauch et al., 2021). The concentrations of phosphorus, iron, cobalt, zinc, arsenic, and molybdenum within the OECD stock were lower than previous water chemistry analyses of the same stock water (Luu et al., unpublished results). Stock OECD water for this experiment was made during the spring melt to early summer. During this time, there may be an influx of dissolved organic matter in water which can complex with the low concentrations of these elements present (Weng et al., 2002). Dissolved organic matter would be filtered out by the water treatment facilities to produce the dechlorinated tap water used to make the OECD stock. Many elemental concentrations in the 0.1% FPW stock were similar in concentration to the uncontaminated OECD water (Table 3-1). Therefore, the salt ions and metals were not elevated in the 0.1% FPW sample.

4.2 *FPW causes Death and Delayed Maturation Time*

There was a higher number of deaths in F_0 FPW exposed treatments compared to the other generations (F_1, F_2, F_3) in clean water (control and recovery) and FPW (chronic) (Tables 3-2 and

3-3). Survivorship after F_0 FPW exposure was at 65%-72% in this study at 0.1% FPW (Tables 3-2 and 3-3). This study had a decreased total FPW concentration compared to the same FPW sample at a higher concentration of 0.75% (Boyd et al., 2021). In Boyd et al. (2021), there was only 20% survival after a 21-day exposure to 0.75% FPW. As FPW concentration decreased, mortality also decreased. Although mortality decreased, surviving F_0 daphnids exposed to FPW had delayed maturation rates.

F_0 FPW exposure delayed the time to first brood in *D. magna* in the recovery and chronic treatments (Figure 3-2). This is consistent with previous FPW studies in *D. magna* (Blewett et al., 2017a; Boyd et al., 2021). Unlike the previous studies (Blewett et al., 2017a; Boyd et al., 2021), there was no decreased reproductive output in this study. This study used the same FPW well sample as Boyd et al. (2021) but at a 7.5-fold decrease in concentration. Concentrations of salts and metals were similar between the OECD stock and the 0.1% FPW, therefore it is unlikely that these chemical groups in FPW were responsible for the delayed time to first brood. In previous chronic studies, the toxicity and impairments in *D. magna* were attributed to the organic fraction in FPW (Blewett et al., 2017a; Boyd et al., 2021). Although organic characterization was not analyzed in this study, there are qualitative commonalities in the organics (e.g., PAHs) found in FPW samples where organics were characterized. Common PAHs include naphthalene, phenanthrene, anthracene, and acenaphthene (Folkerts et al., 2020b; He et al., 2018, 2017a). Phenanthrene has been found to delay time to first brood by ~ 3 days in *Daphnia pulex* at 0.36 mg/L levels (Geiger and Buikema, 1982). It was hypothesized that the mechanism at which reproduction was affected was through steroid metabolism, where PAHs may act as a competitive inhibitor to a typical steroid on a receptor protein (Geiger and Buikema, 1982). This competition induces a steroid cascade pathway, influencing glucose and lipid metabolism downstream, and

ultimately affecting energy allocation towards reproduction (Geiger and Buikema, 1982). Another mechanism that PAHs induce is cytochrome p450 activity (Buhler and Williams, 1988). During PAH detoxification involving cytochrome p450 enzymes, toxic metabolites may be produced to cause cytotoxicity or genotoxicity which may not immediately impact survival but can be responsible for impairments in reproduction (Geiger and Buikema, 1982).

Previous studies involving PAHs and FPW (Blewett et al., 2017a; Boyd et al., 2021; Feldmannová et al., 2006; Geiger and Buikema, 1982; Luu et al., unpublished results) have shown that molting and reproduction were not affected in any of the treatments nor generations after FPW exposure (Figures 3-3B and 3-4B). The same FPW sample was used in a previous study at 0.25% FPW and found decreased molting, reduced neonate output, and delayed time to first brood (Luu et al., unpublished results). Luu et al. linked these developmental and reproductive impairments to a decrease in carbohydrate and lipid metabolism. Oxygen consumption, a proxy for metabolism, has also been seen to be negatively impacted with respect to FPW exposure in *D. magna* (Blewett et al., 2017a). In addition, naphthalene, a common PAH in FPW, has been found to decrease oxygen consumption and reduce metabolism in *Daphnia* (Geiger and Buikema, 1981). Metabolism is the sum of all processes that require energy resources, where energy is allocated towards maintenance, growth, or reproduction (McCauley et al., 1990). An explanation to why reproductive outputs were not impacted but time to maturation was delayed may be due to the low concentration of FPW used in this study. At an even lower concentration of FPW (e.g., 0.1%), *D. magna* may still have reduced energy but divert that energy towards growth rather than reproductive outputs. It is of note that this delayed maturation time may be specific to this FPW sample. Previous research has shown when a stressor (e.g., restriction of nutrient resources) is applied, *D. magna* will allocate 40% of their energy towards reproduction compared with 60% in

a no stressor environment (McCauley et al., 1990). It may also be possible that the organics present in the FPW degraded over time as this study was conducted over a year after the Luu et al. study. The various methods of degradation include VOC dissipation, photolysis and oxidation reactions (Shemer and Linden, 2007).

4.3 *Exposure Effects on the Germ Line*

F_0 exposure to FPW had delayed the time to first brood in both the F_0 recovery and chronic treatments but this was not seen in the F_1 and F_2 generations of *D. magna* whether FPW exposure continued (chronic treatment) or not (recovery treatment; Figure 3-2B). However, if FPW exposure continued to the F_3 generation, like in the F_3 chronic treatment, then time to first brood was again delayed (Figure 3-2B). Despite FPW exposure continuing in the chronic F_1 and F_2 generations, it appears that these subsequent exposures did not confer tolerance to FPW in the chronic F_3 generation. In previous multigenerational studies using low concentrations of contaminant (i.e., 1-12 µg/L copper), sensitivity to the contaminant decreased with each successive generation until the F_3, where it slightly increased albeit not significantly (Bossuyt and Janssen, 2003). This could indicate acclimation to the contaminant and resistance being developed after F_0 exposure to the F_1 embryos and F_2 germ cells, but tolerance is not retained once the F_3 generation is reached. Like Bossuyt and Janssen (2003), these results suggest that increased tolerance to a sublethal concentration of a contaminant can be a rapid process taking only a couple generations, but to permanently adapt for maximal tolerance may require several generations (>4 generations).

4.4 *Low controls*

Of note, there was a lower number of surviving females in the controls within the F_0 control treatment (Table 3-2). Fewer surviving females led to the average number of neonates produced

being lower in the F_0 compared to subsequent generations (Figure 3-3A). Although the number of neonates was reduced in the F_0 control, it was not significantly lower than the subsequent generations (Figure 3-3B). In all control generations, the average number of neonates per surviving reproductive *Daphnia* did not reach 60, which is required for an OECD chronic reproduction test to be valid (OECD, 2012). A possible explanation for lower neonate production may be because the neonates from the experiment were those obtained from the colony reared during the spring melt. During the spring melt, there is a decrease in water quality with increased ammonia-nitrogen (Li et al., 2020) as the influx of water creates a strain on water treatment facilities. Ammonia can cause stress and damage to the gills in fish (Francis-Floyd et al., 2009) and cause mortality and impairments in reproduction in *D. magna* (Gersich and Hopkins, 1986). It may be possible that during the spring melt, increased dissolved organic matter complexed with essential metals (e.g., zinc, iron, cobalt, molybdenum) and was filtered out prior to entering the Edmonton municipal water system. This may explain the decreased metal concentrations that could impact the water quality and thus the reproductive health of *D. magna* (Table 3-1). *D. magna* reared in lower quality water would likely produce neonates of a reduced quality despite the experimental daphnids being raised in water after the spring melt.

5.0 Conclusion

This study highlights the importance of conducting chronic studies over acute studies. Acute studies can only show the concentration of FPW which is toxic to the organism but cannot measure an organism's reproductive fitness through endpoints like neonate production, time to maturation, and molting behaviour. Multigenerational studies not only measure an organism's reproductive fitness but can help understand the mechanism of tolerance against a stressor over time. Epigenetic factors may contribute to the mechanism of tolerance inherited through prior

generations. After F_0 exposure to 0.1% FPW, *D. magna* experienced a delayed time to first brood. Regardless of continued FPW exposure in the F_1 and F_2 generation, after F_0 exposure to FPW, *D. magna* were able to recover from the effects of FPW. However, if FPW exposure continued to the F_3 generation, like it did in the chronic F_3 treatment, the time to first brood would again be delayed. Despite the F_1 and F_2 generations being unaffected from parental FPW exposure, FPW spill remediation must be completed rapidly to prevent fewer subsequent generations being negatively affected.

Table 3-1. Measured inorganic water chemistry of stock solutions for OECD water and 0.1% FPW (Well ID: 02-12-81-18 W6, Well O, 2-hour shut-in time) over the course of the 10-week experiment. Water samples were taken once a week with 3 replicates for each sample point. Data were averaged between 3 replicates taken weekly over the 10-week experiment (n=30). Concentrations were represented as mean ± SEM in mg/L. BDL indicates concentrations below the detection limit for that element.

	Element	Control (OECD)	0.1% FPW Stock
Concentration (mg/L)	Li	BDL	BDL
	Na	33.4 ± 0.3	40.6 ± 0.4
	Mg	25.5 ± 0.4	25.3 ± 0.3
	Al	0.0119 ± 0.0009	0.0131 ± 0.0013
	Cl	141 ± 3	125 ± 2
	K	3.14 ± 0.05	3.48 ± 0.04
	P	BDL	BDL
	S	35.7 ± 0.7	36.4 ± 0.8
	Ca	88.2 ± 1.1	88.8 ± 0.9
	Ti	BDL	BDL
	Cr	BDL	BDL
	Mn	BDL	BDL
	Fe	BDL	BDL
	Co	BDL	BDL
	Ni	BDL	BDL
	Cu	BDL	BDL
	Zn	BDL	BDL
	Br	BDL	BDL
	Sr	0.346 ± 0.004	0.464 ± 0.006
	As	BDL	BDL
	Mo	BDL	BDL
	Cd	BDL	BDL
	Ba	0.0524 ± 0.0018	0.0547 ± 0.0018
	Pb	BDL	BDL
	U	0.00275 ± 0.00010	0.00277 ± 0.00011

Table 3-2. Survivorship of daphnids and proportion of males across the F_0, F_1, F_2 and F_3 generations for each treatment: Control (Ctrl), Recovery (Rec), and Chronic for the physiological experiment. Twenty daphnids were used at the beginning of the experiment. Two representative third brood neonates were chosen to follow for the F_1 generation while one neonate was followed for the F_2 and F_3 generations. Males were not counted for any filial generation as they were discarded when choosing the representative neonate to follow.

	Generation											
	F0			F1			F2			F3		
	Ctrl	Rec	Chronic	Ctrl	Rec	Chronic	Ctrl	Rec	Chronic	Ctrl	Rec	Chronic
Dead	3	6	7	2	0	2	0	0	4	2	0	1
Male	6	7	9	-	-	-	-	-	-	-	-	-
Total	20	20	20	20	12	10	19	10	9	18	10	7

Table 3-3. Survivorship of daphnids and proportion of males across each generation for each treatment: Control (Ctrl), Recovery (Rec), and Chronic for the molecular experiment. Twenty-five daphnids were used at the beginning of the experiment. Up to twenty-five third brood neonates were chosen to follow for the next generation, if produced on a single day.

	Generation											
	F0			F1			F2			F3		
	Ctrl	Rec	Chronic	Ctrl	Rec	Chronic	Ctrl	Rec	Chronic	Ctrl	Rec	Chronic
Dead	12	50	42	23	52	66	15	8	21	5	20	27
Male	61	59	61	2	0	0	0	0	0	0	11	0
Total	150	150	150	127	127	111	76	116	90	94	124	100
n	6	6	6	6	6	5	5	5	4	5	5	4

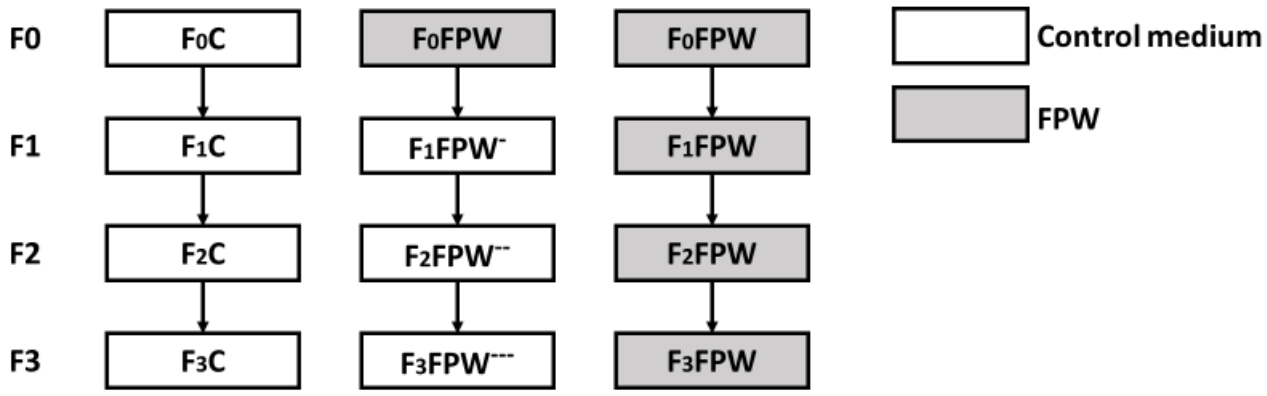

Figure 3-1. Summary of the multigenerational experimental design. F0, F1, F2, and F3 represent the parental generation, first filial generation, second filial generation, and third filial generation, respectively. Arrows indicate the next generation produced from the third brood neonates. White rectangles represent the control/clean water medium while gray rectangles represent 0.1% FPW diluted in the control/clean water medium. The "-" present in a control medium indicate the number of generations removed after 0.1% FPW exposure.

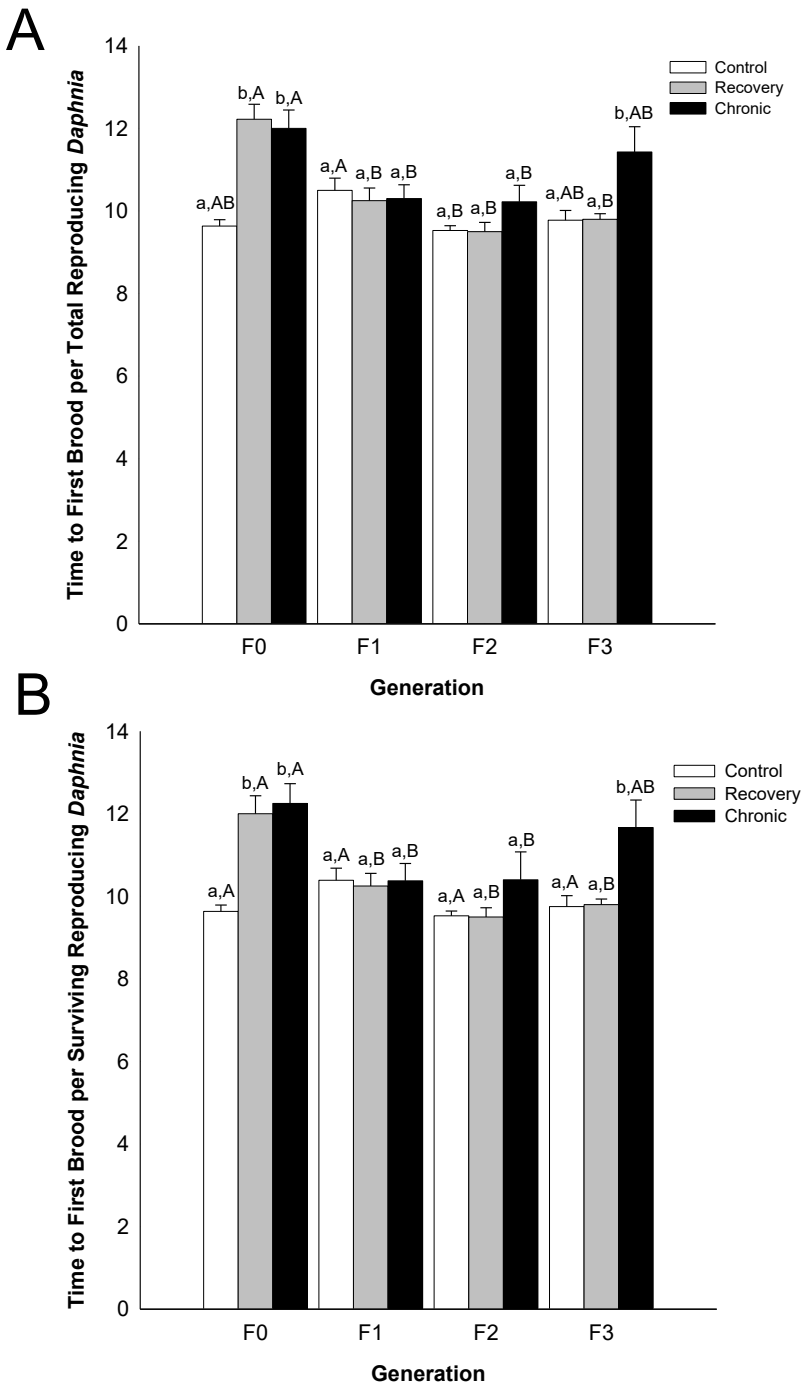

Figure 3-2. The time to first brood of <24-h third brood *Daphnia magna* neonates over the four-generation experiment for **(A)** all reproducing daphnids **(B)** for only surviving reproductive daphnids. Bars represent the mean ± SEM. See Table 2 for sample sizes (n = 4-20). Lowercase letters indicate a significant difference within a generation, whereas capital letters indicate a significant difference across generations (Two-Way ANOVA, Holm-Sidak, $p < 0.05$).

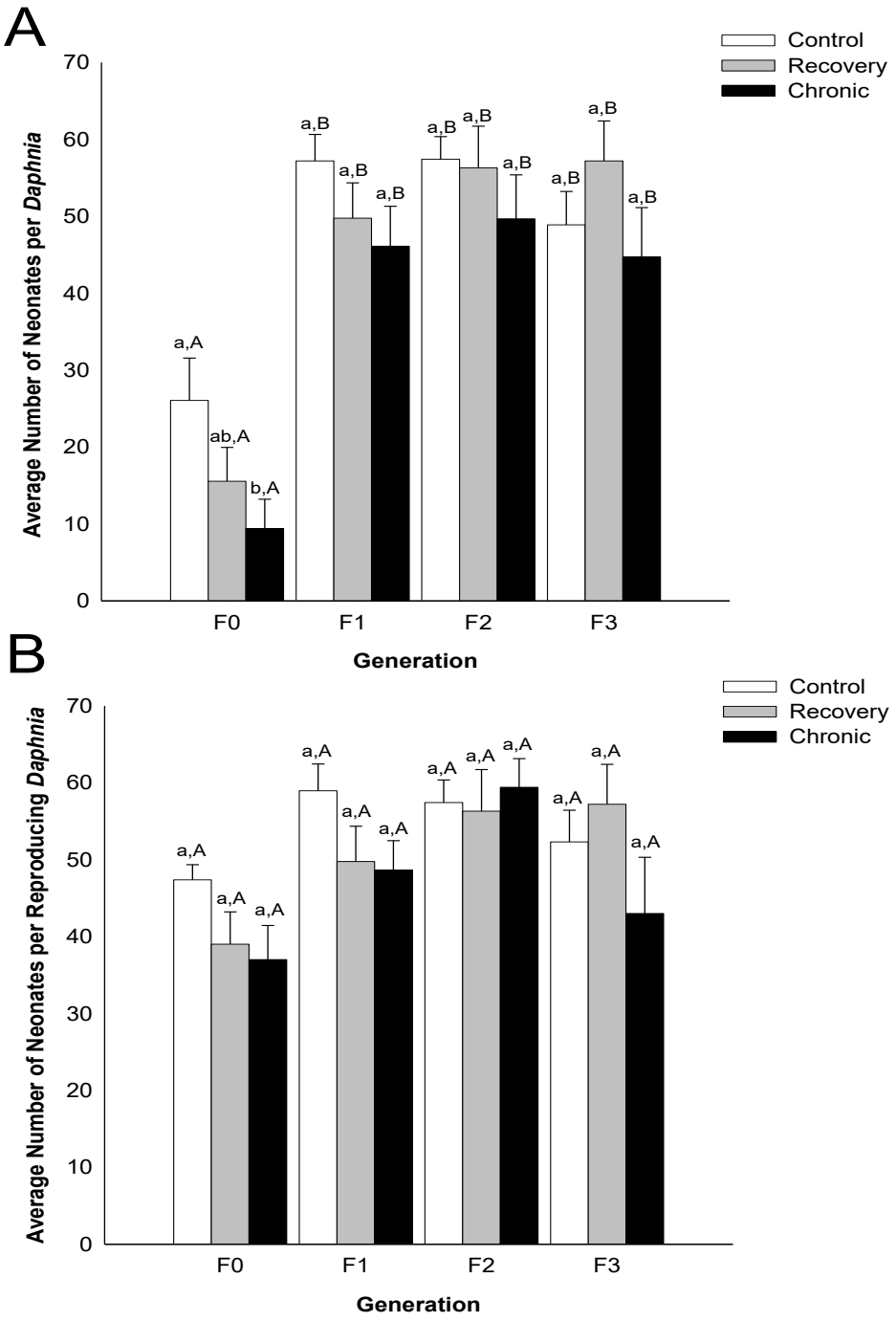

Figure 3-3. The average number of neonates produced by female third brood *Daphnia magna* over the four-generation experiment for **(A)** all daphnids and **(B)** for only surviving reproductive daphnids. Bars represent the mean ± SEM. See Table 2 for sample sizes (n = 4-20). Lowercase letters indicate a significant difference within a generation, whereas capital letters indicate a significant difference across generations (Two-Way ANOVA, Holm-Sidak, $p < 0.05$).

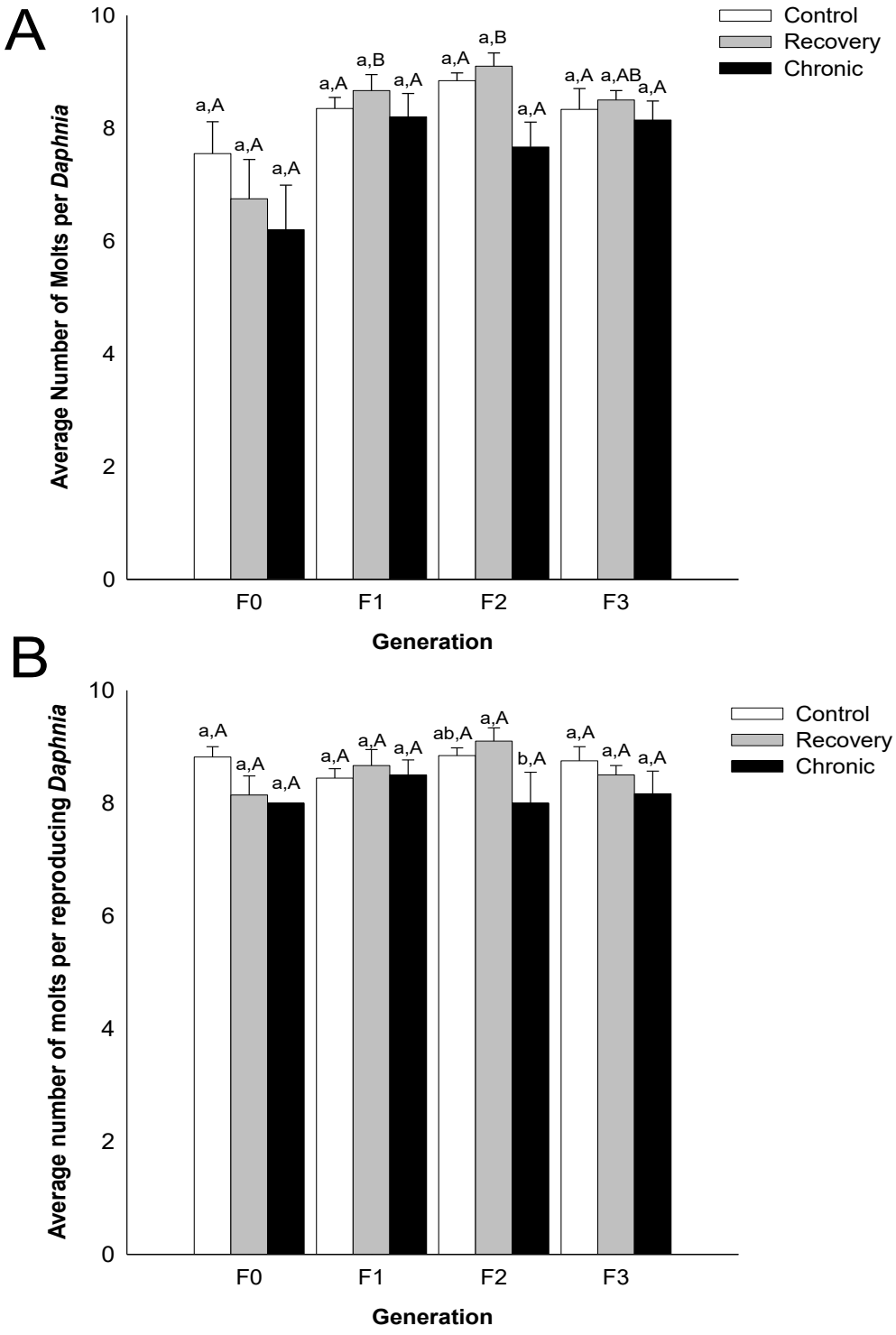

Figure 3-4. The average number of molts produced by all third brood *Daphnia magna* over the four-generation experiment for **(A)** all daphnids and **(B)** for only surviving reproductive daphnids. Bars represent the mean ± SEM. See Table 2 for sample sizes (n = 4-20). Lowercase letters indicate a significant difference within a generation, whereas capital letters indicate a significant difference across generations (Two-Way ANOVA, Holm-Sidak, $p < 0.05$).

Chapter 4 – General Discussion

Aquatic ecosystems are under constant threat from anthropogenic pollutants in their surrounding environment (Groh et al., 2022). It is therefore important to understand not only the immediate toxic consequences, but also the long-term influence of these pollutants on a population of aquatic organisms. A method to determine ecosystem health is the use of a model indicator species sensitive to pollutants, such as *Daphnia magna* (Hickey, 1989; Miner et al., 2012). Therefore, the goal of this thesis was to understand how *Daphnia magna* respond to an anthropogenic stressor (e.g., FPW) over different temporal scales, and to investigate biological recovery after the stressor was removed. Molting, reproduction, and molecular endpoints in response to low concentrations of FPW were evaluated. Notably, it was determined that after an acute exposure to low FPW concentrations (i.e., 0.25% and 0.75%), the first generation was unable to recover from the negative effects on reproduction, growth, and metabolism in this FPW sample. The results of my thesis also indicated that after a chronic exposure to a low concentration of this FPW sample (i.e., 0.1%), the F_0 generation, the F_1 and F_2 generations saw no effect from continued FPW exposure. As discussed below, not only do these results offer valuable information regarding the generational influence of anthropogenic pollutants, but also shed light on the pitfalls and future directions of this research.

Summary of chapter 2: lack of recovery after acute 48-hour FPW exposure

This chapter investigated the effects of an acute 48-hour FPW exposure to one generation of *D. magna* over a 21-day period. Survival, time to first brood, molting, and neonate output of *D. magna* was analyzed after 48 hours of 0.25% and 0.75% FPW exposure and a 19-day

recovery period in clean water. After an acute exposure to FPW, survival, molting, and neonate output were decreased and the time to first brood was delayed. Notably, the concentration of salts and metals in the FPW sample were lower than reported LC50 values for *D. magna* (Arambašić et al., 1995; Biesinger and Christensen, 1972). This suggests that the organic compounds in FPW were primarily responsible for the toxicity, with salts and metals possibly increasing the toxicity caused by the organics (Blewett et al., 2017a). Latent mortality was observed in *D. magna* exposed to FPW with many individuals surviving the initial exposure but dying later in the experiment. It was therefore speculated that latent mortality was likely caused from the PAHs present in the organic fraction of FPW, although PAH concentration was not measured in this chapter. PAHs are known to cause genotoxicity inducing the formation of DNA adducts (Pampanin et al., 2016), which may not be acutely lethal to organisms but can increase carcinogenicity, eventually leading to impairments in growth, reproduction, and ultimately survival.

Whole organism endpoints were analyzed in parallel to targeted protein analysis after 48 hours of FPW exposure. It was determined that in the experimental groups, the proteins expressed in *D. magna* displayed similar patterns at both 0.25% and 0.75% concentrations of FPW exposure but were vastly different from the control. After FPW exposure, there was a decrease in the abundance of metabolic proteins (e.g., carbohydrate and lipid) but an increase in transcriptional proteins. Decreased lipid metabolism may be linked to reduced reproduction and molting given the importance of lipids in molting and egg formation (Tessier and Goulden, 1982). Meanwhile transcription proteins may be increased to produce conserved stress proteins. Key molting proteins affected include chitinases, which are known to break down chitin (Qi et al., 2018). As a result, decreasing chitin levels could translate to the decreased molting seen on

the whole organism level in this study. In addition, glutathione S-transferase, an enzyme involved in detoxification reactions, was also decreased and suggests an increase in the presence of ROS. The resultant increase in ROS can damage DNA, lipids, and proteins (Kurutas, 2016), impairing reproductive function or causing organism mortality. While these results offer insight regarding acute FPW impacts on organisms, spill cleanup is not perfect which leads to chronic low exposures of FPW in aquatic ecosystems.

Summary of chapter 3: recovery possible after chronic FPW exposure

This chapter investigated a chronic low exposure of FPW over multiple generations of *D. magna.* Recovery after a 21-day chronic parental exposure to FPW was investigated in *D. magna* over a total of four generations ($F_0 – F_3$). Survival, time to first brood, molting and neonate production were evaluated in each generation, with only the time to maturation being delayed in the F_0 and F_3 generations in response to FPW exposure. Similar to the results of Blewett et al., (2017a), the time to first brood was delayed in response to sublethal FPW exposure. In contrast to the Blewett et al., (2017a) study, reproductive outputs remained unaffected. The high variability between individual FPW samples make direct comparisons challenging, but many chemicals in FPW remain consistent between the samples. The consistency between samples may suggest that delayed maturation could be a uniform effect of FPW toxicity. Throughout FPW exposure, organisms will divide their energy towards either maintenance, reproduction, or growth (McCauley et al., 1990), although it is unclear which pathway energy will be allocated to. Therefore, as cellular energy allocation is linked to whole-organism growth and maturity (Goodchild et al., 2019), FPW may be inhibiting pathways related to energy production, and in turn delaying maturation. Recent studies have shown oxygen consumption – a surrogate measure of metabolic rate – was decreased in response to FPW exposure (Blewett et al., 2017a).

Decreased metabolic rate could indicate inhibition of energy production in relation to oxidative metabolism (Blewett et al., 2017a). A decreased metabolic rate in combination with the aforementioned acute lipid and carbohydrate metabolism decrease could explain the delayed maturation of daphnids when exposed to FPW. Interestingly, *D. magna* exhibited a decrease in molts and neonate output after acute FPW exposure, which was not seen throughout a chronic exposure. This discrepancy may be due to the lower FPW concentration used in the chronic analyses, where toxic thresholds may not have been reached yet. At a concentration of 0.1% FPW, maturation but not reproduction was impacted, where concentrations higher than 0.1% may impair fecundity. These results provide information towards the effects of FPW persistence in the environment but require molecular analyses to further understand the mechanisms at which recovery from FPW may be possible.

Pitfalls during experimentation

The greatest pitfall throughout this thesis was the onset of the COVID-19 pandemic and related issues, greatly impacting my ability to conduct research on campus. Throughout the shutdowns, limited personnel were granted working access to university facilities. As such, it became challenging to complete any on-campus research. Therefore, research became the largest constraint since work on campus was halted countless times to enhance safety procedures. Upon the initial return to campus, multiple attempts to prepare a sequenceable library offered little success. During that period, time was spent troubleshooting and optimizing successful library generation, but there was high variability in whether library generation was possible using the Illumina TruSeq Nano Kit. Changes in DNA extraction kits and tissue homogenization methods, optimization of DNA shearing volumes and times to increase input DNA, and testing of different purification beads in the library preparation protocol still led to large losses of DNA. When

bisulfite conversion was included, this process was very harsh on the DNA, leading to an unsuccessful library generation. Due to the time constraint and these setbacks with my research, I was unable to complete the second half of my experiment for chronic exposures, which involved an epigenetic component. Epigenetic changes would have been monitored to determine the effects of FPW exposure on DNA methylation and if recovery can occur in the epigenome. Currently, only the F_0 and F_3 generations of the control and recovery generations are being assessed for DNA methylation. If the results indicate that there are changes in the epigenome at the F_3 level, then determining the generation at which the changes first occurred would be a helpful avenue to explore.

FPW is a complex chemical mixture and varies greatly between samples, making it difficult to determine a specific adverse outcome pathway. As FPW is a CCM that contains multitudes of organic constituents and inorganic elements, the mechanism of action is difficult to elucidate even when all chemicals can be characterized and assessed. Indeed, I did not run saltwater or activated charcoal controls to isolate for the effects produced by the salt, metal, or organic portion of FPW. Given the complexity and time required to characterize all the compounds, organic characterization of the FPW sample was not conducted. Constrained by time, FPW was considered as a mixture for the purpose of my thesis and although the sample was considered a mixture, I did not specifically test for additive, synergistic, or antagonistic toxicity.

Future directions and conclusions

Despite the pitfalls of this thesis, it was demonstrated that recovery was possible in *D. magna* after a chronic low dose FPW exposure, which could be beneficial to the other populations of organisms that depend on *D. magna* as a food source or as a predator. Further

investigations could involve collecting wild Albertan *D. magna* in the field and comparing the responses to FPW exposure with laboratory-reared *Daphnia.* This would elucidate the differences between their life-history traits and determine if a laboratory colony is representative of a real-life spill scenario. While not the scope of this thesis, other non-model organisms from freshwater ecosystems should also be monitored (e.g., microorganisms, algae, arctic grayling) to determine the full effects of FPW exposure on an ecosystem. The effects of FPW should be evaluated in other organisms to expand our understanding of its effects. In addition, future studies on the biokinetics of FPW could be evaluated. This can be studied through FPW bioaccumulation in higher tropic level organisms through dietary uptake of FPW exposed *D. magna*. Studying FPW bioaccumulation is important because it elucidates the effects of biomagnification on organisms, which can potentially affect human populations. It would also be beneficial to study the chronic effects of multiple FPW samples to see how they would compare with the one used in this thesis. Fractionation work could be used to isolate the effects attributable to the organics, metals, and salts within FPW to better understand the adverse outcome pathways of FPW.

In conclusion, my findings show that after an acute 48-hour FPW spill, there are long term effects on the exposed generation of *D. magna* that could lead to changes at the population level, ultimately affecting the ecosystem therein. This result highlights that FPW spills need to be prevented such that immediate deleterious effects do not occur. Oftentimes, full strength FPW is spilled so low doses of FPW can be found after cleanup. After a chronic low dose 21-day FPW spill, only the F_0 generation is affected and the F_3 generation if the spill continued. Thus, when a low FPW exposure occurs, it is not disastrous to *D. magna* populations and only needs to be completely remediated before the F_3 generation. Considering there are already toxic effects of

FPW on aquatic organisms, biotic and abiotic stressors may further exacerbate these effects. One "hot" stressor that is of concern for aquatic organisms is climate change. Climate change is linked to increased water temperatures, decreased dissolved oxygen levels, and acidification. These climate change related stressors in conjunction with FPW could not only affect *D. magna* but also cause a trophic cascade.

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Appendices

Supplemental Tables

Table S2-1. Measured elements, MS/MS masses, and used collision/reaction gases in water chemistry analysis

Element	Q1 → Q2	Gas	Element	Q1 → Q2	Gas
Li	7 → 7	-	Fe	56 → 56	H2
B	11 → 11	-	Co	59 → 59	He
Na	23 → 23	He	Ni	60 → 60	He
Mg	24 → 24	He	Cu	63 → 63	He
Al	27 → 27	He	Zn	66 → 66	He
Si	28 → 28	H2	Br	79 → 79	H2
P	31 → 47	O2	As	75 → 91	O2
S	32 → 48	O2	Sr	88 → 88	He
Cl	35 → 37	H2	Mo	95 → 95	He
K	39 → 39	He	Cd	114 → 114	He
Ca	40 → 40	H2	Ba	137 → 137	He
Cr	52 → 52	He	Pb	208 → 208	-
Mn	55 → 55	He	U	238 → 238	-

Table S2-2. The summarized gene ontology (GO) terms and descriptions of the biological processes (BP) assigned to the general function "Transport". The highlighted terms were those that were included in the main table of enriched GOBPs that were summarized as transport processes. The non-highlighted terms were grouped into the highlighted GOBP that precedes them.

Term ID	GOBP Description
GO:0016192	vesicle-mediated transport
GO:0015031	protein transport
GO:0008104	*protein localization*
GO:0045184	*establishment of protein localization*
GO:0071705	nitrogen compound transport
GO:0006810	*transport*
GO:0071702	*organic substance transport*
GO:0051234	*establishment of localization*

Table S2-3. The summarized GO terms and descriptions of the BPs assigned to the general function "Cell Organization". The highlighted terms were those that were included in the main table of enriched GOBPs that were summarized as transport processes. The non-highlighted terms were grouped into the highlighted GOBP that precedes them.

Term ID	GOBP Description
GO:0071840	cellular component organization or biogenesis
GO:0007010	*cytoskeleton organization*
GO:0044085	*cellular component biogenesis*
GO:0065003	*protein-containing complex assembly*
GO:0043933	*protein-containing complex subunit organization*
GO:0006996	*organelle organization*
GO:0022607	*cellular component assembly*
GO:0022613	*ribonucleoprotein complex biogenesis*
GO:0016043	*cellular component organization*
GO:0022618	*ribonucleoprotein complex assembly*
GO:0071826	*ribonucleoprotein complex subunit organization*
GO:0034622	*cellular protein-containing complex assembly*
GO:0051179	localization
GO:0033036	macromolecule localization
GO:0061024	membrane organization

Table S2-4. The summarized GO terms and descriptions of the BPs assigned to the general function "Metabolism". The highlighted terms were those that were included in the main table of enriched GOBPs that were summarized as transport processes. The non-highlighted terms were grouped into the highlighted GOBP that precedes them.

Term ID	GOBP Description
GO:0006629	lipid metabolic process
GO:0008152	metabolic process
GO:0009056	catabolic process
GO:0071704	organic substance metabolic process
GO:0044238	*primary metabolic process*
GO:0005975	carbohydrate metabolic process
GO:0044281	small molecule metabolic process
GO:1901576	organic substance biosynthetic process
GO:0034645	*cellular macromolecule biosynthetic process*
GO:0009059	*macromolecule biosynthetic process*
GO:0044271	*cellular nitrogen compound biosynthetic process*
GO:1901566	*organonitrogen compound biosynthetic process*
GO:0044249	*cellular biosynthetic process*
GO:0006412	*translation*
GO:0043604	*amide biosynthetic process*
GO:0006518	*peptide metabolic process*
GO:0043043	*peptide biosynthetic process*
GO:0043603	cellular amide metabolic process

Table S2-5. The summarized GO terms and descriptions of the BPs assigned to the general function "Transcription". The highlighted terms were those that were included in the main table of enriched GOBPs that were summarized as transport processes. The non-highlighted terms were grouped into the highlighted GOBP that precedes them.

Term ID	GOBP Description
GO:0006397	mRNA processing
GO:0016071	*mRNA metabolic process*
GO:0006396	*RNA processing*
GO:0016070	*RNA metabolic process*
GO:0010467	gene expression

Table S2-6. Summary of pathway enrichment of the whole proteome based on the OmicsBox pathway analysis. The pathway with the number of protein sequences that fall under it were totaled. Tabulated are the results of pathways with a FDR < 0.05 and a *p-value* < 0.05.

Whole Proteome					UpDn Enrichment			Up OR Dn Enrichment		
Database	Pathway	ID	Species	#Seqs	Fisher FDR	Fisher P-Value	#Seqs	Up/Dn FDR	Up/Dn P-value	#Seqs
KEGG	Starch and sucrose metabolism	ko00500	None	48	0.004	0.000	10	0.000	0.000	10
Reactome	Phase I - Functionalization of compounds	R-DME-211945	Drosophila melanogaster	12	0.008	0.000	6	0.001	0.000	6
KEGG	Glutathione metabolism	ko00480	None	82	0.013	0.000	12	0.001	0.000	11
KEGG	Galactose metabolism	ko00052	None	54	0.016	0.000	9	0.001	0.000	9
KEGG	Other glycan degradation	ko00511	None	32	0.016	0.000	7	0.001	0.000	7
Reactome	Synthesis of PC	R-DME-1483191	Drosophila melanogaster	18	0.048	0.000	6	0.010	0.000	6
Reactome	Glutamate and glutamine metabolism	R-XTR-8964539	Xenopus tropicalis	3	0.048	0.000	3	0.013	0.000	3
Reactome	Metabolism of Angiotensinogen to Angiotensins	R-DME-2022377	Drosophila melanogaster	41	0.140	0.000	8	0.013	0.000	8
KEGG	Glycosphingolipid biosynthesis - ganglio series	ko00604	None	7	0.067	0.003	3	0.025	0.001	3
Reactome	L13a-mediated translational silencing of Ceruloplasmin expression	R-DRE-156827	Danio rerio	18	0.530	0.007	4	0.026	0.000	4
Reactome	GTP hydrolysis and joining of the 60S ribosomal subunit	R-DRE-72706	Danio rerio	18	0.530	0.007	4	0.026	0.000	4
Reactome	L13a-mediated translational silencing of Ceruloplasmin expression	R-DME-156827	Drosophila melanogaster	50	1.000	0.021	6	0.026	0.000	6
Reactome	Formation of a pool of free 40S subunits	R-DME-72689	Drosophila melanogaster	50	1.000	0.021	6	0.026	0.000	6
Reactome	Eukaryotic Translation Elongation	R-RNO-156842	Rattus norvegicus	2	0.194	0.002	2	0.044	0.000	2
Reactome	SRP-dependent cotranslational protein targeting to membrane	R-DME-1799339	Drosophila melanogaster	43	1.000	0.038	5	0.044	0.000	5
Reactome	GTP hydrolysis and joining of the 60S ribosomal subunit	R-DME-72706	Drosophila melanogaster	42	1.000	0.035	5	0.044	0.000	5

Table S2-7. Summary of key differentially expressed proteins from the proteomic analysis.

ProteinID	LogFC_0.25	LogFC_0.75	Description
A0A164YDL7	3.376	3.292	chitinase-3-like protein 1
A0A164YDK2	1.382	1.416	chitinase 15
A0A0P6D3J7	-1.020	3.041	WD repeat domain phosphoinositide-interacting protein 2 isoform X2
A0A0P5VMY1	-1.091	-1.235	probable chitinase 10
A0A164VDW4	-1.144	-1.083	glutathione S-transferase
A0A162Q5W2	-1.441	-1.432	glutathione S-transferase 1-like
A0A164Y7C0	-1.606	-2.152	probable chitinase 10
A0A0P5AHY8	-1.881	-1.138	chitinase 1 precursor
A0A164YAJ9	-2.051	-3.822	probable chitinase 10
A0A0P6CX50	-2.526	-2.203	glutathione S-transferase 1-like
A0A164LPT9	-3.461	-3.862	probable chitinase 10
A0A164U2A9	-6.349	-6.134	sphingomyelin phosphodiesterase-like