Toxicological Characterization of Hydraulic Fracturing Flowback and Produced Water Exposures to Aquatic Organisms

by

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Abstract

Hydraulic fracturing is an unconventional oil and gas extraction method used to liberate oil and gas reserves from low permeability geological formations. Hydraulic fracturing techniques involve injecting large quantities of fracturing fluids into the well-bore under high pressures, where once a well comes into production, fracturing chemical mixtures (along with waters and other chemical components from the formation) return to the surface and are separated from targeted resources. The resulting wastewater mixture is termed flowback and produced water (FPW). Although many environmental concerns surround the process of hydraulic fracturing, spills and releases of FPW to surface bodies of water is an environmental topic gaining increasing public attention. FPW is a highly complex, heterogeneous mixture comprised of numerous organic chemical compounds, naturally occurring radioisotopes, high concentrations of salt-related ions, metals, and transformation products resulting from chemical reactions within the well-bore under high heat and high pressures. Interestingly, relatively few studies have investigated FPW-mediated organismal toxicity.

Understanding basic toxicological properties and responses of organisms exposed to FPW is essential when attempting to tease apart more fine sub-lethal responses of exposures. I determined that numerous freshwater species are extremely sensitive to FPW, and that saline components of the solution are responsible for a considerable proportion of the induced lethality experienced by freshwater species, including *Daphnia magna*, *Lumbriculus variegatus,* zebrafish, and rainbow trout. However, many of these species exhibited significantly greater lethality when exposed to FPW versus saline control exposures, indicating that other chemical constituents (e.g., metal species and numerous toxic dissolved organic compounds, including polycyclic aromatic hydrocarbons [PAHs], organophosphorus compounds, and alkyl ethoxylate carboxylate surfactants) besides saline components appreciably contribute to the overall toxicological potential of FPW, with a pronounced toxicological emphasis associated with the organic fraction of FPW.

To begin elucidating sub-lethal responses and physiological systems impacted by FPW exposure, liver and gill tissue from FPW exposed rainbow trout was analyzed and determined to have significant induction of cytochrome P450 1A transcript expression (*cyp1a*; a major family of monooxygenase detoxification enzymes) and ethoxyresorufin-o-

deethylase activity (a biomarker of induced aryl hydrocarbon receptor and CYP1A activity). These results imply that a significant biotransformative response is mounted in exposed organisms to detoxify organic compounds (specifically PAHs). Induction of UDP-glucoronosyl transferase and glutathione-s-transferase phase II biotransformation enzyme transcripts further emphasize the detoxification strains induced following FPW exposure. Increased expression of the oxidative response genes glutathione peroxidase and superoxide dismutase, along with increased thiobarbituric acid reactive substance formation (indicating lipid peroxidation) in trout gill, liver, and kidney tissue, suggest that increased reactive oxygen species from FPW exposure is another major physiological stress encountered in exposed organisms. Elevated expression of vitellogenin and estrogen receptor α2 genes in trout liver tissue also hint at the presence of endocrine disrupting compounds (in particular, xenoestrogens) in FPW, demonstrating possible endocrine disruptive effects in exposed organisms.

Organisms are suspected to be placed in a compromised energy homeostatic position due to the metabolic costs associated with xenobiotic detoxification processes following FPW exposure. In support of this notion, it was observed that gross hepatic histological alterations and significantly increased glucose uptake in isolated rainbow trout hepatocytes occured following acute whole-organism FPW exposure. Interestingly, overall plasma glucose levels were not impacted in exposed fish. Furthermore, no clear pattern concerning transcriptional expression and activity of enzymes associated with hepatic metabolic pathways was observed. However, altered enzymatic activities of phosphoenolpyruvate carboxykinase (gluconeogenesis) and glutamate dehydrogenase (amino acid catabolism), along with increased hepatocyte glucose uptake, suggest that an immediate deviation in nutrient handling occurs following/during FPW exposure in trout. Following a 3-week recovery in freshwater conditions, however, all indices of measured energy homeostatic dynamics returned to control levels, indicating that nutrient handling and energy homeostatic systems are processes acutely affected by FPW exposure without lasting effects.

Embryonic exposure studies revealed that the development and function of cardio-respiratory systems in fish are severely impacted by acute FPW exposures. In both zebrafish and rainbow trout, embryonic exposures to FPW significantly increased rates of

numerous developmental deformities at hatch (including rates of pericardial edema) and decreased heart rates in zebrafish embryos. Embryo metabolic O_2 consumption (MO_2) analyses additionally displayed that embryonic FPW-mediated impacts persist through development past initial acute exposures. In zebrafish, embryonic/larval MO_2 was significantly inhibited at all timepoints up to 96 hours post fertilization (hpf) following acute FPW exposures. In rainbow trout, however, varied embryonic MO_2 responses were observed, wherein embryos exposed to FPW at 3 days post fertilization (dpf) displayed significantly elevated MO_2 up to 15 dpf compared to control embryo rates and significantly decreased MO_2 from 15 to 26 dpf. Similar trends in MO_2 were additionally recorded in trout embryos chronically exposed to FPW for 28 days. Such species-specific differential results are theorized to be a product of differences in developmental timing between zebrafish and rainbow trout (and therefore timing of developmental metabolic analyses).

In both zebrafish and rainbow trout embryos acutely exposed to FPW, elevated expression of the critical homeobox transcription factor *nkx2.5* (required for proper cardiogenic differentiation) was additionally observed. Furthermore, rainbow trout embryos exposed to FPW displayed elevated expression the key cardiac tissue development T-box transcription factor *tbx2b*. Both species of fish also exhibited altered expression of *atp2a2a*, a cardiac Ca^{2+} ATPase responsible for sequestering Ca back into the sarcoplasmic reticulum following an excitation-contraction coupling event. Rainbow trout embryos additionally displayed altered expression of the electrochemically related genes *scn5lab* (Na^+ channel responsible for invoking cellular depolarization during an action potential), *kcnh6* (delayed rectifying K^+ channel responsible for repolarizing membrane potentials during an action potential), and *kir2.1* (an inward-rectifying K^+ channel crucial for setting and maintaining resting membrane potential). Expressional changes to key functional structure genes (e.g., *tnnt2a* and *vmhc* in zebrafish and rainbow trout, respectively) were also affected following FPW exposure.

FPW-mediated impacts to cardio-respiratory processes during development may persist and in part be responsible for latent impacts at later-stage developmental periods. I have identified in both zebrafish and rainbow trout that embryonic exposures to FPW significantly decrease juvenile fish swimming performance (U_{crit}), with corresponding decreases in maximum metabolic rates and aerobic scope without any impacts to standard

metabolic rates. It was further identified in juvenile rainbow trout that embryonic FPW exposures reduce ventricular compact myocardium thickness; an added response which may contribute to the latent effects of embryonic FPW exposure on juvenile fish swimming performance and respirometry. Impacts of FPW exposure on cardio-respiratory function was also observed at the cellular level. In isolated cardiomyocytes from ventricles of *mahi mahi* (*Coryphaena hippurus*), exposure to dilute, filtered FPW solutions altered sarcomere contraction size and relaxation indices. Paired with swimming analyses and observations of significantly reduced U_{crit}, MMR, and aerobic scope in juvenile *mahi* acutely exposed to FPW, it is suggested that decreases in cardiomyocyte contractile function following FPW exposure reduce fitness in acutely exposed juvenile fish.

Implications of FPW timing and severity of induced sub-lethal cardio-respiratory toxicity was also investigated presently. In rainbow trout embryos exposed to FPW earlier in development (3dpf) versus those exposed later (10 dpf), significantly increased rates of developmental deformities and altered embryonic metabolism was observed. Interestingly, 10 dpf embryos displayed a greater number of differentially expressed cardiac-related genes following acute FPW exposure, while 3 dpf exposed embryos only had *atp2a2a* altered of the selected genes analyzed. Regarding latent impacts on juvenile fish fitness and aerobic scope, 3 dpf embryonic exposures resulted in reduced U_{crit} and aerobic scope regardless of FPW dilution used, while 10 dpf exposures of 2.5% FPW did not result in juvenile fish impacts. Collectively, these results suggest that FPW exposures earlier within the cardiac developmental window produce exaggerated impacts to developing cardio-respiratory systems in rainbow trout, resulting in more lasting and adverse effects on juvenile fish swimming behaviour and aerobic capacity.

Overall, these results highlight the considerable toxicity of FPW to organisms of aquatic environments and reveal cardio-respiratory development and function in fish as targeted processes affected by FPW exposure.

Preface

This thesis is an original work by Erik John Folkerts. The research project, of which the thesis herein is a part, received ethics approval from the University of Alberta Animal Care and Use Committee governed under the following protocols: "LC50 Protocols to Supplement Sub-lethal Experiments on Various AUPs", University of Alberta, AUP#: 00002352 (2015-2021); "Effects of Hydraulic Fracturing Flowback Fluids and Produced Water on Aquatic Organisms", University of Alberta, AUP#: 00001334 (2015-2021).

Some of the research conducted for this thesis forms a part of international research collaborations led by Professor G.G. Goss of the University of Alberta. I (Erik John Folkerts) performed the majority of the work presented in this original Ph.D. thesis. However, owing to collaborative efforts which were fundamental to work completed, the terms "we" or "our" are used throughout this thesis. Seven chapters of this thesis have been previously published. Biographical details for the introduction and data chapters are both listed below and indicated at the beginning of each chapter. The roles of all authors for each chapter along with specific experimental contributions of certain co-authors are briefly described below.

Chapter 1:

Folkerts, E.J., Goss, G.G. and Blewett, T.A. (2020). Investigating the potential toxicity of hydraulic fracturing flowback and produced water spills to aquatic animals in freshwater environments: A North American perspective. In:. *Reviews of Environmental Contamination and Toxicology* (Continuation of Residue Reviews). Springer, Cham. DOI: 10.1007/398_2020_43.

This book chapter was conceived and designed by EJF, GGG, and TAB. Roles and authorship contribution for each author are explained below: EJF - Conceptualization, Data Collection, Formal Analysis, Investigation, Writing - Original Draft, Visualization, Writing

- Edited Draft, Editing; GGG - Resources, Funding Acquisition, Supervision, Editing; TAB - Conceptualization, Investigation, Writing – Original Draft, Supervision, Project Administration, Editing.

Chapter 2:

Folkerts, E.J., Blewett, T.A., Delompré, P., Mehler, W.T., Flynn, S.L., Sun, C., Zhang, Y., Martin, J.W., Alessi, D.S. and Goss, G.G. (2019). Toxicity in aquatic model species exposed to a temporal series of three different flowback and produced water samples collected from a horizontal hydraulically fractured well. *Ecotoxicology and Environmental Safety*, 180, 600 – 609.

This study was conceived and designed primarily by EJF, with additional input from GGG. Roles and authorship contribution for each author are explained below: EJF - Conceptualization, Methodology, Investigation, Data Collection, Formal Analysis, Writing - Original Draft, Visualization, Writing - Edited Draft, Editing; TAB – Investigation (*Daphnia*), Data Collection; PD – Investigation (*Daphnia*), Data Collection; WTM – Investigation (*Lumbriculus*), Data Collection; SLF – Investigation (*Inorganic* Characterization), Data Collection; CS – Investigation (*Organic Characterization*); YZ – Investigation (*Organic Characterization*), Data Collection; JWM – Funding Acquisition (*Organic Characterization*); DSA – Funding Acquisition (*Inorganic Characterization*); GGG - Resources, Funding Acquisition, Supervision, Editing, Project Administration.

Chapter 3:

He, Y., **Folkerts, E.J.**, Zhang Y., Martin J.W., Alessi, D.S., Goss, G.G. **(**2017). Effects on biotransformation, oxidative stress, and endocrine disruption in Rainbow Trout (*Oncorhynchus mykiss*) exposed to hydraulic fracturing flowback and produced water. *Environmental Science and Technology*, 51, 940 – 947.

This study was published with YH and EJF as co-first authors, with joint equal efforts of study conception and design made by YH and EJF. Roles and authorship contribution for each author are explained below: YH - Conceptualization, Methodology, Investigation,

Data Collection, Formal Analysis, Writing - Original Draft, Visualization, Writing - Edited Draft, Editing; EJF - Conceptualization, Methodology, Investigation, Data Collection, Formal Analysis, Writing - Original Draft, Visualization, Writing - Edited Draft, Editing; YZ – Investigation (*Organic Characterization*), Data Collection; JWM – Funding Acquisition (*Organic Characterization*); DSA – Funding Acquisition (*Inorganic Characterization*); GGG - Resources, Funding Acquisition, Supervision, Editing, Project Administration.

Chapter 4:

Weinrauch, A.M., **Folkerts, E.J.,** Alessi, D.S., Goss, G.G., and Blewett, T.A. (2021). Changes to hepatic nutrient dynamics and energetics in rainbow trout (*Oncorhynchus mykiss*) following exposure to and recovery from hydraulic fracturing flowback and produced water. *Science of the Total Environment*, 764, 142893.

This study was published with AMW and EJF as co-first authors, with joint equal efforts of study conception and design made by AWM and EJF. Roles and authorship contribution for each author are explained below: AWM - Conceptualization, Methodology, Formal Analysis, Investigation, Writing - Original Draft, Writing - Edited Draft, Editing; EJF - Conceptualization, Methodology, Formal Analysis, Investigation, Writing - Original Draft, Visualization, Writing -
Edited Draft, Editing; DSA - Resources, Funding Acquisition (*Inorganic Characterization*), Editing; GGG - Resources, Writing - Original Draft, Funding Acquisition, Supervision, Editing; TAB - Conceptualization, Investigation, Writing - Original Draft, Supervision, Project Administration, Editing.

Chapter 5:

Folkerts, E.J., Blewett, T.A., He, Y., Goss, G.G. (2017). Cardio-respirometry disruption in zebrafish (*Danio rerio*) embryos exposed to hydraulic fracturing flowback and produced water. *Environmental Pollution*, 231, 1477 – 1487.

This study was conceived and designed primarily by EJF, with additional input from GGG. Roles and authorship contribution for each author are explained below: EJF - Conceptualization, Methodology, Investigation, Data Collection, Formal Analysis, Writing - Original Draft, Visualization, Writing - Edited Draft, Editing; TAB – Investigation (*qPCR*), Data Collection; YH – Investigation (*qPCR*), Data Collection; GGG - Resources, Funding Acquisition, Supervision, Editing, Project Administration.

Chapter 6:

Folkerts, E.J., Blewett, T.A., He, Y., Goss, G.G. (2017). Alterations to juvenile zebrafish (*Danio rerio*) swim performance after acute embryonic exposure to sub-lethal exposures of hydraulic fracturing flowback and produced water. *Aquatic Toxicology*, 193, 50 – 59

This study was conceived and designed primarily by EJF, with additional input from GGG. Roles and authorship contribution for each author are explained below: EJF - Conceptualization, Methodology, Investigation, Data Collection, Formal Analysis, Writing - Original Draft, Visualization, Writing - Edited Draft, Editing; TAB – Investigation (*qPCR*), Data Collection; YH – Investigation (*qPCR*), Data Collection; GGG - Resources, Funding Acquisition, Supervision, Editing, Project Administration.

Chapter 7:

Folkerts, E.J., Sniher, K. N., Zhang, Y., Martin, J.W., Alessi, D.S., Goss, G.G. Persistent cardio-respiratory impairments in rainbow trout (*Oncorhynchus mykiss*) following embryonic exposure to hydraulic fracturing flowback and produced water. *To be submitted*.

This study was conceived and designed primarily by EJF, with additional input from GGG. Roles and authorship contribution for each author are explained below: EJF - Conceptualization, Methodology, Investigation, Data Collection, Formal Analysis, Writing - Original Draft, Visualization, Writing - Edited Draft, Editing; KNS – Investigation (*Inorganic Characterization*), Data Collection; YZ – Investigation (*Organic Characterization*), Data Collection; JWM – Funding Acquisition (*Organic*

Characterization); DSA – Funding Acquisition (*Inorganic Characterization*); GGG - Resources, Funding Acquisition, Supervision, Editing, Project Administration.

Chapter 8:

Folkerts, E.J., Heuer, R.M., Flynn, S.L., Stieglitz, J., Benetti, D.D., Alessi, D.S., Goss, G.G., and Grosell, M. (2020). Exposure to hydraulic fracturing flowback water impairs Mahi-mahi (*Coryphaena hippurus*) cardiomyocyte contractile function and swimming performance. *Environmental Science and Technology*, 54, 13579 – 13589.

This study was conceived and designed primarily by EJF, with additional input from GGG and MG. Roles and authorship contribution for each author are explained below: EJF - Conceptualization, Methodology, Investigation, Data Collection, Formal Analysis, Writing - Original Draft, Visualization, Writing - Edited Draft, Editing; RMH – Investigation, Supervision, Editing; SLF – Investigation (*Inorganic Characterization*), Data Collection; JS – Resources, Funding Acquisition; DDB – Resources, Funding Acquisition; DSA – Funding Acquisition (*Inorganic Characterization*); GGG - Resources, Funding Acquisition, Supervision, Editing, Project Administration; MG - Resources, Funding Acquisition, Supervision, Editing, Project Administration.

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List of Symbols, Nomenclature and Abbreviations

α	Alpha
β	Beta
Σ	Sigma; summation
°C	Degrees celcius
π	Pi
$\bar{X}$	Average
2-DR	2-deoxy-d-ribose
7-ER	7-ethoxyresorufin
^{14}C	Carbon-14
^{40}K	Potassium-40
^{226}Ra	Radium-226
^{228}Ra	Radium-228
^{234}U	Uranium-234
^{235}U	Uranium-235
^{238}U	Uranium-238
AB	Alberta
AC	Activated charcoal/carbon
ADP	Adenosine diphosphate
AEO	Alkyl ethoxylates
AER	Alberta Energy Regulator
Ag	Silver
AGI	American Geological Institute

AhR	Aryl hydrocarbon receptor
Al	Aluminum
AMR	Active metabolic rate
ANOVA	Analysis of variance
anp	Atrial natriuretic peptide
As	Arsenic
AS	Aerobic scope
ASE	Accelerated solvent extraction
ATP	Adenosine triphosphate
atp2a2a	Cardiac sarcoplasmic reticulum Ca^{2+} ATPase 2a2a
ATU	Acclimated thermal unit
B	Boron
Ba	Barium
BaP	Benzo[a]pyrene
BC	British Columbia
BCOGC	British Columbia Oil and Gas Commission
BHT	Butylated hydroxytoluene
BL	Body length
BL/s	Body length per second
BL % Peak Height	Baseline percent peak height
Br	Bromine
BT	Brown trout

BTEX	Benzene, toluene, ethylbenzene, and xylene (collective term)
Ca	Calcium
$CaCl_2$	Calcium chloride
$CaCO_3$	Calcium carbonate
CAN	Canada
Cd	Cadmium
CD	*Ceriodaphnia dubia*
cDNA	Complimentary deoxyribonucleic acid
CEQ	Canadian environmental quality
CI	Confidence interval
Cl	Chloride
cm	Centimeter
cm/s	Centimeters per second
CMC	Critical micelle concentration
cmlc1	Cardiac myosin light chain 1
cmlc2	Cardiac myosin light chain 2
CO_2	Carbon dioxide
COD	Chemical oxygen demand
CPM	Counts per minute
CT	Critical threshold
Cu	Copper
$CuCO_3$	Copper carbonate
CYP	Cytochrome P450

-d/d*t*	Relaxation velocity
d/d*t*	Contraction velocity
DBCM	Dibromochloromethane
DBT	Dibenzothiophene
DCM	Dichloromethane
DEP	Department of Environmental Protection
DEPC	Diethyl pyrocarbonate
DM	*Daphnia magna*
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DO	Dissolved oxygen
DOC	Dissolved organic carbon
DP	*Daphnia pulex*
dpf	Days post-fertilization
dph	Days post-hatch
dU	Swim speed increment
E-C	Excitation contraction
EC_{50}	Half maximal effective concentration
EDTA	Ethylenediaminetetraacetic acid
e.g.	*exempli gratia*
elf1a	elongation factor 1a
EOG	Electro-olfactogram
EPO	Erythropoietin

epoa	Erythropoietin alpha
ER	Estrogen receptor
EROD	Ethoxyresorufin-O-deethylase
F-rtAC	Factorial real-time aerobic capacity
Fe	Iron
FF	Force frequency
fhl2	Four and a half LIM domain 2
FHM	Fathead Minnow
EPA	Environmental Protection Agency
FPW	Flowback and produced water
FPW-AC	Activated charcoal treated FPW
FPW-S	Sediment containing FPW
FPW-SF	Sediment-free FPW fraction
g	Gram
g/L	Gram per litre
gal	Gallon
gapdh	Glyceraldehyde 3-phosphate dehydrogenase
gata5	GATA binding protein 5
GC-MS	Gas chromatography-mass spectrometry
GDH	Glutamate dehydrogenase
GoM	Gulf of Mexico
gpx	Glutathione peroxidase
gst	Glutathione-s-transferase

h	Hour(s)
H&E	Hematoxylin and eosin
H^+	Hydrogen ion/proton
H_2	Hydrogen gas
H_2O	Dihydrogen oxide/water
HA	*Hyalella azteca*
hbbe1.1	Embryonic hemoglobin beta 1.1
HCB	Hexachlorobenzene
HCl	Hydrochloric acid
HCO_3^-	Bicarbonate
He	Helium
HEPES	2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid
HEWAF	High energy water accommodated fraction
HF	Hydraulic fracturing
HF-FW	Hydraulic fracturing flowback
HIF	Hypoxia inducible factor
hif1aa	Hypoxia inducible factor 1aa
hpf	Hours post-fertilization
HPG/I	Hypothalamic-pituitary-gonadal/interrenal
HPLC	High performance liquid chromatography
HR	Heart rate
Hz	Hertz
ICP-MS	Inductively coupled plasma-mass spectrometry

IDEM	Indiana Department of Environmental Management
i.e.	*id est*
I_{kl}	Non-voltage-gated inward rectifying potassium channel currents
I_{Ks}	Slow delayed rectifying potassium channel currents
I_{kr}	Rapid delayed rectifying potassium channel currents
ILCM	Interlamellar cell mass
J	Uptake
Juv.	Juvenile
K	Potassium
KCl	Potassium chloride
kcnh6	Voltage gated K^+ channel subfamily H, member 6
KCNQ1	Slow delayed rectifying potassium channels
kg	Kilogram
KH_2PO_4	Monopotassium phosphate
kir2.1	Inwardly-rectifying K^+ channel 2.1
KOW	Octanol-water partitioning coefficients
L	Litre
LC	Lethal concentration
LC_{50}	Median lethal concentration causing 50% death
LC-MS	Liquid chromatography-mass spectrometry
LDH	Lactate dehydrogenase
Li	Lithium
m	Meters

m^3	Meters cubed
MΩ	Megaohm
MDA	Malondialdehyde
MDEQ	Michigan Department of Environmental Quality
MDL	Method detection limit
mg	Milligram
Mg	Magnesium
$MgCl_2$	Magnesium chloride
mg/L	Milligrams per litre
$MgSO_4$	Magnesium sulfate
min	Minute(s)
mm	Millimeter
mM	Millimolar
mmol	millimol
mmol L^{-1}	Millimol per litre
MMR	Maximum metabolic rate
Mn	Manganese
$MnCl_2$	Manganese chloride
MO_2	Metabolic oxygen consumption
Mod.	Moderately
mOsm	milliosmoles
mRNA	Messenger ribonucleic acid
mS	Millisiemen

MS	Mass spectrometry
MS-222	Tricaine methanosulfonate
mtβ	Metallothionein beta
mV	Millivolt
MYA	Million years ago
mylc2	Myosin regulatory light chain 2
n	Replicate number
N	Number of collective samples
Na	Sodium
NAD^+	Nicotinamide adenine dinucleotide
NADH	Reduced nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
$NaHCO_3$	Sodium bicarbonate
$NaHPO_4 \cdot H_2O$	Sodium phosphate, monobasic, monohydrate
Na_2HPO_4	Disodium phosphate
Na_2SO_3	Sodium sulfite
NaCl	Sodium chloride
N_{agg}	Average micelle aggregation
NCX1h	Na^+/Ca^{2+} exchanger
n.d.	No date
N.D.	No data
ng	Nanogram
Ni	Nickel

nkx2.5	Homeobox protein Nkx2.5
nM	Nanometer
NOS	Nitric oxide synthase
nppa	Natriuretic peptide a
NYS DEC	New York State Department of Environmental Conservation
O_2	Oxygen
OECD	Organisation for Economic Co-operation and Development
OFR	Office of the Federal Registration
OH^-	Hydroxide ion
OPE	Octylphenol ethoxylate
OSPW	Oil sands process-affected water
PAC	Polycyclic aromatic compound
PAH	Polycyclic aromatic hydrocarbon
Pb	Lead
pCi/L	Picocuries per litre
PCB	polychlorinated biphenyl
PCP	Pentachlorophenol
PCR	Polymerase chain reaction
PE	Pericardial edema
PEG	Polyethylene glycol
PEPCK	Phosphoenolpyruvate carboxykinase
PK	Pyruvate kinase
PM	Red Seabream (*Pagrus major*)

ppb	Parts per billion
ppm	Parts per million
ppt	Parts per thousand
psi	Pounds per square inch
PXR	Pregnane X receptor
QA	Quality assurance
QC	Quality control
qPCR	Quantitative real-time polymerase chain reaction
Q-RT-PCR	Quantitative real-time polymerase chain reaction
RBC	Red blood cell
RMR	Routine metabolic rate
RNA	Ribonucleic acid
ROS	Reactive oxygen species
rpl7l1	Ribosomal protein L7 -like 1
rpm	Revolutions per minute
rps25	Rribosomal protein S25
RT	Rainbow Trout
RXR	Retinoid X receptor
RYR	Ryanodine receptor
S	Sulfer
SA	Surface area
SC	Saline control
scn5lab	Voltage gated Na^+ channel, type 5ab

SD	Standard deviation
SEM	Standard error of the mean
SERCA2a	Sarcoplasmic Ca^{2+} ATPase
SF	Sediment-free
SMR	Standard metabolic rate
SO_4^{2-}	Sulphate
sod	Superoxide dismutase
SPE	Solid phase extraction
Sr	Strontium
SR	Sarcoplasmic reticulum
SW	Salt control
t	Time interval of each step
T	Time spent at the maximum achieved swim velocity
tata12	TATA-box binding protein associated factor 12
tataaf12	TATA-box binding protein associated factor 12
TBARS	Thiobarbituric acid reactive substances
tbx2b	T-box transcription factor 2b
tbx5	T-box transcription factor 5
TDS	Total dissolved solids
TENORM	Technologically enhanced naturally occurring radioactive materials
TIE	Toxicity identification evaluation
Tl	Thallium
TN	Total nitrogen

tnnt2a	Cardiac troponin T type 2a
TOC	Total organic carbon
TPGS	Tocopheryl polyethylene glycol succinate
TSC	Tail/spine curvatures
t to BL	Times to peak relaxed states
t to Peak	Times to peak contraction states
µCi/ml	Micrcocuries per millilitre
U_{crit}	Critical swimming speed
udpgt	Uridine 5'-diphospho-glucuronosyl transferase
U_f	Highest maintain swim velocity
µg	Microgram
µg/L	Microgram per litre
uL	Microlitre
µm	Micrometer
µM	Micromolar
U_{max}	Maximum swimming speed
U of A	University of Alberta
µS	Microsiemen
UOG	Unconventional oil and gas
US	United States
USA	United States of America
U.S. EPA	United States Environmental Protection Agency
v/v	Volume per volume

vmhc	Ventricular myosin heavy chain
vnp	Ventricular natriuretic peptide
vtg	Vitellogenin
VOC	Volatile organic compound
W	Watt
WET	Whole effluent toxicity
YSE	Yolk-sac edema
ZF	Zebrafish
Zn	Zinc

CHAPTER 1: Introduction

Parts of this introduction have been taken from previously published work:
Folkerts, E.J., Goss, G.G. and Blewett, T.A. (2020). Investigating the potential toxicity of hydraulic fracturing flowback and produced water spills to aquatic animals in freshwater environments: A North American perspective. In:. *Reviews of Environmental Contamination and Toxicology* (Continuation of Residue Reviews). Springer, Cham. DOI: 10.1007/398_2020_43.

1.1 INTRODUCTION

Extraction of hydrocarbons from unconventional resources is increasing globally as a result of technological advances in horizontal drilling (Gagnon et al., 2016; Vengosh et al., 2014). Hydraulic fracturing is one such advance where chemically formulated fluids are used to fracture low permeability formations under high pressure and temperature (Stringfellow et al., 2014). This process increases the permeability of shale, tight sands, coalbeds, and other gas and oil containing strata, facilitating extraction of hydrocarbon resources from reserves that would otherwise be uneconomic (Stringfellow et al., 2014). Hydraulic fracturing fluids are injected over a short period of time and use large quantities of water per well injection (10,000–100,000 m^3). Injected fluids are comprised of chemical additives that maximize efficiency and output of oil and gas from any given well. These additives include proppants (e.g., ceramic beads, sand – to prevent fracture reclosure), biocides (to prevent microbial degradation of end-product resources and maintain well viability), gelling and foaming agents, pH adjustors, clay stabilizers, and surfactants (DiGiulio and Jackson, 2016; Ferrer and Thurman, 2015; FracFocus, n.d.; Lester et al., 2015). When pressure along the length of the fracture is released, these complex chemical mixtures of hydraulic fracturing fluid return to the surface, and this fluid is termed either flowback or produced water. A general depiction of the horizontal hydraulic fracturing process can be found in Figure 1-1. The delineation of "flowback" versus "produced water" is often subject to well operator discretion. Flowback typically is considered the earliest fluid which returns to the surface and which most closely resembles the composition of the initial injection fluid. Produced water is the fluid returning from the subterranean environment following longer periods of well production. This fluid is often saline in nature and contains high gas and oil content, which is then operationally separated (U.S. EPA, 2016). The distinction between these two types of fluid is not clear, since mixing occurs in the formation, so for the purposes of this review, we will refer to the hydraulic fracturing wastewater as flowback and produced water (FPW) (Stringfellow et al., 2014) with the recognition that toxicological properties of FPW change over the course of flowback production. FPW is a chemically complex heterogeneous mixture that contains highly variable concentrations of organic compounds (e.g., polycyclic aromatic hydrocarbons, PAHs), naturally occurring radioisotopes (e.g., radium), ions (e.g., calcium,

magnesium, chloride, sodium, potassium), metals (e.g., barium, thallium, lead), and transformation products resulting from chemical reactions under high temperature and high pressure (DiGiulio and Jackson, 2016; He et al., 2017a; Lester et al., 2015) (further expansion in section 1.2). The specific composition of any given FPW is unique, depending on many factors including the geology of the formation, well shut-in length, phase of flowback collection, and the composition of the initial fracturing fluid additives used for operation (Alessi et al., 2017; Goss et al., 2015; Stringfellow et al., 2017). FPW may be recycled but must eventually be disposed of.

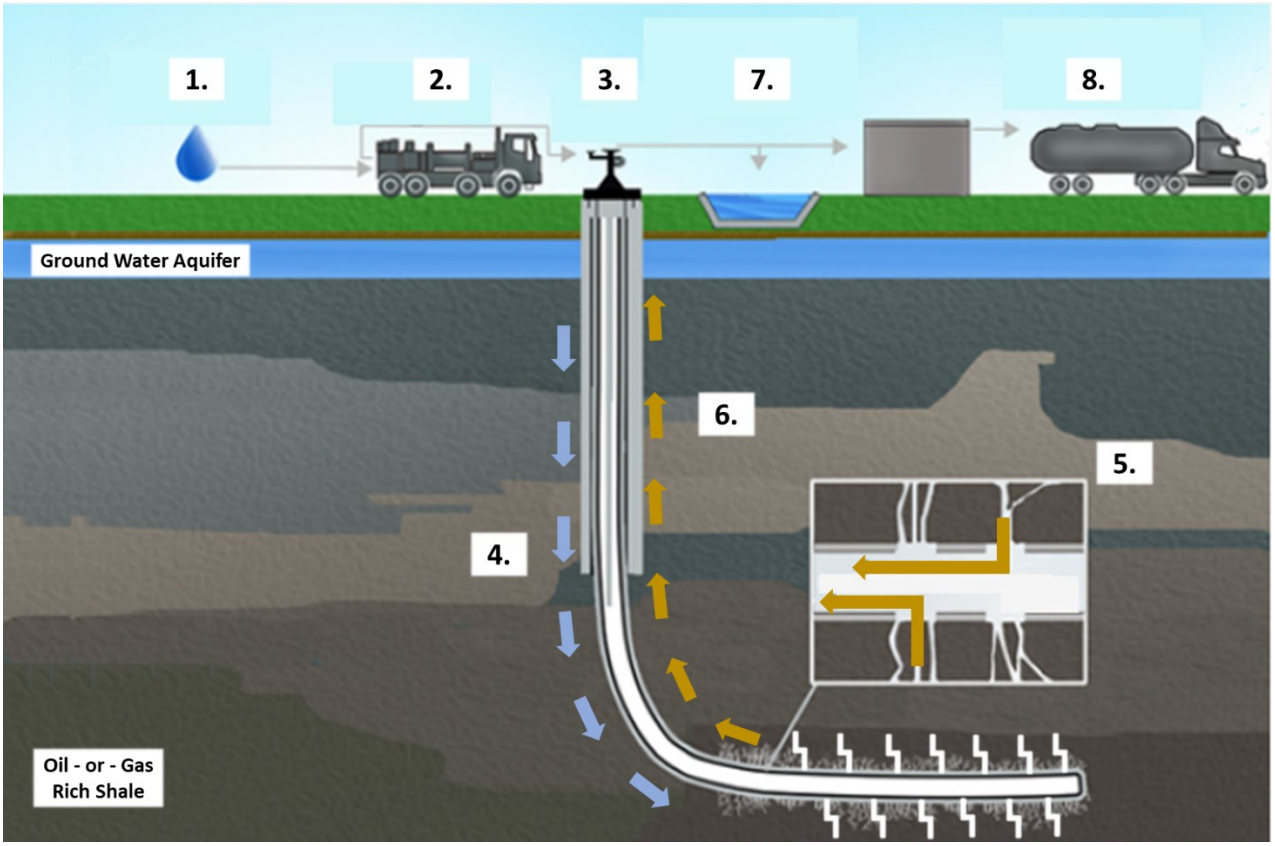


Figure 1- 1. Schematic of the general hydraulic fracturing process for a horizontally drilled well. 1.) Water is typically acquired from surface water sources (e.g. rivers, streams, lakes, reservoirs). 2.) Collected water is mixed with proppants and other fracturing additives prior to injection. 3.) Water-fracturing chemical mixture is pumped down into the drilled well at high pressures. 4.) Pressurized fracturing fluid travels the length of the well until it reaches the desired resource-rich formation 5.) Pressurized injected fracturing fluid is then sealed and capped in the well-bore. Fissures in the targeted formation are subsequently formed as a result of the pressurized injected fracturing fluid, releasing oil or gas from the shale

formation. 6.) Once capping is removed, pressurized fracturing fluid, oil or gas, and flowback and produced waters return to the surface for collection. 7.) Flowback and produced water may be temporarily stored on site. 8.) Ultimately, flowback and produced waters are transported for wastewater treatment and recycling or for disposal. Image modified from AGI, 2021.

It is estimated that in the USA, unconventional production of natural gas will account for nearly half of newly developed gas production by 2035 (Gagnon et al., 2016). Considering this expected rise in unconventional oil and gas (UOG) activity and the large quantities of FPW created from these processes, management strategies for FPW and potential hazards to the environment are two growing concerns facing the industry and regional governments. One of the key risks associated with hydraulic fracturing is the potential for spills during the generation and transport of large volumes of FPW. Such risks include ground and surface water contamination during pipeline leaks, truck transportation, and injection well integrity issues (Ferrar et al., 2013). Determination of ecological impacts of FPW is considered a top science priority to inform energy policy, conservation, and management of natural systems (Jones et al., 2015). As such, government, industry, landowners, and environmental groups have scrutinized the current methods of storage, transportation, and remediation protocols when spills/leaks occur of this wastewater (Boudet et al., 2014; Gehman et al., 2016; Theodori et al., 2014).

The amount of FPW produced by hydraulic fracturing activities in North America is substantial and growing. For example, it is estimated that 210 billion gallons of FPW were produced from 2005 to 2014 in the USA alone (Kondash and Vengosh, 2015). In Canada, formations exploited for shale gas and tight oil extraction using UOG technologies primarily occur in the western provinces of British Columbia (BC) and Alberta (AB). These formations include, among others, the Montney and Duvernay. In the Montney formation, approximately 50–100% of initial fracturing fluid/water injected is recovered as FPW (Rivard et al., 2014), and between 2011 and 2013, it was estimated that 10,000–25,000 m^3 of wastewater was produced per well in this formation (Goss et al., 2015). The volumes of FPW produced from the Duvernay formation are less well characterized, but estimates of water use in 2013 found volumes of 10,000–60,000 m3 of water per well (Goss et al., 2015). FPW generation and management are growing concerns among

regulatory bodies in regions utilizing UOG practices, and aspects of UOG development pertaining to FPW characterization and spill hazard assessment are only recently being analyzed and studied more intimately (reviewed in Alessi et al., 2017; Goss et al., 2015). Considering UOG activities are anticipated to increase in prevalence in North America (Gagnon et al., 2016; Vengosh et al., 2014), determining how to effectively gauge the severity of spills and the extent of impact to the environment is a topic of increasing importance.

Despite regulation and controls in all North American jurisdictions on the handling of FPW, spills still occur. Common routes of FPW contamination to surface water sources include accidental releases during FPW transport (i.e., pipeline or truck transport), inadequate FPW treatment and subsequent release, and intentional illegal releases by operators (Goss et al., 2015; Vengosh et al., 2014). From 2005 to 2010, it was determined that within the Marcellus and Fayetteville shale plays of the eastern USA, the average distance of drilled wells from freshwater streams was ~300 m (Entrekin et al., 2011). Given the close association of hydraulic fracturing activities with freshwaters and the growth of this extraction approach, the potential for releases to surface bodies of water is expected to rise. In the province of Alberta, Canada alone, the Alberta Energy Regulator (AER; the main energy policy and regulating agency in the province) compliance dashboard reported 205 spills/releases of salt/produced water associated with UOG or crude oil production from January 2017 to July 2018 (AER, n.d.). The BC Oil and Gas Commission (BCOGC) reported that nine spills of emulsion (oil, gas, water, and condensate) and three spills of brine from UOG activities occurred during 2017 in and around the Montney shale formation of upper northeast BC (BCOGC, n.d.). Furthermore, in the state of Colorado, there were a reported 257 spills of produced water in 2016 (data from the Center for Western Priorities 2016). To exemplify how spills are of concern and FPW management strategies need to be more carefully considered among regulatory agencies and operators, a report was released by the United States Environmental Protection Agency (U.S. EPA) in 2015 studying hydraulic fracturing spills from January 2006 to April 2012 in the states of Colorado, Pennsylvania, Oklahoma, Arkansas, Texas, Louisiana, New Mexico, Wyoming, Utah, West Virginia, and North Dakota. This report found a total of combined 24,000

hydraulic fracturing-related reported spills at sites located at a distance from well pads and an additional 457 spills at well pad sites (U.S. EPA, 2016).

Depending on the state/province, a spill or release of FPW warranting report can vary in terms of volume of spill, spill type, and time required to report a spill. For example, in both North Dakota and Colorado, spills escaping secondary containment of ≥ 42 gal must be reported, with written report of said spills mandated within at least 10 days (Patterson et al., 2017). In Pennsylvania, however, spills of ≥ 5–15 gal (depending on FPW TDS measurements) are required within 24 h, whereas in New Mexico, only spills ≥ 210 gal require written notification within 15 days (Patterson et al., 2017). In Alberta, the AER requires spills of ≥ 2000 L (~ 530 gal) to be reported within 24 h (AER, n.d.). Regarding spill frequency, in Alberta, an estimated >2,500 FPW spills occurred from 2005 to 2012 with more than 113 of those spills entering into freshwater lakes and streams (Alessi et al., 2017; Goss et al., 2015). Analysis of data from 2005 to 2014 representing >30,000 UOG wells in the states of Colorado, North Dakota, Pennsylvania, and New Mexico showed that 50% of spills were associated with storage or transport of FPW and that 2–16% of wells reported a spill every year, the largest singular FPW spill recorded at 3,756 m^3 (Patterson et al., 2017). However, in this same study, it was found overall that across the three states of Colorado, North Dakota, and Pennsylvania, annual spill rates are either being sustained or decreasing, with only New Mexico showing an increasing annual spill rate (Patterson et al., 2017). With increased use of UOG technologies on the rise recently, however, it is only expected that spills of FPW will continue and become more prevalent. Furthermore, risks associated with FPW are not only from spills but from direct application for dust suppression or de-icing on roads. Currently, 13 states in the USA allow spreading of FPW on roads as an inexpensive alternative to other dust suppressants (Tasker et al., 2018). FPW released to the environment in this manner has the potential to leach from roads during rain events into ground and surface water and cause toxicity to aquatic biota. In Northern Pennsylvania alone, over 280,000 L of FPW was spread on roads in 2015 (Pennsylvania DEP, 2016a) resulting in a mean radium concentration of 14.5 pCi/L in surface waters associated with the contaminated roadways, significantly higher than national recommended regulatory limits for radium in drinking water (5 pCi/L) (Tasker et al., 2018).

1.2 MAJOR FPW CHEMICAL CONSTITUENT GROUPS

Three major classes of chemical species were taken into consideration when analyzing the composition of FPW samples: major ions and other general water characteristics, organic constituents, and trace metals. Although radioactivity associated with FPW has also been determined to be of potential concern to natural environments (Haluszczak et al., 2013; Tasker et al., 2018), analysis of FPW radioactivity is not discussed in further detail presently. However, a table recording types and levels of radioactivity associated with FPW can be found in the Supplemental Information (Table S1-1). Furthermore, analyses have been restricted to studying only those constituents that are commonly reported across studies of FPW composition from North American hydraulic fracturing operations. In total, 15 different sources of detailed chemical analysis of strictly FPW (not affected surface waters or effluent discharges) were compiled to create a single database of over 5,000 data points of targeted inorganic and organic chemicals. These sources can be found in Table 1-1. It should be noted that the analyzed FPW samples detailed in the literature vary with respect to the timing of collection (flowback period sampled), well location, and shale formation being exploited – all factors which influence FPW chemistry. Indeed, many reports do not offer sufficient or complete details regarding these variables. Consequently, it is therefore acknowledged that the current review is not able to offer detail regarding how the chemical composition and toxicity of FPW samples differ with respect to time spent in the well or source geology. For information on hydraulic fracturing-associated wastewaters from drilling operations in other non-North American formations and on potential other toxicities pertaining to the initial fracturing fluid used to induce fractures, please see reviews by Faber et al., 2017 and Annevelink et al., 2016.

Table 1-1. References used for compiling chemical characterization data.

Reference	Type of Document	Analytical Methods and QA/QC Stated?	Location/Formation of FPW Sampled	Time-Period of FPW Sample(s) (Days Post-Well Stimulation)

U.S. EPA, (2015) Atlas Response_211 419	Analytical Study Report	Yes	Unknown (Marcellus suspected)	Unknown
Blauch et al., (2009)	Regional Meeting Paper	No	Pennsylvania / Marcellus	0 - 55
T. A. Blewett et al., (2017a)	Academic Paper	Yes	Alberta / Duvernay	10
Pennsylvania DEP, (2016b)_TENORM	Analytical Study Report	Yes	Pennsylvania / Marcellus	Unknown
Dresel and Rose (2010)	Geological Survey Report	Yes	Pennsylvania / Marcellus	Unknown
Hayes (2009)	Analytical Study Report	Yes	Pennsylvania / Marcellus	0, 1, 5, 14, and 90
He et al., (2017a)	Academic Paper	Yes	Alberta / Duvernay	7
Lauer et al., (2016)	Academic Paper	Yes/No	North Dakota / Bakken	Unknown
Lester et al., (2015)	Academic Paper	Yes	Colorado / Denver-Julesburg Basin	Unknown
Maguire-Boyle and Barron (2014)	Academic Paper	Yes	Pennsylvania / Marcellus Texas / Eagle Ford Barnett / New Mexico	Unknown
NYS DEC (2015)	Environmental Impact Statement Proceeding	Yes/No	Pennsylvania – West Virginia / Marcellus	Unknown
Pennsylvania DEP (2010)_Inorganics Report	Analytical Study Report	No	Unknown (Marcellus suspected)	Unknown
Rosenblum et al., (2017)	Academic Paper	Yes	Colorado / Niobrara	1, 4, 7, 15, 22, 80, 130, 220, and 405
Rosenblum et al., (2017a)	Academic Paper	Yes	Colorado / Niobrara	1, 4, 7, 15, 22, 55, 80, 130, and 220
Ziemkiewicz et al., (2014)	Academic Paper	Yes	Pennsylvania / Marcellus	Unknown

1.2.1 Major Ions

Cations and anions/salt-related ions (see Table 1-2) are major components of FPW and are primarily responsible for the high total dissolved solid (TDS) concentrations that are often observed in FPW samples. During the fracturing process, many classes of chemical compounds present in the initial fracturing fluid pumped down the wellbore (e.g., acids, breakers, and stabilizers) contain ionic chemical species that enhance operational efficiency and maximize resource recovery (FracFocus, n.d.). These include chemicals such as hydrochloric acid (used to dissolve minerals and initiate fissure formation), sodium and calcium chloride (breakers used to stabilize geogenic products and clay formations), magnesium peroxide (a breaker used to delay gel break down), and many others. These

ions, following parent compound reactions, reside in the formation fluid until they are brought back up to the surface in FPW.

However, another source of salt ionic species contributing to the commonly seen high FPW TDS concentrations is the geological environment being targeted/exploited during hydraulic fracturing activities. During the mid-late Cretaceous era (~100 million years ago; MYA), North America was divided into two land masses by a large inland sea which stretched from the Gulf of Mexico to the Arctic Ocean (Nicholls and Russell, 1990). Correspondingly, many of the sedimentary formations exploited for hydraulic fracturing purposes have a marine origin, and as a consequence, formation waters and FPW from these formations following natural resource production contain high levels of major ions/salts (Chen and Osadetz, 2013; Connolly et al., 1990; Kahrilas et al., 2016; Li et al., 1997; Rice, 2003).

Table 1-2. General water quality and ion concentrations in hydraulic fracturing flowback and produced water samples and current acute (and chronic) guideline concentrations for the protection of freshwater aquatic life according to Canadian Environmental Quality Guidelines (CEQ) and the Unites States Environmental Protection Agency (USEPA). N= number of collective samples used to establish range. All values in mg/L.

Chemical	$\bar{X}$ Conc.	Median Conc.	Range	N	CEQ Guidelines		USEPA Guidelines	
					Acute	Chronic	Acute	Chronic
Bromide	610	472	0.2 - 2240	147	ND	ND	ND	ND
Calcium	9252	7630	35.2 - 41600	164	ND	ND	ND	ND
Chloride	60293	43800	64.2 - 207000	193	640	120	860	230
Magnesium	982	710	7.1 - 13000	164	ND	ND	ND	ND
Manganese	9	4	0 – 96.5	160	ND	ND	ND	ND
Potassium	705	253	2.7 - 17043	148	ND	ND	ND	ND
Sodium	26669	21510	45.9 - 95500	165	ND	ND	ND	ND
Sulphate	94	46	0 - 1010	167	ND	ND	ND	ND
TDS	108562	91405	680 - 345000	148	ND	ND	ND	ND

Nitrate	2.049	1.3	0.081 – 15.9	74	550	13	ND	ND
Nitrite	12.03	4.7	0.045 - 146	72	ND	60	ND	ND
Nitrogen (total)	116	96	5.6 - 498	69	ND	ND	ND	ND
Carbon (total)*	1200	110	1.2 - 58550	77	ND	ND	ND	ND
pH	6.3	6.5	3.4 – 10.1	126	ND	6.5 – 9.0	ND	6.5 – 9.0

* Carbon (total) includes measurements of total organic carbon, total carbon, and dissolved organic carbon.

ND = No Data

1.2.2 Trace Metals

FPW metal profiles depend greatly on the geology of the formation being exploited. As seen in Table 1-3, numerous different trace metals may be present in FPW and at varying concentrations. The trace metals present in FPW which will be discussed in this section are from multiple different chemical groups, including the alkali and alkaline earth metals (e.g., barium and strontium – both of which are commonly found at high concentrations in FPW), classically defined "transition" metals (e.g., iron, zinc, cadmium, etc.), post-"transition" metals (e.g., aluminum, lead, thallium, etc.), and metalloids (e.g., boron, arsenic, etc.). Due to their toxicity at relatively low concentrations (Wood, 2012a), many of these metals may pose significant hazards to aquatic systems if present (detailed in section 1.3.2).

Table 1-3. Trace metal species present in hydraulic fracturing flowback and produced water samples and current acute (and chronic) guideline concentrations for the protection of freshwater aquatic life according to Canadian Environmental Quality Guidelines (CEQ) and the Unites States Environmental Protection Agency (USEPA). N= number of collective samples used to establish range. All values in mg/L.

					CEQ Guidelines		**USEPA Guidelines**	
Chemical	**$\bar{X}$ Conc.**	**Median Conc.**	**Range**	**N**	**Acute**	**Chronic**	**Acute**	**Chronic**

Antimony	0.25	0.1	0 – 9.9	71	ND	ND	ND	ND
Arsenic	0.37	0.077	0 – 26.1	93	ND	0.005	0.34	0.15
Barium	1177.32	355	0 - 13900	157	ND	ND	ND	ND
Beryllium	0.031	0.04	0 – 0.1	89	ND	ND	ND	ND
Cadmium	0.024	0.01	0 – 0.13	93	0.001	9.0×10^{-5}	0.0018	7.2×10^{-4}
Copper	0.21	0.071	0 – 4.15	125	ND	0.002 – 0.004[a]	ND	0.0065 – 0.034[a,b]
Iron (total)	203	46	0 - 18432	163	ND	0.3	ND	1.0
Lead	0.11	0.03	0 – 3.48	118	ND	0.001 – 0.007[a]	0.082	0.0032[a]
Mercury	0.18	0.0002	0 – 14.6	83	ND	2.6×10^{-5}	0.0014	7.7×10^{-4}
Strontium	1632.73	912	0.58 - 13100	160	ND	ND	ND	ND
Thallium	2.34	0.1	0.0049 - 151	69	ND	8.0×10^{-4}	ND	ND
Zinc	8	0.1	0 - 685	128	0.037	0.007	0.12	0.12

[a]Concentrations dependent on water hardness

[b]Concentrations based on a Biotic Ligand Model analyses

ND = No Data

1.2.3 Organic Chemicals

Similar to major ions, organic chemicals present in FPW are derived from two primary sources: from chemicals originally added to fracturing fluids for the purposes of inducing formation fractures and maintaining well viability but also from the formations themselves. Organic chemicals present in FPW samples derived from the formations being exploited are petrogenic in nature. As noted previously, certain drilling practices add specific organic chemicals to the fracturing fluid to aid extraction (e.g., biocides for

antifouling properties and gelling agents such as ethylene glycols and differing petroleum distillates). Many of these fracturing fluid additives and their purposes can be found on the open-access website FracFocus.org. Although the majority of fracturing fluid organic additives are depleted during drilling operations, trace amounts of organic additives may still be present in collected FPW samples. However, much of the reported data on FPW does not include analysis of these organic additives. Thus, for the sake and purpose of this review, only organic chemicals originating from the formations that are present in FPW will be discussed.

The organic species present in FPW are both numerous and diverse. Although only organic chemicals that are common to most FPW samples are reported in Table 1-4, potentially thousands of organic chemical species may be present (He et al., 2017a). One of the more commonly measured organic chemicals in FPW are PAHs, a class of organic compounds that have high toxicity, and which elicit negative impacts on both biota and ecosystems through many different mechanisms. This group encompasses both small organic chemicals, such as the double to 4-ring PAHs (e.g., naphthalene, phenanthrene, and pyrene), and relatively larger 5 to 10-ring PAH molecules (e.g., benzo[a]pyrene, perylene, and ovalene). It should be noted, however, that many larger PAH compounds (≥ 6 rings) are often insoluble in water and sorb to organic carbon in aquatic systems (de Maagd et al., 1998; Ma et al., 2010). Accordingly, such larger PAHs are immediately less bioavailable to aquatic organisms (although accumulation within sediments may become a toxicological concern).

Table 1-4. Organic chemical species present in hydraulic fracturing flowback and produced water samples and current acute (and chronic) guideline concentrations for the protection of freshwater aquatic life according to Canadian Environmental Quality Guidelines (CEQ) and the Unites States Environmental Protection Agency (USEPA). N= number of collective samples used to establish range. All values in µg/L.

Chemical	$\bar{X}$ Conc.	Median Conc.	Range	N	CEQ Guidelines		USEPA Guidelines	
					Acute	Chronic	Acute	Chronic
Polycyclic Aromatic Hydrocarbons								

Naphthalene	68.66	5	0 - 1400	69	ND	1.1	ND	ND
Acenaphthylene	8.084	1.9	0 - 190	69	ND	ND	ND	ND
Acenaphthene	8.34	1.9	0 - 190	69	ND	5.8	ND	ND
Fluorene	7.89	1.9	0 - 190	69	ND	3	ND	ND
Phenanthrene	7.64	1.9	0.15 - 190	69	ND	0.4	ND	ND
Fluoranthene	7.67	1.9	0 - 190	69	ND	0.04	ND	ND
Pyrene	7.71	1.9	0.037 - 190	69	ND	0.025	ND	ND
Benzo[a]anthracene	8.085	1.9	0 - 190	69	ND	0.018	ND	ND
Chrysene	8.026	1.9	0 - 190	69	ND	ND	ND	ND
Benzo[b]fluoranthene	8.21	1.9	0 - 190	69	ND	ND	ND	ND
Benzo[k+j]fluoranthene	8.15	1.9	0 - 190	69	ND	ND	ND	ND
Benzo[a]pyrene	8.18	1.9	0 - 190	69	ND	0.015	ND	ND
Indeno[1,2,3-cd]pyrene	8.25	1.9	0 - 190	69	ND	ND	ND	ND
Benzo[g,h,i]perylene	7.89	1.9	0 - 190	69	ND	ND	ND	ND
Dibenz[a,h]anthracene	8.27	1.9	0 - 190	69	ND	ND	ND	ND
(1 or 2)-Methylnaphthalene	11.19	1.9	0.11 - 190	69	ND	ND	ND	ND
Volatiles								
1,1,1-Trichloroethane	46.1	5	0 - 500	68	ND	ND	ND	ND
1,1,2-Trichloroethane	46.1	5	0 - 500	68	ND	21	ND	ND
1,2-Dichloropropane	46.1	5	0 - 500	68	ND	ND	ND	ND
1,2,4-Trichlorobenzene	43.35	5	0 - 500	68	ND	24	ND	ND
Benzene	888.6	7.7	0 - 12500	81	ND	370	ND	ND

Carbon tetrachloride	46.1	5	0 - 500	68	ND	13.3	ND	ND
Ethylbenzene	103.9	5	0 - 1540	75	ND	90	ND	ND
Styrene	45.4	5	0 - 500	70	ND	72	ND	ND
Toluene	1293.3	5	0 - 41500	81	ND	2	ND	ND
Vinyl chloride	46.1	5	0 - 500	68	ND	ND	ND	ND
Xylenes (total)	1569.3	15	3 - 73000	76	ND	ND	ND	ND
Synthetics								
Dibromochloromethane	46.1	5	0 - 500	68	ND	ND	ND	ND
Hexachlorobenzene	8.21	1.9	0 - 190	68	ND	ND	ND	ND
Pentachlorophenol	40.98	9.5	0 - 940	68	ND	0.5	19	15

ND = No Data

1.2.4 Wellbore Reaction Products

One area of FPW chemical characterization which has recently begun to gain analytical attention is the presence of transformation products created during reactions involving the initial fracturing fluid compounds and the high-heat, high-pressure environments of a horizontally fractured wellbore. Albeit highly variable, and dependent on numerous factors including depth of formation, length of well, formation geology, etc., temperatures and pressures within a bore of a horizontally fractured well can reach up to ~200 °C and 10,000 psi, respectively (Kahrilas et al., 2016; Nelson and Santus, n.d.; Shaffer et al., 2013). This creates an ideal environment for the transformation of organic chemicals incorporated into the initially injected fluid. These wellbore-created chemical species may hold differing toxicities than the original parent compounds (He et al. 2017a; Kahrilas et al. 2016). Although theorized to constitute a relatively small proportion of the total FPW chemical makeup, these wellbore-transformed compounds pose challenging problems to operators and regulatory agencies when attempting to develop risk assessment and remediation protocols. Currently, targeted analysis of select compounds is slowly

elucidating the extent of these transformational reactions and the occurrence of these wellbore reaction products. However, non-targeted approaches (such as non-targeted orbitrap LC-MS studies) comparing FPW and initial fracturing fluids may better determine what and how many products are formed in a given fracturing operation. Industry proprietary constraints, however, often impede these types of studies and hinder further expansion of this topic.

1.3 MODES OF FPW CHEMICAL CONSTITUENT TOXICITY IN AQUATIC SYSTEMS

1.3.1 Major Ion Toxicity

Salinity in produced waters is likely to be the most significant source of acute toxicity associated with FPW exposure to aquatic organisms (Folkerts et al. 2019). The levels of salts such as Na (sodium), K (potassium), Mg (magnesium), Ca (calcium), and Cl (chloride) in many FPWs are extremely high compared to natural freshwaters. For example, the levels of Na in FPW may be > 6,000-fold higher than Na concentrations in some freshwater lakes and rivers (Table 1-2; Allen, 2008). As observed with trace metals, there is a large variation within concentrations of these major ions. For example, Na concentrations in FPW can range from 45.9 to 95,599 mg/L, and Cl values range from 64.2 to 207,000 mg/L depending on the FPW sample (Table 1-2). In assessing the potential for major ion toxicity, it is worth noting that it may be difficult to isolate the effects of specific ions or salts, as elevations of multiple ions co-occur, and this is a phenomenon likely to influence toxicological outcomes. However, as a general metric, salinity can be a useful guide as to the anticipated toxic outcome, and concentrations of major ions in FPW often surpass Canadian Environmental Quality (CEQ) and United States Environmental Protection Agency (USEPA) water quality guideline concentrations and LC_{50} values for numerous aquatic organisms (see Tables 1-2 and 1-5). A sudden influx of highly saline water, such as a saline FPW spill, into a lake or stream will initially cause toxicity due to osmoregulatory distress. All freshwater organisms must closely maintain ionic homeostasis, but the species most at risk are stenohaline species that may lack the capacity to osmoregulate beyond their normal environmental salinity range (Evans et al., 2005).

Similar to metals, the gill is a primary target for the effects of salts associated with an FPW spill. The gill is a multifunctional organ that is involved with ion regulation and is the first line of defense against toxicants. Morphologically, the gill epithelium is composed of several different cell types, which undergo changes in response to physiological stress and environmental challenges. Of these cell types, interlamellar cells (cells between the lamellae of gills) may grow and change to meet physiological demands, such as in situations where environmental dissolved oxygen concentrations are altered (Sollid and Nilsson, 2006), toxicant exposure occurs (Tamzin A. Blewett et al., 2017), or when the fish is osmotically challenged (Blair et al., 2016). Changes to interlamellar cell mass (ILCM) is a commonly used index for observing respiratory stress in fish (Sollid and Nilsson, 2006). Previous studies have observed altered responses of the ILCM as a response to high salt exposure (Blair et al., 2016; T. A. Blewett et al., 2017b) In Arctic grayling (*Thymallus arcticus*) and rainbow trout (*Oncorhynchus mykiss*) exposed to salinities of 17,000 and 13,500 mg/L, respectively, for 24 and 48 h, histological analysis of the gill filaments showed structural changes. In the stenohaline Arctic grayling, ILCM recordings showed signs of hyperplasia and overgrowth of the protruding lamellae, decreasing the surface area of the gills dramatically. This gill remodeling is thought to be beneficial as a defense mechanism against exposure to high saline FPW as it would limit the uptake of salts and minimize water loss across the epithelium. Conversely, during the same exposure, the ILCM was reduced in rainbow trout, likely because of the greater osmoregulatory capacity of this euryhaline species was sufficient to handle the change in environmental concentrations.

Another acute effect observed in fish exposed to high saline waters is the generation of an oxidative stress response. In trout exposed to the salinity component of FPW alone, alterations in key oxidative stress enzyme activities were observed in the gill and liver of rainbow trout (T. A. Blewett et al., 2017b). More specifically, there was both an increase in the enzyme catalase activity and a decrease in the enzyme superoxide dismutase activity. These enzymes are part of a critical antioxidant defense system which operates to scavenge free oxygen radicals, preventing damage to cellular constituents. For an extensive review on salinity induced oxidative stress, see Lushchak, (2011). Daphnia magna, a freshwater crustacean, showed even more extreme sensitivity to the major ions present in FPW (T. A.

Blewett et al., 2017a). In this study, the authors exposed the animals to dilutions of a saline solution which was made up to match the major ion composition of a specific FPW sample. The median lethal concentration occurred when the saline solution was diluted to a concentration representing just 1.5% (~2,700 mg/L) of the raw FPW sample salinity. Previous reports have shown that total offspring production in Daphnia decreases under NaCl exposure (Ghazy et al., 2009; Martínez-Jerónimo and Martínez-Jerónimo, 2007). Blewett et al., (2017b) showed that after chronic exposure, both salinity and FPW caused decreased reproduction and metabolic rate. Furthermore, chronic exposure to salinity can cause changes in physiological homeostasis such as plasma-enhanced stress-related hormones (e.g., prostaglandins), stimulation of energy metabolism, alteration of electrolyte equilibrium, enhanced reactive oxygen species generation, and altered reproductive fitness (Lushchak, 2011).

Table 1- 5. Reported acute lethal toxicities (LC_{50} in mg/L) of major ions associated with hydraulic fracturing flowback and produced water to aquatic species.

Chemical	Species	LC_{50}		Water Temp. (°C)	Hardness (mg/L $CaCO_3$)	pH	Ref.
		48 h	96 h				
Bromide	Juv. DM	1.0[a]		22	173	8	(LeBlanc, 1980) †
		8.1[a]		20	170	7.5	(Baird et al., 1991)†
			> 100.0[a]	20	130	7.5	(Ewell et al., 1986) †
		11000.0[a]		20			(Canton et al., 1983) †
		13500.0[a]		19	100		(Hermens et al., 1984) *
	Juv. FHM		> 100.0[a]	20	130	7.5	(Ewell et al., 1986) †
	FHM	17800.0[a]	16500.0[a]	12	107	7.8	(Alexander et al., 1981) †
Calcium	Juv. DM	52.0[a]		18	45	7.7	(Biesinger and Christensen, 1972)*
	Juv. DM	2770.0[a]		20	Mod. Hard	7.5 – 9.0	(Mount et al., 1997)†
	FHM		4630.0[a]	25	Mod. Hard	7.5 – 9.0	(Mount et al., 1997)†
Magnesium	Juv. DM	140.0[a]		18	45	7.7	(Biesinger and Christensen, 1972) *
	Juv. DM	1330.0[a]		20	Mod. Hard	7.5 – 9.0	(Mount et al., 1997)†
	FHM		2120.0[a]	25	Mod. Hard	7.5 – 9.0	(Mount et al., 1997)†
Manganese	Juv. DM	9.8[a]		18	45	7.7	(Biesinger and Christensen, 1972) *
Potassium	Juv. DM	93.0[a]		18	45	7.7	(Biesinger and Christensen, 1972) *

	Juv. DM	660.0[a]		20	Mod. Hard	7.5 – 9.0	(Mount et al., 1997)[†]
	FHM		880.0[a]	25	Mod. Hard	7.5 – 9.0	(Mount et al., 1997)[†]
Sodium	Juv. DM	1640.0[a]		18	45	7.7	(Biesinger and Christensen, 1972) *
	Juv. DM	4770.0[a]		20	Mod. Hard	7.5 – 9.0	(Mount et al., 1997)[†]
	FHM		6390.0[a]	25	Mod. Hard	7.5 – 9.0	(Mount et al., 1997)[†]
Sulphate	CD	2050.0[a]		25	94	7.9	(Soucek and Kennedy, 2005)*
		2526.0[a]		25	107	7.9	(Soucek and Kennedy, 2005)*
	HA	512.0[a]		22	94	7.9	(Soucek and Kennedy, 2005)*
		2855.0[a]		22	107	7.9	(Soucek and Kennedy, 2005)*

[a] Static/Semi-static Exposures

[b] Flow-Through Exposures

* Measured chemical concentrations used for LC_{50} calculations

[†]Nominal chemical concentrations used for LC_{50} calculations

CD - *Ceriodaphnia dubia*

DM – *Daphnia magna*

FHM – Fathead Minnow (*Pimephales promelas*)

HA – *Hyalella azteca*

Juv. – Juvenile-aged organisms used. DM = within 24 hrs. RT = ≤ 1-year post hatch.

1.3.2 Trace Metal Toxicity

It is difficult to quantify the toxicity caused by individual trace metals present in FPW, as they are present in mixtures with other trace metals and chemical additives, which modify bioavailability, bioaccumulation, and toxicity. Below, we identify certain trace metals of toxicological concern commonly found in characterized FPW samples by comparing concentrations to LC_{50} values for multiple model aquatic animal species. Furthermore, we outline potential mechanisms of toxicity of individual trace metals to aquatic organisms and the role of metal speciation in mediating toxicity. Most common trace metals found within many different FPW samples studied include barium (Ba), strontium (Sr), iron (Fe), manganese (Mn), thallium (Tl), zinc (Zn), copper (Cu), and lead (Pb). Although these metals are commonly found in FPW samples, their concentrations

will vary considerably as a consequence of the geology of the formation being exploited for resources. Nevertheless, water quality criteria for dissolved metals are typically in the µg/L range (Wood, 2012a), while trace metals present in raw (i.e., undiluted) FPW samples are present at concentrations in the mg/L range. Moreover, FPW frequently reaches metal concentrations well above both the current ambient water quality criteria for Canada, the USA, and the European Union (Wood, 2012a) (Table 1-3) and above LC_{50} concentrations for numerous aquatic organisms (Table 1-6).

Metal toxicity can be exerted over either an acute time frame or a chronic time frame, and the mechanisms of effect may differ between these two periods of exposure. Metals can cause toxicity through a plethora of mechanisms, including structural damage, disruption of enzymatic function, reacting as redox catalysts in the production of reactive oxygen species (ROS), disruption of ion regulation, and initiating the formation of DNA and protein adducts (Liu et al., 2008). Over acute exposure periods, the lethality of metals is often associated with damage at the gill. The gills of fish and invertebrates represent up to 70% of the total surface area of an organism that is directly exposed to the external environment and are organs designed for physiological transport processes (Evans et al., 2005; Hughes, 1972). Previous evidence has shown that exposure to various trace metals can cause profound morphological changes in the gills, often characterized by an inflammatory response (Wood, 2001). This response can elicit rapid lethality due to edematous swelling, cellular lifting, necrosis, and lamellar fusion – changes that result in a significantly increased water-to-blood diffusion distance, which in turn reduces the ability of the animal to effectively take up oxygen (Wood, 2012a). There are, however, mechanisms of toxicity that are specific to certain trace metals. Some metals impose toxicity via ionic mimicry whereby metals target the active ionic uptake pathways in the gill of aquatic organisms (Bury, 2003; Büsselberg, 1995; Clarkson, 1993; Wood, 2012a). For example, silver (Ag) and Cu mimic Na (Goss et al., 2011; Grosell, 2012; Grosell and Wood, 2002; Wood, 2012b), while Pb (Mager, 2012), Zn (Hogstrand, 2012; Hogstrand et al., 1994), and cadmium (Cd) (McGeer et al., 2012; Niyogi and Wood, 2004) will mimic Ca. This mimicry results in these metal contaminants disrupting ion homeostasis, through both decreased uptake of the nutrient ions and through metal effects on sensitive ion transport enzymes. For example, Cu can inhibit the basolateral Na/K ATPase, an enzyme

that energizes the active uptake of Na and Cl for ionic regulation (Wood, 2012a). Active uptake of Na, Cl, and Ca is essential for freshwater animals, which constantly lose these ions by diffusion from their relatively salt-rich bodies to the much more dilute environment. Consequently, death via hyponatremia or hypocalcemia occurs as a result of metals inhibiting these enzymes. Cu can also inhibit carbonic anhydrase, the enzyme that hydrates CO_2 to produce H^+, which is also critical for acid-based ion regulation (Grosell, 2012). Similarly, Zn, Pb, and Cd compete with Ca for entry through apical Ca channels and can inhibit the basolateral Ca-ATPase that powers Ca transport within the gills (Hogstrand, 2012; Mager, 2012; McGeer et al., 2012).

In terms of chronic toxicity from lower-level exposures, similar mechanisms of toxicity may be observed. However, under chronic exposure conditions, a suite of mechanisms are usually altered which ultimately result in decreased survival, growth, or reproduction. For example, small molecular weight thiol-containing proteins such as metallothionein and glutathione may be induced by certain metal exposures and act to bind to intracellular metals and prevent their reaction with sensitive cellular sites (Wood, 2012a). However, this may act to limit bioavailability of other essential metals for metalloenzymes. Chronic toxicity of metals may also be associated with increased oxidative stress. Increased metal exposure can induce oxidative stress through two mechanisms, the first is related to the generation of free radicals by ions with changeable valences (e.g., Fe, Ni, Cu) and the second through damage of antioxidant enzymes that are normally used to prevent reactive oxygen species damage (for detailed review of these processes, see Lushchak, 2011).

The thresholds for acute and chronic effects of metals will vary as a function of metal concentration and length of exposure. However, in FPW spill scenarios, metals will be present in aquatic settings in the presence of other chemicals that have the potential to mitigate and enhance their toxicity. This occurs either through altering metal speciation or through interactions with effect pathways. The speciation of a trace metal is critical to its toxicity and is significant for understanding trace metal bioavailability and environmental fate (Wood, 2012a). Commonly, speciation refers to the distribution of the different metal chemical species within a system. For most metals, the free ion form is considered to be the most toxic, as it is the species that is thought to be the most bioavailable, gaining access to

the organism through dedicated ion transporters on epithelial surfaces (as described above; Wood, 2012a). In aquatic systems, trace metal bioavailability is affected by complexation (anionic and/or organic) and competition (trace metals and major essential ions [Na, Ca, K, Mg] that may share a pathway; Wood, 2012a) making interpretation of the bioavailability in the complex mixture that is FPW extremely challenging.

Anionic complexation involves the association of a trace element with anions such as SO_4^{2-} and $Cl^-$and OH^- and HCO_3^- (Wood, 2012a). The association of any given metal with any given anion is dependent on several factors, including the physicochemical characteristics of both the metal cation and the anion. In FPW, however, concentrations of complexing anions are high. For example, FPW SO_4^{2-} concentrations display a median value of 46.3 mg/L but ranges from 0 to 1,010 mg/L, while the median value for Cl^- is 43,800 mg/L with a range from 64.2 to 207,000 mg/L (Table 1-2). This suggests that anion complexes of metals are likely to be significant in FPW and in turn presumably affect and alter bioavailability. However, other water chemistry factors play an important role. For example, in freshwater, the chemical speciation of trace metals is greatly influenced by pH. FPW is generally acidic (e.g., median pH of 6.5, Table 1-2) but can vary greatly from pH 3.4 to 10.1 (Table 1-2). More alkaline waters will favor bicarbonate or carbonate-based complexes, thereby decreasing bioavailability and toxicity. However, it is important to note that some anion complexes of metals may be bioavailable and induce effects associated with toxicity. For example, the formation of $CuCO_3$ has been associated with the toxic effects of Cu in Daphnia magna (De Schamphelaere et al., 2002). However, to date, the toxicological significance of anions and anion complexes has received little attention.

Organometallic complexes are also found in FPW. Metals are known to bind to organic carbon with varying degrees of binding affinities (Aiken et al., 2011; Lores and Pennock, 1998; Playle et al., 1993). Again, because of the complexity associated with organics present in hydraulic fracturing effluents, we are unable to individually associate metals with specific organic complexes. However, it is known that PAHs bind to certain metals through cation-π interactions (Gauthier et al., 2014) resulting in lipophilic organometallic complexes. These organometallic complexes may diffuse across the epithelia of aquatic organisms, and the potential for toxic effects associated with this bioaccumulation has been documented (Boullemant et al., 2009; Parthasarathy et al., 2010,

2008). However, to date, the toxicities of these complexes in FPW have not been extensively investigated. However, it must also be noted that metal toxicity in aquatic environments may also be mitigated by complexation with some organics. For example, dissolved organic carbon (DOC) is ubiquitous in natural waters and is formed by the microbial breakdown of plant and animal materials (Thurman, 1985). DOC has a high affinity for metals, and by binding to them, bioavailability of trace metals to target surfaces such as the gills is reduced (Wood et al., 2011). Similarly, the high salinity of these effluents (Alessi et al., 2017; Goss et al., 2015) may likely outcompete metals for binding to ion transporters on the gill, reducing uptake, bioavailability, and toxicity (reviewed in (Blewett and Leonard, 2017).

Table 1-6. Reported acute lethal toxicities (LC_{50} in mg/L) of metals associated with hydraulic fracturing flowback and produced water to aquatic species.

Chemical	Species	LC_{50}		Water Temp. (°C)	Hardness (mg/L $CaCO_3$)	pH	Ref.
		48 Hr	96 Hr				
Antimony	DM	>530.0[a]		22	173	8	(LeBlanc, 1980)[†]
Antimony ($^{+3}$)	Juv. PM	15.5[a]	12.4[a]	20	Seawater	~6.5	(Takayanagi, 2001)*
Antimony ($^{+4}$)	Juv. PM	0.93[a]	0.93[a]	20	Seawater	8	(Takayanagi, 2001)*
Arsenic	Juv. DM	2.5[a]		21	Modified M4 Medium		(Tišler and Zagorc-Končan, 2002)*
		7.4[a]		18	45	7.7	(Biesinger and Christensen, 1972)*
	Juv. RT	23.2[a]	15.3[a]	12	250	8	(Tišler and Zagorc-Končan, 2002)*
Barium	Juv. DM	14.5[a]		18	45	7.7	(Biesinger and Christensen, 1972)*
	DM	410[a]		22	173	8	(LeBlanc, 1980)[†]
Beryllium	Juv. DM		0.4[a]	20	130	7.5	(Ewell et al., 1986)[†]
	DM	1.0[a]		22	173	8	(LeBlanc, 1980)[†]
	Juv. FHM		>100.0[a]	20	130	7.5	(Ewell et al., 1986)[†]
Cadmium	Juv. DM	0.03[a]		19	100		(Canton and Slooff, 1982)*
		0.046[a]		19		7.9	(Slooff et al., 1983)[†]
	DM	0.035[a]		20	29	7.8	(Nelson et al., 1984)[†]
	ZF	7.0[b]		20	170		(Canton and Slooff, 1982)*
	Juv. FHM	2.2[a]		20	140		(Slooff et al., 1983)[†]
	Juv. RT		<0.0005[a]	15	9.2	7	(Cusimano et al., 1986)*

			0.0025[b]	13	20	7.2	(Hollis et al., 2000)*
			0.018[b]	15	140	8	(Szebedinszky et al., 2001)*
		0.091[a]		15	20	7.2	(Calamari et al., 1980)*
		0.15[a]		15	285	7.5	(Slooff et al., 1983)[†]
		0.36[a]		15	80	7.2	(Calamari et al., 1980)*
	RT		0.022[b]	14	140	8	
Copper	Juv. DM	0.0098[a]		18	45	7.7	(Biesinger and Christensen, 1972)*
	DM	0.017[a]		20	250	7	(Park et al., 2009)[†]
	DP	0.011[a]		20	37	7.4	(Boeckman and Bidwell, 2006)*
	Larval FHM		0.078[b]	22		8.1	(Erickson et al., 1996)*
			0.11[a]	22		7.9	(Erickson et al., 1996)*
	FHM		0.075[b]	25	31	7	(Mount and Stephan, 1969)*
			0.43[a]	23	200	8	(Mount, 1968)*
			0.47[a]	22	~170		(Dwyer et al., 2005)[†]
	Juv. RT		0.0028[a]	15	9.2	7	(Cusimano et al., 1986)*
			0.1[b]	14	120	8	(Taylor et al., 2000)[†]
	RT		0.03[a]	15	30	9	(Howarth and Sprague, 1978)*
			0.031[a]	15	102	7	(Howarth and Sprague, 1978)*
			0.3[a]	15	361	7	(Howarth and Sprague, 1978)*
Iron ($^{+3}$)	Juv. DM	9.6[a]		18	45	7.7	(Biesinger and Christensen, 1972)*
Lead	Juv. DM	0.45[a]		18	45	7.7	(Biesinger and Christensen, 1972)*
	Juv. RT		1.0[a]	11	120	8	(Rogers et al., 2003)*
	RT		1.17[a]	11	28	7	(Davies et al., 1976)*
Mercury	Juv. DM	0.003[a]		20		7.9	(Canton and Adema, 1978)[†]
		0.005[a]		19		7.9	(Slooff et al., 1983)[†]
		0.005[a]		18	45	7.7	(Biesinger and Christensen, 1972)*
	Juv. FHM	0.05[a]		20	140		(Slooff et al., 1983)[†]
	FHM		0.17[a]	23.5	45	7.4	(Snarski and Olson, 1982)*
	Juv. RT	0.65[a]		15	285	7.5	(Slooff et al., 1983)[†]
Strontium	Juv. DM	125.0[a]		18	45	7.7	(Biesinger and Christensen, 1972)*
Thallium	Juv. DM	2.2[a]		22	173	8	(LeBlanc, 1980)[†]
Zinc	Juv. DM	0.1[a]		18	45	7.7	(Biesinger and Christensen, 1972)*
			0.75[a]	20	130	7.5	(Ewell et al., 1986)[†]

Juv. FHM	0.6[b]	25	46	7.5	(Benoit and Holcombe, 1978)*
	9.2[b]	23	203	7.7	(Brungs, 1969)*
	17.0[a]	20	130	7.5	(Ewell et al., 1986)[†]
Juv. RT	0.103[a]	10	10	5.8	(Alsop and Wood, 1999)*
	0.17[a]	15	31	7	(Bradley and Sprague, 1985)*
	0.869[a]	15	120	8	(Alsop et al., 1999)*
	4.46[a]	15	386	8	(Bradley and Sprague, 1985)*
RT	0.066[a]	15	9.2	7	(Cusimano et al., 1986)*

[a] Static/Semi-static Exposures

[b] Flow-Through Exposures

* Measured chemical concentrations used for LC_{50} calculations

[†]Nominal chemical concentrations used for LC_{50} calculations

DM – *Daphnia magna*

DP – *Daphnia pulex*

FHM – Fathead Minnow (*Pimephales promelas*)

RT – Rainbow Trout (*Oncorhynchus mykiss*)

ZF – Zebrafish (*Brachydanio rerio*)

PM – Red Seabream (*Pagrus major*)

Juv. – Juvenile-aged organisms used. DM = within 24 hrs. RT = ≤ 1-year post hatch.

1.3.3 Organic Chemicals

In this review, we have categorized organics from analyzed FPW samples into three different subgroups: polycyclic aromatic hydrocarbons, volatiles, and synthetics Although numerous other organics (and associated groupings) may be present in FPW, analyses performed to date have most readily and reliably identified these main groupings of organic compounds and, thus, will be the focus of discussion in this section.

1.3.3.1 Polycyclic Aromatic Hydrocarbon Toxicity

Polycyclic aromatic hydrocarbons (PAHs) have long been identified as a toxicological concern in aquatic environments, with numerous studies investigating the different pathways and processes that PAHs affect and how perturbation of these pathways

elicits toxic responses. Our analysis indicates that numerous PAHs exist in FPW samples (Table 1-4), often at concentrations near or above water quality guidelines for the protection of aquatic life (Table 1-4) and LC_{50} values for many model aquatic toxicological species (Table 1-7). As alluded to earlier, many of the PAH compounds present in FPW are petrogenic and derived from the formations being drilled into during horizontal fracturing activities (unless the process uses diesel fuel in the fracturing fluids; FracFocus, n.d.) (Neff et al., 2011; Orem et al., 2014; U.S. EPA, 2016). PAHs are generally classified into lower molecular weight (2–3 fused rings) and higher molecular weight (four and above fused rings) compounds with each subgroup having unique physiochemical characteristics associated with their toxicodynamics and toxicity potential. In aquatic systems, lower molecular weight molecules are associated with both the sediment and water matrices, whereas higher molecular weight PAHs generally associate (both physically and chemically) with the sediment (Chiou et al., 1998; Collier et al., 2013; Wang et al., 2001). Degradation via photooxidation and microbial communities occurs for both subcategories of PAHs, although generally, lower molecular weight PAHs have been demonstrated to degrade more rapidly than high molecular weight PAHs, meaning that higher molecular weight PAHs are more environmentally persistent (Bertilsson and Widenfalk, 2002; Juhasz and Naidu, 2000; Lima et al., 2005; Meador et al., 1995).

Although the presence of both low and high molecular weight PAHs in FPW is readily observable (Table 1-4), the toxicological consequences of these chemicals in FPW are less well studied. However, for many PAHs, average concentrations found in FPW are at or near LC_{50} values for certain aquatic species (Table 1-7). Furthermore, for all PAHs identified in FPW samples, the recorded upper-range concentrations are at or above LC_{50} values for almost all species studied. Accounting for the persistent nature of these chemical species, we conclude that a significant PAH hazard exists when FPW is released into an aquatic system.

PAHs are known to cause numerous effects in exposed organisms. A substantial component of the toxicity associated with PAHs results from both the PAHs and their metabolite intermediates created during organismal biotransformation. For example, intermediate PAH epoxides formed during initial phase I oxidation steps are highly reactive, and these epoxides nonenzymatically form toxic phenol derivatives (Buhler and

Williams, 1988; Livingstone, 1998). Susceptibility to PAH toxicity is highly species dependent, with developmental stage known to be a factor influencing PAH toxicity (James, 1989; Meador et al., 1995; Varanasi, 1989). When compared to vertebrates, invertebrates exhibit lower rates of both organic contaminant metabolism and elimination of contaminants. As a result, they tend to bioaccumulate metabolizable contaminants such as PAHs to a greater extent (James, 1989; Meador et al., 1995). Regardless, biomarkers of PAH biotransformation are still observed in invertebrate species, suggestive of some limited PAH metabolism capacity and potential for PAH intermediate-mediated toxicity (James, 1989; Meador et al., 1995; Varanasi, 1989). In fish, PAH exposure has been shown to induce hepatic neoplasia and lesions, and many PAHs are known hepatocarcinogens (Baumann et al., 1996; Johnson et al., 1993; Kelly et al., 1993; Pinkney and Harshbarger, 2006).

Many vertebrate and invertebrate studies have shown that PAHs negatively impact reproductive processes. In fish, PAHs are known to elicit endocrine disrupting effects, although the mechanisms behind these PAH effects are notably diverse and complex. One pathway by which PAHs disrupt the endocrine system is through interactions with the aryl hydrocarbon receptor (AhR) and downstream response elements (such as the dioxin response element and the estrogen response element, among others) that regulate endocrine processes (Navas and Segner, 2001; Villeneuve et al., 2002; Williams et al., 1998). In addition, PAHs interact with other elements responsible for the expression of several nuclear receptors, including retinoic acid receptors and retinoid X receptors (Bilbao et al., 2010; Cheshenko et al., 2008). Furthermore, PAHs have been demonstrated to influence cytochrome P450 aromatase activity and expression – a key enzyme involved in the conversion of androgens to estrogens (see reviews Cheshenko et al., 2008 and Le Page et al., 2011). PAHs may also influence fish neuronal endocrine processes by altering expression of monoaminergic neurotransmitters such as dopamine and serotonin (Gesto et al., 2009, 2006). These neurotransmitters are instrumental for proper homeostatic regulation of a wide range of physiological processes including reproduction (Rahman et al., 2011). PAH mechanisms of action on invertebrate reproduction are much less well characterized. However, a few studies on invertebrates have shown effects of PAHs on reproductive output and other whole organism markers used as indices for altered organism

development including hatching success rate, recruitment, and time to first reproductive event (Bellas and Thor, 2007; Eom et al., 2007; Feldmannová et al., 2006).

PAHs are also known to induce oxidative stress and oxidative stress responses in both invertebrates and vertebrate species following exposure (Dalton et al., 2002; Han et al., 2014; Sun et al., 2006; Valavanidis et al., 2006). Reactive oxygen species (ROS) are produced endogenously in cells, primarily through oxidative metabolism in the mitochondria. However, exposure to contaminants such as PAHs can upset the delicate balance between those processes generating ROS and those responsible for scavenging ROS. Monooxygenase metabolic processes during CYP1a induction following PAH exposure create numerous ROSs including superoxide anions and hydroxyl radicals which are known to deleteriously interact with a host of cellular components (Dalton et al., 2002; Zangar, 2004). Some of the more common mechanisms of ROS toxicity are the reaction with, and deterioration of, cell membranes (potentially creating more ROS and mutagenic metabolites), inhibitory impacts on proteins and enzymes, and damage of DNA (Dalton et al., 2002; Kappus, 1987; Köhler et al., 2002; Zangar, 2004). Compounding the oxidative stress problems associated with the presence of PAHs in aquatic settings is the photoactivation of PAH molecules in the presence of UV, a process which increases the reactivity and toxic potential of some PAHs (Lampi et al., 2006; Spehar et al., 1999; Yu, 2002).

A recent development in the understanding of PAH toxicity is the identification of cardiac defects in exposed fish. PAHs are associated with numerous morphological deformities in developing larval fish – including pericardial edema (Barron, 2004; Incardona et al., 2014, 2013, 2011). Further analyses have found that cardiac beat frequency, stroke volume, and stroke power are all affected in crude oil-exposed fish (Incardona et al., 2009; Khursigara et al., 2017; Nelson et al., 2016). It has been proposed that PAH exposure during embryonic stages does not cause morphological deformities per se, but rather the changes in cardiac physiology precede the noted morphological defects (Incardona et al., 2004). For example, following exposure to crude oil and to differing PAHs, Brette et al., (2014) determined that certain ionic currents vital for regulating proper myocyte action potential propagation and progression are altered. More specifically, these authors identified PAH-induced blockade of the K^+ currents responsible for cardiomyocyte

repolarization and disruption of Ca^{2+} cycling in the sarcolemma and sarcoplasm reticulum (Brette et al., 2014). These effects on cardiomyocytes change the contractile properties of myocyte sarcomeres, causing the changes in cardiac function observed in organisms following PAH exposure. Similarly, three-ringed phenanthrene has been shown to interact with specific voltage-gated ion channels instrumental for generating ionic currents required for cardiomyocyte action potentials (Brette et al., 2017), lending support to this hypothesis.

Table 1-7. Reported acute lethal toxicities (LC_{50} in mg/L) of polycyclic aromatic hydrocarbons associated with hydraulic fracturing flowback and produced water to aquatic species.

Chemical	Species	LC_{50}		Water Temp. (°C)	Hardness (mg/L $CaCO_3$)	pH	Ref.
		48 Hr	96 Hr				
Naphthalene	Juv. DM	2.16[a]		20	140	7.8	(Millemann et al., 1984)*
		24.1[a]		20	140	7.8	(Parkhurst et al., 1981)*
	DM	2.19[a‡]		20			(Muñoz and Tarazona, 1993)[†]
		8.6[a]		22	173	8	(LeBlanc, 1980)[†]
		22.6[a]		12	134	7.6	(Eastmond et al., 1984)[†]
	Juv. DP	4.66[a‡]		20	170	~7.5	(Smith et al., 1988)[†]
	DP		1.0[a]	15		7.5	(Trucco et al., 1983)*
		3.4[a]		20	43	7.2	(Geiger and Buikema Jr., 1982)*
	Juv. FHM		1.99[a]	20	140	7.8	(Millemann et al., 1984)*
	FHM		7.9[b]	15			(DeGraeve et al., 1982)*
	Juv. RT		0.11[b]	13.5	100	7.8	(Black et al., 1982)*
			1.6[b]	15			(DeGraeve et al., 1982)*
			4.5[a]	12	175		(Edsall, 1991)[†]
Acenaphthene	DM	1.28[a‡]		20			(Muñoz and Tarazona, 1993)[†]
		41.0[a]		22	173	8	(LeBlanc, 1980)[†]
	Larval FHM		0.61[b]	25	35	7.4	(Cairns and Nebeker, 1982)*
	Juv. FHM		1.6[b]	23	43	7.5	(Holcombe et al., 1983)*
	Juv. RT	1.13[b]	0.67[b]	12	46	7.3	(Holcombe et al., 1983)*

	Larval BT	0.65[b]	0.58[b]	12	46	7.3	(Holcombe et al., 1983)*
Fluorene	Juv. DP	0.21[a‡]		20	170	~7.5	(Smith et al., 1988)[†]
Phenanthrene	Juv. DM	0.7[a‡]		20	~165	~7.5	(Lampi et al., 2006)[†]
		0.7[a]		20	140	7.8	(Millemann et al., 1984)*
		1.0[a]		20	140	7.8	(Parkhurst et al., 1981)*
	DM	0.38[a‡]		20			(Muñoz and Tarazona, 1993)[†]
		0.84[a]		19	134	7.6	(Eastmond et al., 1984)[†]
	Juv. DP	0.35[a‡]		20	170	~7.5	(Smith et al., 1988)[†]
	DP		0.1[a]	15		~7.5	(Trucco et al., 1983)*
		1.1[a]		20	43	7.2	(Geiger and Buikema Jr., 1982)*
	Juv. RT		0.04[b]	13.5	100	7.8	(Black et al., 1982)*
			3.2[a]	12	175		(Edsall, 1991)[†]
Fluoranthene	Juv. DM	0.12[a]		22.5	~195	7.8	(Spehar et al., 1999)*
	DM	0.11[a]		20	80	~7	(Suedel and Rodgers Jr., 1996)*
		320.0[a]		22	173	8	(LeBlanc, 1980)[†]
	Larval FHM		0.009[b]	25	285	7.1	(Diamond et al., 1995)*
	Juv. FHM		>0.21[a]	22.5	~54	7.8	(Spehar et al., 1999)*
	Juv. RT		0.091[a]	17	~53	7.8	(Spehar et al., 1999)*
Benz[a]anthracene	Juv. DM	0.0015[a‡]		20	165	~7.5	(Lampi et al., 2006)[†]
	DP		0.01[a]	15		7.5	(Trucco et al., 1983)*
Benzo[a]pyrene	Juv. DM	0.0016[a†]		20	165	~7.5	(Lampi et al., 2006)[†]
	DP		0.005[a]	15		7.5	(Trucco et al., 1983)*

[a] Static/Semi-static Exposures

[b] Flow-Through Exposures

[‡]EC_{50} Measurement

* Measured chemical concentrations used for LC_{50} calculations

[†]Nominal chemical concentrations used for LC_{50} calculations

DM – *Daphnia magna*

DP – *Daphnia pulex*

FHM – Fathead Minnow (*Pimephales promelas*)

RT – Rainbow Trout (*Oncorhynchus mykiss*)

BT – Brown Trout (*Salmo trutta*)

Juv. – Juvenile-aged organisms used. DM = within 24 hrs. RT = ≤ 1-year post hatch.

1.3.3.2 Volatile Toxicity

Despite their high aqueous solubility and moderate Henry's law constant (a proportionality constant describing the ability of a gas to be dissolved within a liquid given the partial pressure of the gas in the liquid; Mackay and Shiu, 1981), volatile organic compounds (VOCs) have relatively low boiling points and high vapor pressures and therefore do not persist in the aquatic environment (Borden et al., 2002; Short, 2003). During crude oil releases, there is a systematic and rapid loss of smaller organic compounds, such as benzene, toluene, ethylbenzene, and xylene (collectively termed BTEX), along with their alkyl homologues and other smaller ringed PAHs as weathering increases (Mackay and McAuliffe, 1989; Roques et al., 1994). However, volatile chemicals have been shown to elicit potent acute toxicological effects in aquatic organisms (Mayer and Reichenberg, 2006; Neff et al., 2000) and deserve attention as potential chemicals of concern worth reviewing in the context of immediate hazards to organisms associated with surface water FPW contamination events. In this review, we identified the VOCs found in FPW to include differing chlorocarbons, benzene (and related benzene compounds), carbon tetrachloride, styrene, toluene, and xylene (Table 1-4). Of the above stated VOCs, most are above CEQ environmental water quality guidelines for the protection of aquatic life (Table 1-4). However, only toluene has been recorded at average concentrations in FPW above LC_{50} values for larval rainbow trout (Table 1-8). However, benzene concentrations have been found at or near rainbow trout LC_{50} values, while upper-range concentrations of toluene and xylenes (total) occur at or surpass LC_{50} values for almost all aquatic organisms analyzed in this review (Table 1-8). Despite the highly variable nature of FPW, concentrations found in FPW suggest VOCs are more likely to toxicologically impact aquatic organisms over acute exposure periods (pending on the turbidity and flow dynamics of the water systems FPW may be released into).

When exposed to water-accommodated fractions of gasoline containing high levels of BTEX, tested species of fish have displayed CYP1a induction and oxidative stress (Schein et al., 2009; Simonato et al., 2011; Stagg et al., 2000). Oxidative stress in these studies was measured in both liver and gill tissue, indicating that multiple organ systems in exposed fish are compromised following exposure to BTEX chemicals (Simonato et al., 2011). Monocyclic aromatic hydrocarbons, like BTEX, are thought to elicit most of their toxicity through induced nonpolar narcosis, a pathology characterized by the insertion of these chemicals into the membranes of cells and subsequent disruption of membrane homeostasis and integrity (Peterson, 1994; Ren, 2002). As a result, VOC exposures elicit many different physiological forms of toxicity in multiple cell, tissue, and organ systems. For example, in spermatozoa exposed to toluene, toxicity has been measured via the production of 7,8-dihydro-20-deoxyguanosine (8-oxodG), a marker of oxidative DNA damage (Nakai et al., 2003), while benzene has been shown to be a potent inducer of ROS including the superoxide radical anions, hydroperoxyl radicals, hydrogen peroxide (H2O2), and the highly reactive hydroxyl radicals (Ho and Witz, 1997; Wiemels and Smith, 1999; Winn, 2003). In nonaquatic species studies, these radicals then affect numerous other biological systems including, but not limited to, embryonic development (reviewed in Badham et al., 2010). Interestingly, in fish, BTEX components of crude oil have not been shown to have major discernible effects on embryonic development (Anderson et al., 2009; Jung et al., 2013; Marty et al., 1997). VOCs associated with FPW are also known to be both induced and metabolized by multiple different CYP monooxygenase systems, specifically the CYP2 family and to a lesser extent CYP1A (Nakajima, 1997; Stagg et al., 2000). Again, these enzymes may generate reactive oxygen species leading to increased oxidative stress (Dalton et al., 2002; Zangar, 2004).

BTEX effects on endocrine-related activities in aquatic species are relatively less well studied. In marine crabs, benzene delays molting, an effect likely mediated through endocrine disruption (Zou, 2005). In experimental and epidemiological studies of nonaquatic species, differing BTEX compound exposures have been reported to both stimulate hypothalamic pituitary adrenocortical activity and adrenocorticotropin release and decrease pituitary gonadotropin activity. These findings highlight the diverse nature of BTEX effects on the hypothalamic-pituitary system and suggest that further investigation

to elucidate mechanistic modes of action is required (Baccarelli et al., 2000; Verma and Rana, 2009).

In humans, male workers exposed to BTEX compounds exhibit reduced semen quality and vitality (Xiao et al., 2001). Repeated exposure to ethylbenzene and benzene has also been shown to induce cancerous tumors and lesions in the testes, respectively, while both toxicant exposures were associated with decreased sperm counts, lowered sperm mobility, and increased sperm DNA damage in male workers (Chan et al., 1998; Katukam et al., 2012; Ward et al., 1985). Toluene has also been shown to induce spermatozoa DNA damage and to play a role in reproductive toxicity (Nakai et al., 2003). In female human and rodent species, BTEX compounds are theorized to affect luteal functions of the ovaries, while benzene has been specifically identified as causing ovarian toxicity and carcinogenicity (Maronpot, 1987; Reutman et al., 2002). Therefore, despite the paucity of studies on the effects of VOCs on aquatic organism endocrine systems and reproductivity, the effects described above are likely to also manifest in aquatic organisms should levels reach effective concentrations. However, given the volatility of BTEX compounds, it is expected that for many of these endocrine disrupting and reproductive effects (among other toxicological end points) to manifest in aquatic organisms following FPW release, repeated or extended exposures would need to occur.

Table 1-8. Reported acute lethal toxicities (LC_{50} in mg/L) of volatile and synthetic organic chemicals associated with hydraulic fracturing flowback and produced water to aquatic species.

Chemical	Species	LC_{50}		Water Temp. (°C)	Hardness (mg/L $CaCO_3$)	pH	Ref.
		48 Hr	96 Hr				
1,1,1-Trichloroethane	DM	> 530[a]		22	173	8	(LeBlanc, 1980)†
1,1,2-Trichloroethane	DM	11.0[a]		22	~115	7.8	(U.S. EPA, 1978)*
		18.0[a]		22	173	8	(LeBlanc, 1980)†
		43.0[a]		20	Hard	8	(Adema and Vink, 1981)*
		186.0[a]		20	~54	~7.2	(U.S. EPA, 1984)*
	Juv. FHM	81.6[b]	81.6[b]	25	45	7.4	(U.S. EPA, 1984)*

			81.7[b]	25	56	7.5	(Veith et al., 1983)*
1,2-Dichloroethane	DM	220.0[a]		22	173	8	(LeBlanc, 1980)[†]
		268.0[a]		20	~54	~7.2	(U.S. EPA, 1984)*
	Juv. FHM	118.0[b]	116.0[b]	25	45	7.4	(U.S. EPA, 1984)*
			118.0[b]	25	56	7.5	(Veith et al., 1983)*
	Larval RT		~34.0[b]	13	104	8	(Black et al., 1982)*
1,2-Dichloropropane	Juv. DM	52.0[a]		22	173	8	(LeBlanc, 1980)[†]
	DM	52.5[a]		22	~115	7.8	(U.S. EPA, 1978)*
	Juv. FHM	154.0[b]	140.0[b]	25	45	7.4	(U.S. EPA, 1984)*
1,2,4-Trichlorobenzene	DM	2.09[a]		20	~54	~7.4	(U.S. EPA, 1984)*
		50.0[a]		22	173	8	(LeBlanc, 1980)[†]
	Juv. FHM		2.76[b]	25	45	7.4	(U.S. EPA, 1984)*
			2.9[b]	25	56	7.5	(Veith et al., 1983)*
	Juv. RT		1.52[b]	12	~54	~7.4	(U.S. EPA, 1984)*
Benzene	Juv. DM	400.0[a]		19		7.9	(Slooff et al., 1983)[†]
		426.0[a]		20		7.9	(Canton and Adema, 1978)[†]
	DM	200.0[a]		22	173	8	(LeBlanc, 1980)[†]
		682.0[a]		19	134	7.6	(Eastmond et al., 1984)[†]
	DP		15.0[a]	15		7.5	(Trucco et al., 1983)*
	Juv. FHM	32.0[a]	32.0[a]	25	360	8.2	(Pickering and Henderson, 1966)[†]
		35.1[a]	33.5[a]	25	20	7.5	(Pickering and Henderson, 1966)[†]
		84.0[a]		20	140		(Slooff et al., 1983)[†]
	FHM		~15.1[b]	15			(DeGraeve et al., 1982)*
	Larval RT		8.25[b]	13	94	7.8	(Black et al., 1982)*
	Juv. RT		5.3[b]	15			(DeGraeve et al., 1982)*
		56.0[a]		15	285	7.5	(Slooff et al., 1983)[†]
Carbon Tetrachloride	DM	10 – 100[a]		22	~115	7.8	(U.S. EPA, 1978)*
		35.0[a]		22	173	8	(LeBlanc, 1980)[†]
	Larval FHM		4.0[b]	20	96	7.8	(Black et al., 1982)*
	Larval RT		1.97[b]	13	104	7.9	(Black et al., 1982)*
Ethylbenzene	DM	75.0[a]		22	173	8	(LeBlanc, 1980)[†]
	Juv. FHM	42.3[a]	42.3[a]	25	360	8.2	(Pickering and Henderson, 1966)[†]

		48.5[a]	48.5[a]	25	20	7.5	(Pickering and Henderson, 1966)[†]
Styrene	DM	23.0[a]		22	173	8	(LeBlanc, 1980)[†]
	Juv. FHM	53.6[a]	46.4[a]	25	20	7.5	(Pickering and Henderson, 1966)[†]
		62.8[a]	59.3[a]	25	360	8.2	(Pickering and Henderson, 1966)[†]
Toluene	Juv. DM	19.6[a‡]		20	26	~7.9	(Pearson et al., 1979)[†]
	DM	310.0[a]		22	173	8	(LeBlanc, 1980)[†]
	Juv. FHM		12.6[b]	20	26	~7.9	(Pearson et al., 1979)[†]
		46.3[a]	34.3[a]	25	20	7.5	(Pickering and Henderson, 1966)[†]
		56.0[a]	42.3[a]	25	360	8.2	(Pickering and Henderson, 1966)[†]
	Larval RT		0.02[b]	14	106	7.8	(Black et al., 1982)*
	Juv. RT		24.0	12			(Johnson and Finley, 1980)[†]
Xylene(s)	Juv. FHM	27.7[a]	27.7[a]	25	20	7.5	(Pickering and Henderson, 1966)[†]
		28.8[a]	28.8[a]	25	360	8.2	(Pickering and Henderson, 1966)[†]
	Larval RT		3.77[b]	13	96	7.8	(Black et al., 1982)*
	Juv. RT		8.2	12			(Johnson and Finley, 1980)[†]
Dibromochloromethane	Juv. DM	58.1[a]	46.8[a]	25	Hard	~7.8	(Fisher et al., 2014)*
Hexachlorobenzene	FHM		22.0	20			(Johnson and Finley, 1980)[†]
	RT	< 0.03[a]		15	320	7.4	(Calamari et al., 1983)*
	ZF	< 0.03[a]		23	320	7.4	(Calamari et al., 1983)*
Pentachlorophenol	Juv. DM	0.28[a]		19	100		(Hermens et al., 1984)*
			0.32[a]	20	130	7.5	(Ewell et al., 1986)[†]
		0.48[a]		20		7.9	(Canton and Adema, 1978)[†]
		0.48[a]		19		7.9	(Slooff et al., 1983)[†]
		0.6[a]		20		7.9	(Adema, 1978)[†]
		1.05[a]	0.8[a]	20	Hard	8	(Adema and Vink, 1981)*
	DM	0.68[a]		22	173	8	(LeBlanc, 1980)[†]
		1.4[a]	0.68[a]	20	Hard	8	(Adema and Vink, 1981)*
	Juv. FHM	0.21[a]		20	140		(Slooff et al., 1983)[†]
			0.32[a]	20	130	7.5	(Ewell et al., 1986)[†]

	7.9[b]	0.23[b]	25	~46	~7.9	(Phipps et al., 1981)*
FHM		0.25[a]	22	~ 170		(Dwyer et al., 2005)[†]
Juv. RT	0.2[a]		15	285	7.5	(Slooff et al., 1983)[†]
	0.093		11	45	7.6	(McKim et al., 1987)*

[a] Static/Semi-static Exposures

[b] Flow-Through Exposures

* Measured chemical concentrations used for LC_{50} calculations

[†]Nominal chemical concentrations used for LC_{50} calculations

[‡]EC_{50} Measurement

DM – *Daphnia magna*

DP – *Daphnia pulex*

FHM – Fathead Minnow (*Pimephales promelas*)

RT – Rainbow Trout (*Oncorhynchus mykiss*)

ZF – Zebrafish (*Brachydanio rerio*)

Juv. – Juvenile-aged organisms used. DM = within 24 hrs. RT = ≤ 1-year post hatch.

1.3.3.3 Synthetic Compound Toxicity

Our analysis of FPW chemical composition literature has identified that organochlorines are the most characteristic and consistently present synthetic organic compounds in FPW. These compounds include the trihalomethane compound dibromochloromethane (DBCM) and the chlorobenzene compounds hexachlorobenzene (HCB) and pentachlorophenol (PCP). HCB and PCP are typically employed as pesticides, whereas DBCM is a disinfection by-product from the reaction of chlorine with organic matter and bromide ions but has also been used as a solvent and flame retardant (Bailey, 2001; Cirelli, 1978; Munson et al., 1982). Although we have classified DBCM as "synthetic," it should be noted that DBCM may be produced in small quantities by certain species of marine algae (Itoh and Shinya, 1994), and thus, natural production of the compound may occur. As their anthropogenic origin suggests, these synthetic compounds found in FPW are most probably components of the original fracturing fluids injected into the well during the production stage of drilling practices. Of these three synthetic species (HCB, PCP, and DBCM), average concentrations are below concentrations expected to

induce significant lethality to aquatic biota (Tables 1-4 and 1-8). However, HCB upper-range FPW concentrations are found to correspond to LC_{50} values for rainbow trout and zebrafish, while PCP upper-range FPW concentrations are found at or above LC_{50} values for most aquatic model species examined (Table 1-8). While limited CEQ and USEPA water quality guideline data exists for DCBM and HCB, PCP concentrations in FPW were found to be above guideline concentrations (Table 1-4). The toxicity of HCB and PCP are likely driven to a significant extent by their relatively high octanol-water partitioning coefficients (KOWs) and corresponding potential for bioaccumulation (Chamberlain et al., 1996; Sabljić et al., 1995). Thus, a synthetic organic compound toxicological hazard to organisms will likely exist if spills or releases of FPW to surface waters occur.

HCB and PCP toxicity to aquatic organisms has been studied for many years (Goodnight, 1942; Johnson et al., 1974). However, only a few sublethal studies pertaining to endocrine system function and reproduction in aquatic species have been performed. In the Chinese rare minnow (*Gobiocypris rarus*), PCP exposure invokes upregulation of certain EDC-related genes (e.g., estrogen receptor alpha and beta, androgen receptor, and vitellogenin) in male hepatic and gonadal tissues. PCP also affects the hypothalamic-pituitary-gonadal/interrenal (HPG/I) axis, resulting in changes to plasma steroid hormone levels and spermatogenesis rates. In female rare minnows, PCP negatively impacted the HPG/I axis and increased estradiol and testosterone plasma concentrations while decreasing cortisol levels. Correspondingly, degenerate ovaries were also characterized (Yang et al., 2017). Changes in vitellogenin in PCP-exposed male Japanese medaka (*Oryzias latipes*) have also been observed, in association with decreased fecundity, fertility, and gonadal health in both male and female fish (Zha et al., 2006). Similarly, serum testosterone levels significantly increased in crucian carp (*Carassius carassius*) following sublethal PCP exposure over 7 and 15 days (Zhang et al., 2008), and detrimental effects to gonad growth and development have been observed in multiple other freshwater fish species (Hanson et al., 2007). In vitro studies have also shown that PCP exhibits estrogenic, antiestrogenic, antiandrogenic, and antagonistic retinoid X receptor effects in a variety of tissue and cell types (Jung et al., 2004; Li et al., 1997; Orton et al., 2009; Suzuki et al., 2001).

In nonaquatic species, HCB has been found to negatively affect steroidogenesis in monkey ovarian tissue, possibly due to peroxidation of the mitochondrial lipid membrane (Foster, 1995). In porcine ovarian follicle tissue cultures, HCB inhibits both testosterone and estrogen secretion and reduces expression of key steroidogenic enzymes, such as CYP17, CYP17β-hydroxysteroid dehydrogenase, and CYP19 (Gregoraszczuk et al., 2011). Furthermore, an interplay between HCB and the estrogen receptor α (ERα) has been reported. Following exposure in healthy rats, HCB increases ERα protein expression and 17-β estradiol and prolactin levels and reduces progesterone, follicle-stimulating hormone, and luteotrophic hormone concentrations in mammary tissues (Peña et al., 2012). Interestingly, in vitro studies have also shown that breast cell proliferation and subsequent tumor formation following HCB exposure are ERα dependent, further exemplifying the interplay between HCB and the ERα (García et al., 2010).

Similar to VOCs, nonpolar narcosis is one theorized mode of toxicity associated with HCB and PCP exposure in aquatic organisms (Call et al., 1985; Ren, 2002). These narcotic effects may result in a myriad of impacts to biological systems due to their deleterious effects on cell lipid membranes and lipid membrane functions. PCP is also a known oxidative phosphorylation uncoupler and causes mitochondrial membrane damage (Weinbach, 1954; Weinbach and Garbus, 1969). Accordingly, PCP (and its metabolite tetrachlorohydroquinone) has been shown to induce ROS in rainbow trout hepatocyte cell lines while also causing lipid peroxidation, DNA damage, and ROS induction in zebrafish larvae (*Danio rerio*) (Fang et al., 2015; Pietsch et al., 2014). Similarly, HCB has been shown to cause oxidative stress in common carp (*Cyprinus carpio*), where brain tissue in exposed organisms displayed elevated levels of ROS, thiobarbituric acid reactive substances (indicative of lipid peroxidation), and nitric oxide synthase (NOS) activity (Song et al., 2006). While PCP has not been conclusively shown to bind the AhR, HCB has been shown to weakly associate with the AhR, and both PCP and HCB are shown to induce CYP1A and ethoxyresorufin-O-deethylase (a marker for CYP1A induction) activity in a variety of organisms and tissue/cell models (Mundy et al., 2012, 2010; Starek-Świechowicz et al., 2017; Zha et al., 2006; Zhang et al., 2008). Again, induction of these monooxygenase detoxification pathways may contribute to the overall increased oxidative stress observed in organisms exposed to these two chemicals.

1.3.4 FPW-Related Toxicity

To date, very few studies have examined the toxicity of raw FPW samples. Some groups have taken characterized components of FPW and reconstituted them into mixtures in an attempt to simulate FPW from a representative well (Boulé et al., 2018; Kassotis et al., 2018b). Other studies have sampled for water affected by UOG operations and tested these waters for adverse effects on organisms (Tasker et al., 2018). However, the highly complex nature of FPW fluids may render studies examining reconstituted solutions or waters affected by UOG activities less practical for accurately estimating full toxicological effects and determining mechanistic modes of toxicity. Under laboratory settings, whole effluent toxicity (WET) studies are better able to control for environmental variables, determine accurate dose effects, and observe real-time events leading to toxicity in exposed organisms (Chapman, 2000). Examples of where such WET studies have significantly contributed to the understanding of specific detrimental environmental incidents are studies pertaining to oil spills, such as the Exxon Valdez and the Deepwater Horizon releases (Alloy et al., 2016; Heintz et al., 1999). Because of such WET studies, our understanding of crude oil impacts on multiple levels of biological organization (cell, tissue, organs, and whole organism) has significantly advanced, and risk assessment and adverse outcome pathway analyses now exist should future spills of crude oil occur (Ankley et al., 2010; Incardona and Scholz, 2016; Short, 2003). Thus, investigation on whole, raw FPW samples is required to gain a better understanding of the toxicological impacts to the biota when releases of FPW to the environment occur.

Studies which utilize either simulated or field-collected UOG-affected samples may, however, offer insight into possible pathways of toxicity which are induced when FPW exposure to organisms occurs. In a study using groundwater samples collected from regions experiencing horizontal hydraulic fracturing activities in Wyoming, USA, greater ER antagonist activity and increased progesterone receptor antagonist activity were exhibited using a human endometrial cell gene reporter assay (Kassotis et al., 2018b). These groundwater samples contained organic contaminants, such as 2-ethylhexanol and styrene, which were suggested to be responsible for the bioactivity observed. Supporting

the notion that development and endocrine activity may be impacted following exposure to FPW, a review performed by Webb et al., (2014) identified other compounds in FPW which may cause sublethal developmental and endocrine disruption effects.

Other endpoints associated with exposure to FPW include markers of xenobiotic metabolism and an altered immune system. Using surface samples of FPW collected following spreading on roads for de-icing or dust suppression purposes, increased bioassay activity for both AhR and pregnane X receptor (PXR) activity was observed in both daphnid and in reporter cell lines (Tasker et al., 2018). Similarly, using a mixture of 23 chemicals associated with UOG operations, developmental exposures in mice were found to significantly alter sex-specific immunological responses (e.g., T cell-dependent allergic airway disease responses and influenza A virus infection leukocyte recruitment) and intensify autoimmune encephalitis responses in exposures as low as 0.1 and 1.0 μg/mL dosages (Boulé et al., 2018). Furthermore, some of these effects were observed to persist weeks post-exposure. A similar simulated FPW mixture consisting of 23 distinct FPW-related chemicals was used to expose *Xenopus laevis* tadpoles which had contracted the ranavirus FV3. Mixture exposures of <5.0 μg/L significantly affected tadpole homeostatic myeloid lineage gene expression and increased viral load (Robert et al., 2018). Changes in the key immunological genes, TNF-α, IL-1β, and Type I IFN, were also observed. These findings suggest that FPW-associated contaminants may acutely disrupt immune function in organisms exposed at low doses.

Another pathway demonstrated to be affected following exposure to simulated FPW is metabolic function. Adipose tissue function in mice was examined using a 23-compound simulated mixture of chemicals associated with UOG operations and a small subset of surface water samples contaminated with UOG wastewater from Colorado and West Virginia (Kassotis et al., 2018a). These researchers showed that adipogenic activity (both triglyceride accumulation and pre-adipocyte proliferation) in a mouse adipose cell line was potently induced following exposure. Furthermore, exposure to contaminated surface water samples resulted in increased peroxisome proliferator-activated receptor gamma activity (a receptor critical for regulating fatty acid storage and glucose metabolism) in a human embryonal kidney cell line reporter assay (Kassotis et al., 2018a).

1.4 PERSPECTIVES AND OBJECTIVES OF THE THESIS

Given the paucity of actual FPW toxicological studies, and the estimated rise of future UOG activities and hydraulic fracturing processes worldwide, it is imperative that more directed efforts are made into better understanding the environmental hazards associated with complex FPW waste solutions. Within the context of biological systems, the intimate relationship fracturing activities (and subsequently produced FPW) have with aquatic environments located in and around regions experiencing such industrial stresses renders investigations of how FPW releases and exposures affect aquatic organisms vital. Understanding which physiological processes are affected by FPW exposure, and how they are being affected, is a natural and logical first step in addressing environmental concerns associated with FPW waste management.

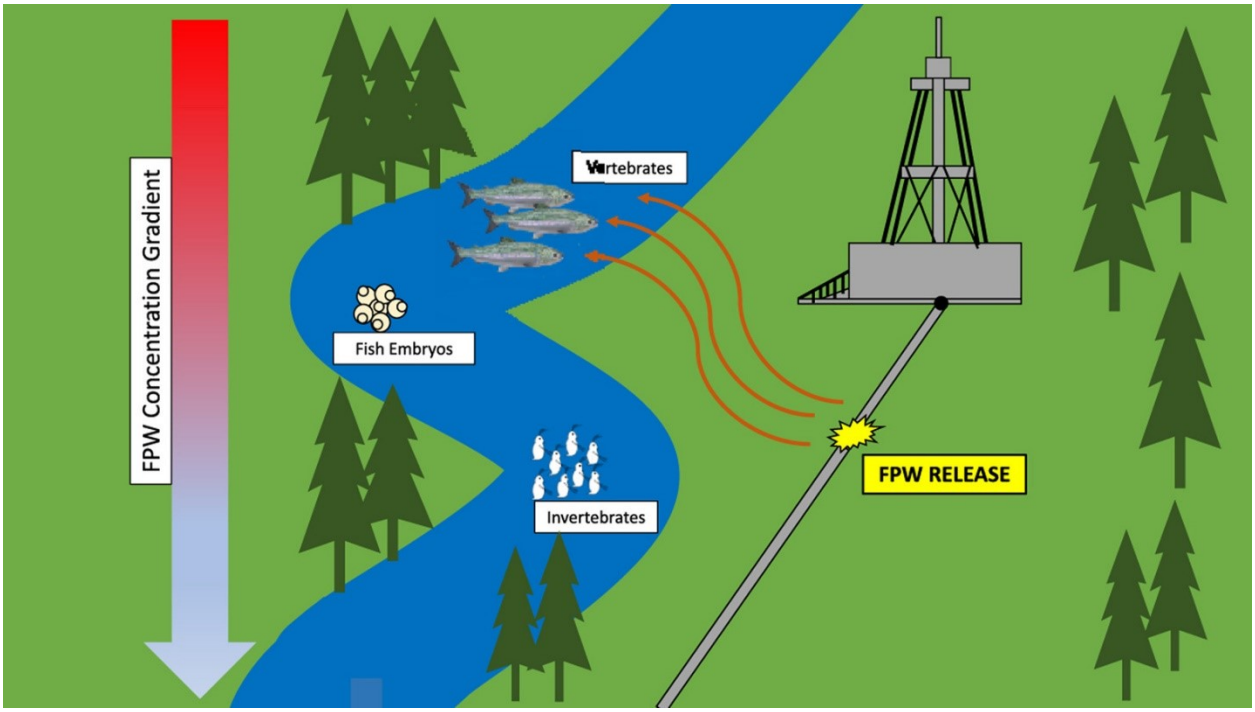
The objectives of this thesis were to attempt to address some of these FPW toxicological knowledge gaps by investigating responses in numerous aquatic species and using multiple levels of biological organization to tease apart specific physiological processes implicated in sub-lethal FPW toxicity. By using this multi-perspective approach to address such a vast and intricately complex topic, our understanding of how FPW exposure elicits its toxicological effects on aquatic organisms has been greatly enhanced. Chapter 2 examines baseline toxicity of a temporally differing set of FPW samples from a single horizontal hydraulically fractured well in a suite of freshwater toxicological model species. Goals of this study were to establish baseline toxicity associated to FPW exposure and comparatively determine differing toxicological sensitivities amongst species. Furthermore, chemical characterization of the temporally different FPW samples allowed us to gain more insight into the potential toxicological influence and impact of certain chemical constituents. To begin assessing physiological processes affected and detoxification mechanisms induced from FPW exposure, Chapters 3 examines toxicological responses in rainbow trout acutely exposed to sub-lethal dilutions of FPW, with a particular focus on phase I and II biotransformation processes, oxidation stress responses, and potential endocrine disruption implicated effects. Chapter 4 continues with the theme of investigating FPW biotransformative process in acutely exposed trout, but with an overall goal of determining if organismal nutrient handling and energetics are impacted by exposure. This involved analyzing the activity and gene expression of multiple

key liver enzymes involved in energy substrate catabolism/anabolism pathways and investigating changes to hepatocyte glucose and alanine flux rates. Recovery capacities were also studied to determine if acutely exposed trout were able to return to pre-FPW exposure energetic physiological conditions following a 3-week period of recovery.

Chapters 5, 6, 7, and 8 begin to centralize on investigating the interplay of FPW exposure and cardio-respiratory responses in exposed fish. Chapter 5 examines how acute, sub-lethal FPW exposures during zebrafish embryonic development impact rates of developmental deformities at hatch, embryonic expression of key cardiac development and functional genes, and metabolic rates during embryonic and larval states. Chapter 6 then examines if these same exposures employed in Chapter 5 have lasting, persistent effects on cardio-respiratory parameters by analyzing changes to juvenile zebrafish swimming performance and aerobic capacity following embryonic FPW exposures. Components of Chapters 5 and 6 are mirrored in Chapter 7, except cardio-respiratory impacts are analyzed in the more ecologically relevant species rainbow trout. Juvenile fish heart morphometrics are also examined post rainbow trout embryonic FPW exposures to determine if ventricular-related structural alterations are implicated in observed changes to juvenile swimming performance and aerobic capacity. Chapter 8 then analyzes impacts at the cellular level by investigating changes to cardiomyocyte sarcomere contractile properties during FPW exposure and relating this to swimming performance and aerobic capacity in the marine pelagic fish *mahi mahi* (*Coryphaena hippurus*).

Collectively, this complementary grouping of research is summarized in Chapter 9, wherein I reiterate major findings and suggest future avenues of research which may lead to improvements to not only the understanding of cardio-respiratory focused effects of FPW exposure in organisms, but to the overall understanding of FPW mediated toxicity in aquatic organisms. Implications of this suggested future work may hold great promise for FPW management endeavours and regulatory hazard assessment and risk identification policies in regions experiencing UOG activities.

CHAPTER 2: Toxicity in Aquatic Model Species Exposed to a Temporal Series of Three Different Flowback and Produced Water Samples Collected from a Horizontal Hydraulically Fractured Well



Folkerts, E.J., Blewett, T.A., Delompré, P., Mehler, W.T., Flynn, S.L., Sun, C., Zhang, Y., Martin, J.W., Alessi, D.S. and Goss, G.G. (2019). Toxicity in aquatic model species exposed to a temporal series of three different flowback and produced water samples

collected from a horizontal hydraulically fractured well. *Ecotoxicology and Environmental Safety*, **180**, 600 – 609. DOI: 10.1016/j.ecoenv.2019.05.054

2.1 INTRODUCTION

Technological advancements and development in the oil and gas sectors have greatly increased our ability to access world-wide geological reserves of oil and gas previously thought to be unattainable. Implementation of unconventional oil and gas (UOG) recovery processes, in the form of horizontal hydraulic fracturing, is largely responsible for increased production of these resources, and is only expected to become more commonly utilized by industry (Vengosh et al., 2014; Gagnon et al., 2016). UOG operations have significantly expanded in the last decade due to advances in horizontal drilling and completions technology. However, research on environmental impacts of accidental releases is still limited and many gaps in knowledge exist related to the environmental hazards associated to horizontal hydraulic fracturing activities. One such gap pertains to flowback and produced water (FPW), the wastewater by-product that returns to the surface following hydraulic fracturing activities. Although terminology of "flowback" versus "produced water" is subject to operator discretion, FPW collectively is a highly complex mixture composed of original fracturing fluids, compounds from the geological formation itself, saline constituents derived from deep, groundwater reservoirs, and potential daughter compounds from the transformation of initial fracturing fluid or formation constituents in the high pressure-elevated heat environments of the well-bore-formation interface (DiGiulio and Jackson, 2016; He et al., 2017a; Hoelzer et al., 2016; Lester et al., 2015).

One of the most apparent and immediate hazards posed to the environment from UOG activities and FPW production are surface water contamination events following spills/releases of FPW. In the U.S. alone, 210 billion US gallons (7.95×10^8 m^3) of FPW was produced from 2005 to 2014 (Kondash and Vengosh, 2015). This is especially concerning as a recent study of the Marcellus and Fayetteville shale formations of the eastern U.S. found that on average, wells drilled for hydraulic fracturing purposes were within ~300 m of a freshwater surface body of water, such as streams or rivers (Entrekin et al., 2011). Although not as developed as its American counterparts, Canadian UOG development has also been increasing, resulting in larger volumes of FPW produced and

increased frequencies of accidental releases to the environment (Alessi et al., 2017; Johnson and Johnson, 2012). Thus, understanding effects of FPW releases on the aquatic environment has become essential for government and industry mandates such as spill assessment, clean-up, and remediation protocols. As a result of the paucity of FPW toxicological-related research and high variation in spill mitigation requirements among states and provinces, releases of FPW to surface bodies of water have not historically been considered one of the greatest drivers of FPW environmental risk (Kulander, 2013; Richardson et al., 2013). Furthermore, both Canada and the U.S. do not consider FPW as hazardous waste (Goss et al., 2015). Combining this lack of classification with the decentralized and regionally governed management nature of FPW (Ralston and Kalmbach, 2018), policies and mandates dictating FPW spill and remediation strategies are often variable and may not adequately address the hazards posed to the environment. Thus, more conclusive and thorough FPW guideline development among government and industry is a pressing concern. However, to combat the increased risk of FPW environmental impacts, industry operations more commonly implement greater mitigative efforts to assess and reduce risk at the multiple stages of well operations (e.g. pre-development, operational, and well shut-down) by utilization of environmental health and safety regulatory matrices, more structured and transparent spill reporting and clean-up measures, and more thorough reclamation practices. In Alberta, Canada, this has resulted in a distinct reduction over time in number of reported spills per well (Alberta Energy Regulator; AER, n.d.) and we assume similar reductions have occurred in other jurisdictions.

Recently, efforts to understand the impact of FPW on aquatic organisms have found that the primary sub-lethal effects observed in organisms following FPW exposure are oxidative and biotransformative stress, negative reproductive impacts, and deleterious effects on the cardio-respiratory system (T. A. Blewett et al., 2017a, 2017b; Folkerts et al., 2017a, 2017b; He et al., 2017b). There is also evidence that endocrine disruptive effects may additionally be induced in organisms exposed to FPW (He et al., 2018a, 2018b). However, references providing strict lethality (measured via varying lethal concentration analyses) toxicological information of FPW on freshwater organisms, as required for guideline development, are still largely unavailable.

One of the complications involved with performing any toxicological study related to FPW is the highly complex and chemically diverse nature of the wastewater (Flynn et al., 2019). Compounding this complexity, the chemical makeup of FPW is highly variable depending on the formation being exploited, initial fracturing fluids used, and well operation characteristics (e.g. temperature, pressure, shut-in times, period of flowback) (S. Kim et al., 2016). Thus, an appreciable amount of work is required to obtain any toxicological knowledge of this wastewater, or of the hazards it poses to the environment. To begin addressing if there are significant toxicological concerns in a legislative and policy-driven context, an evaluation of FPW using standard based whole effluent toxicity protocols (termed WET tests) (Chapman, 2000) will provide information about whole-mixture effects on the environment and results subsequently may be included in government and industry protocols and policies.

In the present study, acute FPW toxicity was measured in four different common toxicological model species using samples of FPW collected at different time points during production from a horizontally fractured well in the Duvernay shale play in central Alberta, Canada. These different time points of sampling cover three operational shifts during the fracturing resource recovery process and FPW produced from these operational phases are hypothesized to contain differing chemical profiles and toxicological potentials, with an increased saline and ion presence predicted in later FPW samples. Early FPW from a producing well is theorized to contain a greater number of initial fracturing additives and may be more toxic to aquatic organisms following exposure. However, FPW samples extending into the production phase of a well (> 120 h post well production, as observed in previous studies and those preliminarily made by our group) (Goss et al., 2015; Zolfaghari, 2014) tend to increase drastically in saline content. Among the freshwater aquatic organisms tested, less saline tolerant species (e.g. *Daphnia* and zebrafish) are hypothesized to have greatest sensitivity to FPW, although inter-species responses to FPW exposure are expected to be greatly varied considering the split between invertebrate and vertebrate species and varying lifestyles amongst species tested. Our analyses indicate that temporal variation in FPW chemical characteristics - inorganics, non-targeted organics, and polycyclic aromatic hydrocarbons (PAHs) - can significantly alter toxicity to organisms, and that there is great inter-species variability in lethality responses to FPW. Our chemical

characterization and toxicological results further the notion of FPW complexity and serve as a reminder that species-specific differences should be considered for understanding the implications of accidental FPW releases to the environment.

2.2 MATERIALS AND METHODS

2.2.1 FPW Samples and Animal Maintenance

FPW samples were collected from a single horizontal hydraulically fractured well post-stimulation. Three timepoints for FPW collection (and subsequent analysis) were chosen: a 1 h, 20 min sample (1.33 h), a 3-day sample (72 h), and a 9-day, 12 h sample (228 h) (see Table 2-1). A saline solution matching the major cation and anion concentrations of the most saline FPW sample (228 h) was also created to control for saline-induced responses (see Table S2-1 for ionic composition information).

A total of four model test species were chosen for toxicological experimentation, including two invertebrate and two vertebrate species. Our two chosen invertebrate species included the freshwater cladoceran, *Daphnia magna* (neonates), and the oligochaete *Lumbriculus variegatus*. Our two vertebrate species were zebrafish (*Danio rerio*; embryos) and rainbow trout (*Oncorhynchus mykiss*; embryos and juveniles). All invertebrate species were cultured and maintained in dechlorinated City of Edmonton (Edmonton, AB, CAN) tap water (moderately hard: $[Na^+]$=14.6 mg/L, $[Ca^{2+}]$=55.9 mg/L, $[Mg^{2+}]$=15.3 mg/L, $[K^+]$=2.5 mg/L, pH: 7.9, conductivity: 395 ± 0.5 µS/cm, dissolved oxygen: 7.5 ± 0.5 mg/L, general hardness: 186 mg/L as $CaCO_3$, salinity: 0 ppt) at 20 ± 1 °C. Zebrafish embryos (wildtype strain AB) were collected from adult fish housed in 30 L tanks (~25 fish/tank, 14 h light: 10 h dark photoperiod) in the University of Alberta zebrafish aquaculture facility filled with zebrafish facility water (pH: 7.4, conductivity: 553 µS/cm, temperature: 28.5 ± 1 °C, dissolved oxygen: 7.3 mg/L, hardness: 174 mg/L as $CaCO_3$, salinity: 0 ppt) treated by a RiOs 100 (reverse osmosis) water purification system. Diploid rainbow trout embryos were collected from the Raven Creek Trout Brood Station (Caroline, AB, CAN), brought to the University of Alberta, and maintained at 10 °C in heath-tray stacks connected to a recirculating water chiller apparatus containing 150 L of dechlorinated City of Edmonton tap water filtered and treated by a bioball purification layer (to treat for nitrogenous waste products) and ultra-violet light sterilization. Recirculating water for rainbow trout embryos

was constantly aerated and maintained at dissolved oxygen levels of 8.1 ± 0.3 mg/L. Periodic water top-ups to the heath-tray-recirculation system were additionally performed. Juvenile rainbow trout (2.38 ± 0.15 g) were maintained in aerated flow-through 400 L tanks filled with dechlorinated City of Edmonton tap water at 10 ± 1 °C. All animal use was approved by the University of Alberta Animal Care Committee under protocols AUP00001334 and AUP00002352. All previously stated variation was calculated to one standard deviation.

2.2.2 Inorganic FPW Characterization

2.2.2.1 Inductively coupled plasma (ICP) -MS/MS analysis

ICP-MS/MS analysis was used to quantify the concentration of cations, bromide, and total sulfur (Figure 2-1 and Table S2-1). Before analysis all samples were filtered through a 0.2 µm nylon filter membrane and diluted by a factor of 850 for Na analysis and 85 for all other elements with 18MΩ cm ultrapure water. Diluted 10 ml samples were then acidified with 12 µL using 15.698 normal trace metal grade nitric acid. For analysis, an Agilent 8800 Triple Quadrupole ICP-MS (ICP-QQQ) was used with a RF power of 1550 W, a RF reflected power of 18 W, a microMist nebulizer and nickel/copper cones. Samples and external standards were analyzed in high matrix mode, in which samples were diluted inline with 8 mL/min argon. Additionally, analysis was performed in MS/MS mode for greater mass resolution and a collision gas reaction cell was used with He gas (3 mL/min), O_2 gas (10% maximum flow) or H_2 (5 mL/min) gas to overcome matrix interferences (Table S2-2.). Instrumental drift was accounted for using 0.5 ppm solution of indium, which was added to each sample using an inline internal standard addition system. For quality assurance and control, a standard solution was run at the start, middle, and end of each run (every 10 sample for n=3) to quantify the run precision and percent recovery for each element (Table S2-2).

2.2.2.2 Anion, total organic carbon and total nitrogen analysis

Ion chromatography was used to determine the concentration of chloride (Figure 2-1 and Table S2-1). Before analysis, samples were filtered through a 0.2 µm nylon filter membrane and diluted by a factor of 2000 using 18MΩ cm ultrapure water. Analysis was

then performed on a Dionex Ion chromatography DX 600 with a 4mm analytical column (AS9-HC), guard column (AG9-HC), and a 4mm ASRS Ultra suppressor. For total non-purgeable organic carbon (TOC) and total nitrogen (TN) a subsample of the filter sample was diluted by a factor of five and analyzed using on a TOC analyser (Shimadzu model TOC-V-CHS/CSN). For quality assurance and control for IC a control standard solution was run every five samples (n=4) to quantify the run precision and percent recovery for each element (Table S2-3).

2.2.3 Organic FPW Characterization

2.2.3.1 Extraction of organics

For each FPW sample, 150 mL of FPW was vacuum filtered through a glass fiber membrane (90mm diameter, pore size: 0.4 μm), then subsequently freeze-dried for 48 h. Approximately 50 mL of the aqueous filtrate was then liquid-liquid extracted using a repeated process of 20 mL of dichloromethane (DCM) and shaking for 3 min twice. The combined extracts (40 mL) for each sample were concentrated by nitrogen gas evaporation and reconstituted in 1 mL methanol for High Performance Liquid Chromatography/Orbitrap Mass Spectrophotometry (HPLC/Orbitrap-MS) analysis. The internal standard mix (10 ng of each, Wellington Laboratories, ON, CAN) was spiked into the remaining (unextracted) 100 mL of aqueous filtrate, extracted and evaporated as described above, and reconstituted in 3 mL hexane for further cleanup. Accelerated solvent extraction (ASE) was used for PAH extraction from particulates in the freeze-dried glass filters sediments. A different, separate glass fiber filter was added at the bottom of the extraction cell, and ~ 2–3 g of florisil (pre-cleaned by DCM) was added, followed by dried sediment filters, to which the internal standard mix was then spiked (10 ng of each). ASE cells were filled with solvent (hexane/DCM 4:1 v:v; Optima™, Fisher Scientific, NH, USA), pressurized to 14 MPa, and heated to 80 °C within 6 min. Pressure and temperature were held for 5 min (static extraction), followed by rinsing with cold solvent (50% of the cell volume) and purging with nitrogen gas for 90 s. This extraction cycle was repeated once more. Approximately 40 mL of total extract was gently concentrated by nitrogen gas evaporation and reconstituted in 3 mL of hexane for further cleanup.

2.2.3.2 High Performance Liquid Chromatography/Orbitrap Mass Spectrometry

Spectrophotometry (HPLC/Orbitrap - MS) Dissolved organic compounds in FPW aqueous filtrate extracts (section 2.2.3.1) were analyzed via HPLC/Orbitrap-MS. Briefly, 10 µL of diluted organic extract (equivalent to 50 µL of the respective original FPW sample) was used for targeted organic compound identification and semi-quantification using reverse phase LC-Orbitrap-MS. Orbitrap MS was operated in positive electrospray ionization mode, and acquisition was in full scan mode (m/z 100 to 2000) at 2.3 Hz, with resolving power set to 120,000 at m/z 400. Tandem mass analysis of targeted compounds was also performed using collision-induced dissociation or high collision dissociation. Please see our previous study for further detail (He et al., 2018a). The identification of target compounds, as listed in Figure 2-2, was achieved through accurate mass measurement and tandem mass spectra and later confirmed by the corresponding reference standards. The semi-quantification was achieved by an external linear calibration curve analysis of each of the reference standards. Please see a previous study for a more in-depth account of orbitrap characterization, reference standard chemical concentrations and recoveries, and all other quality assurance and control information (C. Sun et al., 2019).

2.2.3.3 Polycyclic aromatic hydrocarbon (PAH) analysis

To characterize and measure FPW sample PAHs, copper powder and anhydrous sodium sulfate (pre-cleaned with DCM) were added into the 3 mL extracts (section 2.2.3.1) and vortexed. Solid phase extraction was used for cleanup of all the sample extracts. A Sep-Pak Silica 6 cc Vac cartridge (1 g; Waters, MA, USA) was conditioned with 5 mL solvent (hexane/DCM 7:3 v:v; Optima™, Fisher Scientific, NH, USA), followed by 5 mL of hexane. Extracts (3 mL) were loaded into cartridges and washed with 4 mL of hexane. PAHs were finally eluted with 5 mL of hexane/DCM 7:3 (v/v) which was subsequently concentrated to 200 µL for gas chromatography-mass spectrometry (GC-MS) analysis. Details of PAH standards, internal standard corrected recoveries of the detectable PAHs based on accelerated solvent extraction method (range from 60.0 to 114%) and liquid-liquid extraction (range from 82.9 to 179%), and the GC–MS instrumental method have been described in previous work (Zhang et al., 2016). Similarly, details of PAH analyte detection limits can be found previously (He et al., 2018b).

2.2.4 Lethal Concentration (LC) Analyses and Toxicity Determination

LC analyses (LC_{10}, LC_{20}, and LC_{50}) were performed according to Organisation for Economic Co-operation and Development (OECD) guidelines (OECD, 2013, 2004, 1992) with some slight adjustments. Dechlorinated City of Edmonton tap water was used for all exposures and FPW dilutions. Fluorescent lighting on a ratio of 14:10 h light:dark was applied for all exposures. For 96-h LC analyses, static-renewal exposures were employed where 50% solution changes were made every 48 h. Dead organisms were immediately removed from exposure containers. A total of seven different FPW concentration dilutions (created by mixing raw FPW with dechlorinated tap water) - including a freshwater control - were used for each LC analysis with six replicates for each individual FPW time-point sample at each dilution being employed to ensure statistical robustness. A series of range-finding tests were performed for all species prior to acute lethal toxicity analyses to ensure proper dilutions of FPW were being used to accurately capture LC values of each FPW sample.

All invertebrate exposures were acute 48-h, static exposure lethal analyses occurring in 30 mL of FPW containing mediums at 20 ± 0.5 °C in 50 mL glass beakers (10 organisms/beaker). *Daphnia* neonates were immediately collected from adult organisms and exposed within 24 h following adult brooding. Adult *Lumbriculus variegatus* were collected from cultures and held overnight before being used in bioassays. Fish (both zebrafish and rainbow trout) embryo lethal analyses occurred over a 96-h, static-renewal exposure period and used ten embryos/beaker for exposures. Observations every 24 h under a stereomicroscope were performed to determine mortality. Zebrafish embryos collected from adult fish were immediately placed into petri dishes containing fresh zebrafish facility water and were subsequently observed under a Leica Zoom 2000 stereomicroscope (Leica Camera Co., GER) to determine embryo fertilization. After confirming fertilization (~2 h post fertilization; hpf) and viable status, embryos were transferred to 100 mL of respective FPW solutions in 250 mL glass beakers maintained at 25 ± 0.5 °C for 96-h LC_{50} analyses.

Rainbow trout embryos (7–12 days post fertilization; dpf) removed from the recirculating heath-tray chiller unit were immediately placed into 200 mL of respective FPW solutions in 400 mL beakers maintained at 10 ± 1 °C under constant aeration for LC

analyses. Observations every 24 h under a stereomicroscope were performed to determine embryo mortality. For juvenile rainbow trout 96-h LC_{50} analyses, static-renewal exposures occurred in 10 L glass tanks containing 8 L of respective FPW solutions under constant aeration and were maintained at 10 ± 1 °C. All tanks were allowed to equilibrate with FPW exposure solutions 24 h prior to onset of LC analyses. Fish were fasted for 24 h prior to experimentation and were not fed for the duration of the 96-h LC analysis. For each exposure, 7 fish were used per tank at each concentration. Observations every 24 h were performed to determine fish mortality.

2.2.5 Statistical Analyses and Calculations

All LC analyses and associated 95% confidence intervals (CI) were calculated using a three-parameter probit model (TRAP; Toxicity Relationship Analysis Program, v1.30a, U.S. EPA). Compared LC values which did not have overlapping 95% CIs were considered significantly different. All graphing and non-linear curve-fitting procedures were performed using the statistic and graphing program Prism (GraphPad Software Inc., CA, USA).

2.3 RESULTS

2.3.1 Inorganic Characterization of FPW

ICP-MS/MS inorganic analyses revealed a predicted increasing ion concentration gradient with increased FPW sampling period. This was highlighted in many of the major salt ion characterizations; ions such as Cl, Na, Ca, K, and Mg, which were all shown to increase in concentration as the timing of FPW sampling progressed (Figure 2-1 and Table S2-1). Accordingly, a similar increase in total dissolved solid (TDS) readings was observed, as the highest recorded value was found in the 228 h sample at ~175700 mg/L. Interestingly, total organic carbon (TOC) values dropped as FPW sampling progressed (Table S2-1), while total nitrogen (TN) measurements did not follow any apparent trend. Regarding metals, high concentrations of Fe, Sr, and Ba were found in all FPW samples, although only Sr displayed a rough observable trend of increasing concentration with FPW sampling time (Table S2-1). Outside these constituents, Br was also found in FPW samples at high concentrations (up to~270 mg/L), but with no obvious trend (Table S2-1).

2.3.2 Organic Characterization of FPW

Following HPLC/Orbitrap-MS/MS analyses, the organic chemical profiles revealed distinct differences between FPW sampling periods. However, the organic composition of FPW is complex and the lack of reference standards makes quantification difficult, thus, only the organic compounds that were identified with high confidence (i.e. comparison with corresponding reference standards) are semi-quantified and presented in this study. Polyethylene glycols (PEGs) were found in all FPW samples, with the highest concentrations found in the 1.33 h sample (63.5 ng/L; Fig. 2). Similarly, C10-alkyl ethoxylates (C10-AEO) and octylphenol ethoxylates (OPE) were present in all FPW samples, with the highest concentration of C10-AEO (111 ng/L) found in the 1.33 h sample and the concentration of OPE highest in the 72 h FPW sample (85.2 ng/L). Beyond these chemicals, triphenyl phosphate, tri (2-butoxyethyl) phosphate, 3-(dodecanoylamino)-N,N-dimethylpropan- 1-amine oxide, and 2-[dimethyl-[3 (tetradecanoylamino)propyl]azaniumyl] acetate were found at varying levels across the FPW samples analyzed, although all were found at very low concentrations overall (Figure 2-2).

HPLC-MS/MS analyses of 22 PAHs (16 identified by the USA EPA as priority PAHs of toxicological concern; Office of the Federal Registration, OFR, 1982) in the aqueous and sediment phases of the FPW samples revealed that all samples contained PAHs; the majority being either of 3 and 4-ringed analytes (Table 2-2). In particular, the 3-ringed PAH phenanthrene was commonly found at the highest concentrations, with largest total (aqueous and sediment phase summation) concentration of 36.9 µg/L phenanthrene found in the 1.33 h FPW sample. Phenanthrene (and phenanthrene related compounds such as 1- methylphenanthrene and 3,6-dimethylphenanthrene) were also found to compose the majority of the PAH compounds present in FPW samples, comprising 66%, 63%, and 71% of the 22 total PAHs analyzed in the 1.33 h, 72 h, and 228 h FPW samples, respectively (Table 2-2). Overall, all PAH compounds followed the general trend of having the highest concentration in the 1.33 h sample, and lowest in the 72 h FPW sample, resulting in total PAH concentrations of 112.7, 11.6, and 25.1 µg/L in 1.33 h, 72 h, and 228 h FPW samples, respectively.

2.3.3 Aquatic Species Lethality Responses

For clarity and succinctness, all statements of LC analyses and discussion of results will pertain to LC_{50} analyses unless otherwise stated. However, information on LC_{10} and LC_{20} analyses can be found in Table 2-3. From our inorganic analyses, we anticipated the inherently high salinity of FPW to be a significant contributor to the toxicological hazard of FPW spills posed to freshwater aquatic organisms. However, our organic chemical characterizations also revealed a substantial amount and diversity of organic compounds to be present. Thus, when compared to our saline control exposures, not all organismal toxic responses to the experimental FPW series samples are observed to be strictly implicated to the high salt concentrations of the FPW samples.

Daphnia magna responses to our FPW samples were observed to be most sensitive of the four species tested and displayed the greatest acute toxicity regardless of sample collection timepoint. Comparing the daphnid LC_{50} FPW dilution values of 1.58, 0.76, 0.94, and 2.81% for our 1.33 h, 72 h, 228 h, and salt control, respectively (Figure 2-3, Table 2-3), to the observed LC_{50} values observed in rainbow trout embryos (8.82, 12.26, 10.83, and 16.87% for 1.33 h, 72 h, 228 h, and salt control, respectively) highlights the large variability in aquatic organism response to FPW. Relatively moderate LC_{50} responses of zebrafish embryos (3.03, 2.22, 2.15, and 2.11%), *Lumbriculus* (3.13, 4.18, 3.80, and 4.11%), and juvenile rainbow trout (7.14, 10.51, 9.02, and 14.24%) when exposed to the FPW series (1.33 h, 72 h, 228 h, and salt control, respectively) (Figure 2-3, Table 2-3) were additionally observed. These results again highlight both the diverse mechanisms and toxic nature of FPW solutions.

2.4 DISCUSSION

A significant contribution to the differences in toxicity between the FPW samples analyzed can be attributed to noted differences in inorganic and organic chemical profiles. The FPW samples chosen for analysis from the horizontal hydraulically-fractured well studied spanned a time series hypothesized to hold varying toxicological potentials, as all three time points represented a shift in general well production states.

When individually assessing toxicity amongst species, it was observed that *Daphnia magna* were overall the most sensitive species (lowest LC values) of those tested

to FPW samples at all collection time points (Figure 2-3, Table 2-3). Interestingly, *Daphnia* were most sensitive to our 72 h FPW sample when all other species (save for zebrafish embryos) were least sensitive to this particular sample. However, differential *Daphnia* responses to the three FPW samples were insignificant (overlapping LC_{50} 95% confidence intervals), but all elicited significantly higher toxic responses (lower LC_{50} values with non-overlapping 95% CIs) compared to the salt controls, indicating that something besides salinity in FPW (presumably organics) causes an additional significant toxic effect. Zebrafish embryos were found to be the second most sensitive to FPW exposures amongst all species tested. However, zebrafish embryo responses generally contrasted other species responses, as the saline control elicited the greatest toxicity in zebrafish embryos and was significantly more toxic compared to the 1.33 h sample (non-overlapping LC_{50} 95% CIs). These results suggest that zebrafish embryos are, above all, most sensitive to high osmotic conditions, and that salinity dominated to a greater degree FPW toxicity in zebrafish embryos, perhaps masking any other effects. Considering their current natural ecological niche and no recent evidence of a diadromous lifestyle within the evolution of their species (Engeszer et al., 2007; Imoto et al., 2013), the high sensitivity of zebrafish to hyperosmotic conditions is a logical response. *Lumbriculus* were found to be the third most overall sensitive species to FPW exposure, although general trends of sensitivity in this species were mirrored in both embryonic and juvenile rainbow trout exposures (Figure 2-3, Table 2-3). When analyzing these organism response trends to FPW exposure, it was observed that the 1.33 h sample elicited greatest toxicity, while the 72 h sample was least toxic. In contrast to zebrafish, the family Salmonidae, of which rainbow trout are a member, have a relatively recent evolutionary history of anadromy (natural transfer between freshwater and seawater environments) (Alexandrou et al., 2013; Crespi and Fulton, 2004; Docker and Heath, 2003). Thus, a more saline-tolerant response to FPW was expected and found for rainbow trout. Interestingly, responses to different FPW samples within each distinct rainbow trout exposure series were significantly different from one another (no 95% C.I. overlap for LC_{50} analyses). However, juvenile rainbow trout were observed to be significantly more sensitive to FPW exposures compared to embryonic forms; a result consistent with earlier reports suggesting that embryonic life-stages are often more resistant to environmental stressors than larval stages (immediately following hatching) and juvenile

forms (Embry et al., 2010; Woltering, 1984). It is thought that the enveloping chorion in ovo acts as a barrier to toxicants and other environmental stressors, thereby protecting the developing organism within the embryo (Cotelli et al., 1988; Denluck et al., 2018; Embry et al., 2010; Pelka et al., 2017).

As demonstrated by the total ion chromatograms of HPLC/Orbitrap-MS and PAH analyses of FPW extracts, there are clear differences in both diversity and abundance of organic compounds among samples (Figure 2-2, Table 2-2). Of the semi-quantified compounds identified, class C10-AEO non-ionic surfactants were highest in our 1.33 h sample. This particular class of surfactants have known bioaccumulation potentials (Cheng et al., 2005; Müller et al., 1999a), with nonspecific narcosis as the suspected primary toxicological mode of action (Dorn et al., 1997; Müller et al., 1999b). Another semi-quantified class of surfactants identified were OPEs. These are non-ionic surfactants which may mimic endogenous hormones and cause endocrine disruption upon exposure (Nimrod and Benson, 1996; White et al., 1994). These surfactants are degraded/or metabolized into octylphenols (OPs), which are often more toxic and more persistent in the environment (Ahel and Giger, 1993; Ying et al., 2002). Although the concentrations of C10-AEOs were below water quality guidelines of the Canadian Environmental Protection Act (70 μg/L; Environment and Climate Change Canada, 1999) and OPEs in the 1.33 h sample were lowest amongst the FPW samples, the toxicity of surfactants in the 1.33 h sample may still play a larger role in producing greater effects compared to the other two FPW samples, particularly when other characteristics of the mixture are taken into account. Specifically, PEGs were found at highest levels in the 1.33 h sample. Although not inherently toxic to fish individually, PEG physiochemical properties may alter the toxicity of other compounds found in the FPW mixture. In relation to surfactants, PEGs are shown to increase surfactant critical micelle concentrations (CMC), decrease average micelle aggregation (N_{agg}), and increase surfactant polydispersity (Nagarajan and Wang, 2000), while surfactants which incorporate PEGs directly into their chemical structure (such as tocopheryl polyethylene glycol succinate surfactants; TPGS) increase CMCs and decreased N_{agg} values compared to traditional non-ionic surfactants without PEGs associated (Sadoqi et al., 2009). These TPGS surfactants have been shown to be an effective pharmaceutical agent for drug deliveries by increasing drug absorption in tissues (Ismailos et al., 1994; Z.

Zhang et al., 2012). Increased PEG presence in our 1.33 h FPW sample may therefore increase the bioavailability of the surfactants present in the 1.33 h sample and allow them to interfere and react to a greater degree with biological surfaces of exposed organisms to induce greater toxicity.

PAH analysis of the current samples similarly affirmed TOC analyses depicting the 1.33 sample to contain the greatest amount of organic compounds (Table 2-2). Of the PAH compounds investigated, all present/detectable forms were highest in concentration in the 1.33 sample, resulting in the highest Σ16 and Σ22 PAHs concentrations. Specifically, phenanthrene-related PAH compounds were highest in the 1.33 h sample, although in both the 72 and 228 h samples these were also the major PAHs identified. PAHs are known to cause numerous toxic effects in exposed organisms. Although PAH toxicity can be species dependent, given that invertebrates are generally considered to exhibit lower PAH metabolic and elimination capabilities, other factors such as developmental stage are also known to influence PAH toxicity (James, 1989; Meador et al., 1995; Varanasi, 1989). Regardless, toxicity associated with PAH exposure may come from the parent molecule itself or, as is more often the case, from metabolite intermediates formed during biotransformation processes. These intermediates may include highly reactive epoxides formed during phase I cytochrome P450 (CYP) monooxygenase oxidation steps which may also be transformed into toxic phenol derivatives depending on the original substrate (Buhler and Williams, 1988; Livingstone, 1998). The creation of these intermediate metabolites through CYP systems (and the effects they have on biological systems) are mediated at differing levels depending on the specific PAH by as many as 70 nuclear receptors, including the aryl hydrocarbon receptor (AhR), retinoid X receptor (RXR), and others (Honkakoski and Negishi, 2000; Xu et al., 2005).

Alternatively, PAHs may non-specifically interfere with other cellular processes via nonspecific narcosis (Barron, 2004; Di Toro et al., 2007; Wezel and Opperhuizen, 1995) or other cellular processes/receptors not yet studied. PAHs are also known to produce oxidative stress responses in both invertebrates and vertebrate species (Dalton et al., 2002; Lemaire et al., 1994; Penning et al., 1996; Sun et al., 2006; Zangar, 2004). Considering phenanthrene, phenanthrene-related compounds, and other 3-ring PAHs (such as fluorene and dibenzothiophene) in our FPW samples are generally found at the highest

concentrations compared to other PAHs, we suspect that significant cardiotoxic potentials are associated with FPW, as recent research often associates 'blue-sacs disease' (a condition characterized by larval craniofacial and tail/spine deformities, as well as pericardial and yolk-sac edemas) (He et al., 2012a; Spitsbergen et al., 1991) with exposure to these 3-ring PAHs in larval fish species. Exposure to 3-ring PAHs in fish has recently also been shown to directly alter cardiomyocyte function by specifically affecting Ca^{2+} and K^{+} currents via blockade of K^{+} channel and disruption of sarcolemmal and sarcoplasm reticulum Ca^{2+} cycling (Brette et al., 2017, 2014). Detriments to cardiac function would undoubtedly place higher energetic demands and stress in exposed organisms and may be another toxicological hazard warranting assessment when releases of FPW occur.

2.5 CONCLUSION

Research on FPW and wastewaters associated with hydraulic fracturing activities has only recently begun in a manner which assesses the toxicological hazards associated with such fluids. In this study, we aimed to not only determine baseline toxicities of FPW in multiple relevant toxicological model species, but also determine how toxicity (and chemical characterizations) change depending on duration of early stage flowback from a well. These data are of value to the reassessment of risk and remediation strategies/requirements and will overall contribute to a better understanding of the potential impacts of FPW releases. Despite the complexity and variations in FPW chemical and toxicological makeup (dependent on numerous geological and operational factors), understanding basic toxicological and chemical characteristic tendencies will help shape governmental and industrial FPW management policies and hazard assessments.

In the present study, we have determined that FPW toxicity is not only species-dependent but is also influenced by the length of time that a well has been producing FPW. A significant component of the toxicity of FPW to freshwater organisms is mediated by the high salinity and organic make-up (from either initial fracturing fluid constituents and formation derived organics) of the wastewater, with even very high dilutions of FPW inducing toxicity in all species tested. However, all species also demonstrated significantly different toxic responses to at least one of the FPW samples tested separate of saline-related effects, suggesting that other components of FPW are contributing to overall toxic potentials. In particular, earlier samples of FPW collected closer to the beginning of the

flowback period (e.g. our 1.33 h sample) were determined to contain the highest organic contaminant concentrations and were also generally most toxic to the organisms studied. Considering these earlier samples contained overall lower salt concentrations, and metal concentrations that were either lower or equal to later FPW samples, it is assumed that organic contaminants were responsible for most of the added toxicity observed. However, all FPW samples contained significant toxic potential and should be considered hazardous regardless of time post-well stimulation when accidental releases to the environment occur.

Collectively, we show that FPW produced from an active hydraulically fractured well is i.) overall toxic to a range of freshwater aquatic species, and ii.) elicits differential toxicity depending on the species, the developmental time point of the organism, and the stage at which the well was producing FPW (earlier versus later FPW sampling timepoints). Concerning the chemical compositional changes across the FPW samples at the differing timepoints, toxicological responses may be in response to the changing salinities and other chemical components (e.g. organics and metals). Future research is needed to tease apart exactly which compounds are contributing (and to what degree) towards the toxicological responses observed. Furthermore, it is unknown the type and degree to which certain sub-lethal toxicological stresses are being propagated in FPW exposed organisms. Therefore, further research on potential sub-lethal toxicities induced following FPW exposure is warranted.

Table 2- 1. General overview of flowback and produced water (FPW) samples analyzed for toxicological and chemical characterizations in the current study.

FPW Sample Analyzed[a]	General Sample Designation
1.33 hr	Flowback
72 hr	Flowback-Produced Water Mix
228 hr	Produced Water
Saline Control	Matches FPW 228 hr Major Ion Content

[a]All times indicated refer to time of collection post well-stimulation

Table 2- 2. Polycyclic aromatic hydrocarbon (PAH) analysis of FPW sample 1.33 hrs (1.33), 72 hrs (72), and 228 hrs (228). All measurements are in µg/L. Total PAH content of a specific compound determined via summation of both aqueous (A) and sediment (S) phase measurements of the specified compound in the respective FPW sample. Total summation of US EPA 16 PAHs (shaded compounds) and all PAHs analyzed (shaded + un-shaded compounds) for respective FPW samples identified as **Σ16 PAHs** and **Σ22 PAHs**, respectively. N.D. = not detected.

	FPW Sample								
PAHs	**A – 1.33**	**S – 1.33**	**1.33 Total**	**A – 72**	**S - 72**	**72 Total**	**A - 228**	**S - 228**	**228 Total**
Naphthalene	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
Acenaphthlene	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
Acenaphthene	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
Fluorene	3.49	4.68	8.17	0.0401	0.29	0.33	0.045	0.51	0.56
Phenanthrene	16.44	20.48	36.92	0.052	0.82	0.87	0.14	1.53	1.67
Anthracene	0.44	0.51	0.95	0.0089	0.021	0.030	0.028	0.093	0.12
Fluoranthene	0.095	0.11	0.21	0.0031	0.068	0.071	0.027	0.095	0.12
Pyrene	1.99	1.88	3.87	0.0054	0.93	0.94	0.090	1.63	1.72
Benz[a]anthracene	0.78	0.83	1.61	0.0012	0.2	0.201	0.003	0.22	0.22
Chrysene	1.19	1.42	2.61	0.0045	0.79	0.80	0.015	1.043	1.058
Benzo[b]fluoranthene	N.D.	0.23	0.23	N.D.	0.11	0.11	N.D.	0.21	0.21
Benzo[k+j]fluoranthene	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
Benzo[a]pyrene	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
Indeno[1,2,3-cd]pyrene	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
Benzo[g,h,i]perylene	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
Dibenz[a,h]anthracene	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
Dibenzothiophene	2.093	7.080	9.17	0.0008	0.0093	0.0101	0.0044	0.020	0.024
Retene	0.70	0.98	1.68	N.D.	0.28	0.28	N.D.	0.42	0.42
1-Methylfluorene	2.36	5.36	7.72	0.024	1.10	1.12	0.031	1.94	1.97
1-Methylphenanthrene	10.43	13.89	24.32	0.013	3.083	3.096	0.057	8.041	8.098
3,6-Dimethylphenanthrene	5.70	7.83	13.53	0.0033	3.34	3.34	0.021	8.064	8.085
1-Methylpyrene	0.86	0.87	1.73	0.0004	0.38	0.38	0.0054	0.76	0.77

	Σ16 PAHs	**54.55**	**Σ16 PAHs**	**3.35**	**Σ16 PAHs**	**5.68**
	Σ22 PAHs	**112.69**	**Σ22 PAHs**	**11.58**	**Σ22 PAHs**	**25.05**

Table 2- 3. Comparison of lethal concentrations (LC's) and 95% confidence interval (C.I.) values among multiple species exposed to flowback and produced water samples from a horizontal hydraulically fractured well.

	LC_{10}*	95% C.I.	LC_{20}*	95% C.I.	LC_{50}*	95% C.I.
FPW 1.33 Hr						
Daphnia[a]	0.31	-1.30 - 1.92	0.74	-0.43 – 1.90	1.58	0.95 - 2.21
Lumbriculus[a]	1.30	0.73 – 1.87	1.91	1.46 – 2.36	3.13	2.81 – 3.45
***Danio* Embryos**[b]	0.36	-1.41 – 2.14	1.26	-0.23 – 2.74	3.03	2.10 – 3.97
***Oncorhynchus* Embryos**[b]	4.59	3.14 – 6.03	6.01	4.87 – 7.14	8.82	8.06 – 9.59
Juv. *Oncorhynchus*[b]	3.07	1.99 – 4.14	4.43	3.58 – 5.29	7.14	6.46 – 7.83
FPW 72 Hr						
Daphnia[a]	0.19	-0.60 – 0.99	0.38	-0.17 – 0.93	0.76	0.50 – 1.01
Lumbriculus[a]	3.30	3.05 – 3.56	3.60	3.37 – 3.82	4.18	3.98 – 4.38
***Danio* Embryos**[b]	0.58	-0.23 – 1.40	1.13	0.46 – 1.81	2.22	1.78 – 2.66
***Oncorhynchus* Embryos**[b]	7.61	6.10 – 9.11	9.16	7.90 – 10.43	12.26	11.34 – 13.17
Juv. *Oncorhynchus*[b]	8.30	7.53 – 9.07	9.04	8.41 – 9.67	10.51	9.95 – 11.07
FPW 228 Hr						
Daphnia[a]	0.37	-0.032 – 0.77	0.56	0.29 – 0.83	0.94	0.75 – 1.12
Lumbriculus[a]	2.82	2.49 – 3.16	3.15	2.87 – 3.43	3.80	3.58 – 4.02
***Danio* Embryos**[b]	0.85	0.15 – 1.54	1.28	0.82 – 1.75	2.15	1.85 – 2.44
***Oncorhynchus* Embryos**[b]	7.03	5.72 – 8.34	8.30	7.26 – 9.34	10.83	10.09 – 11.58
Juv. *Oncorhynchus*[b]	7.13	6.10 – 8.16	7.76	7.00 – 8.51	9.02	8.48 – 9.55
SALINE CONTROL						
Daphnia[a]	1.45	1.11 – 1.80	1.91	1.55 – 2.26	2.81	2.50 – 3.12
Lumbriculus[a]	3.28	3.04 – 3.53	3.56	3.35 – 3.77	4.11	3.92 – 4.30
***Danio* Embryos**[b]	0.87	0.40 – 1.35	1.29	0.90 – 1.68	2.11	1.85 – 2.37
***Oncorhynchus* Embryos**[b]	14.95	14.23 – 15.67	15.59	14.97 – 16.21	16.87	16.46 – 17.27

Juv. *Oncorhynchus*[b]	10.76	9.72 – 11.80	11.93	11.07 – 12.78	14.24	13.55 – 14.92

[a] 48-hr LC_{50} analysis

[b] 96-hr LC_{50} analysis

* All LC values listed as % dilutions of FPW

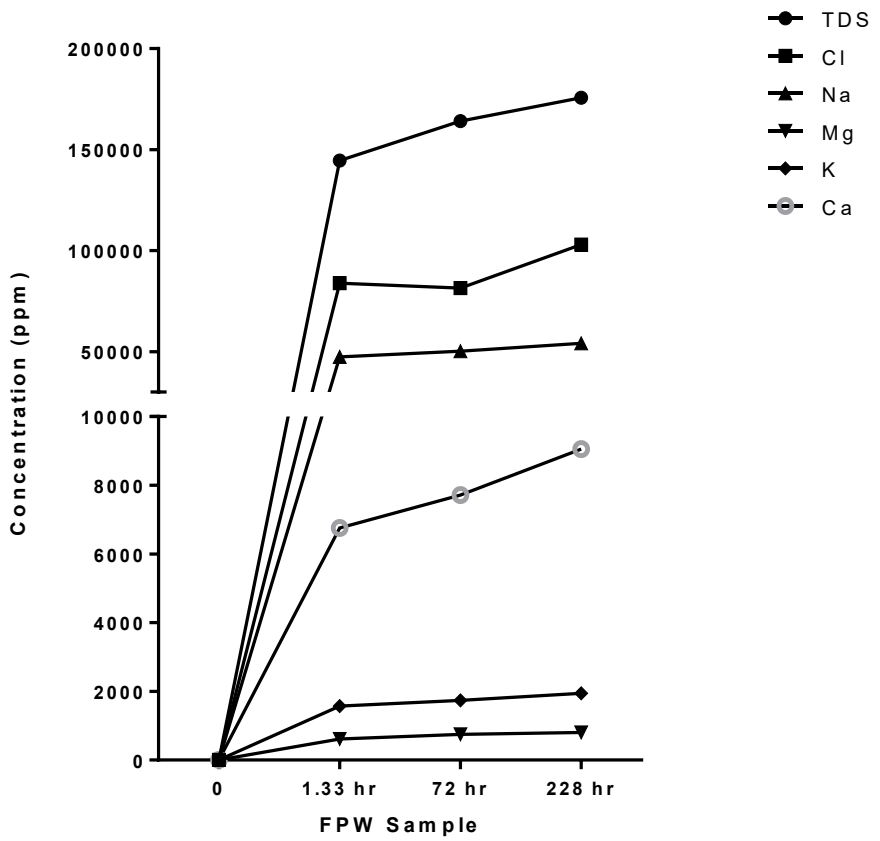
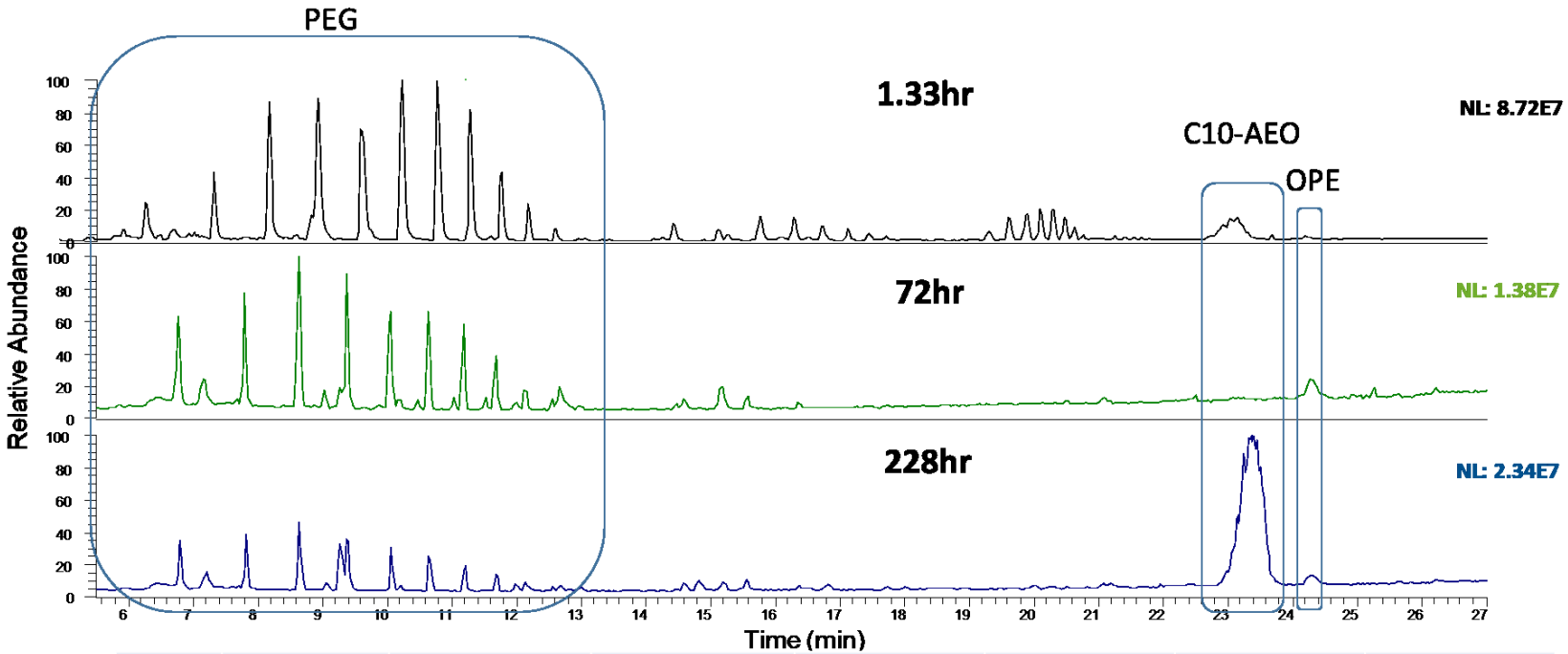


Figure 2- 1. Concentrations of major cation, anion, and total dissolved solids (TDS) of flowback and produced water samples from a horizontal hydraulic fractured well measured by ICP-MS/MS and ion chromatography.



FPW Sample	Polyethylene Glycol (PEG)	C10-Alkyl Ethoxylates (C10-AEO)	Octylphenol Ethoxylates (OPE)	Triphenyl phosphate	Tri (2-butoxyethyl) phosphate	3-(dodecanoylamino)-N,N-dimethylpropan-1-amine oxide	2-[dimethyl-[3-(tetradecanoylamino)propyl]azaniumyl]acetate
1.33 HR	63.5	111	65.9	8.01	2.85	1.76	2.23
72 HR	45.1	76.2	85.2	4.65	N/A	N/A	N/A
228 HR	25.7	87.1	75.3	N/A	N/A	N/A	N/A

Figure 2- 2. HPLC-Orbitrap MS total ion chromatogram analysis of flowback and produced water samples collected at 1.33, 72, and 228 hours from a horizontal hydraulic fractured well. Semi-quantified compounds identified are listed in table below chromatograms. All table concentration values are reported as ug/L. NL = normalized total ion abundance.

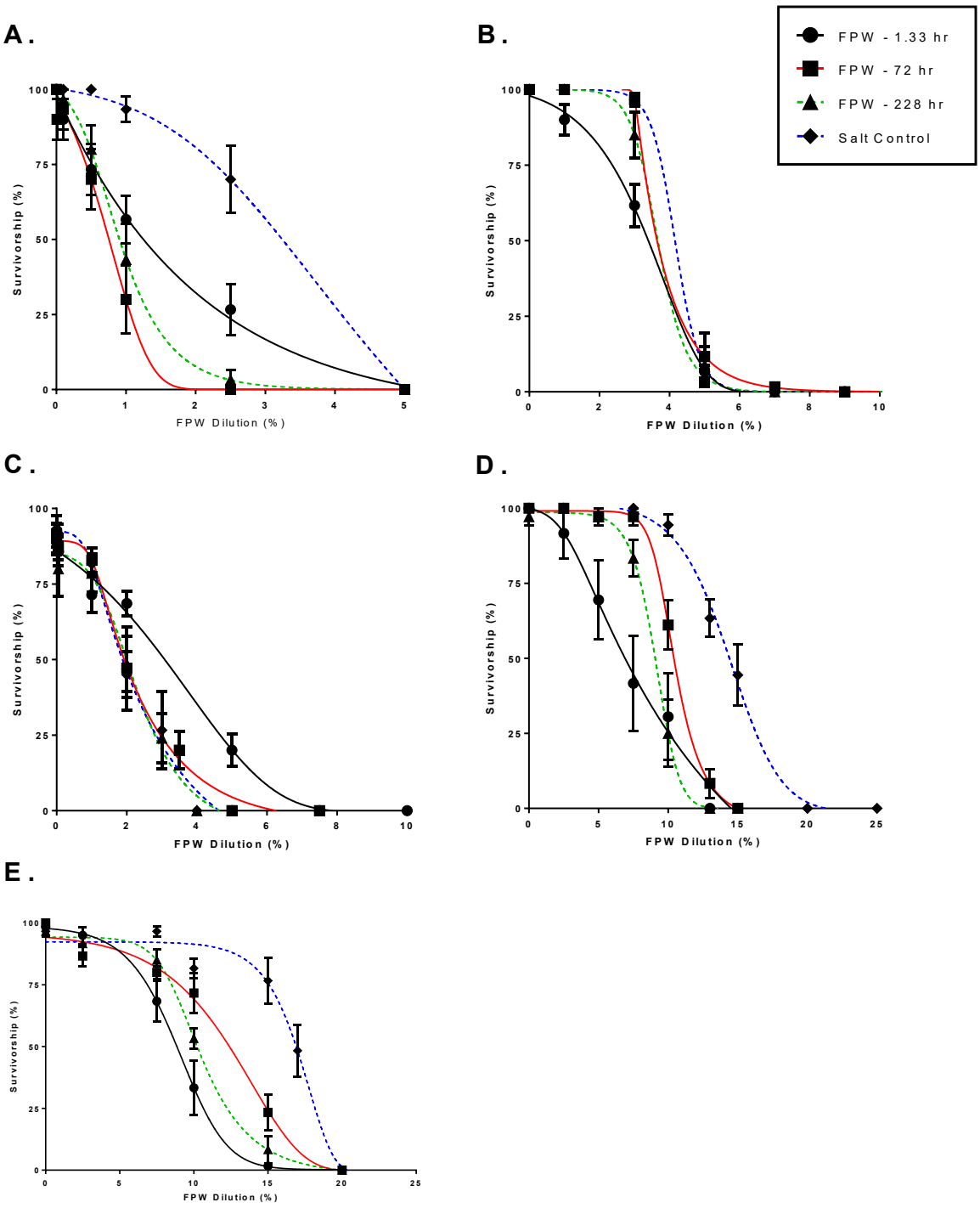
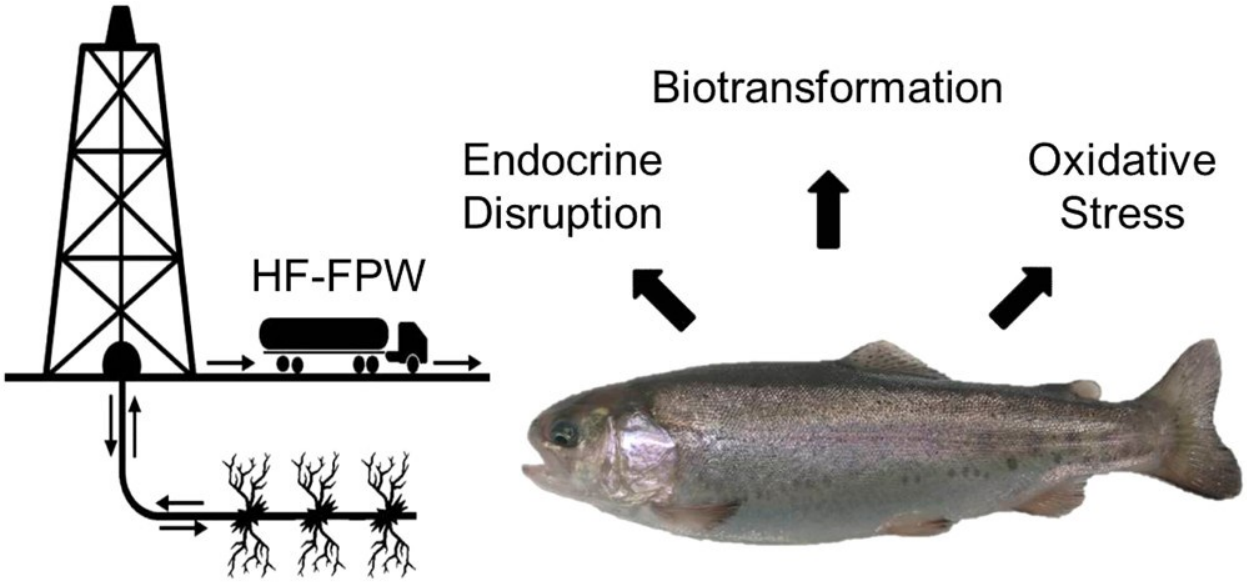


Figure 2- 3. Aquatic freshwater species survivorship non-linear regression curve responses to differing flowback and produced water (FPW) dilution exposures. FPW was collected from a horizontal hydraulic fractured well. Species tested are A.) Juvenile *Daphnia magna*, B.) *Lumbriculus variegatus*, C.) *Danio rerio* embryos, D.) Juvenile *Oncorhynchus mykiss*,

and E.) *Oncorhynchus mykiss* embryos. Per species, $n = 6$ for each dilution for each individual FPW sample. SEM plotted for each dilution data point.

CHAPTER 3: Effects on Biotransformation, Oxidative Stress, and Endocrine Disruption in Rainbow Trout (*Oncorhynchus mykiss*) Exposed to Hydraulic Fracturing Flowback and Produced Water*



He, Y., Folkerts, E.J., Zhang Y., Martin J.W., Alessi, D.S., Goss, G.G. (2017). Effects on biotransformation, oxidative stress, and endocrine disruption in Rainbow Trout (*Oncorhynchus mykiss*) exposed to hydraulic fracturing flowback and produced water. *Environmental Science and Technology*, **51**, 940 – 947. DOI: 10.1021/acs.est.6b04695.

*Co-first Authorship

3.1 INTRODUCTION

Horizontal drilling with high-volume hydraulic fracturing (HF) is a practice being used in Alberta/Canada for improving the extraction of oil and gas from tight reservoirs. Energy production from these resources in North America is expected to continue, with estimated increases of 45% and 25% above current production levels for the US and Canada, respectively, in the next 25 years (Natural Resources Canada, 2015; U.S. EIA, 2015). The rapid expansion of HF practices, together with its large quantity of water usage and process affected water production, poses potential environmental hazards to the environment, including contamination of surface and shallow groundwater aquifers via discharges and spills (Entrekin et al., 2011; Lauer et al., 2016; Vengosh et al., 2014; Vidic et al., 2013), as well as subsurface gas migration (Darrah et al., 2014; Kargbo et al., 2010; Llewellyn et al., 2015; Osborn et al., 2011; Wilson and VanBriesen, 2012).

However, there remain significant knowledge deficits on the environmental impacts and risks of the flowback and produced water (HF-FPW) to aquatic ecosystems (Harkness et al., 2015). HF-FPW is a complex, tripartite mixture of injected HF fluid components, deep formation water, and secondary by-products of downhole reactions with the formation environment (DiGiulio and Jackson, 2016; Drollette et al., 2015; Lester et al., 2015; Llewellyn et al., 2015). HF-FPW brine may contain numerous inorganic and organic constituents, including high levels of metals (e.g., barium, strontium, chromium, cadmium, lead), radionuclides (e.g., radium and uranium), and a complex profile of organic compounds, theorized to be additive components in HF fluid, natural organics related to *in situ* formation hydrocarbons (e.g., polycyclic aromatic hydrocarbons (PAHs)), and even secondary chemical products from the interaction between the fracturing environment in the well (elevated temperature and/or pressure) and the deep saline groundwater (Ferrar et al., 2013; Gordalla et al., 2013; He et al., 2017a). Correspondingly, the concentrations of metals, radionuclides, and PAHs detected in FPW are often well above the maximum contamination level for water quality guidelines (Alberta Environment & Sustainable Resource Development (ESRD), 2014; Lauer et al., 2016; Vengosh et al., 2014). Together, these chemicals return to the surface with released oil and gas, and the HF-FPW is separated for treatment/reuse, or disposed in deep, subsurface injection wells (Warner et al., 2013). Risks of ground and/or surface fresh water contamination are principally

associated with on-site fluid handling, transportation of HF-FPW to disposal wells, and well integrity issues (Vengosh et al., 2014). Accidental release of HF-FPW in certain regions is well documented, with more than 2500 spills in Alberta from 2011 to 2014 (Goss et al., 2015). It has been suggested that the presence of endocrine disrupting chemicals in HF wastewater may be linked to reproductive and developmental impairment in laboratory animals based on the systematic evaluation of chemicals used in HF fluids (Elliott et al., 2017). While the potential biological risk and impacts of chemicals used during the fracturing process have been predicted and documented in several reviews (Kahrilas et al., 2015; Stringfellow et al., 2014), there is very limited information regarding the toxicity of any real HF-FPW samples and the potential toxicological impacts of HF-FPW spills on freshwater organisms.

The lack of available hazard assessment for HF-FPW spills in Canada and the United States hinders environmental impact and risk assessment of hydraulic fracturing activities (Gagnon et al., 2016). Mandatory disclosure of the chemical constituents of fracturing fluids for example, through the chemical disclosure registry, FracFocus, has somewhat improved our understanding but the toxicity data of many chemicals is often missing (Gagnon et al., 2016; Stringfellow et al., 2014). The environmental fates of those chemicals are further complicated by potential down-hole reactions and generation of secondary products (He et al., 2017a). Therefore, there exists an obvious need to investigate the toxicity on aquatic organisms. In this study, juvenile rainbow trout (*Oncorhynchus mykiss*), commonly used as a biologically relevant freshwater model for regulatory science, were used to determine responses to potential spills and leaks of HF-FPW in the aquatic environment. Acute exposures (48 h) were conducted followed by measurements of a variety of endpoints including hepatic and branchial ethoxyresorufin-O-deethylase (EROD) activity, thiobarbituric acid reactive substance (TBARS) formation in various tissues, and mRNA abundance of a battery of genes related to biotransformation, oxidative stress, and endocrine disruption by quantitative real-time polymerase chain reaction (Q-RT-PCR). This is one of the first studies to investigate the physiological responses to HF-FPW exposure in a whole organism. Our study will help address the ecotoxicological hazards associated with HF-FPW and provide potential biomarkers for water quality monitoring in areas affected by hydraulic fracturing activities.

3.2 MATERIALS AND METHODS

3.2.1 HF-FPW Collection

The HF-FPW sample analyzed in this study was collected at 7 days post-stimulation from a horizontal, hydraulically fractured well in the Devonian-aged Duvernay Formation (Fox Creek, Alberta, Canada). In this study, HF-FPW-S (abbreviated as S in figures, the same below) refers to the original, raw sample containing sediment and/or suspended particles. A summary of key compositional information on this sample is presented in Table 3-1. Detailed geological and chemical information on this sample is reported in a companion study (He et al., 2017a). All tests were conducted within 60 days of sample acquisition and samples were stored at room temperature to best reflect on-storage conditions. Sediment-free (HF-FPW-SF, or SF) was prepared by vacuum filtration of the raw sample through a 0.22 μm membrane, which also greatly reduced the levels of total organic contaminants in the sample. An activated-charcoal treated (HF-FPW-AC, or AC) sample was also prepared by treating the raw sample with activated charcoal, followed by vacuum filtration through a 0.22 μm membrane. This resulted in most of the organic contaminants being removed. We acknowledge that vacuum filtration may also have caused a loss of volatile dissolved components in SF and AC sub-samples, but this was necessary to remove the sediment fraction. Once we aliquoted a raw sample for either treatment or direct exposure (HF-FPW, HF-FPW-SF, and HF-FPW-AC), these aliquots were stored at 4 °C until the start of the exposure period. Details of the sample preparation are described in a companion study (He et al., 2017a).

3.2.2 Chemicals

All the chemicals were purchased from Sigma-Aldrich (USA). Details are provided in the Supporting Information.

3.2.3 *Fish*

Rainbow trout embryos were obtained from the Raven Brood Trout Station (Caroline, AB, Canada) and grown to the appropriate size (24.9 ± 10.1 g) as juveniles for

experimentation. Juvenile fish were maintained indoors in flow-through 450 L tanks supplied with aerated and dechlorinated facilitate water (hardness as $CaCO_3$, 1.6 mmol/L; alkalinity, 120 mg/L; NaCl, 0.5 mmol/L; pH 8.2, temp, 10 ± 1 °C). Fish were fed ground dry commercial trout pellets (Purina trout chow) once daily and kept on a 14 h/10 h day/night photoperiod. All animal use was approved by the University of Alberta Animal Care Committee under Protocol AUP00001334.

3.2.4 Exposure Design

Exposure was conducted in 8 L glass tanks filled with 4 L of aerated control/treatment water. Water temperature was maintained at 10 ± 1 °C by partial immersion of tanks in a water bath with constant facility water flow; 50% control/treatment water changes were made in each tank every 24 h. Fish were fasted 3 days prior to and during experimentation. Fish were exposed to control/treatment waters in triplicate tanks, with each tank containing two individuals. The concentrations of low dose (2.5%) and high dose (7.5%) were selected based on the results of preliminary range finding test of HF-FPW-S using finger-length juvenile rainbow trout (Figure S3-1). Fish were exposed to facility water as a control (Ctl), HF-FPW-AC (2.5% and 7.5% dilutions in facility water, the same below), HF-FPW-SF (2.5% and 7.5%) and HF-FPW-S (2.5% and 7.5%), as well as benzo[a]pyrene (BaP) (0.33 and 1 μM) as a positive control for 24 and 48 h. At the end of exposure, fish were euthanized by cephalic blow and decapitated. Gill filament and liver samples were collected and immediately assayed for EROD activity. Subsamples (0.25 g) of gill, liver, and kidney in all 48 h exposure groups were placed into 1.5 mL Eppendorf tubes containing 500 μL of phosphate buffer (100 mM KH_2PO_4, 5 mM EDTA, pH = 7.5), frozen in liquid nitrogen and stored at −80 °C for TBARS assay. Subsamples of liver in all 48 h exposure groups were frozen and stored at −80 °C for Q-RT-PCR assay. The exposure water from each treatment was sampled and stored in the dark at 4 °C prior to PAHs analysis.

3.2.5 PAH Analysis

Subsamples of exposure water at 24 and 48 h (500 mL), including control, AC (2.5% and 7.5%), SF (2.5% and 7.5%), and S (2.5% and 7.5%), were collected for

polycyclic aromatic compound analysis by the liquid–liquid extraction method as described previously (Zhang et al., 2016). Detailed methodology is provided in the Supporting Information and in Table S3-1.

3.2.6 Hepatic and Branchial EROD Assays

The hepatic EROD activity was determined via a modified method previously established (Hodson et al., 1991). The gill-filament-based EROD assay was performed following methods previously described in an other study (Jönsson et al., 2002). Details are provided in the Supporting Information.

3.2.7 TBARS Assay

TBARS assay using fish tissues was performed following a previous study with minor modification (Boyle et al., 2013). Briefly, the supernatant of the homogenized samples were treated with thiobarbituric acid and TBARS formation was quantified by fluorescence measurement at 531 and 572 nm (excitation and emission, respectively). In addition, the intrinsic oxidative potentials of HF-FPW fractions alone were also determined via a modified protocol from a previous study (Biaglow et al., 1997). HF-FPW samples were incubated with a final concentration of 4 mM 2-deoxy-d-ribose (2-DR) for 1 h at 25 °C under ambient room light, followed by fluorescence measurement of TBARS formation. Details are provided in the Supporting Information.

3.2.8 Quantitative Real-Time PCR assay

Total RNA was extracted from liver samples and cDNA was prepared for quantitative real-time PCR (Q-RT-PCR) measurement using SYBR Green master mix system (Applied Biosystems, CA). Details of RNA extraction, cDNA synthesis, and Q-RT-PCR reactions were provided in the Supporting Information. Eleven genes representing biotransformation, oxidative stress, and endocrine disruption in rainbow trout were selected for screening. Changes in abundances of transcripts of target genes were quantified by normalizing to *elongation factor 1a* (*elf1a*). There was no difference in the expression of *elf1a* among all the exposure groups (Figure S3-2). Gene name, abbreviation, sequences of primers, efficiency, and GeneBank reference number are listed in Table S3-2.

3.2.9 Statistical Analysis

Juvenile rainbow trout were exposed to the control/treatment solutions in triplicate tanks, with each tank containing two fish. No differences in responses to the same treatment between fish in the same tanks were observed. Therefore, each individual fish is considered an experimental unit. Statistical analyses were conducted by use of SPSS16.0 (SPSS, Chicago, IL). All data are expressed as mean ± standard error mean. Log transformation was performed if necessary to meet the assumptions. Statistical differences were evaluated by one-way ANOVA followed by *posthoc* Tukey test. Differences were considered significant at $\rho < 0.05$.

3.3 RESULTS AND DISCUSSION

3.3.1 PAH Analysis

Nominal and measured exposure concentrations of parent PAHs and total PAHs (parent + alkylated PAHs) are presented in Table 3-2. Detailed results of individual analytes are presented in Table S3-3. Generally, the measured PAH concentrations in the sediment free fraction exposure water (HF-FPW-SF dilutions) were lower or equivalent compared to nominal levels; however, PAH concentrations in the raw exposure water (HF-FPW-S dilutions) were higher than nominal (Table 3-2), suggesting the possibility of chemical desorption from the sediment particles present in the HF-FPW sample during the exposure period. Since HF-FPW is a complex mixture of HF fluid and formation water, it is likely that not only PAHs but a variety of other contaminants could desorb from sediment particles, resulting in more adverse effects on exposed fish compared to those exposed to a sediment-free fraction.

3.3.2 EROD Assays

Exposure to HF-FPWs caused significant induction of EROD activity in both hepatic and branchial tissues in rainbow trout. The hepatic and branchial EROD assays were modified from previous studies using S-9 fraction (Hodson et al., 1991) and gill filament (Jönsson et al., 2002), respectively. Assay validation was performed by using a

parallel BaP positive control (Figure 3-1). The relative fold of induction in hepatic and branchial EROD activity in 24 and 48 h exposure groups are presented in Figure 3-1.

Significant induction of EROD activity has been widely used as a biomarker of exposure to aryl hydrocarbon receptor (AhR) agonistic contaminants, including, PAHs, dioxins, polychlorinated biphenyls (PCBs), and various petroleum-related extracts (Sarkar et al., 2006). BaP is a prototype and well documented PAH widely used as positive control in EROD activity assay (Agency for Toxic Substances and Disease Registry (ATSDR), 1995). Previously, significant EROD induction was demonstrated in zebrafish larvae exposed to HF-FPWs using a nondestructive assay (Noury et al., 2006), indicating that the presence of PAHs and possibly other contaminants acting as AhR agonists may play a significant role in HF-FPW toxicity (He et al., 2017a). In the current study, hepatic and branchial EROD activities were measured in juvenile rainbow trout exposed to HF-FPWs by using liver S-9 fractions and gill filaments as indicator tissues. The results clearly demonstrate that exposure to HF-FPWs significantly induces EROD activity in both liver and gill tissues in juvenile rainbow trout, which is consistent with the companion study (He et al., 2017a). In addition, on the basis of induction fold data, branchial tissues seem to display more sensitive EROD activity responses than hepatic tissue after HF-FPW exposure (Figure 3-1A,B). Hepatic EROD inductions greater than 2.5-fold were not observed, and significant induction only occurred in tissue exposed to HF-FPW-S. Conversely, significant branchial EROD induction was detected in the 2.5% dilution of the HF-FPW-SF group, and in general, induction of EROD activity in gill tissues was greater than those in hepatic tissues. For example, exposure to 7.5% of HF-FPW-S for 48 h resulted in an EROD induction of 9.30 ± 0.31-fold in branchial tissue while a 2.41 ± 0.25-fold increase was found in hepatic tissue. Since the gill is an organ characterized by high surface areas, a thin membrane, concentrated vascularization, and is directly exposed to water-born contaminants, it is likely gill filaments receive higher levels of exposure compared to liver tissue, resulting in a more significant and sensitive EROD activity response. In addition, since most of the organic content has been removed by activated charcoal treatment (He et al., 2017a), the absence of significant effects on EROD activity in all AC exposure groups clearly supports the hypothesis that the organic contaminants were the major components responsible for EROD induction. Considering the dilution

factors and the low total PAH levels detected in the sample, the results observed in the current study confirmed that EROD activity in fish is sensitive to highly diluted HF-FPW exposures and could be used as efficient biomarker for an HF-FPW spill or in a post-spill monitoring program.

3.3.3 TBARS Assays

HF-FPW exposure resulted in elevated TBARS formation in juvenile rainbow trout tissues indicating oxidative stress. The highest TBARS tissue levels were observed in fish exposed to 7.5% HF-FPW-S (2.95 ± 0.66, 2.12 ± 0.18, and 3.11 ± 0.97-fold for kidney, gill, and liver tissues, respectively). Exposure to 2.5% of HF-FPW-S also significantly increased TBARS formation in rainbow trout kidney tissue (1.99 ± 0.55-fold). There were no additional significant oxidative stress effects observed in either high or low exposure of HF-FPW-SF and HF-FPW-AC groups. Significant increases in TBARS formation were also seen in our positive control (BaP) treatments, validating our methodologies (Figure 3-2). Overall, it was observed that HF-FPW-S displayed increased TBARS formation, with highest oxidative stress responses seen in higher HF-FPW concentrations.

Interestingly, the results of the TBARS assay demonstrated significant intrinsic oxidative potential of HF-FPW samples (Figure 3-3). Incubation of 2.5% and 7.5% of HF-FPW-S with 4 mM 2-DR at 25 °C for 1 h resulted in significantly elevated TBARS formation by 8.68 ± 1.41, and 13.74 ± 0.59-fold, respectively, compared to control water. Similarly, 2.5% and 7.5% of HF-FPW-SF (incubation with 4 mM 2-DR at 25 °C for 1 h,) also resulted in significantly elevated TBARS formations (9.01 ± 1.12 and 13.13 ± 1.32-fold, respectively). No TBARS formation was observed in HF-FPW-AC (Figure 3-3). Both HF-FPW-SF and HF-FPW-S contain various organic contaminants including PAHs, while organic contaminants, including PAHs, in HF-FPW-AC are below detection limits (Table 3-2) (He et al., 2017a). Our results suggest that significantly increased oxidative potential is inherent to both HF-FPW-S and HF-FPW-SF, but not to HF-FPW-AC, presumably due to organic compounds present in HF-FPWs.

Oxidative stress is one of the major adverse effects on aquatic organisms of many contaminants (Valavanidis et al., 2006). Our study determined that raw HF-FPW at concentrations of 7.5% significantly increased TBARS formation (lipid peroxidation) in

kidney, gill, and liver tissue after acute exposures to HF-FPW (Figure 3-2). While endogenous production of reactive oxygen species (ROS) is essential to many cellular functions, increased ROS production by exogenous influences may overwhelm cell oxidative homeostatic and antioxidant defense systems, negatively impacting cell and organism physiological health (Valavanidis et al., 2006). The TBARS assay detects malondialdehyde (MDA), an aldehyde produced via lipid membrane oxidation and damage (Fraga et al., 1988). However, it is important to note that many other reactive lipid peroxide products, such as alkoxyls, alkanes, and lipid epoxides, which are known to be toxic and mutagenic (Esterbauer et al., 1990), may be produced. Many components of HF-FPW are theorized to induce oxidative stress upon exposure. Added oxidant chemicals in the fracturing fluid may elicit oxidative responses, while other chemicals secondarily formed in the subsurface may additionally increase oxidative stress to organisms exposed to HF-FPW. The intrinsic ROS potential of both the SF and S fractions were significantly greater than control as well as AC fraction, suggesting that the organic constituents are responsible for the majority of the ROS activity in HF-FPWs (Figure 3-3). However, we observed significant TBARS formation only in tissues of fish exposed to sediment containing fractions. This indicates that sediment constituents (including all the suspended particles and the chemicals potentially sorbed to them) further contribute to physiological oxidative stress and observed adverse effects in fish exposed to HF-FPWs (Figure 3-2). Our companion study suggested the suspended particles present in FPW might play a synergistic role in causing adverse effects by adhering on the fish body surface acting as a delivering vehicle to enhance exposure rate (He et al., 2017a). Another possibility is that the concentrations of contaminants were better maintained by desorption of chemicals from sediment particles, thus resulting in greater response in adverse effects in HF-FPW fish exposure. This hypothesis is supported by the fact that in HF-FPW samples, PAHs levels in sediment-containing samples were higher than nominal levels potentially due to desorption, while PAH levels in sediment-free samples were lower than nominal levels potentially due to dissipation. This response supports our EROD analyses showing higher EROD activity in gill and liver tissue of fish exposed to sediment-containing HF-FPW fractions over sediment-free fractions (Figure 3-1A and B). Nephritis, characterized by increased lipid peroxidation products, is a well-studied physiological inflammatory response observed in

kidney tissue under oxidative stress (Diamond et al., 1986). Correspondingly, significant TBARS formation in kidney tissue was observed after 48 h exposures to 2.5% and 7.5% HF-FPW-S (1.99 ± 0.55 and 2.95 ± 0.66-fold, respectively). Liver tissues are responsible for many detoxification and antioxidant defense responses to xenobiotics (Xu et al., 2005), and our results similarly displayed elevated liver TBARS formation after exposure to HF-FPW-S (7.5% after 48 h; 3.11 ± 0.97-fold). Gill tissue also displayed increased TBARS formation (2.12 ± 0.18-fold). Although the magnitudes of fold induction of TBARS formation in fish tissues were less significant than the intrinsic oxidative potentials observed in direct measurement of HF-FPWs, this does not necessarily imply that the TBARS formation observed in exposed fish tissues was less biologically significant. In fact, the elevated TBARS formation indicates that the oxidative stress generated by HF-FPWs exposure had already overwhelmed the ROS defense system. Rainbow trout tissue oxidative stress responses after HF-FPW exposure were consistent with various other tissue expression profiles observed in fish exposed to oil-contaminated wastewater effluent and spills (Holth et al., 2014), suggesting oxidative stress likely contributes to the overall adverse effects observed in the current study.

3.3.4 Quantitative Real-Time PCR Assays

Compared to the control, exposure to HF-FPW-S (2.5% and 7.5%) for 48 h significantly induced several tested genes in juvenile rainbow trout liver (Figure 3-4). In 7.5% of HF-FPW-S group, as compared to control, the expressions of *cyp1a* and *udpgt* were significantly elevated by 2.49 ± 0.38 and 2.01 ± 0.31-fold, respectively, indicating the activation of Phase I and Phase II biotransformation genes. In addition, in the 7.5% of the HF-FPW-S group, the expression of *sod* and *gpx* were also significantly elevated by 1.67 ± 0.09 and 1.58 ± 0.10-fold, respectively, indicating the activation of oxidative stress response genes. Moreover, the expression of *vtg* and *erα2* were significantly elevated by 7.60 ± 1.77 and 5.37 ± 1.70-fold, respectively, in the 2.5% of HF-FPW-S group, and 19.33 ± 4.89 and 12.33 ± 1.56-fold, respectively in the 7.5% of HF-FPW-S group, suggesting exposure to HF-FPW-S has the potential to alter endocrine metabolism in fish. There were no significant changes of mRNA abundance in the genes tested in the groups of AC and SF (2.5% and 7.5%) (Figure S3-3). Low and high doses of BaP (0.33 and 1 μM) were also

applied as positive controls to confirm the dose-dependent response of *cyp1a* in tested fish (Figure S3-4).

In the current study, mRNA abundance of seven genes related to biotransformation and oxidative stress were determined in juvenile rainbow trout exposed to HF-FPWs for 48 h. Metallothionein β (*mtβ*) is an enzyme important for metal metabolism, and is commonly used as bioindicator of metal exposure (Cobbett and Goldsbrough, 2002). Although there were nominal amounts of heavy metals detected in our sample, with the dilution applied in this study, the concentrations and predicted toxicity would be very limited as compared to other HF samples; (He et al., 2017a; Lauer et al., 2016), therefore, it was not surprising that exposure to HF-FPWs did not affect the expression of *mtβ*. Cytochrome p450 1A (*cyp1a*) and 3A (*cyp3a*) are Phase I biotransformation enzymes which are responsible for hydroxylation modification of xenobiotics (Xu et al., 2005). UDP-glucoronosyl transferase (*udpgt*) and glutathione transferase (*gst*) are Phase II biotransformation enzymes responsible for detoxifying reactive electrophiles and producing more polar metabolites for active transport (Xu et al., 2005). Exposure to HF-FPW-S caused significant induction in *cyp1a*. Together with the observed gill and liver EROD induction, we theorized the presence of AhR agonist(s) in HF-FPW caused activation of Phase I biotransformation. The absence of increased expression of *cyp3a* indicated that there were no or very low concentrations of *cyp3a* agonists (for example, dexamethasone) in the HF-FPW-S sample. However, a significantly increased expression of *udpgt* and *gst* suggests that exposure to HF-FPW-S also triggers Phase II enzymes, which taken together with Phase I enzyme induction results, indicates activation of biotransformation and detoxification processes of xenobiotics in juvenile rainbow trout, which may be used as a biomarker in future monitoring programs. Additionally, mRNA abundance of two oxidative response genes, glutathione peroxidase (*gpx*) and superoxide dismutase (*sod*) was also elevated in the HF-FPW-S exposure group. Oxidative stress in aquatic organisms is one of the most common adverse effects caused by exposure of petroleum-related contaminants in aquatic environments (He et al., 2012a; Valavanidis et al., 2006). In the current study, ROS may be generated from either activated xenobiotic metabolites generated in the Phase I biotransformation by cytochrome P450 and other oxidation enzymes, or potentially via the oxidizing chemicals originally presented in the fracturing fluid. In this well, ammonium

sulfate, a strong oxidizing agent, was added into the fracturing fluid to break the added antioxidant, Irgafos 168, and thus promote the fracturing process. The small but significant elevated expression of *sod* and *gpx* (magnitudes <2-fold), demonstrated the potential activation of ROS defense enzymes in response to HF-FPW exposure. These may be due to oxidative stress from either or both xenobiotic metabolism and oxidizing chemicals originally present in HF-FPW sample. Together with the TBARS results, the activation of *sod* and *gpx* demonstrated the oxidative stress response in juvenile rainbow trout exposed to HF-FPWs.

In this study, mRNA abundance of five endocrine disruptive genes, including *vitellogenin* (*vtg*) and four estrogen receptor isoforms (*erα1*, *erα2*, *erβ1*, and *erβ2*) were also measured. Vitellogenin, the precursor of lipoproteins and phosphoproteins which comprises most of the protein content of fish yolk, has been widely used as a sensitive biomarker on endocrine disrupting chemicals (EDCs) exposure in oviparous species (Ankley and Johnson, 2004; He et al., 2012b; Miller et al., 2007). It has been demonstrated that exposure to petroleum related contaminants can induce significant elevation of *vtg* transcripts and protein in fish (Ankley and Johnson, 2004; He et al., 2012b; Holth et al., 2014). Xenoestrogens can interact with the estrogen receptor (ER) to exert their endocrine disrupting effects in organisms. Rainbow trout has two distinct ER subtypes, ERα and ERβ, each of which has two distinct isoforms (Menuet et al., 2001; Nagler et al., 2007). In this study, Q-RT-PCR primers of four estrogen receptor isoforms were designed to determine differential receptor isoform responses to potential EDC exposure. Interestingly, exposure to HF-FPW resulted in significantly higher mRNA abundance of *vtg*, as well as *era2*, but not the other three *er* isoforms. In rainbow trout, the full-length estrogen receptor alpha (*era1*) is widely expressed in liver, brain, pituitary, and ovary. However, expression of the alternative splicing isoform resulting in a truncated A domain (*era2* in this paper) is primarily in the liver (Menuet et al., 2001). These results suggest a role for *era2* in the development and/or maintenance of the vitellogenesis process unique to the liver (Menuet et al., 2001; Nagler et al., 2007). In this study, the elevated expression of *vtg* and *era2* were consistent in rainbow trout exposed to HF-FPW-S, indicating the presence of EDCs (in particular, xenoestrogens) in HF-FPW that are able to disrupt and/or alter vitellogenesis synthesis in oviparous species. This study is the first

research reporting elevation of *vtg* and *era2* expression in fish caused by HF-FPW exposure. Significantly elevated expression of *vtg* and *era2* demonstrate possible endocrine disruptive effects derived from HF-FPW exposure and the potential hazards to the aquatic organisms from a HF-FPW spill. Future research should focus on the potential endocrine disruptive properties of HF-FPW including advanced chemical analyses to determine the potential EDC(s) in HF-FPW.

3.4 CONCLUSION

In conclusion, the current study demonstrates the complexity and diverse mechanisms of adverse effects of HF-FPW using rainbow trout as a model organism. Adverse effects were observed at high dilutions in both SF and/or S fractions exposure groups, rather than AC fractions exposure groups. This indicates that the organic contaminants rather than the salts *per se* were the major contributor in acute exposure of diluted HF-FPW in fish. Analysis of multiple biomarkers and gene expression for key markers of adverse effects reveal HF-FPW exposure in a biologically relevant fish elicits responses in a variety of pathways, including biotransformation, oxidative stress, and endocrine disruption. Our results further suggest that sediment found in HF-FPW is an important component in causing adverse effects related to biotransformation and oxidative stress pathways, in agreement with our earlier studies (He et al., 2017a). An alternative hypothesis is that the exposure concentration of various contaminants present in HF-FPW was elevated by chemical desorption from sediment particles, thus enhancing the exposure rate. Future study is needed to address the potential adverse effects derived from sediments of HF-FPW, and special attention should be paid to the sediment residues in spill response and the remediation process. This study is also the first to demonstrate potential endocrine disruptive effects associated with HF-FPW thereby warranting future investigation in this area.

Table 3- 1. A Summary of key composition of a HF-FPW sample. Data are obtained from a companion study (He et al., 2017a).

HF-FPW sample	
Time (Post-Stimulation)	7 days
pH	4.78
Total Dissolved Solid	242 624 mg/L
Total Nitrogen	498 mg/L
Total Organic Carbon	211 mg/L

Table 3- 2. Parent PAHs and Total PAHs (parent + alkylated) in HF-FPW-AC, HF-FPW-SF, and HF-FPW-S exposure waters at 24 and 48 h[a].

Sample		**Dilutions**	**Nominal Concn. (ng/L)**	**Measured Concn. (ng/L) At 24 h**	**At 48 h**
HF-FPW-AC	**Parent**	**2.5%**	N.D.	N.D.	N.D.
		7.5%	N.D.	N.D.	N.D.
	Total	**2.5%**	N.D.	N.D.	N.D.
		7.5%	N.D.	N.D.	N.D.
HF-FPW-SF	**Parent**	**2.5%**	13	30	19
		7.5%	39	31	17
	Total	**2.5%**	29	34	20
		7.5%	87	39	19
HF-FPW-S	**Parent**	**2.5%**	24	89	64
		7.5%	73	220	160
	Total	**2.5%**	51	150	110

	7.5%	150	390	300

[a]Nominal concentrations are calculated based on the results from a companion study (He et al., 2017a). All the PAHs in control and HF-FPW-AC samples were below the detection limit. N.D. means not detected.

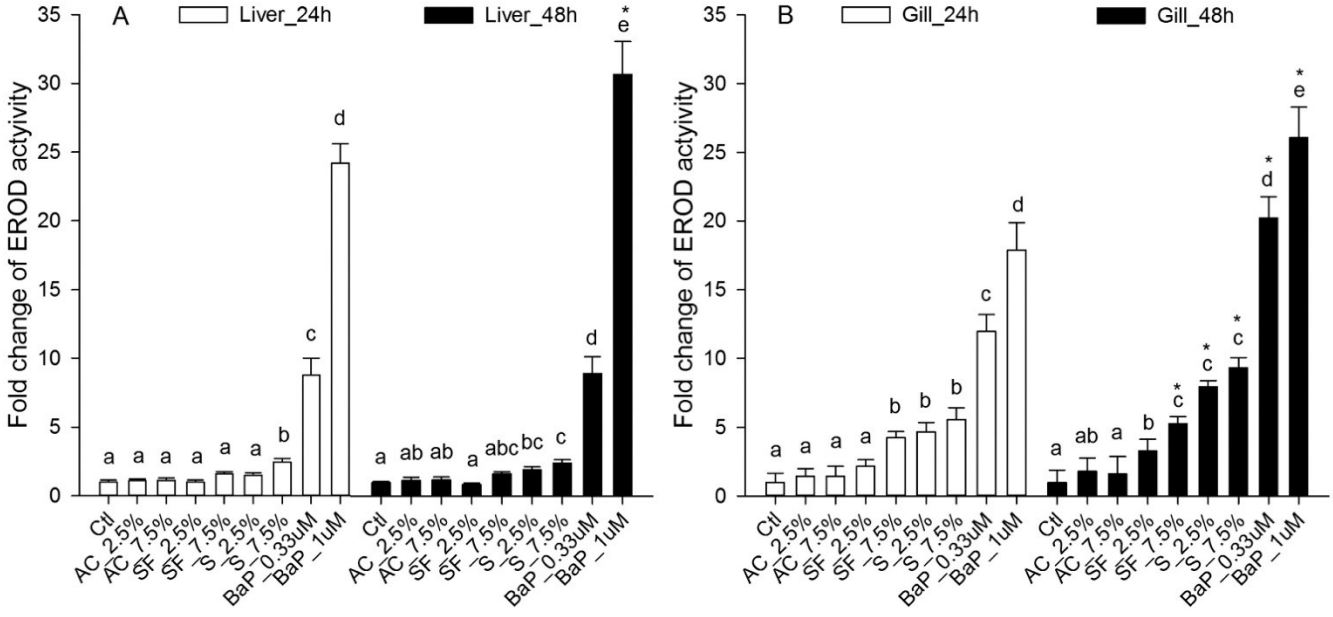


Figure 3- 1. Induction of (A) liver and (B) gill EROD activity in rainbow trout exposed to HF-FPWs at 2.5% and 7.5% dilutions for 24 and 48 h. BaP (0.33 and 1 μM) was used as positive control. Different letters indicate significant differences within the same treatment time in liver or gill tissues (n = 6, $\rho < 0.05$,). Asterisks indicate significant differences between 24 and 48 h treatments (n = 6, $\rho < 0.05$).

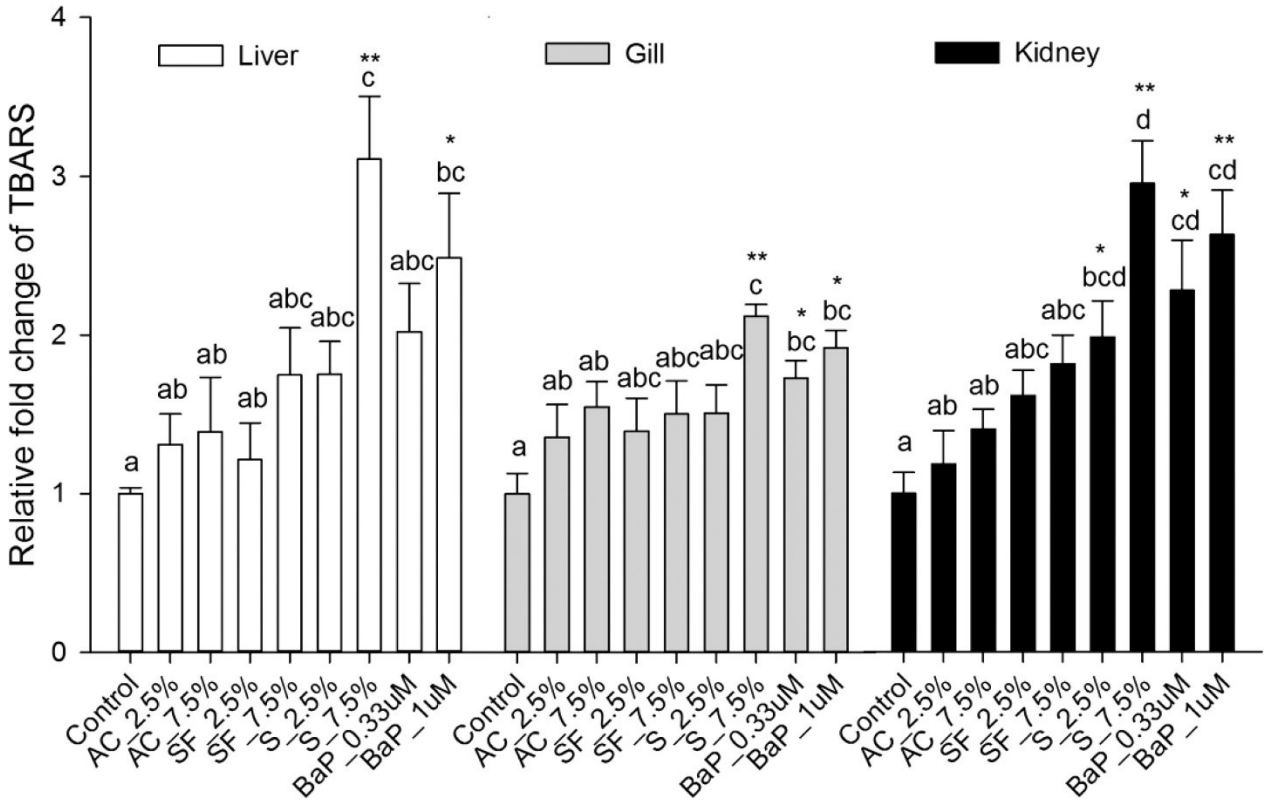


Figure 3- 2. TBARS in the liver, gill, and kidney tissue of rainbow trout exposed to control, HF-FPW-AC, HF-FPW-SF, and HF-FPW-S at 2.5% and 7.5% dilutions for 48 h. Asterisks ($n = 6$, *$\rho < 0.05$; ** $\rho < 0.01$) indicate significant differences from control. Different letters indicate significant differences within the same treatment time in liver or gill tissues ($n = 6$, $\rho < 0.05$).

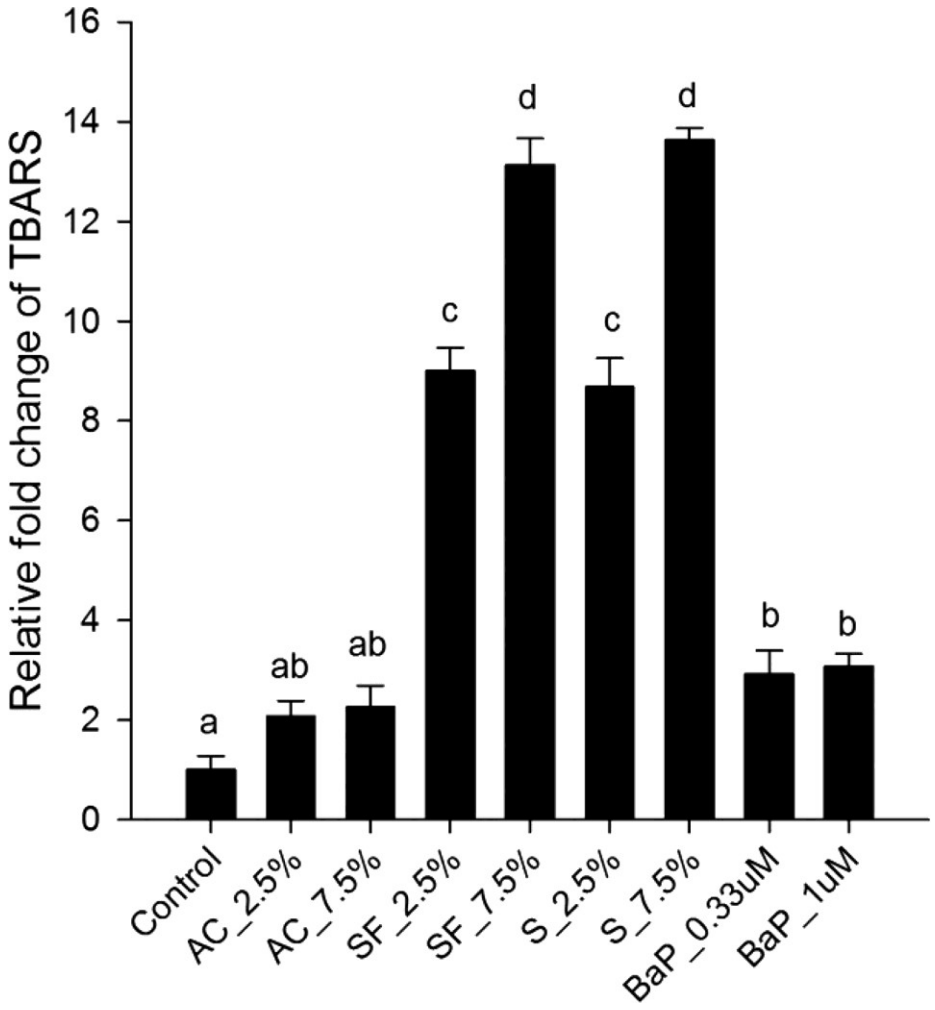


Figure 3- 3. TBARS formation in control as well as HF-FPW-AC, HF-FPW-SF, HF-FPW-S at 2.5% and 7.5% dilutions spiked with 4 mM 2-deoxy-d-ribose and samples taken for TBARS measurement after 1 h incubation under ambient light at 25 °C. Different letters indicate significant differences between treatment groups ($n = 6$, $\rho < 0.05$).

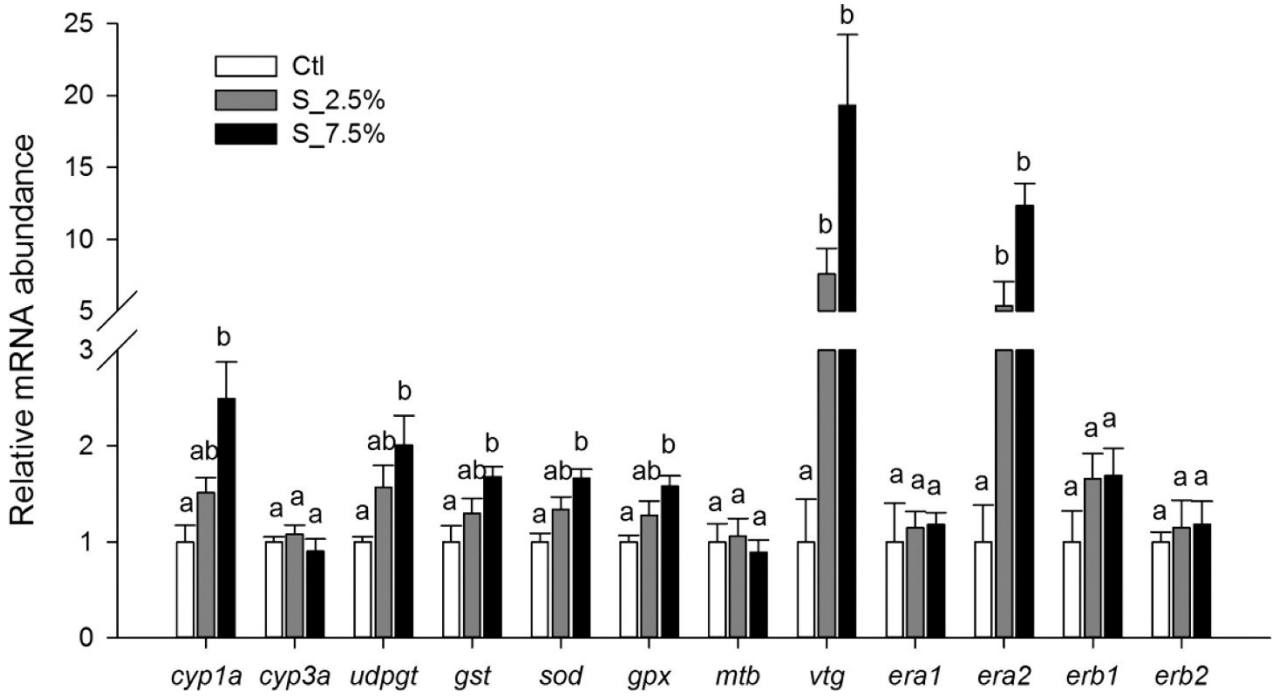
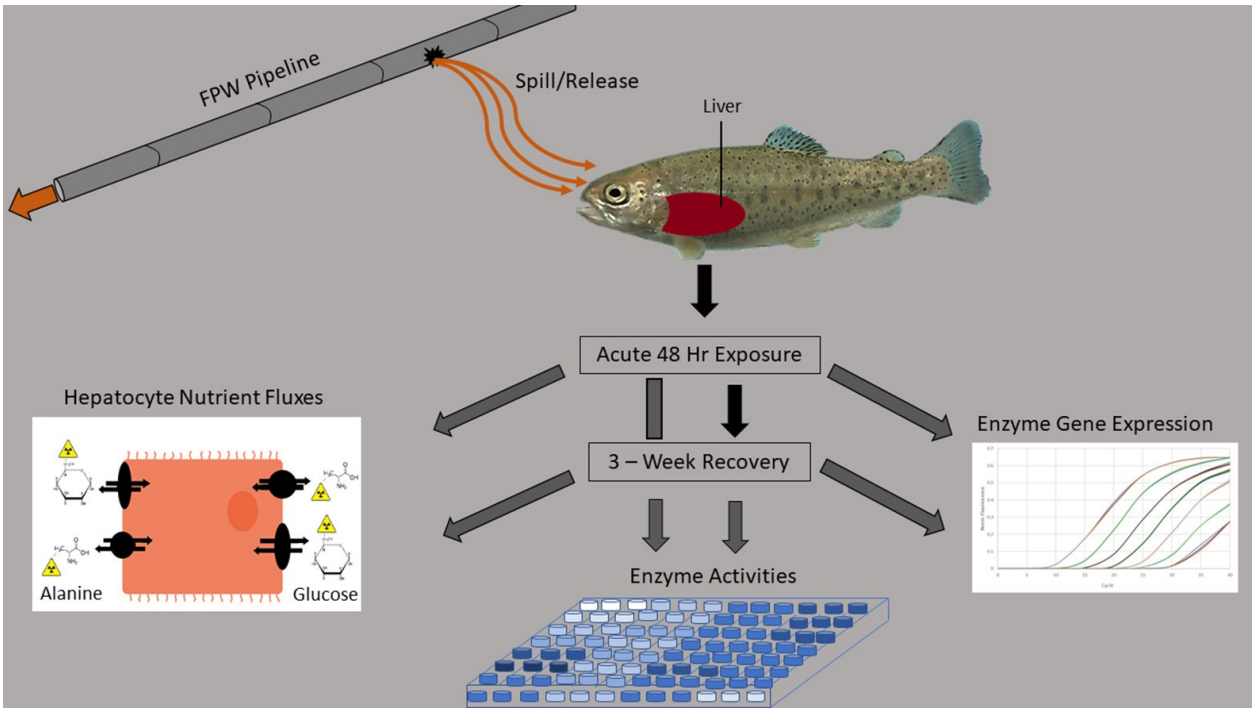


Figure 3- 4. Hepatic mRNA expression in rainbow trout exposed to control and HF-FPW-S (2.5% and 7.5%) for 48 h. Different letters indicate significant differences within the group (n = 6, $\rho < 0.05$).

CHAPTER 4: Changes to Hepatic Nutrient Dynamics and Energetics in Rainbow Trout (*Oncorhynchus mykiss*) Following Exposure to and Recovery from Hydraulic Fracturing Flowback and Produced Water*



Weinrauch, A.M., Folkerts, E.J., Alessi, D.S., Goss, G.G., and Blewett, T.A. (2021). Changes to hepatic nutrient dynamics and energetics in rainbow trout (*Oncorhynchus mykiss*) following exposure to and recovery from hydraulic fracturing flowback and produced water. *Science of the Total Environment*, **764**, 142893. DOI: 10.1016/j.scitotenv.2020.142893.

*Co-first Authorship

4.1 INTRODUCTION

Environmental impacts of unconventional oil and gas (UOG) operations have become a topic of increased environmental interest over the past couple of decades. The global use of UOG horizontal hydraulic fracturing practices has grown substantially and is projected to continue expanding in prevalence (Gagnon et al., 2016; Kondash et al., 2018). Potential environmental issues related to these hydraulic fracturing practices include impacts to groundwater aquifers (Osborn et al., 2011), increased seismic activity during drilling and disposal activities (Atkinson et al., 2016; Davies et al., 2013), and releases of hazardous volatile compounds into the air (Annevelink et al., 2016; Vinciguerra et al., 2015). However, an additional concern is the accidental release of flowback and produced water (FPW), wastewater by-products from hydraulic fracturing processes, to surface bodies of water (Folkerts et al., 2020a). Given the vast quantities of the initial injection fracturing fluid and maintenance water (a single well can use 10,000–100,000 m^3 of initial fluid) (Alessi et al., 2017), FPW returning to the surface from hydraulic fracturing activities can be quite substantial. It is estimated that between 2005 and 2014, 210 billion gallons of FPW was produced in the US alone (Kondash and Vengosh, 2015). Although hydraulic fracturing companies typically employ some form of FPW recycling/treatment regimen to lessen practice water burdens (Alessi et al., 2017), FPW must ultimately be treated, recycled, or transported for disposal. While strict water management practices banning environmental releases are in place for most jurisdictions, there are numerous incidences each year of accidental releases of FPW (Folkerts et al., 2020a; Goss et al., 2015; Ralston and Kalmbach, 2018). In shale plays of the eastern US between 2005 and 2010, average distances of drilled hydraulic fracturing wells from freshwater streams was determined to be ~300 m (Entrekin et al., 2011). This close proximity to freshwaters, coupled with large FPW production, emphasizes the need to critically assess the hazards of FPW release into aquatic systems.

FPW is a complex heterogenous hypersaline solution composed of the injection fracturing fluid chemicals, geogenic-derived metals and organic chemical species, and potential transformation chemical products from well-bore conditions (*i.e.*, high heat and pressure) (DiGiulio and Jackson, 2016; Folkerts et al., 2020a; He et al., 2017a; Lester et al., 2015). Many sub-lethal effects on aquatic organisms following FPW exposure have been

characterized and documented, including increased developmental deformities (Folkerts et al., 2017a; He et al., 2018a), impacts to cardio-respiratory functioning and performance (Folkerts et al., 2017a, 2017b, 2020a), reduced reproductive output (T. A. Blewett et al., 2017a), and altered gill morphology (T. A. Blewett et al., 2017b). The earliest reports of FPW-induced sub-lethal toxicities observed were of increased oxidative stress and xenobiotic metabolism (T. A. Blewett et al., 2017b; He et al., 2017a, 2017b). The vertebrate liver is the primary site of detoxification processes, where xenobiotics such as organic chemical species are metabolized and eventually excreted following Phase I and/or Phase II reactions (Grant, 1991; van der Oost et al., 2003; Xu et al., 2005). Exposure to FPW dilutions of 2.5% in zebrafish embryos (*Danio rerio*) and 7.5% in rainbow trout (*Oncorhynchus mykiss*) have been shown to increase ethoxyresorufin-O-deethylase (EROD) activity (T. A. Blewett et al., 2017b; He et al., 2017a, 2017b), an extensively used biomarker assay for the cytochrome P450 1A (CYP1A) metabolic enzyme and aryl hydrocarbon receptor (AhR) agonistic activity (Whyte et al., 2000). Liver tissue from trout exposed to FPW has also shown increased levels of *cyp1a*, *udpgt* (uridine 5′-diphospho-glucuronosyltransferase), and *gst* (glutathione *S*-transferase) mRNA expression (He et al., 2017b), indicating Phase I and II metabolism induction. In addition to xenobiotic transformation, the liver is a key metabolic organ involved in the storage and mobilization of energy (Rui, 2014). Glucose is stored post-prandially as glycogen but may also be converted to fatty acids or amino acids for downstream energetic use. Furthermore, glycogenolysis and glycolysis result in the catabolism of glycogen and glucose, respectively, into pyruvate for ATP generation (Rui, 2014). During periods of fasting, *de novo* glucose synthesis (gluconeogenesis) occurs from multiple precursors, including pyruvate, lactate, and/or amino acids, the latter of which can also be utilized to provide energy directly (Rui, 2014). Given that the liver is a multifaceted organ involved in multiple roles from Phase I and II metabolic processes to energy production and storage, we hypothesized that following FPW exposure, there is a metabolic trade-off wherein hepatic nutrient metabolism is altered as energy is diverted towards detoxification.

Rainbow trout are a useful bioindicator species for aquatic pollution and have an environmentally relevant habitat range that geographically overlaps with potential hydraulic fracturing activities and FPW spills (Alessi et al., 2017; Kondash et al., 2018;

MacCrimmon, 1971). To assess the metabolic capacity of the liver, we used radiotracers to measure the hepatocyte nutrient uptake capacity of both glucose and alanine in rainbow trout. We complemented these studies with measurements of gene expression and activity of key enzymes involved in hepatic metabolism (pyruvate kinase [PK] - glycolysis; phosphoenolpyruvate carboxykinase [PEPCK] – gluconeogenesis; glutamate dehydrogenase [GDH] – amino acid catabolism; lactate dehydrogenase [LDH] – anaerobic metabolism). Further assessment of cellular health was conducted by histological analysis of liver tissues. To examine the potential for effects of exposure and also for the ability to recover, we analyzed the above endpoints after both an acute 48 h FPW exposure and after a 3-week recovery period in control freshwater.

4.2 MATERIALS AND METHODS

4.2.1 Hydraulic Fracturing FPW Sample

For this study, a hydraulic fracturing FPW sample collected from a horizontal hydraulically fractured well in the Devonian-aged Duvernay formation (around Fox Creek, AB, CAN) was employed for all whole effluent FPW exposures. This FPW sample was collected 5 h-post well stimulation (when the well was brought into production) and put into 20 l, air-tight sealed industrial food-grade poly-ethylene plastic buckets, transported to the University of Alberta, and stored at room temperature until use. Inorganic characterization of the FPW sample (Table S4-1) was performed *via* inductively coupled plasma (ICP) – MS/MS using an Agilent 8800 Triple Quadrapole ICP-MS-MS. FPW polycyclic aromatic hydrocarbon (PAH) analysis (Table S4-2) was performed using gas chromatography – MS (GC–MS) with selective ion monitoring. Specifications concerning both ICP-MS/MS and GC–MS analyte quantification methods, instrument details, detection limits, and precision/accuracy can be found in the SI (see also Tables S4-3–5).

4.2.2 Animal Maintenance

Juvenile rainbow trout (hepatocyte fluxes: 136.5 ± 31.2 g, 23.0 ± 2.4 cm; enzymatic and molecular assays: 13.7 ± 8.2 g, 10.4 ± 1.8 cm) originally obtained as embryos from the Raven Creek Trout Brood Station (Caroline, AB, CAN) were reared indoors in flow-

through 500 l tanks in dechlorinated University of Alberta facility freshwater (moderately hard: $[Na^+]$ = 14.6 mg/l, $[Ca^{2+}]$ = 55.9 mg/l, $[Mg^{2+}]$ = 15.3 mg/l, $[K^+]$ = 2.5 mg/l, pH: 7.9, conductivity: 395 ± 0.5 µS/cm, dissolved oxygen: 7.5 ± 0.5 mg/l, general hardness: 186 mg/l as $CaCO_3$, salinity: 0 ppt). Rearing/maintenance tank flow-through water was kept at 10 ± 1 °C with constant aeration and on a 14 h light:10 h dark photoperiod. Trout were fed daily a satiating amount of EWOS Vita 2 mm (smaller fish for enzymatic and molecular analyses) or 4 mm (larger fish for flux analyses) pellets (Cargill Inc., MN, USA). All animal use was approved by the University of Alberta Animal Care Committee (protocol #AUP00001334).

4.2.3 Exposure Design

For all exposures, rainbow trout (fasted 48 h prior) were exposed for 48 h in 10 l tanks (2 fish per tank for hepatocyte flux exposures, 5 fish per tank for enzymatic analyses exposures) to either control freshwater, a low 2.5% or high 7.5% diluted FPW solution or saline control (SC) which contained the same major cation and anion concentrations (Na^+, Ca^{2+}, Mg^{2+}, K^+, and Cl^-) as the FPW sample (see Table S4-1) and was diluted to 7.5% to ionically match the higher FPW exposure. Exposures to SC were performed to control for any potential salinity-induced effects. Chosen FPW dilutions match what have been used in previous studies which are both sub-lethal and, at 7.5%, have induced toxicological effects before (T. A. Blewett et al., 2017b; Folkerts et al., 2019; He et al., 2017b). All dilutions (FPW and/or SC) were made using dechlorinated University of Alberta facility water. During exposures, all tanks were outfitted with constant aeration, and checks for fish mortality were made every 24 h. Details on water exposure chemistry and organism morality can be found in Table S4-6. FPW dilutions were chosen based on FPW lethal concentration causing 50% mortality (96 h LC_{50}) values of similar FPW samples and dilutions used in previous studies (Folkerts et al., 2019; He et al., 2017b). All exposures were conducted with six replicates per treatment at 10 ± 1 °C and on a 14 h light:10 h dark photoperiod, and no feeding occurred. Following 48 h exposures, fish were immediately euthanized *via* cephalic blow and decapitation (and subsequently liver tissue was harvested for analyses), or they were transferred to 'recovery' tanks (85 l and 200 l tanks for enzymatic/molecular fish and hepatocyte flux fish, respectively). Recovery tanks were

maintained on fresh, flow-through dechlorinated University of Alberta facility water for 3-weeks at 10 ± 1 °C. Seventeen days into this recovery period, fish were fasted to ensure that by the end of the recovery period, equal periods of fasting occurred between the two exposure-type groups (48 h fasting + 48 h exposure = 96 h total). At the end of 48 h and 3-weeks of recovery, fish were euthanized, and tissues were harvested. For enzymatic/molecular fish, livers were immediately harvested and flash-frozen in liquid nitrogen, whereas hepatocyte flux fish had caudal blood samples collected prior to liver perfusions and hepatocyte isolation (see section 4.2.4).

4.2.4 Primary Hepatocyte Isolation

Primary hepatocytes were collected using previously established protocols (Mommsen et al., 1994). Chemicals used for all employed assays and analyses were obtained from Millipore Sigma (MS, USA) unless otherwise stated. Briefly, following perfusion with modified Hank's medium (Sol 1 in mM: 137 NaCl, 5.4 KCl, 0.81 $MgSO_4$, 0.44 KH_2PO_4, 0.33 Na_2HPO_4, 5 $NaHCO_3$, 10 HEPES, pH 7.6) into the portal vein, collagenase-containing (~750 U/ml) Hank's medium (Sol. 2) was perfused through for 15 min. The liver was then minced with a razor blade and incubated on a shaking plate at room temperature for 1 h. The cells were passed sequentially through coarse (250 μm), then fine (50 μm) filters, and then centrifuged (200 ×*g*, 4 °C, 4 min). The supernatant was then discarded, and the pellet gently resuspended in modified Hank's solution containing 2% bovine serum albumin and 1.5 mM $CaCl_2$ (Sol 3). The cells were washed and centrifuged (200 ×*g*, 4 °C, 4 min) an additional four times, with the supernatant discarded each time and the pellet resuspended in Sol 3. After the final wash, cells were assessed for viability using Trypan blue exclusion, counted using a hemocytometer, and diluted to the desired concentrations.

4.2.5 Hepatocyte Nutrient Fluxes

Immediately following isolation, the hepatocytes were mixed (1:1, v:v) with the appropriate nutrient fluxing solution. Initially, concentration-dependent studies were run with 0–8 mM glucose in Sol 3 with 0.5 μCi/ml ^{14}C d-glucose (Perkin Elmer) and 0–1 mM alanine in Sol 3 with 0.2 μCi/ml ^{14}C L-alanine (Perkin Elmer). These experiments showed

concentration-dependent uptake (data not shown). Therefore, all subsequent fluxes were conducted using 2 mM glucose or 0.8 mM alanine (*i.e.*, concentrations that yielded less than the measured half-maximal transport rate to ensure increases/decreases in transport could be observed). To terminate the flux period, 1 volume of stop solution (glucose = 100 mM glucose with 4 mM phloretin in Sol 3, (Craik and Elliott, 1979); alanine = 100 mM alanine in Sol 3) was added to the hepatocyte mixture. The solutions were gently mixed by pipetting and centrifuged (300 ×*g*, 30 s). The supernatant was discarded, and the pellet was resuspended in 1 ml of stop solution before second centrifugation (750 ×*g*). The supernatant was removed entirely, and the weight of the remaining pellet was determined. The cells were then suspended in minute volumes of Sol 1 and transferred to vials containing 4 ml Optiphase scintillation cocktail (Perkin Elmer, USA). The samples were vigorously agitated and left overnight in the dark prior to recording the counts per minute within each sample (LS6500 Beckman Coulter). The rate of hepatocyte nutrient uptake (J) was calculated as follows: J = CPM/SA×M, where *CPM* is the counts per minute within each sample, *SA* is a measure of the specific activity (CPM per mmol of nutrient), and *M* represents the mass of the pellet (mg).

4.2.6 Hepatic Enzyme Assays

For all enzyme assays (unless otherwise noted), frozen liver tissue was pulverized under liquid nitrogen (Mommsen et al., 1980). Pulverized samples were homogenized in ice-cold homogenization/extraction buffers and centrifuged. Aliquots (5–40 µl depending on the assay) were then plated in triplicate into 200 µl of assay buffer. Kinetic assays measuring the conversion of NADH to NAD^+ were measured using a microplate spectrophotometer (Spectromax 190, Molecular Devices, Sunnyvale, CA, USA) in clear, flat-bottom 96-well microplates for all enzymatic assays except for EROD assays. For EROD assays, aliquots were plated in 96-black well, clear bottom plates and background fluorescence was read at 535 nm excitation/590 nm emission using a Victor3V Multilabel Counter (Perkin Elmer, USA). Values are expressed as relative fold-change to control. All enzymatic activity was normalized to each respective sample's homogenate protein content using Bradford reagent as per manufacturer's instructions (Millipore Sigma, USA).

Specifics pertaining to each kinetic assay procedure can be found in the Supporting Information (SI).

4.2.7 Determination of Plasma Glucose Content and Osmolarity

Plasma (20 µl) was plated alongside 250 µl glucose assay buffer (22 mM $MgCl_2$, 1.5 mM NAD, 2 mM ATP, 2.4 U/ml glucose-6-phosphate dehydrogenase all dissolved in 250 mM triethanolamine hydrochloride) and an initial read was obtained at 340 nm. Hexokinase (5 U/ml) was added to each sample, and following a 15 min incubation period, the plate was re-read at 340 nm. The glucose concentration is proportional to the change in absorbance and is calculated using a glucose standard curve. Plasma osmolality was measured using a Vapro vapour pressure osmometer (Model 5520; Wescor, Logan, UT, USA).

4.2.8 Quantitative Real-Time PCR

Total RNA was extracted from trout liver tissue, and cDNA was prepared for quantitative real-time PCR (qPCR) using SYBR green master mix analyses (Applied Biosystems, USA). Information on RNA extractions, cDNA synthesis, and qPCR reactions can be found in the SI. Information on gene targets, primer sequences, and accession numbers can be found in SI Table 7. Transcript expression changes were determined through ΔΔCT analysis (Schmittgen and Livak, 2008) using TATA-box binding protein associated factor 12 (*tata12*) as an endogenous control. See Figure S4-1 for details of endogenous control stability and primer efficiency validations.

4.2.9 Tissue Fixation and Histology

Upon dissection, liver samples were immediately placed in 4% paraformaldehyde (pH 7.4) on ice and fixed overnight at 4 °C. The following day, samples were placed into two washes of 70% ethanol prior to serial dehydration and paraffin embedding. The samples were sectioned on a microtome (7 µm) and placed on glass slides prior to staining with hematoxylin and eosin (H&E). To eliminate observer bias, a blind set-up was utilized and, stained tissues were viewed under a Zeiss Scope A1 microscope (Zeiss, GER) with images captured on an optronic camera.

4.2.10 Statistics

The normality of all data was assessed using Shapiro-Wilk test, while data was tested for equal variance *via* Brown-Forsythe, and datasets were transformed if necessary. For all statistical and plotting analyses, Prism v.6.0 software (GraphPad Software Inc., USA) or SigmaPlot 13 (Systat software Inc., USA) were used. Data were assessed using one-way ANOVAs with treatment condition as a factor. Holm-Sidak *post-hoc* multiple comparisons were then performed to determine significant differences between treatment conditions, where significance was concluded when $p < 0.05$. All data are presented as mean ± SEM.

4.3 RESULTS

A 48 h exposure to all treatments (SC, both FPW dilutions) resulted in a significant increase in plasma osmolality immediately post-exposure (338 ± 2 mOsm) in comparison to control treatments (317 ± 4 mOsm; $p = 0.001$). Following a 3-week recovery period in control conditions, no significant differences were noted between FPW or SC treatments (Figure 4-1A). Plasma glucose was not altered between exposure groups after either 48 h (1.2 ± 0.04 mM) or 3 weeks of recovery (1.4 ± 0.08 mM; Figure 4-1B).

A significant ~3.2 and 8.8-fold upregulation in EROD activity compared to freshwater controls was observed in trout S9 fractions from FPW 2.5% and 7.5% liver samples, respectively ($p = 0.008$). However, after 3 weeks, EROD activity in all treatments had returned to control levels (Figure 4-2A). Expression of hepatic *cyp1a* was significantly increased in both FPW 2.5% and 7.5% treated fish following 48 h of exposure, with the highest significant expression in FPW 7.5% tissue samples ($p < 0.001$). However, after the 3-week recovery period, *cyp1a* transcript expression returned to control levels. Expression of *cyp1b1* was unchanged between treatment groups of both 48 h and 3-week recovery fish liver samples (Figure 4-2B).

Control and SC-treated livers presented standard histology, including a homogenous basophilic cytoplasm, round nuclei with a centrally located nucleolus, weak nuclear polarization, and occasional ‘rosettes’ of 6–8 hepatic cells (Figure 4-3A–D). Evidence of cytoplasmic vacuolation was observed in the C/SC fish in the 3-week recovery fish (Figure 4-3B, D). Histological aberrations were noted within the FPW-treated fish

wherein cytoplasmic vacuolation was noted in the 2.5% FPW exposed fish after 48 h (Figure 4-3E, F). The morphological abnormalities appeared exacerbated in the fish exposed to the 7.5% FPW and persisted throughout the 3-week recovery (Figure 4-3G, H).

Following 48 h exposures, trout hepatocyte glucose uptake was increased 6.8- and 12.9-fold in FPW 2.5% and 7.5% exposed fish, respectively ($p < 0.001$). Significant decreases (2.4-fold) were also observed in SC exposed fish following 48 h exposure ($p < 0.001$). However, after the 3-week recovery period, no significant differences were noted between treatments (Figure 4-4A). A significant decrease was observed for hepatocyte alanine uptake in FPW 2.5% exposed fish relative to controls after 48 h ($p = 0.002$). However, after the 3-week recovery period, only the 7.5% FPW-exposed fish showed a significant 39% decrease in uptake rate ($p = 0.02$; Figure 4-4B).

Relative PK and LDH activity remained unchanged from controls both after 48 h and 3 weeks (Figure 4-5A, D). FPW exposure generally decreased liver PEPCK activity, with a 30% decrease observed following 48 h exposure to 2.5% FPW ($p = 0.04$) and ~ 20% decrease in FPW 7.5% exposed fish at the end of the 3-week recovery period ($p = 0.03$; Figure 4-5B). Conversely, after the 3-week recovery period, FPW treatment generally increased trout liver GDH activity, with statistically significant increased activity observed in FPW 7.5% samples compared to SC only (~2-fold change, $p = 0.02$; Figure 4-5C). However, no significant differences in GDH activity were observed immediately following the 48 h exposure period (Figure 4-5D).

Despite general trends of reduced hepatic *pepck* transcript expression and increased *gdh* transcript expression in FPW exposed fish, there was no statistically significant determined change in transcript expression of any nutrient/energy dynamic-related enzymes across treatment groups of the 48 h or 3-week recovery trout liver samples (Figure 4-6A–D).

4.4 DISCUSSION

The effects of FPW on fish after an -acute exposure (48 h) were studied to determine how organismal energetics and nutrient handling may be affected by FPW exposure. Furthermore, rainbow trout recovery capacities were additionally investigated by observing changes to fish energetics and nutrient handling following a 3-week recovery

period after initial acute FPW exposures. Exposure to FPW resulted in both an increase in detoxification enzymes and concomitant alterations in hepatic metabolism and nutrient transport. However, within 3 weeks, these effects had been mitigated and generally returned to control values.

The highly saline nature of FPW (> 6× seawater) is well documented and is a significant factor governing toxicity in several freshwater organisms (T. A. Blewett et al., 2017a, 2017b; Delompré et al., 2019; DiGiulio and Jackson, 2016; Folkerts et al., 2019, 2020a; He et al., 2017a, 2017b; Lester et al., 2015). Herein we report the expected outcome of elevated plasma osmolarity following exposure to FPW (high in ionic content; Table S4-1) and the salt-matched controls (Figure 4-1). This osmotic stress was not accompanied by alterations to fish plasma glucose, a result contrasting previous studies of teleost exposure to petroleum products (Simonato et al., 2011, 2008). Vertebrate glucose concentration is a tightly regulated biological process, whereby plasma glucose content is maintained within a narrow range through the mobilization and deposition of glucose stores (reviewed in Polakof et al., 2011). The apparent lack of trout plasma glucose response may indicate that changes to energy store mobilization and dynamics within the exposed organism are still occurring, likely originating at the liver given its role as the principal energy storage organ.

FPW contaminant exposure often elicits changes in the liver as a result of the xenobiotic transformation that occurs within this organ. After exposure to both dilutions of FPW (2.5% and 7.5%), we observed a significant increase in liver EROD activity and *cyp1a* gene expression in the 48 h treatment only (Figure 4-2). This is an expected effect of exposure to FPW, given the presence of PAH's in these samples (Table S4-2). These data are also consistent with previously published reports in this species wherein 48 h exposure induced significant increases in EROD activity after exposure to 7.5% FPW (T. A. Blewett et al., 2017b; He et al., 2017b). Likewise, similar increases in *cyp1a* mRNA expression following FPW exposure in trout have also been observed (He et al., 2017b). Parallel increases in both *cyp1a* mRNA and EROD activity are indications of aryl hydrocarbon receptor (AhR) binding and Phase I metabolism, processes which were rectified following the 3-week recovery period. These biochemical alterations are often accompanied by cytological modifications (Au et al., 1999; Hinton et al., 2008), which may

impact hepatic function and lead to decreased individual fitness. Furthermore, the high concentration of a some metals (*e.g.* Fe, Sr, and Ba) in our FPW sample (see Table S4-1) may have additionally contributed to hepatotoxic effects, as has been seen in other studies (Atli and Canli, 2003; Liu et al., 2019). However, given the dilutions of FPW used presently, metals are thought to have played a reduced role in hepatotoxic events. Histopathological changes were noted after 48 h in the FPW exposed fish only, indicating that those changes were likely driven by the organic content, as SC exposed trout displayed liver parenchyma consistent with that of control animals (Figure 4-3). In the FPW exposed groups, there was evidence of cytoplasmic vacuolation with additional vacuolation evident in the 3-week recovery fish from all treatment groups, including the control. Liver tissue vacuolation may be indicative of energy depletion and inhibition of protein synthesis (Hinton and Lauren, 1990) or be associated with increased glycogen or lipid energy stores (Schwaiger et al., 1997; Wolf and Wheeler, 2018). Given the progressive appearance of vacuolization only observed in FPW-exposed fish following the 48 h exposure, it is reasonable to conclude that these fish may experience heightened energy demands given the added burden of detoxification (leading to depletion of reserves) (Beyers et al., 1999; Gourley and Kennedy, 2009). Vacuolation observed in 3-week recovery control fish may be caused by energy allocation processes during this period of growth. Captive fish tend to have a relatively high abundance of vacuolation due to excess energy provisions (*i.e.,* feed rations) relative to the physical demands of captive life (Ferguson, 2006; Gingerich, 1982; Wolf and Wheeler, 2018). Therefore, regular feedings during this 3-week recovery period may have provided additional 'excess' and resulted in the heightened appearance of vacuolation.

Alterations to liver morphology, however, do not clearly indicate if a metabolic impact is occurring in an organism. If the liver itself is responding to increased energetic demand and a need for energy mobilization, cellular nutrient sensitivity and uptake are expected to change accordingly. Despite more variable flux patterns observed with the amino acid alanine, hepatocytes from FPW exposed trout displayed a general decrease in alanine uptake and a clear trend of increased cellular glucose uptake (Figure 4-4A, B). It is possible the FPW-exposed fish consumed more hepatic glycogen stores to maintain systemic glucose levels during times of increased xenobiotic metabolic processing prior to

flux analyses. This glycogen consumption would limit internal glycogen-mediated cellular glucose production potential and thereby enhance the glucose gradient between the extracellular and intracellular environments when compared to C/SC animals. A larger gradient would stimulate uptake rates to compensate for diminished cellular glucose levels and depleted glycogen stores. Comparatively, within C/SC animals, hepatocytes would continue to liberate glucose within the cell and diminish inward-directed glucose gradients. Alternatively, increased hepatocyte glucose uptake in FPW-exposed fish could be in response to maintaining plasma glucose levels, or an increased cellular energetic need, or a combination of both. To identify whether key metabolic changes were occurring, we analyzed the activity of enzymes important in multiple cellular energy pathways.

Notably, fish were able to maintain aerobic metabolism as LDH activities, and *ldh* expression were unchanged across all exposure regimes (Figures 4-5D, 4-6). Glycolytic activity was also unchanged across treatment groups regardless of time, as evidenced by PK activity and *pk* mRNA abundance (Figures 4-5A, 4-6). It is possible that in this study, hepatocytes (even those isolated from fish exposed to FPW) had sufficient glucose to act as a substrate for glycolysis throughout the exposure regime. Indeed, the consistent levels of plasma glucose suggest that glucose homeostasis was maintained likely *via* gluconeogenesis or glycogenolysis. Gluconeogenesis, measured by the pathway's rate-limiting enzyme PEPCK, was generally decreased following FPW exposure both in terms of activity and mRNA expression (Figures 4-5B, 4-6), although the latter was not statistically significant. It has previously been shown that glucose can disrupt gluconeogenesis while leaving PK activity unaffected (Feliu et al., 1976). However, for glucose to inhibit gluconeogenesis while not affecting PK activity, intracellular levels must be sufficient to maintain metabolism after FPW exposure; a situation seemingly in disagreement with our aforementioned increases in hepatocyte glucose uptake rates, which suggests these cells require additional energy. Perhaps FPW exposure elicits a greater energy demand, leading to the consumption of glycogen in hepatocytes. Then, when given excess glucose, these cells readily acquire glucose to replenish glycogen stores. Indeed, such glycogen depletion is a common adaptive hepatocyte response to xenobiotic exposure (Arnold, 1995; Eurell and Haensly, 1981; Gluth and Hanke, 1985; Hinton et al., 2008). Unfortunately, enzymes contributing to glycogenesis and/or glycogenolysis were not

examined within this study but represent an avenue for potential research. Elevated GDH activities observed after 3 weeks (Figure 4-5C) may likewise contribute to gluconeogenesis by providing additional substrates in the form of amino acids (French et al., 1981; Walton and Cowey, 1982). This compensatory action is likely required as other energy stores are depleted with the added stresses of detoxification alongside decreased PEPCK activity/*pepck* expression.

When considered in an environmental context, it must be recognized that the experimental fish used in the current study were held in a captive environment where nutritional food was consistently provided, and energy exertion was minimal. Most wild fish undergo periods of food deprivation as a result of fluctuations in the food supply, season, and/or spawning migration (Hemre et al., 2002). Rainbow trout populations are found across North American regions which experience seasonal climatic fluctuations. Changes to abiotic factors (such as temperature and sunlight) are known to affect numerous fish behavioral and physiological parameters, including fish feeding rates, activity, spawning behavior, and cellular nutrient dynamics (Berg and Bremset, 1998; Bremset, 2000; Gordon and McLeay, 1978; Jain, 2003; St-Pierre et al., 1998). Further, pre-fasting diets have been shown to have a profound impact on fish metabolism at the onset of fasting (Hilton, 1982). Since hydraulic fracturing practices also occur and are expected to intensify in these same North American regions (Alessi et al., 2017; Gagnon et al., 2016; Kondash et al., 2018), wild fish without sufficient energy stores could react more adversely to acute or chronic FPW exposures. Thus, results observed immediately following acute exposures (and additionally following recovery periods) may be exacerbated in wild organisms.

4.5 CONCLUSIONS

Overall, our results indicate that while acute exposure to FPW may lead to some alterations in hepatic metabolism, metabolic homeostasis is relatively achieved after a 3-week recovery period. By analyzing hepatocyte alanine and glucose flux, recording gross hepatic tissue morphological aberrations, and determining changes to key energy homeostasis enzyme expression and activity, we demonstrate that FPW exposure elicits immediate impacts on the nutrient handling physiology in affected organisms which may be corrected for following sufficient recovery. However, whether these organisms achieve full naivety to the exposure following certain recovery periods is unknown. These are key

ecotoxicological elements to consider when assessing potential impacts of hydraulic fracturing to environments where spills of FPW into surface waters occur. In light of our results, we suggest further examination of potential FPW effects on natural populations should be undertaken to more fully understand and appreciate how FPW exposure changes organism energy dynamics and nutrient handling. This study is the first to examine the effects of FPW on nutrient and energy dynamics in this key indicator species. Our results may serve to better inform regulators of seasonal periods and environmental conditions where even short term non-lethal exposures to hydraulic fracturing (and risks of spills associated with such activity) may prove detrimental to organisms and the environment.

The Chapters of my thesis thus far have focused on investigating aquatic organism baseline toxicity and characterizing numerous crucial sub-lethal toxicological responses of fish acutely exposed to FPW. In the following Chapters of this thesis, focus shifts to investigating the potential for sub-lethal exposures to impact cardio-respiratory development and function in fish, and whether FPW-mediated impacts to cardio-respiratory parameters result in reductions to overall fitness in a variety of fish species. Using tools developed (e.g. swim tunnels) to investigate detrimental effects to fish fitness from even short term sub-lethal exposures, research performed in the following Chapters of this thesis provide a comprehensive examination of immediate and latent effects of FPW exposure on cardio-respiratory physiology in fish at multiple levels of biological organization.

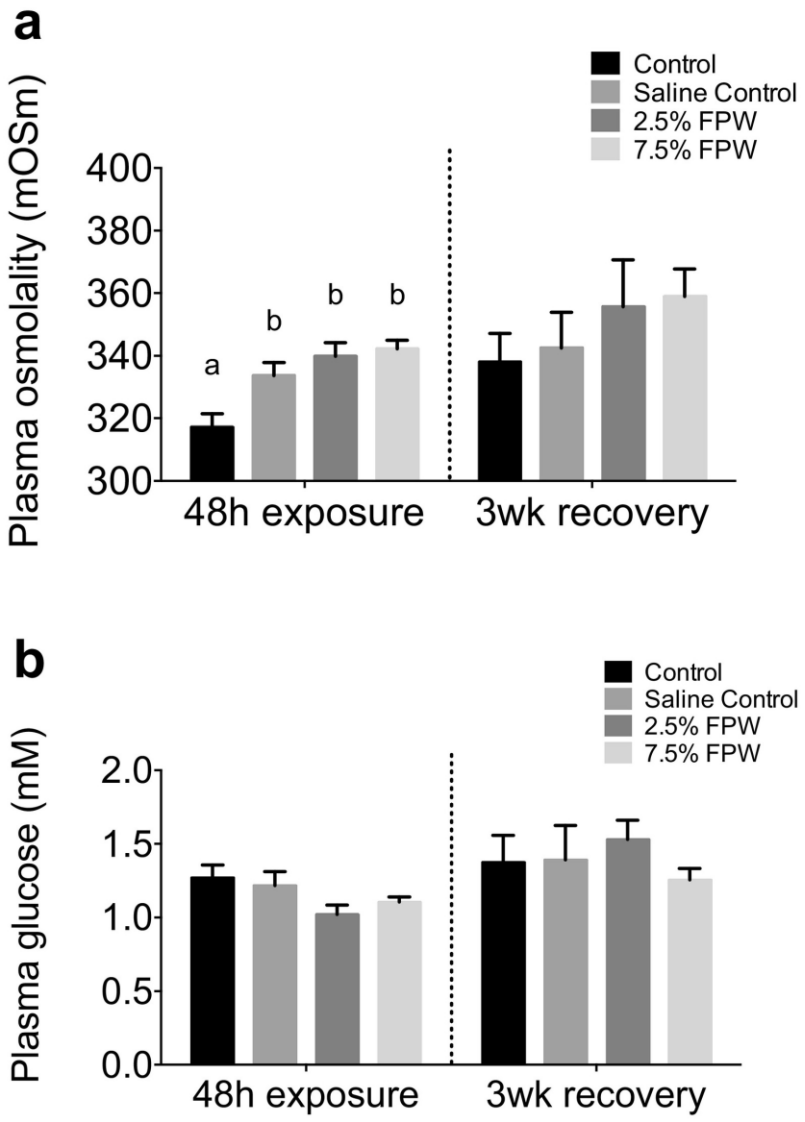


Figure 4- 1. Plasma osmolality (a) and glucose (b) content measured in rainbow trout after a 48 h exposure to control, saline control, a 2.5% dilution of hydraulic fracturing flowback and produced water (FPW), or a 7.5% dilution of FPW immediately following exposure or after a 3-week recovery period in control water. Data are presented as means + SEM (n = 5–6). Different letters denote a significant difference resulting from FPW exposure ($p < 0.05$).

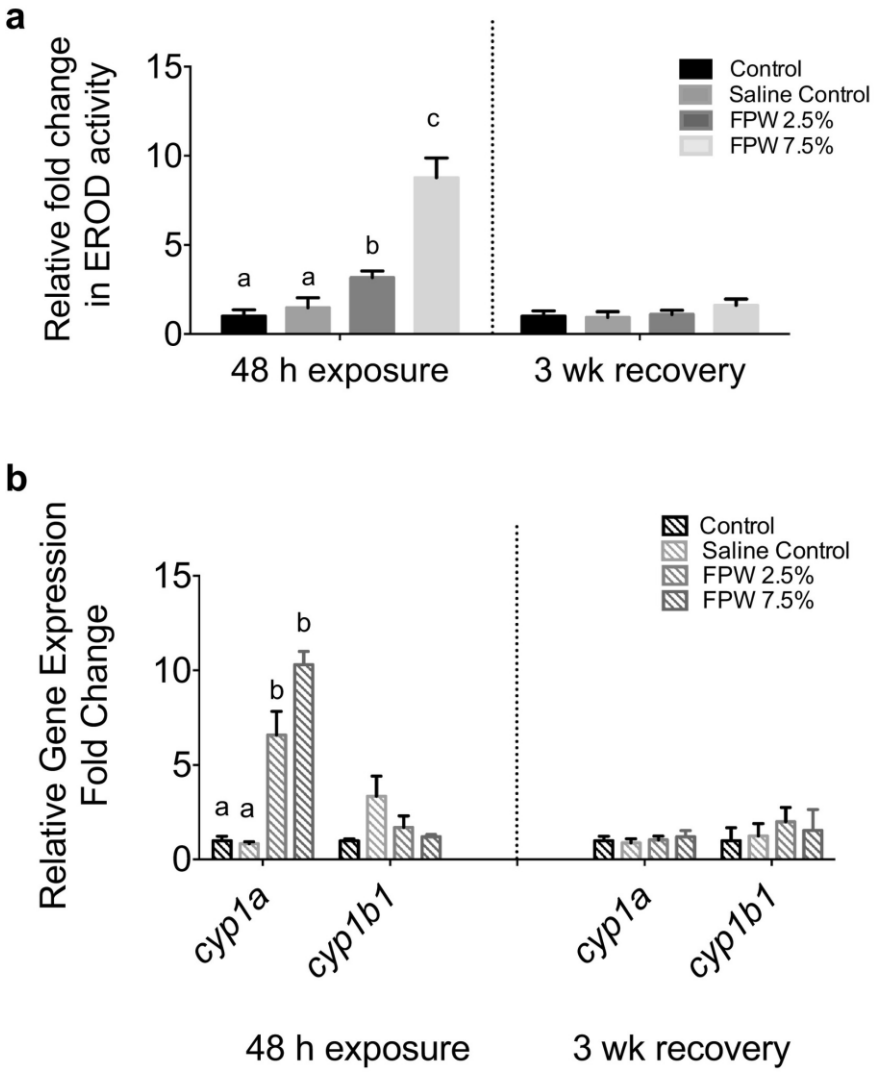


Figure 4- 2. Fold change in ethoxyresorufin-O-deethylase (EROD; a) activities or relative mRNA abundance of cyp1a and cyp1b1 (b) in rainbow trout liver following a 48 h exposure to saline control, 2.5% hydraulically fractured flowback and produced water (FPW), or 7.5% FPW, relative to control. Activity or abundance was measured immediately following exposure (48 h) and after a 3-week recovery period in control water. Data are presented as means + SEM (n = 5–6). Significant differences ($p < 0.05$) are denoted by differing letters.

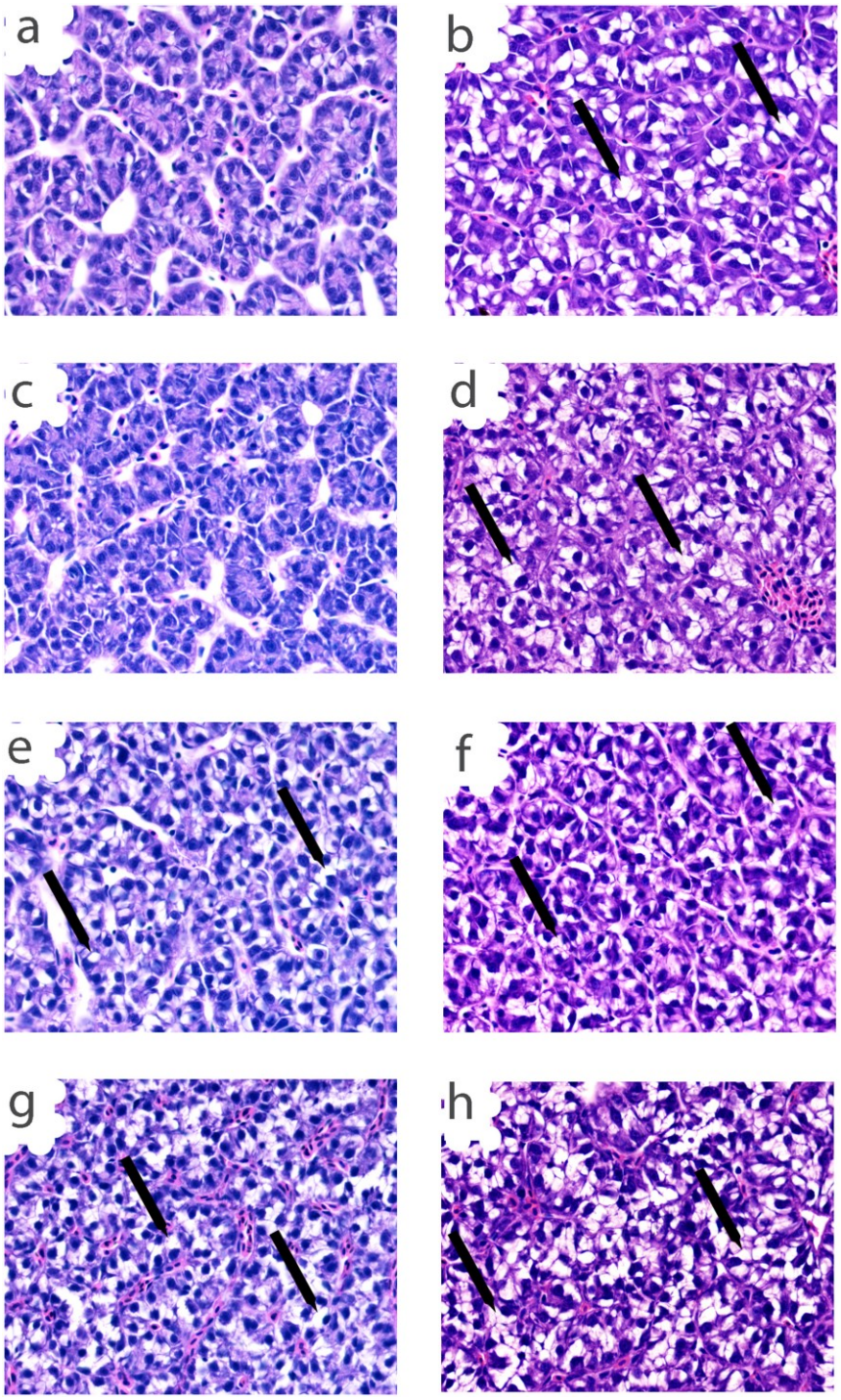


Figure 4- 3. Hematoxylin and Eosin stained light micrographs of representative liver sections from rainbow trout acutely exposed and immediately sampled after 48 h or given a 3 week (w) recovery period following exposure to: control (a = 48 h; b = 3 w), saline

control (c = 48 h; d = 3 w), 2.5% FPW (e = 48 h; f = 3 w), or 7.5% FPW (g = 48 h; h = 3 w). Pointed lines demark vacuolation.

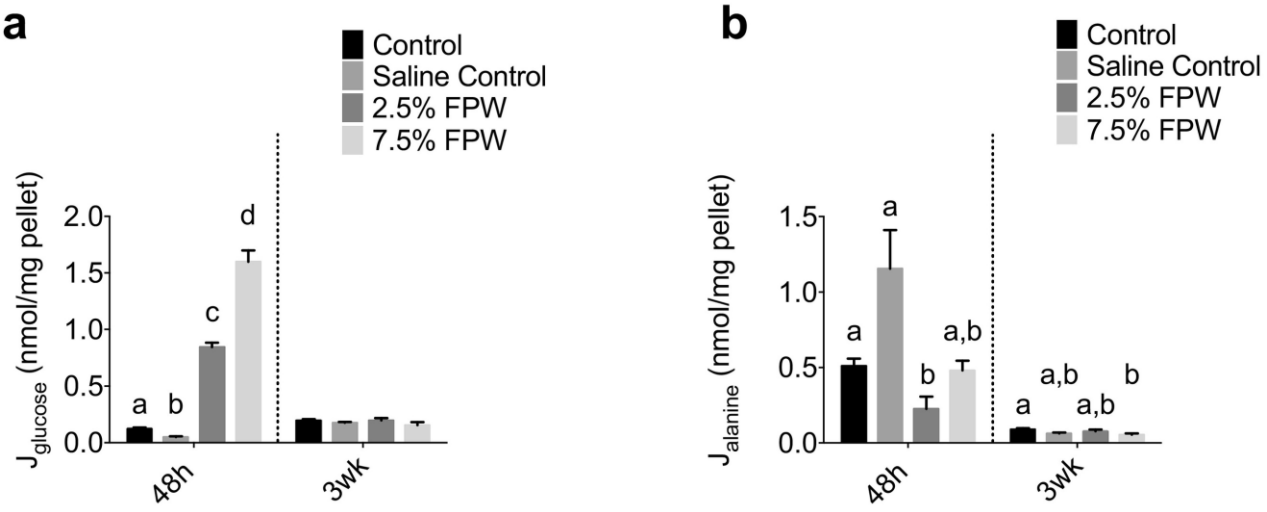


Figure 4- 4. Rates of glucose ($J_{glucose}$; a) and alanine ($J_{alanine}$; b) uptake into hepatocytes retrieved from rainbow trout exposed for 48 h to control, saline control, a 2.5% dilution of hydraulic fracturing flowback and produced water (FPW), or a 7.5% dilution of FPW immediately following exposure or after a 3-week recovery period in control water. Data are presented as means + SEM (n = 4–6). Different letters denote significant differences ($p < 0.05$).

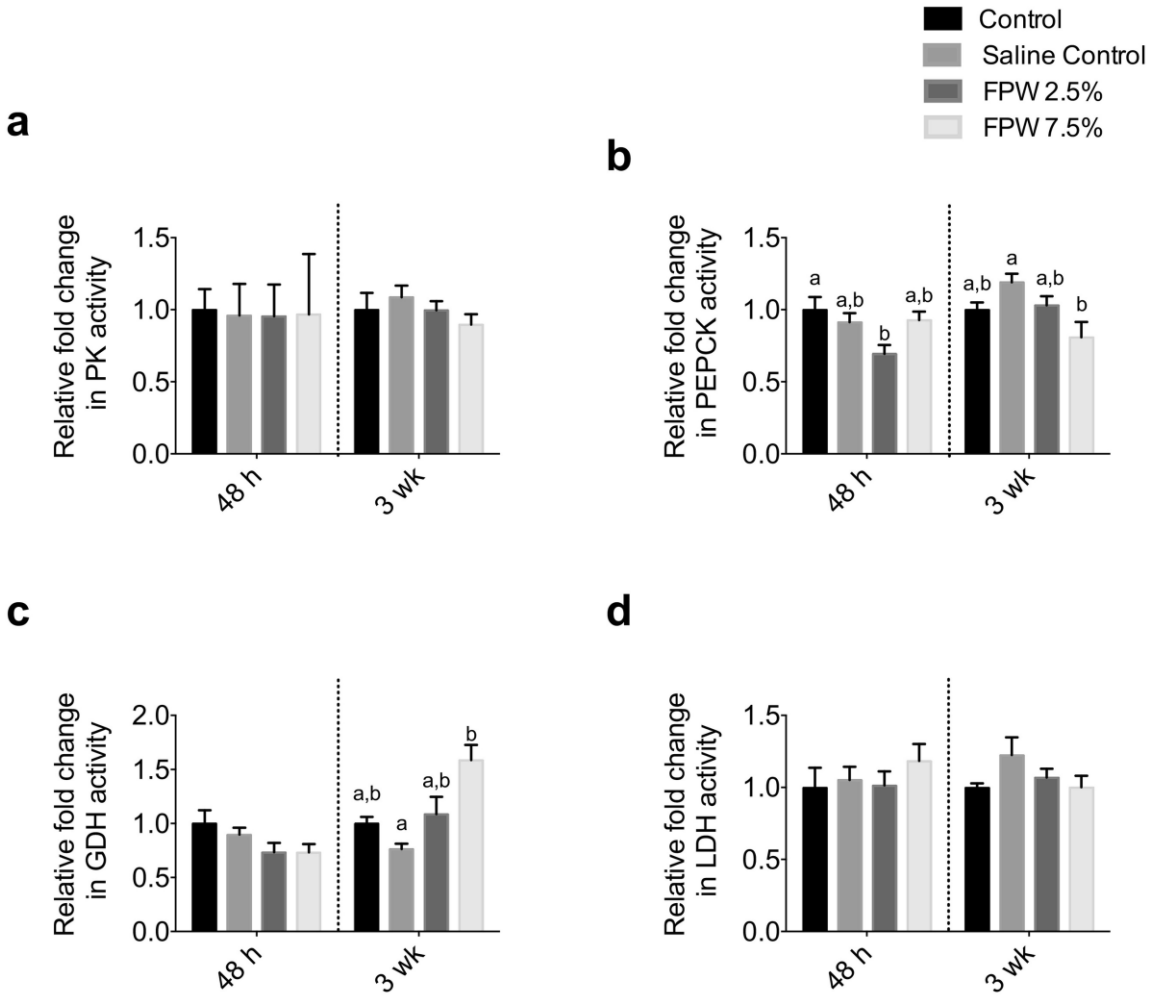


Figure 4- 5. Fold change in pyruvate kinase (PK; a), phosphoenolpyruvate carboxykinase (PEPCK; b), glutamate dehydrogenase (GDH; c), and lactate dehydrogenase (LDH; d) activities in rainbow trout liver following a 48 h exposure to saline control, 2.5% hydraulically fractured flowback and produced water (FPW), or 7.5% FPW, relative to control. Activities were measured immediately after exposure (48 h) or after a 3-week period of recovery in control water. Data are presented as means + SEM (n = 5–6). Significant differences ($p < 0.05$) are denoted by differing letters.

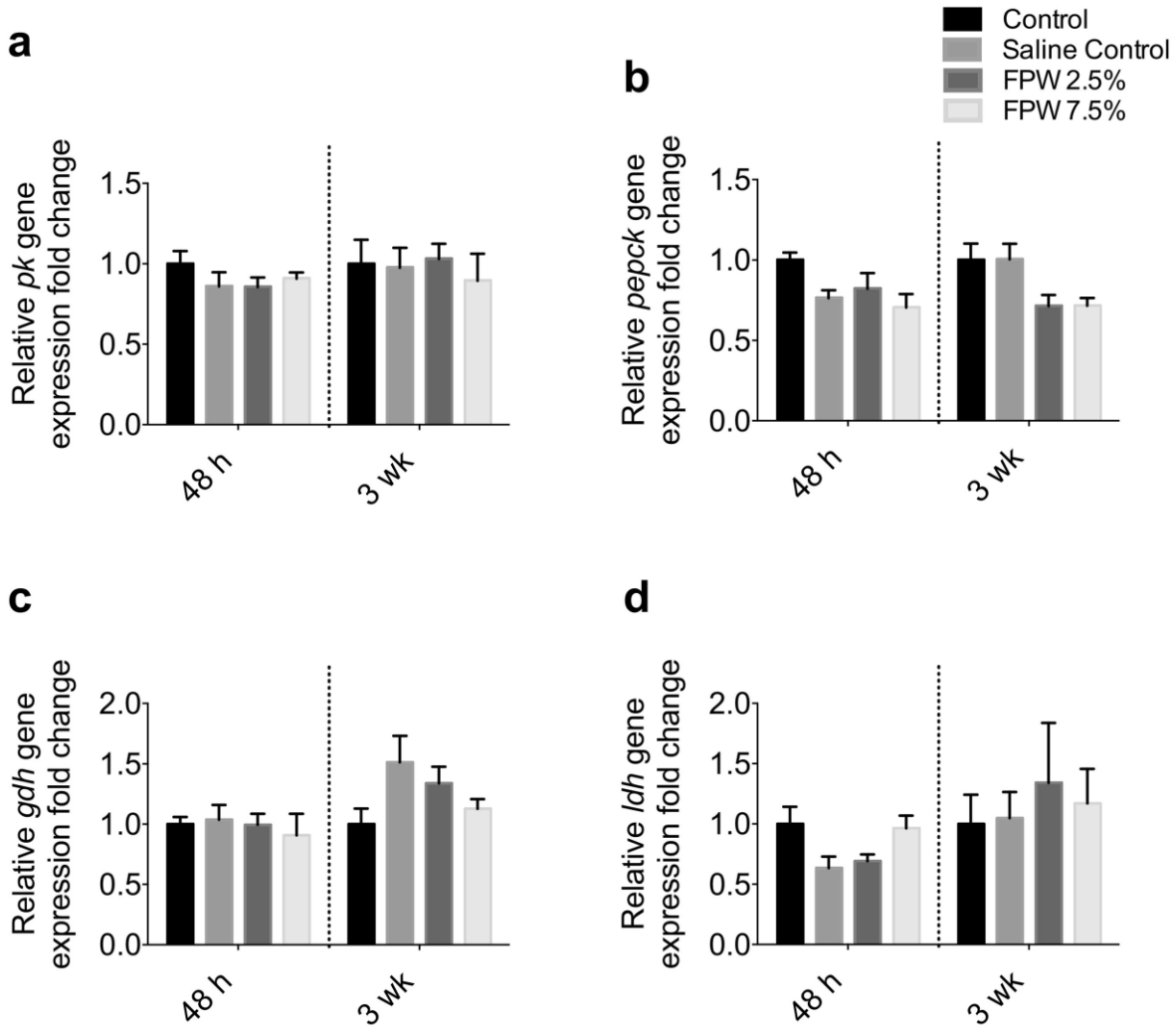
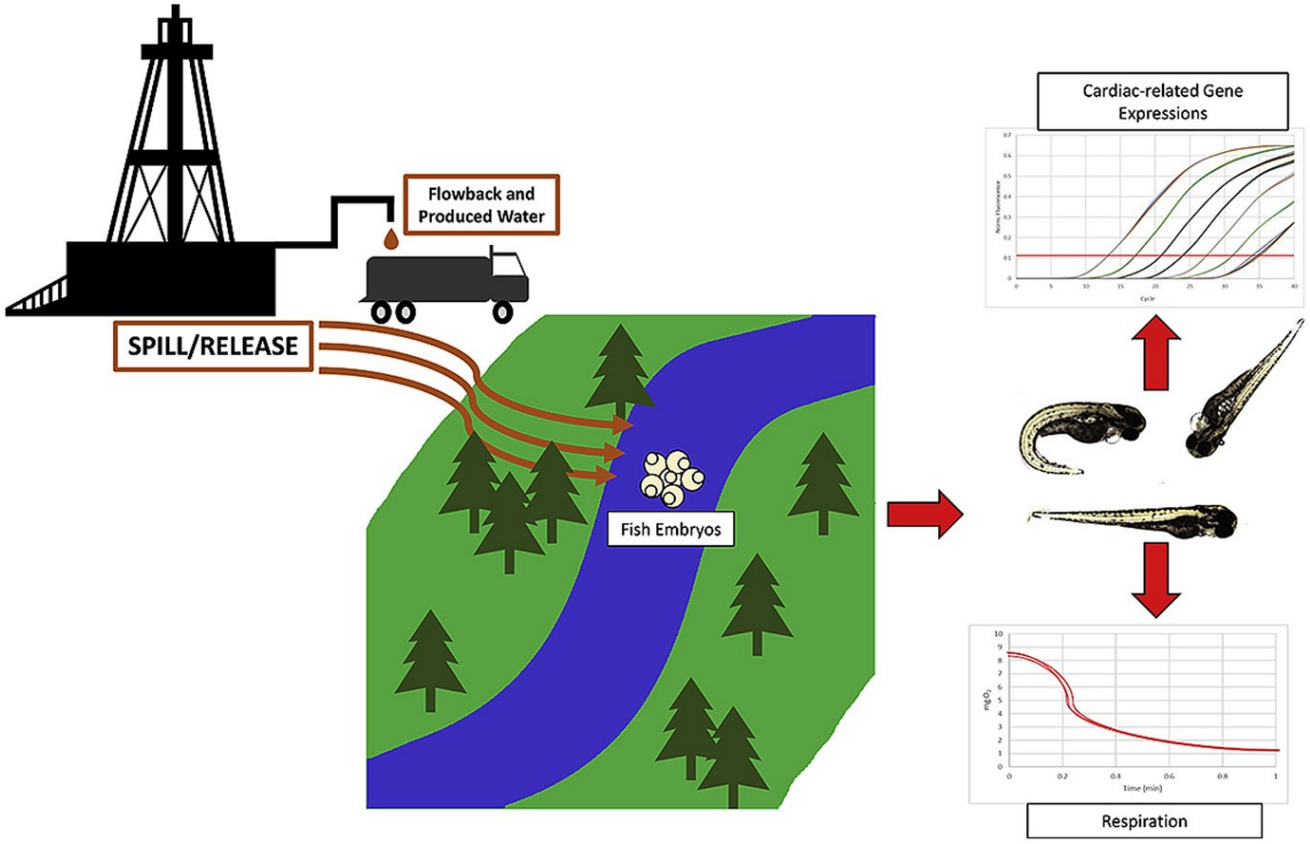


Figure 4- 6. Relative mRNA abundance of a) pyruvate kinase (*pk*), b) phosphoenolpyruvate carboxykinase (*pepck*), c) glutamate dehydrogenase (*gdh*), and d) lactate dehydrogenase (*ldh*), in rainbow trout liver acutely exposed (48 h) to saline control, 2.5% FPW, or 7.5% FPW relative to control. Abundance was measured immediately following exposure (48 h) and after a 3-week recovery period in control water. Data are presented as means + SEM (n = 5–6).

CHAPTER 5: Cardio-Respirometry Disruption in Zebrafish (*Danio rerio*) Embryos Exposed to Hydraulic Fracturing Flowback and Produced Water



Folkerts, E.J., Blewett, T.A., He, Y., Goss, G.G. (2017). Cardio-respirometry disruption in zebrafish (*Danio rerio*) embryos exposed to hydraulic fracturing flowback and produced water. *Environmental Pollution*, **231**, 1477 – 1487. DOI: 10.1016/j.envpol.2017.09.011.

5.1 INTRODUCTION

Hydraulic fracturing processes and their effects on the environment have recently gained public and private interest (Boudet et al., 2014; Gehman et al., 2016; Theodori et al., 2014). Public perceptions and concerns, as well as industry-directed initiatives, have recently accelerated research into how hydraulic fracturing processes affect environmental parameters, such as air, geological, and ground/surface water qualities (Colborn et al., 2014; Entrekin et al., 2011; Kargbo et al., 2010; Lauer et al., 2016; Llewellyn et al., 2015; Osborn et al., 2011; Vengosh et al., 2014; Vidic et al., 2013; Vinciguerra et al., 2015). To date, few studies have investigated the effects and associated potential toxicities of hydraulic fracturing (HF) flowback and produced water (FPW): a wastewater solution formed after a hydraulically fractured well has been opened to produce oil and/or natural gas. FPW is composed of injected HF fluid components (e.g. surfactants, biocides, clay stabilizers, etc.), deep formation water (hyper saline), secondary by-products of downhole reactions within the formation, and petroleum contaminants from the formation (DiGiulio and Jackson, 2016; He et al., 2017a; Lester et al., 2015). In the Duvernay formation (located along the border of eastern British Columbia and western Alberta, Canada), hydraulically fractured wells typically use large volumes of water ranging between 1 and 10 million liters per well, but volumes in excess of 100 million liters have been recorded (Alessi et al., 2017; Kargbo et al., 2010). It is estimated that 30–80% of the volume returns as FPW (Alessi et al., 2017), and thus, storage and transport of this high-volume industrial waste is currently a controversial topic. When spills and releases of FPW to both flowing (rivers, creeks, etc.) and more stagnant (marshes, muskeg, etc.) surface water bodies have been documented, these releases have the potential to be costly in terms of environmental impact and remediation (Alessi et al., 2017; Entrekin et al., 2011; Goss et al., 2015).

FPW salinity from this formation (up to 240 ppt) can far exceed that of sea water (~33 ppt), and is observed to be a major mechanistic mode of toxicity for exposed freshwater organisms (T. A. Blewett et al., 2017a, 2017b; He et al., 2017a, 2017b). However, other potentially harmful chemical species are present in FPW. These may include organic petroleum-related hydrocarbon species (e.g. polycyclic aromatic hydrocarbons; PAHs), metals (lead, arsenic, nickel), radionuclides, and industrial fracturing additives such as biocides, surfactants, and polymers (Drollette et al., 2015; Ferrar et al.,

2013; Harkness et al., 2015; He et al., 2017a; Stringfellow et al., 2014). Moreover, FPW often forms significant iron oxide precipitates upon reoxgenation at the surface and these have been shown to be associated with a different complement of organic chemical species and cause differential toxicity (He et al., 2017a). The aforementioned listed chemicals of concern may also sub-lethally affect organism health, even after acute FPW exposures. Previous studies have shown numerous physiological responses are affected by FPW exposure in multiple freshwater species including alterations to cellular detoxification mechanisms, increases in oxidative stress responses, and decreased reproductive output effects (T. A. Blewett et al., 2017a, 2017b; He et al., 2017a, 2017b), each reflecting the complex and heterogeneous nature of FPW and its inductive hazard potential.

Fish embryos serve as an important exposure toxicological model for human and environmental health as they are excellent indicators of contamination and are generally considered a more resistant life stage than larval (immediately following hatching) and juvenile stages (Embry et al., 2010; Woltering, 1984). Additionally, given that FPW contains significant sediments upon reoxygenation, fish embryos are a good model to examine the effects on benthic organisms of prolonged or chronic exposure to sediment containing mixtures (Baker et al., 1991; Entrekin et al., 2011; Hollert et al., 2000). Industrial waste water and spill solutions have been shown to elicit developmental deformities in exposed fish embryos including pericardial edema (PE) yolk-sac edema (YSE), and tail/spine curvatures (TSC) (He et al., 2012a; Incardona et al., 2013; Philibert et al., 2016; Sfakianakis et al., 2015). These deformities negatively impact physiological functioning of the organism and are often used as indices when determining constituent sub-lethal effects.

Currently, knowledge is very limited on fish embryonic metabolic changes in response to industrial waste and organic contaminant exposure. Organogenesis is a vital period within the embryonic state where disturbances to cardiovascular development may negatively impact embryonic respiration and oxygen handling. In zebrafish, primary heart and related cardiovascular systems are some of the first tissues to develop from 5 to 48 h post fertilization (hpf), but epicardium development extends further into development to 72 hpf (Bakkers, 2011; Glickman and Yelon, 2002). Alterations to cardiac development may

not only affect embryonic survival, but may potentially affect later life-stage fitness parameters. Interestingly, exposure to petroleum-related contaminants has been shown to alter cardiac physiology and cardiomyocyte gene expression profiles responsible for cardiac electrochemical properties (Brette et al., 2014; Edmunds et al., 2015; Zhang et al., 2013), and therefore, embryonic respirometry may serve as a useful sub-lethal endpoint when assessing toxicity of pollutants in the environment.

The prominence and increasing practice of hydraulic fracturing in oil and gas extraction necessitates more thorough investigation into the potential impacts of this process on the environment. Previous investigations have established initial baseline toxicities of FPW in zebrafish embryos (*Danio rerio*), rainbow trout (*Oncorhynchus mykiss*) and *daphnia* (T. A. Blewett et al., 2017a, 2017b; He et al., 2017a, 2017b). However, more rigorous studies exploring sub-lethal responses to FPW are required to thoroughly understand potential impacts of FPW exposure on developmental toxicity in vertebrates, including wild fish and humans. Sub-lethal analyses will add to a mechanistic understanding of FPW hazard, and potentially stimulate development of practical field assays and biomarkers of exposure. This study aims to understand the effects of FPW exposure on respiratory capacity in embryonic zebrafish after acute, sub-lethal exposure at 24 hpf.

5.2 MATERIALS AND METHODS

5.2.1 Animal Species

Adult zebrafish (wild-type, strain AB) housed in 30-L tanks (~25 fish/tank; 14 h light:10 h dark photoperiod) were bred to produce viable embryos in the University of Alberta zebrafish facility. All animal use was approved by the University of Alberta Animal Care Committee under protocol AUP00001334. Fertilized embryos were collected and randomly distributed to multiple Petri dishes incubated at 28 °C in zebrafish facility water (pH: 7.4, conductivity: 168.5 ± 0.5 µS/cm, temperature: 28.5 ± 1 °C, dissolved oxygen: 7.5 ± 0.5 mg/L, general hardness: 175 mg/L as $CaCO_3$, salinity: 0 ppt) until FPW fraction exposures 24 h later.

5.2.2 FPW Sample

A 7-day post stimulation FPW sample from a horizontally-fractured well (Duvernay formation, Fox Creek, Alberta, CAN) was used for fractionations and exposures in the current study. Details on sample inorganic and organic characterization can be found in previous studies (He et al., 2017a, 2017b). Two fractions of our FPW sample were generated and used for zebrafish embryonic exposures. Briefly, a sediment-free fraction (FPW-SF) was created by centrifuging raw FPW in an Eppendorf 5810 R centrifuge (Eppendorf, Hamburg, GER) at 3220 g for 10 min and vacuum filtration through 0.22 μm filter (Sarstedt, North Rhine-Westphalia, GER). Our sediment containing fraction (FPW-S) consisted of the raw FPW sample. Additionally, a salt control (SW) matching major ion concentrations (Na, Mg, K, Ca, and Cl; Sigma- Aldrich, MO, USA) in the raw FPW sample was made (see Table S1 [Table S3-1] of He et al., 2017a, 2017b for composition) to account for any saline influenced responses.

5.2.3 Experimental Design and Exposures

Zebrafish embryos were exposed 24 hpf to the following treatments conditions: FPW-S, FPW-SF, SW (saltwater matched control) and control (zebrafish facility water) at two different dilution concentrations of 1.25% and 2.5% (developmental deformity analyses) and 2.5 and 5% (all other analyses). Zebrafish embryos were then exposed to experimental conditions for 24 and 48 h. All treatments were run in parallel with each other (n = 6 for each fraction exposure treatment for all assays and analyses). Fifty percent water volume replenishments were performed every 24 h for 48 h exposures.

For developmental deformity analyses, 15 embryos were exposed to the 4 treatment conditions (see above) in 100 mL of fraction dilution solutions in 250 mL glass beakers (Corning, NY, USA) (n = 6). Solution pH of control and treatment conditions were 7.3 ± 0.23 during exposure duration. Following exposure termination, embryos were removed from exposure beakers, washed 3 times with clean zebrafish facility water (see above), and placed into respective wells of a 6-well plate (ThermoFisher Scientific, MS, USA) containing 4 mL of fresh zebrafish facility water. Fifty percent water replenishments were made every 24 h in 6-well plates until embryos reach 120 hpf, where developmental deformity recordings were made.

For embryonic real-time quantitative PCR (qPCR) analyses, similar exposure conditions of 15 embryos in 100 mL of fraction dilution treatment solutions in 250 mL glass beakers (n = 6) were used. Following exposure termination, embryos were removed from initial exposure beakers, washed 3 times with clean zebrafish facility water, and snap-frozen in 1.5 mL centrifuge tubes using liquid nitrogen and stored at −80 °C for later qPCR analysis.

Embryonic metabolic rate (MO_2) analyses were performed by exposing 10 zebrafish embryos (24 hpf) to the exposure treatments (see above) in 100 mL of fraction dilution solutions and controls in 200 mL glass beakers. Following exposure termination, 5 embryos were removed from respective exposure beakers, washed 3 times, and assayed for immediate embryonic/larval MO_2 analysis or for MO_2 at specified time intervals post exposure termination. Zebrafish embryonic/larval MO_2 analysis was made at 48, 72, 96, and 120 hpf (n = 6).

5.2.4 Developmental Deformity Analysis

After exposure termination, embryos were washed 3 times and sequentially cultured in 6-well plates until 120 hpf. Observations and removal of dead embryos/larvae were made daily (total of 5 embryos found dead across all replicates). At 120 hpf, larval measurements of percent pericardial edema (PE), yolk sac edema (YSE), tail/spine curvature (TSC), and heart rate (HR) were made. Percent PE, YSE, and TSC was determined by observing larvae under an Observer A1 inverted microscope (Zeiss, Baden-Württemberg, GER) and recording occurrences of malformation. All degrees of larval deformities were included in deformity analysis. Larval heart rate (HR) was determined by recording the number of ventricular contractions per minute using the above stated microscope fitted with a MRm camera and associated AxioVision Release software (V 4.7.2).

5.2.5 Embryonic/Larval Metabolic Rate Analysis

Embryonic/larval MO_2 was determined using a sensor-dish reader (SDR) system (Loligo Systems, Viborg, DEN). Recordings, using PreSens-SDR_v38 software (Loligo Systems, Viborg, DEN), were made immediately after exposure termination and

subsequently in larvae every 24 h to 120 hpf. Larvae not immediately used for MO_2 analysis were kept in 150 mL of zebrafish facility water (50% replenishments made daily) until chosen for analysis at specified time points. Observations and removal of dead embryos were made daily (total of 6 embryos found dead across all replicates). At each 24 h period, 5 washed embryos were placed into wells of a 24-well optical fluorescence glass sensor microplate, wells were filled with 750 µl of fresh, aerated zebrafish facility water, and then immediately sealed using parafilm and a silicon gel pad cover. Sensor microplates with sealed wells were placed onto SDRs and into a 25 °C incubator in complete darkness for 1 h. Using PreSens – SDR_v38 software, embryonic/larval MO_2 calculations were made between 5 and 25 min of well O_2 measurements. Recordings below 70% air O_2 saturation levels were not included in analyses. MO_2 data was corrected for both volume of water and mass of embryos, and expressed as mg O_2 g^{-1} h^{-1}. To control for background levels of respiration, 2–3 wells on the microplate were filled with water with no embryos placed inside.

5.2.6 Embryonic Quantitative Real-Time PCR

Abundance of mRNA for genes coding for proteins and transcription factors of interest were quantified using qPCR. Total RNA was extracted from 10 zebrafish embryos using the MasterPure RNA Purification Kit (Epicentre Biotechnologies, WI, USA). Briefly, recombinant DNase I (Ambion) and 10 × reaction buffer with $MgCl_2$ (ThermoFisher Scientific, MS, USA) were added to embryo samples prior to a 30 min incubation period at 37 °C. The reaction was terminated with 50 mM ethylenediaminetetraacetic acid and samples were stored in nuclease-free water (non-DEPC treated, Ambion) containing SUPERase-In RNase Inhibitor at −20 °C. Total RNA was quantified using a NanoDrop ND-1000 spectrophotometer (v. 3.8.1, NanoDrop Technologies, USA), and RNA integrity was assessed on a 2% formaldehyde – agarose gel with ethidium bromide. Following RNA extraction, 0.5 µg of RNA samples underwent same-day cDNA synthesis using the RevertAid First Strand cDNA Synthesis Kit (ThermoFisher Scientific, MS, USA) and were stored at −20 °C until further analysis.

Quantitative real-time PCR (qPCR) was performed in 96-well plates using an ABI 7500 Real-Time PCR system (Applied Biosystems, CA, USA). Gene specific primers were

designed against target genes using Primer Express v3.0.1 software (Thermofisher Scientific, MS, USA). Primer gene targets, accession numbers, and sequences can be found in Table 5-1. Primer efficiency validations, determined by serial dilution slope calculation after ΔCT and log input regression analyses, can be found in Figure S5-1. Individual PCR reaction mixtures contained 5 µl of SYBR Green master mix (Applied Biosystems, Foster City, CA, USA), 2.5 µl of 3.2 µM primer sets, and 2.5 µl of cDNA diluted in nuclease-free water. PCR reactions were denatured at 95 °C for 10 min prior to first thermal cycle initiation. A 2-step PCR thermal cycle, consisting of a denaturation step of 95 °C for 2 min and an annealing-extension step of 60 °C for 1 min, was employed for 40 cycles and transcript expression changes determined by ΔΔCT analysis using glyceraldehyde 3-phosphate dehydrogenase (*gadph*) as an endogenous control (Schmittgen and Livak, 2008).

5.2.7 Statistical Analyses

All data were tested for normality by use of the Shapiro-Wilk test and homogeneity of variance assessed by use of Levene's test (SigmaPlot 13.0, Systat Software Inc., CA, USA). When necessary, data were $\log_{10}$-transformed to meet assumptions of parametric tests. Non-transformed data are represented in all figures. Prism 6.0 was used for all other outlined statistical analyses (GraphPad Software Inc., CA, USA). Significant statistical differences found in developmental deformities were evaluated by a one-way ANOVA. Effects of FPW fraction type on embryonic/larval MO_2 at multiple stages of development were tested by use of two-way ANOVA. Effects of FPW dilution and fraction type exposures on gene of interest transcript abundance were tested by use of a two-way ANOVA. No interactions between factors were found for all two-way ANOVAs. Significant differences found during ANOVA analyses were followed up with Holm-Sidak *post-hoc*, multiple range tests. All data are expressed as mean ± S.E.M, and differences were considered statistically significant at $p \leq 0.05$.

5.3 RESULTS

5.3.1 Developmental Deformities

PE, YSE, and TSC deformities (Figure 5-1) were observed in developing zebrafish embryos after sub-lethal exposures to FPW fractions. Exposure to 2.5% dilutions of FPW-SF and FPW-S fractions significantly induced developmental deformities in zebrafish embryos (Figure 5-3). After 24 h exposures to 2.5% S fractions, PE occurrences increased 5 and 8-fold over control and SW fractions, respectively. This trend was amplified after 2.5%, 48 h S exposures, where 47 and 12-fold increases in occurrences of PE were observed over control and SW fractions, respectively. SF fraction exposures followed a similar trend, where significant increases of PE were observed after 2.5%, 48 h exposures compared to control and SW conditions. Significant increases in percent occurrences of YSE and TSC were additionally observed after 2.5%, 48 h SF and S exposures. Furthermore, significant reductions in HR were observed after 2.5%, 48 h SF and S exposures compared to control and SW exposures. No significant changes in developmental deformity occurrences were observed after both 24 and 48 h exposures to 1.25% FPW fraction dilutions. (Figure 5-2).

5.3.2 Embryonic/Larval MO_2 Analyses

Exposure to FPW-SF and FPW-S fractions significantly decreased embryonic/larval MO_2 rates (Figure 5-4). Acute 2.5%, 24 h and 2.5%, 48 h exposures to S fractions significantly reduced respirometry rates at all time points in embryos/larvae compared to control conditions. Larger decreases were observed in 5%, 24 h exposures, where both SF and S fraction exposed embryos had significantly reduced MO_2 compared to control embryos/larvae. Embryos exposed to 5%, 48 h S fractions also displayed significantly reduced MO_2 compared to SW exposed embryos/larvae. Finally, acute 5%, 48 h SF and S exposures significantly reduced embryo/larval MO_2 rates at all time points compared to both control and SW conditions.

5.3.3 Transcript Abundances of Target Genes

Expression of select cardiac and developmental genes were assessed following 2.5% and 5% FPW fraction exposures for 24 and 48 h (Figure 5-5). Significant reductions in *atp2a2a* expression were observed in all FPW-S exposures compared to control and SW exposures, with a largest 5.5-fold *atp2a2a* reduction occurring in 5%, 48 h S exposures. FPW-SF fraction exposures followed a similar trend, with a largest 2.5-fold *atp2a2a* reduction occurring in 5%, 48 h SF exposures. Significant reductions in *tnnt2a* expression were recorded for all 5%, 48 h exposures of SW, SF, and S fractions. Significant inductions of *nkx2.5* were observed for 5%, 24 h SF exposures and 5%, 48 h SF and S exposures compared to control and SW conditions. No significant changes in expression were observed for *cmlc1* in any exposure conditions.

5.4 DISCUSSION

The present study is one of the first to investigate FPW sub-lethal toxicity using developmental analyses, respiratory parameters, and expression of cardiac responsive genes in zebrafish. Dilutions of FPW used for exposures were environmentally relevant and have previously been shown to elicit sub-lethal responses in rainbow trout and zebrafish (T. A. Blewett et al., 2017b; He et al., 2017a, 2017b). Thus, determining how relatively novel contaminants, such as FPW, interact with and affect embryonic physiology is vital for ensuring both human and environmental health. In many environmental spill scenarios of crude oil and other petroleum related chemical species, organics are of major concern. Organic characterization of our FPW sample has revealed numerous PAHs and other organic species to be present (He et al., 2017a, 2017b) in both FPW-SF and S fractions. Similar responses of these two fractions suggests that exposure to organics present in FPW may potentially be responsible for eliciting many of these responses observed in developing vertebrates.

5.4.1 Developmental Deformity Analysis

Characterized by PE, YSE, and TSC, larval zebrafish deformities observed after embryonic FPW exposure at 24 hpf are similar to those seen in fish larvae exposed to similar oil and gas industry wastewaters and spill scenarios, such as oil sands produced

waters (OSPW) and oil exposures (He et al., 2012a; Incardona et al., 2015, 2013). Chemicals known to cause these deformities in developing fish after such spills and exposures are primarily organic derived, such as PAHs and dioxins (He et al., 2017a, 2017b; Incardona et al., 2013; Philibert et al., 2016; Raimondo et al., 2014; Sørhus et al., 2015). These compounds are known agonists of the aryl hydrocarbon receptor (AhR), resulting in specific cytochrome P450 inductions and activation, namely CYP1A, and to a lesser extent, CYP3A family monooxygenases (Luckert et al., 2013; Naspinski et al., 2008; Tseng et al., 2005; Xu et al., 2005). Activation of CYP1A and CYP3A gene pathways and subsequent substrate transformation has been shown to generate reactive oxygen species (ROS) (Penning et al., 1996; Zangar, 2004), potentially causing endothelial cell damage and apoptosis, tissue necrosis, and overall organ system compromise (Cantrell et al., 1998; Fernandez-Salguero et al., 1996; Incardona et al., 2004; Tithof et al., 2002). To add to the toxicological complexity of FPW, it should be noted that smaller PAHs found in FPW, such as the 3-ringed PAH phenanthrene, may illicit development deformities independent of the AhR pathway in multiple different developing fish tissue and organ systems (Brette et al., 2017; Incardona et al., 2005), resulting in shared developmental phenotypes such as PE and bradycardia.

In the current study, significant inductions of developmental deformities in zebrafish larvae at 120 hpf were observed in all FPW fractions treatments at 2.5% dilutions for 48 h acute exposures (Figure 5-3). Additionally, significant inductions of PE only were observed when 2.5% dilutions of FPW fractions were exposed to embryos for 24 h (Figure 5-3A). This suggests occurrence of PE may be a more sensitive developmental marker for FPW exposure compared to YSE and TSC. Furthermore, acute 48 h FPW exposures (24–72 hpf) cover a larger interval of time during the zebrafish critical tissue and organ developmental "window", as total zebrafish embryonic organogenesis is known to occur between 5 and 72 hpf (Bakkers, 2011; Glickman and Yelon, 2002), and therefore 48 h exposures are theorized to be responsible for the increased occurrences of developmental defects. Thus, PAH induced impairment of fish cardiac function (Hicken et al., 2011; Incardona et al., 2006; Yu et al., 2015) may be caused by tissue damage and disturbances to excitation-contraction coupling events in cardiomyocytes (Brette et al., 2014). Additionally, recent transcriptomic analysis of weathered crude oil exposure on

Atlantic haddock (*Melanogrammus aeglefinus*) embryo development (Sørhus et al., 2017) has revealed biological pathways related to PAH exposure toxicity including effects on cardiac form and function, ion regulation, and fluid balance. These results further support the notion of PAHs in chemical complex mixtures, such as FPW, are largly responsible for developmental deformities observed in exposed larvae. Correspondingly, 2.5% dilutions of FPW after 48 h exposures significantly reduced HR in zebrafish larvae compared to both fresh water and salt control conditions. Mechanisms linking decreases in larval HR to pericardial edema remain unresolved, although HR is know to be affected by numerous other factors including heart weight, cardiac cell number, arrhythmia, and heart structure (Antkiewicz et al., 2005; Henry et al., 1997; Incardona et al., 2009, 2004; Teraoka, 2002). Clearly, more analysis is needed to determine the dominating factors contributing to lowered larval zebrafish HR after FPW exposure.

5.4.2 Embryonic MO_2 Alterations

As previously stated, embryos, and benthic aquatic species in general, are useful exposure models in many industrial spill scenarios due to many pollutants containing sediment and settling physiochemical characteristics (Baker et al., 1991; Hollert et al., 2000). Embryos offer a unique perspective on toxicological potential and adverse outcome pathways, as their relatively high surface area-to-volume ratio characteristics may enhance toxicant bioavailability. As a result, embryos are a particularly susceptible exposure model system. The development of PE after FPW exposure clearly implicates cardiac stress as an outcome, while observations of reduced HRs further support the hypothesis that cardiotoxicity is a major contributor to FPW toxicity in zebrafish embryos. Embryonic metabolism and respiration, measured frequently by yolk sac disappearance and flow chamber water O_2 decreases, respectively (reviewed in Rombough, 1988 and Kamler, 2008), are two essential and intimately linked physiological processes for embryo survival, and are particularly sensitive to numerous environmental disturbances (Barrionuevo and Burggren, 1999; Finn et al., 1995; Firat et al., 2003; Hardy and Litvak, 2004; Hunter et al., 1979; Ojanguren et al., 1999; Rombough, 1988b).

Exact mechanistic modes of toxicity on cardiac function and morphology during development after PAH and other petroleum related compound exposures are relatively

unknown. However, recent efforts to determine molecular and physiological properties responsible for PAH developmental toxicity have been undertaken and are beginning to reveal biological mechanisms responsible for developmental effects observed (reviewed in Incardona, 2017). Aside from general organic metabolite oxidative tissue damage as outlined earlier, PAH and organic toxicity is thought to be structure dependent. Low molecular weight PAHs (four or fewer rings) are widely considered to elicit cardiovascular toxicity via nonpolar narcosis: membrane integrity disruption caused by the incorporation of hydrophobic organic compounds (reviewed in Bunn et al., 2000; Wezel and Opperhuizen, 1995). Originally thought to perturb lipid bilayers and actions of ligand-gated ion channels in central nervous system cell types (Escher and Hermens, 2002; Franks and Lieb, 1999), it is now suggested that that 3 and 4-ringed PAHs also act specifically to disrupt cardiac function (Brette et al., 2017; Incardona et al., 2004; Park, 2002). PAH analysis of our FPW sample revealed the presence of 3 and 4 ring PAH's (He et al., 2017a, 2017b), suggesting the above mentioned PAH-induced cellular sub-lethal effects may occur after zebrafish embryonic exposure.

In the present study, sediment containing fraction exposures significantly decreased zebrafish MO_2 rates compared to control conditions in all exposure regimens (Figure 5-4), while FPW-SF exposures only showed significant effects at 5% dilutions. These results agree with many recently published studies demonstrating cardiovascular and respiratory alterations in fish exposed to organic petroleum-related compounds or oil fractions (He et al., 2017a; Incardona et al., 2015, 2009; Mager et al., 2014). Although salinity has been observed to affect metabolic processes in fish embryos (Brown et al., 2012; Ern et al., 2014; Morgan et al., 1992), research into salt effects on embryo respiration is limited. Of the studies which have recorded O_2 consumption over increasing salinity, no effects have been observed in fish embryos (Brown et al., 2012; Kinne and Kinne, 1962). Furthermore, across multiple adult fish species, salinity does not appear to affect oxygen uptake rates in any observable trend (reviewed in Morgan et al., 1992). Thus, it is not surprising we did not see any significant effect of our salt control treatments on zebrafish embryo respiration rates. Our findings that FPW-SF and FPW-S exposures induced significant reductions in embryonic/larval MO_2 compared to SW controls at higher FPW fraction concentrations further supports our hypothesis that organics (overview of chemical characterizations

available in He et al., 2017a, 2017b) in our FPW fractions, and not salinity, are eliciting cardiovascular toxic effects in developing fish. Collectively, our developmental and MO_2 analyses suggest FPW exposure induce cardiac defects in developing zebrafish embryos and larvae which alter early embryonic respiration. Given that fractions containing both sediment and organic contaminants elicit greatest MO_2 disturbances, organics are theorized to be main chemical components causing the metabolic impairment upon FPW exposure.

5.4.3 qPCR and Gene Transcript Expression Changes

To further characterize that the impairment of normal cardiac functioning may be responsible for inhibition in embryonic and larval MO_2 after FPW exposure, transcripts coding for specific cardiac structural and electrophysiological proteins, as well as related transcription factors, were assessed for changes in abundance (Figure 5-5). Low molecular weight PAHs are theorized to alter electrophysiological cardiomyocyte properties by disturbances to the lipid bilayer (Brette et al., 2017, 2014; Escher and Hermens, 2002). However, we demonstrate that changes in key cardiac structural and electrogenic protein expressions after FPW exposure may additionally contribute to altered cardiac function and changes in embryonic/larval MO_2. In vertebrates, calcium (Ca^{2+}) circulation and handling in cardiomyocytes is an essential process responsible for cellular repolarization events and action potentials, resulting in cardiac tissue alternans and heart beats (Incardona et al., 2009; Sato et al., 2006; Weiss et al., 2006). During systole, action potentials initiate a minor flux of Ca^{2+} into the ventricular myocytes though the sarcolemmal L-type Ca^{2+} channel stimulating a major Ca^{2+} release from stores in the sarcoplasmic reticulum (SR) through the ryanodine receptor (RyR) (Fabiato, 1983). During diastole and cardiac muscle relaxation, Ca^{2+} is transferred back into the SR via SR Ca^{2+} ATPases (SERCA2a, encoded by *atp2a2a* in zebrafish) and sarcolemmal sodium (Na^{+})/Ca^{2+} exchangers (NCX1h) (Bers et al., 1996; Fabiato, 1985). Decreased Ca^{2+} uptake and expression of SERCA2a have been observed to be major features of cardiomyocyte dysfunction and heart failure (Chen et al., 2004; Frank, 2003; Zhu et al., 2008). Furthermore, *tnnt2a* is a gene in zebrafish identified as being essential for myosin-actin

activity, sarcomere assembly and cardiomyocyte contractility (Kaplan and Hamroun, 2013; Nesan and Vijayan, 2012; Sehnert et al., 2002).

After exposure to FPW-S at either 2.5% or 5%, zebrafish embryos significantly reduced expression of *atp2a2a* (Figure 5-4A), while for FPW-SF, only the 5% SF fraction exposures resulted in a decrease in *atp2a2a* transcript abundance regardless of exposure duration. Our results agree with previous studies which have shown disruptions to Ca^{2+} release and/or reuptake from internal stores after exposure to water accommodated oil fractions (Brette et al., 2014), where decreases in *atp2a2a* transcript abundance and overall Ca^{2+} ATPase activity were noted after exposure to the smaller, 3-ringed PAH phenanthrene (Edmunds et al., 2015). Similar changes in *tnnt2a* gene expression were noted in both SW controls and each FPW exposure suggesting that this may be a genetic response more sensitive to hypertonicity induced perturbations than FPW.

The homeobox transcription factor, *nkx2.5*, is an evolutionarily conserved and essential factor for development and differentiation of cardiomyocytes (Akazawa and Komuro, 2003; Bodmer, 1993; Zhang et al., 2013). Transgenic *nkx2.5* knockdown and knockout experiments in a number of vertebrate species produce a common cardiac phenotypic fate of arrested heart morphogenesis and retarded cardiogenic growth (Bartlett et al., 2010; Fu et al., 1998; Tanaka et al., 1999). Interestingly, following 5%, 48 h SF and S exposure, significant increases in *nkx2.5* transcript abundance (rather than decreases) were observed in our exposed developing zebrafish embryos (Figure 5-5C), contrasting observations in other embryonic organic contaminant and PAH exposure experiments (Jamali et al., 2001; Y. Zhang et al., 2012a; Zhang et al., 2013). Chemical specifics, exposure onset and duration, and species differential responses may account for these contrasting observations. Further, cultured *nkx2.5* overexpressing mutant rat neonatal cardiomyocytes display increased apoptosis rates (Kasahara et al., 2003), while zebrafish embryos injected with *nkx2.5* RNA developed hyperplastic hearts, a condition associated with deleterious symptoms such as congestive heart failure and arrhythmia, often resulting in death (Chen and Fishman, 1996; Lorell and Carabello, 2000; Zhu et al., 2000). These results validate the association of *nkx2.5* expression and hindered cardiogenesis and functional effects.

5.5 CONCLUSIONS

The present study investigates the potential sub-lethal toxicities of FPW exposure in embryonic zebrafish, with a focus on cardiorespiratory sub-lethal effects. Fractionating FPW into distinct separations has allowed us to begin to parse out the chemical and compositional constituents contributing to the sub-lethal responses observed. Increases in developmental deformities, decreased embryonic respiration, and changes in transcriptional expression of key cardiac related genes reveal that organics present in our FPW sample contribute significantly to the sub-lethal responses observed. Together, our results indicate that even at relatively low, sub-lethal levels of FPW exposure (2.5–5%), significant sub-lethal cardiorespiratory responses in zebrafish embryos are noted. Provided that zebrafish are generally considered a more robust and relatively less sensitive species to contaminants, such as PAHs (Incardona et al., 2014; Philibert et al., 2016), compared to counterpart species in the wild, it is probable that freshwater species which spawn in areas affected by hydraulic fracturing activities may more severely be impacted. Future work should investigate FPW effects on other freshwater species to address this issue to provide ecological relevant context to the growing concern of FPW aquatic exposures. This research will provide vital information to industry, regulatory agencies and governments alike to both manage hazards and to develop appropriate remediation protocols when FPW spills and releases occur. Our results further validate the use of respirometry as a potential assay used for assessing sub-lethal effects of industrial waste and produced water exposures.

Determining the impacts of novel toxicant and anthropogenically derived wastewater exposures on early-life stage organisms (i.e. embryos/larvae) is an important step when beginning an investigation into the toxicity of such compounds and solutions. Detrimental impacts at this basal grouping of the population structure can have significant ramifications on the future health and viability of a species' population and existence in an ecosystem. However, impacts at the embryo/larval stage as a consequence of FPW exposure may not solely remain and stop at the embryonic/larval level; sub-lethal toxicities experienced during these early developmental periods may persist and affect later-life stage developmental periods in organisms. Concerning the current study, impairments to cardio-respiratory development and physiology from embryonic FPW exposures may potentially

produce latent effects in juvenile or adult fish. Indeed, it has been shown that embryonic exposures to chemicals or wastewaters similar to FPW impact juvenile or adult fish fitness and cardio-respiratory biology (Brown et al., 2017; Hicken et al., 2011; Mager et al., 2014). Therefore, future studies regarding zebrafish embryonic FPW exposures aim to determine if impacts during the embryonic stage persist and manifest as deleterious impairments to juvenile fish cardio-respiratory functioning.

Table 5- 1. Gene specific primer sequences used for quantitative real time PCR and analysis of transcript expression.

Target	Abbreviation	Primer Sequence (5' – 3')	Annealing Temp. (°C)	Accession #
Glyceraldehyde 3-Phosphate Dehydrogenase	*gapdh*	F: GTAGATGTGACCCCTTTGCTGTT R: CAGGCACGTGGTGCAAAC	60	NM_001115114.1
Cardiac Sarcoplasmic Reticulum Ca^{2+} ATPase 2a2a	*atp2a2a*	F: TCACGTCTTCGAGTCTCCTTACC R: CTGGTTCTCGGATAGACTGTTGAG	60	NM_200965.1
Cardiac Troponin T Type 2a	*tnnt2a*	F: CTGGTGCCTCCAAAGATTCC R: CAGCGTCTGCAGCTCATTCA	60	NM_152893.1
Homeobox Protein Nkx2.5	*nkx2.5*	F: GACGCTTCAAGCAGCAGAAATA R: GTGTGGAGGTGAGTTTGAGAACAT	60	NM_131421.1
Cardiac Myosin Light Chain 1	*cmlc1*	F: GAGGCGGACCGTCTGATG R: ATGTGCTTGATGAAGGACGTGT	60	NM_131692.1

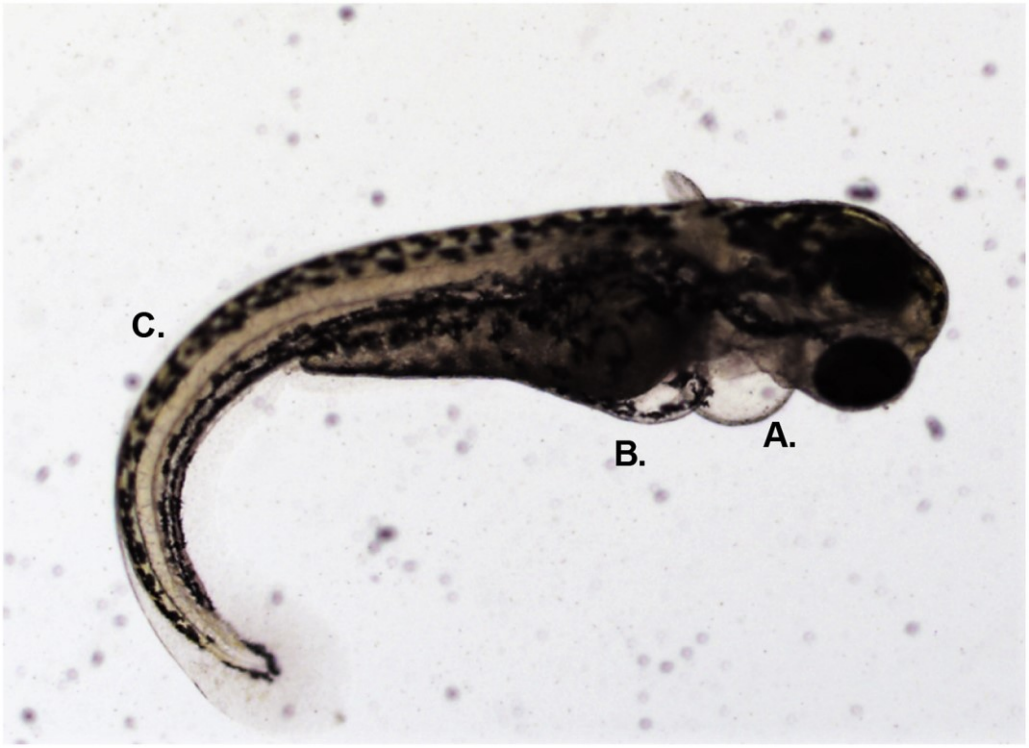


Figure 5- 1. Photograph of measured larval zebrafish developmental deformities. Pericardial edema (A), yolk-sac edema (B), and tail/spine curvatures (C) were measured in zebrafish at 120 hpf after acute embryonic (24 hpf) FPW exposures. Representative shown above was exposed at 24 hpf to 2.5% FPW-S fraction for 48 h. Image was taken at 20× magnification.

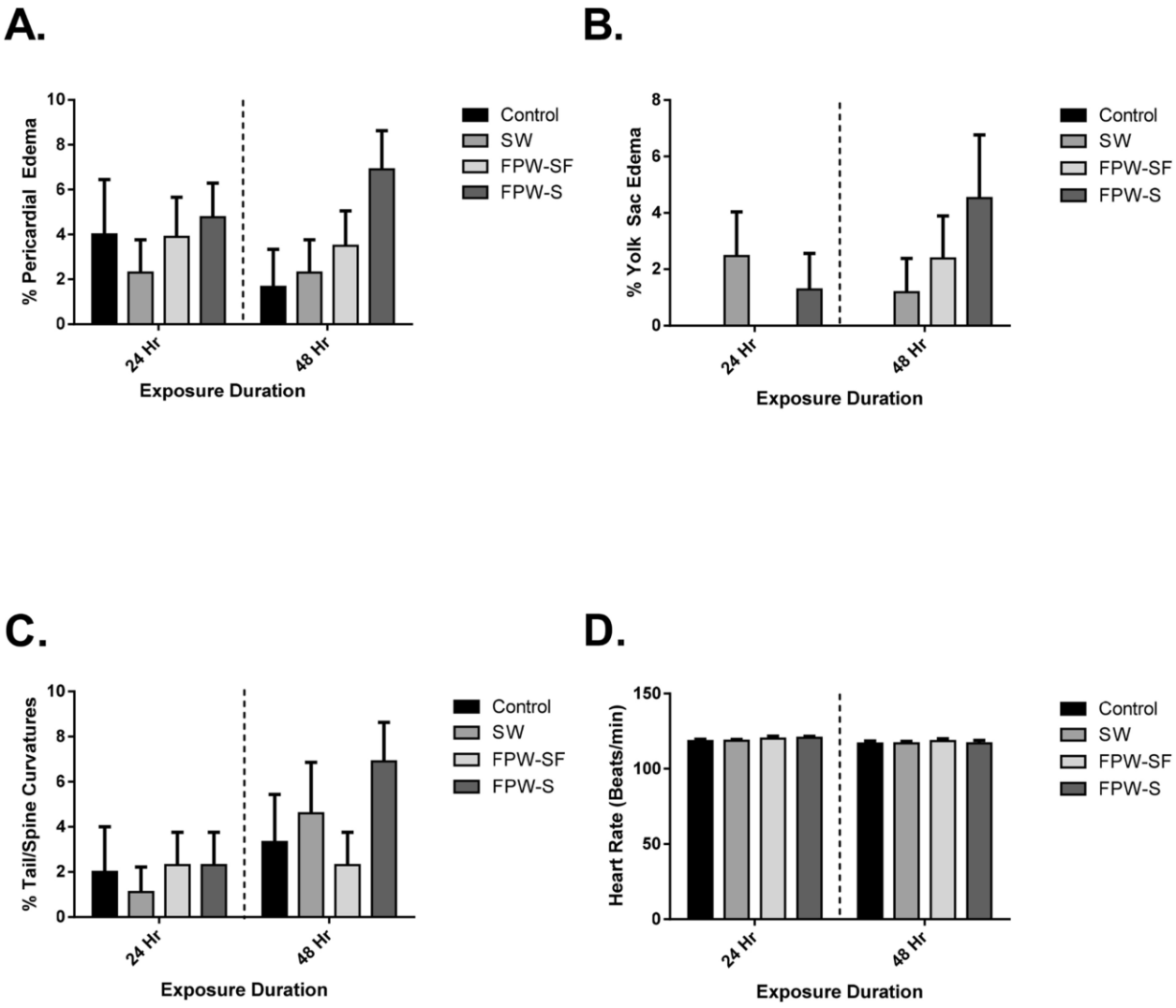


Figure 5- 2. Developmental deformities measured in zebrafish larvae at 120 h post fertilization (hpf) after acute embryonic (24 hpf) exposure to control, salt control (SW), sediment-free (FPW-SF), and raw/sediment containing (FPW-S) hydraulic fracturing flowback and produced water (FPW) fractions at 1.25% dilutions for 24 and 48 h. Deformities analyzed included pericardial edema (panel A), yolk-sac edema (panel B), tail/spine curvature (panel C), and changes to heart rate (panel D). 6 replicates of 15 embryos per treatment exposed in 4 mL wells were performed to obtain results. Data are presented as mean ± SEM. One-way ANOVAs between fraction exposure conditions for each exposure duration followed by Holm – Sidak post hoc tests were performed to determine significant differences ($p < 0.05$) in percent deformity appearance after embryonic FPW exposure.

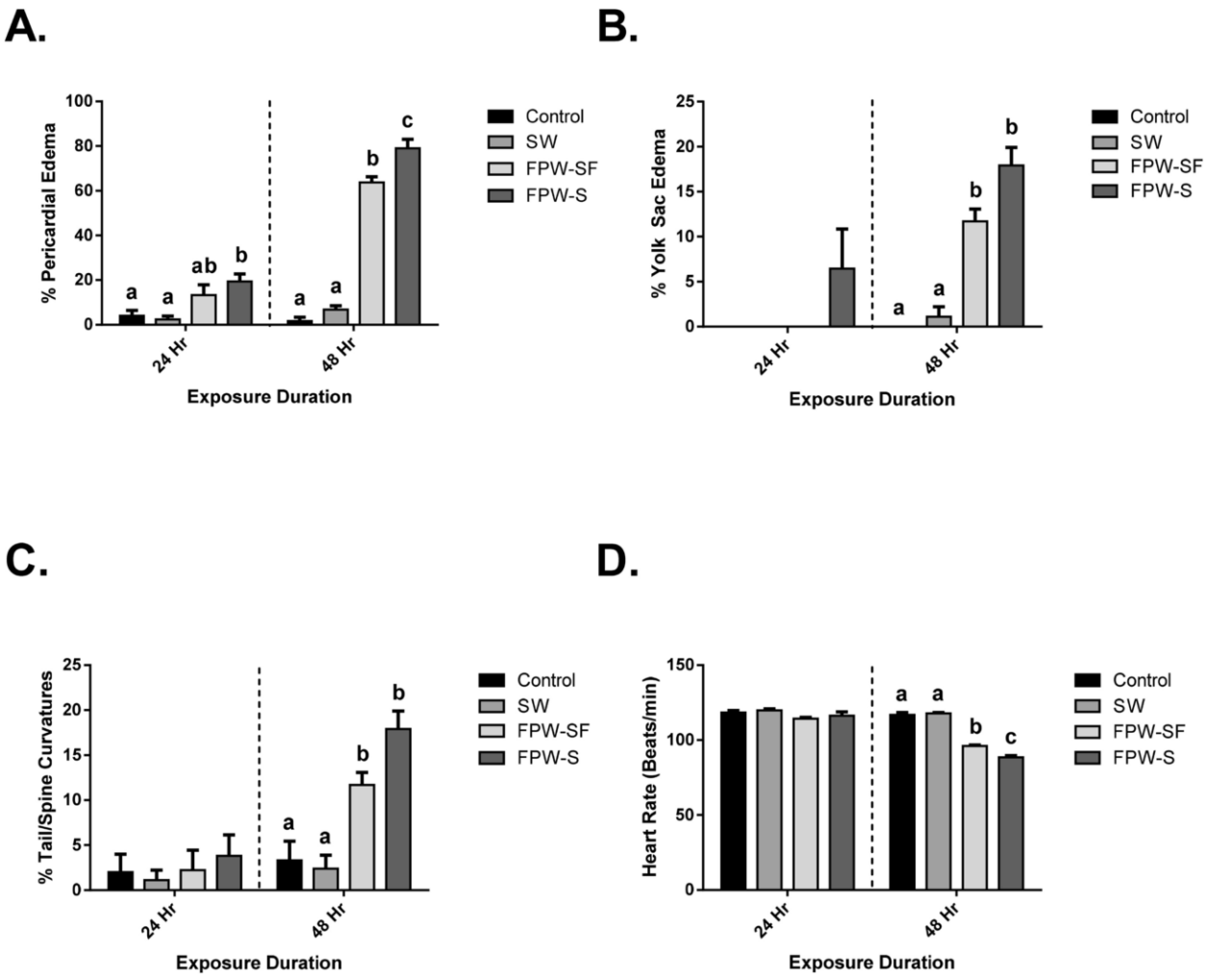


Figure 5- 3. Developmental deformities measured in zebrafish larvae at 120 h post fertilization (hpf) after acute embryonic (24 hpf) exposure to control, salt control (SW), sediment-free (FPW-SF), and raw/sediment containing (FPW-S) hydraulic fracturing flowback and produced water (FPW) fractions at 2.5% dilutions for 24 and 48 h. Deformities analyzed included pericardial edema (panel A), yolk-sac edema (panel B), tail/spine curvature (panel C), and changes to heart rate (panel D). 6 replicates of 15 embryos per treatment exposed in 4 mL wells were performed to obtain results. Data are presented as mean ± SEM. One-way ANOVAs between fraction exposure conditions for each exposure duration followed by Holm – Sidak post hoc tests were performed to determine significant differences ($p < 0.05$) in percent deformity appearance after embryonic FPW exposure.

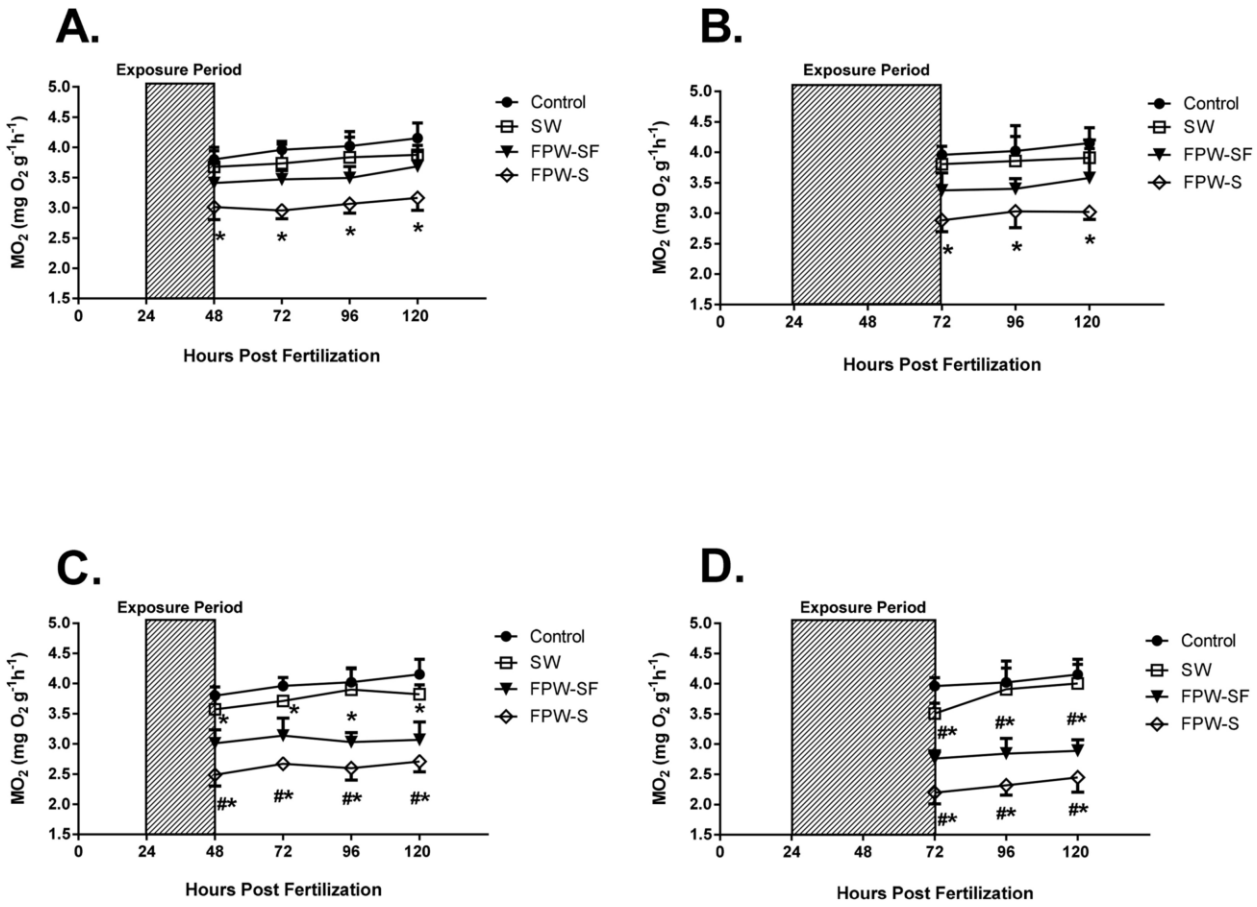


Figure 5- 4. Oxygen consumption (MO_2) measurements in developing zebrafish embryos/larvae at 25 °C after 24 h acute, embryonic (24 hpf) exposures to control, salt control (SW), sediment-free (FPW-SF), and raw/sediment containing (FPW-S) hydraulic fracturing flowback and produced water (FPW) fractions of 2.5% (panels A) and 5% (panels C) dilutions **or** 48 h acute exposures of 2.5% (panel B) and 5% (panel D) dilutions. 6 replicates per treatment per time point of 5 fish per well were used to obtain results. Data are presented as mean ± SEM. One-way ANOVAs for fraction treatment at each developmental period followed by Holm – Sidak post hoc tests were performed to determine significant differences ($p < 0.05$) in O_2 consumption in zebrafish embryos/larvae after FPW exposure. Significant differences from control and SW conditions are indicated with (*) and (#), respectively.

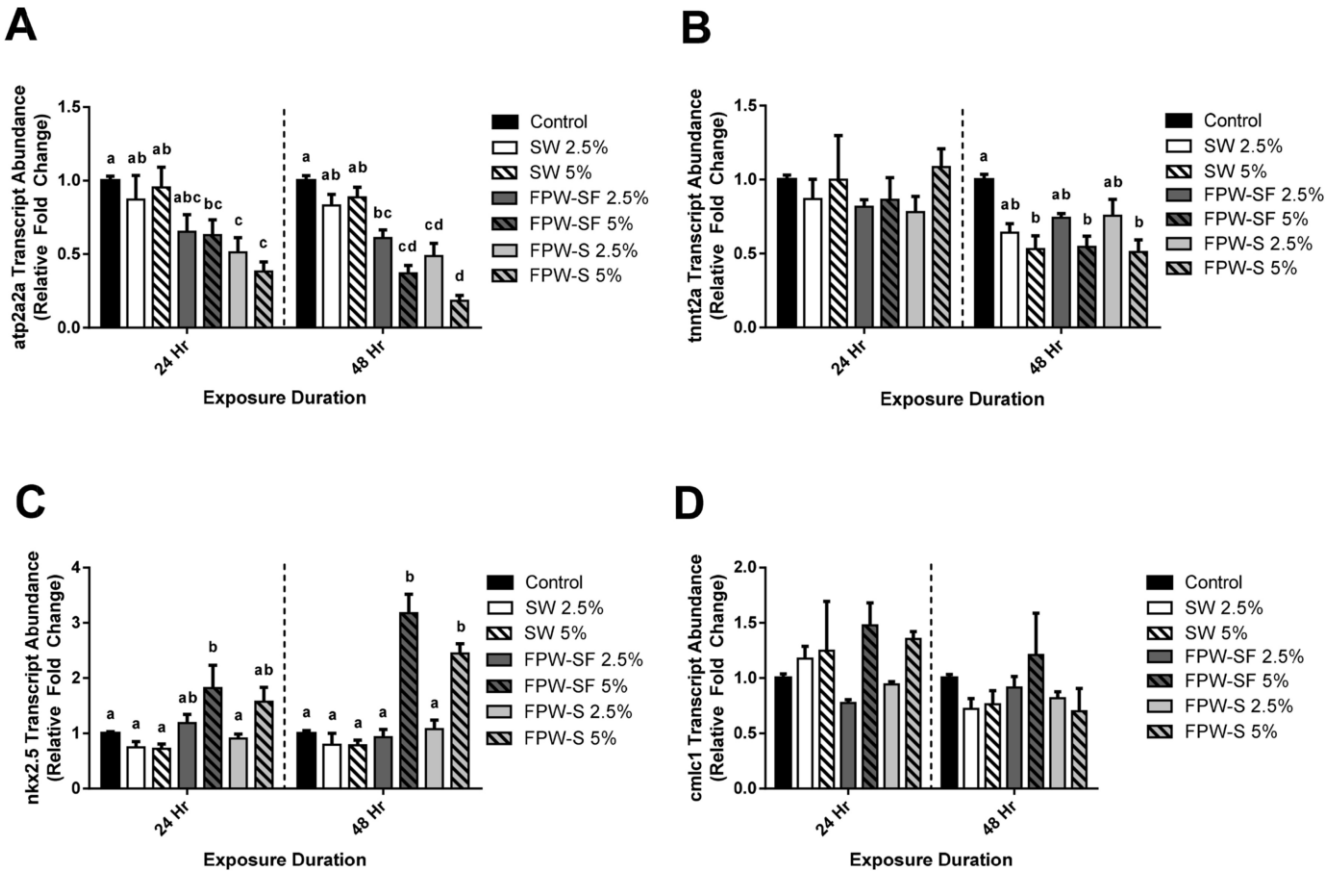


Figure 5- 5. Relative mRNA abundance of (A) cardiac sarcoplasmic reticulum Ca^{2+} ATPase 2a2a (*atp2a2a*), (B) cardiac troponin T type 2a (*tnnt2a*), (C) homeobox protein nkx2.5 (*nkx2.5*), and (D) cardiac myosin light chain 1 (*cmlc1*) in zebrafish embryos (24 hpf) acutely exposed to 24 or 48 h control, salt control (SW), sediment-free (FPW-SF), and raw/sediment containing (FPW-S) hydraulic fracturing flowback and produced water (FPW) fractions at 2.5% or 5% dilutions. Transcript abundance was determined by quantitative real-time PCR using the ΔΔCT method. 6 replicates of each treatment condition were performed to obtain results. Data are presented as mean ± SEM. Two-way ANOVAs using FPW fractions and FPW fraction concentration as factors at each exposure duration followed by Holm – Sidak post hoc tests were performed to determine significant changes ($p < 0.05$) of transcript abundances in zebrafish embryos.

CHAPTER 6: Alterations to Juvenile Zebrafish (*Danio rerio*) Swim Performance after Acute Embryonic Exposure to Sub-lethal Exposures of Hydraulic Fracturing Flowback and Produced Water

Folkerts, E.J., Blewett, T.A., He, Y., Goss, G.G. (2017). Alterations to juvenile zebrafish (*Danio rerio*) swim performance after acute embryonic exposure to sub-lethal exposures of hydraulic fracturing flowback and produced water. *Aquatic Toxicology*, **193**, 50 – 59. DOI: 10.1016/j.aquatox.2017.10.003.

6.1 INTRODUCTION

Horizontal hydraulic fracturing to extract oil and natural gas reserves is predicted to increase in practice in North American energy sectors (Gagnon et al., 2016). Consequently, larger volumes of hydraulic fracturing flowback and produced water (FPW), the wastewater solution produced after a well has begun flowing, is being produced. Increased production of this wastewater has led government, industry, landowners, and environmental groups to scrutinize current methods of storage, transportation, and remediation protocols when spills/leaks occur (Boudet et al., 2014; Gehman et al., 2016; Theodori et al., 2014). In the Duvernay formation located along the provincial borders of eastern British Columbia and western Alberta, Canada, horizontally fractured wells can individually produce millions of liters of FPW, resulting in large volumes of FPW to be properly disposed of (Alessi et al., 2017). With larger volumes comes increased likelihood of spills, resulting in extensive and costly remediation efforts (Entrekin et al., 2011; Goss et al., 2015; Kargbo et al., 2010). FPW is known to be a complex and heterogenous mixture, containing the original hydraulic fracturing (HF) fluid constituents (biocides, polymers, surfactants, etc.), high salt concentrations (from deep formation water sources), petroleum compounds from the formation (DiGiulio and Jackson, 2016; Lester et al., 2015), and products of any downhole reactions (He et al., 2017a). Composition of these different components can vary greatly depending on a multitude of factors including formation geogenic characteristics, well shut-in length, phase of flowback collection (early vs. late), and initial fracturing fluid chemicals used for operation − which are often proprietary (Alessi et al., 2017; Stringfellow et al., 2017). Correspondingly, site location and time of flowback collection can greatly affect FPW composition, although common chemical signatures across FPW samples tested are high salt solute concentrations and presence of geogenic constituents (e.g. organics, alkaline earth metals, metals in general, etc.) (Alessi et al., 2017; Goss et al., 2015; Stringfellow et al., 2017).

Previous studies have identified various markers of sub-lethal toxicity in aquatic organisms following FPW exposure. These markers include oxidative stress, xenobiotic metabolism, and reproductive effects (T. A. Blewett et al., 2017a, 2017b; He et al., 2017a, 2017b). Many of these sub-lethal effects have been attributed to the sediment and organic chemical constituents present in FPW. These observations are consistent with numerous

previous studies on other oil and gas industrial waste solutions and spill scenarios, including oil sands process-affected water (OSPW) (Frank et al., 2009; He et al., 2012a), crude oil solutions (e.g. Exxon Valdez and Deepwater Horizon disasters) (Incardona et al., 2013; Philibert et al., 2016), and FPW (T. A. Blewett et al., 2017a; He et al., 2017a, 2017b). All these previous studies have concluded that organics (e.g. naphthalene acids and polyaromatic hydrocarbons; PAHs), are at least major contributors to the toxicity observed in each species exposed to these industrial waste spill scenarios.

The presence of sediment and sediment associated organic hydrocarbons in our FPW sample (He et al., 2017a, 2017b) creates the physiochemical potential for settling of sediment when spills or leaks of FPW occur. Therefore, benthically located organisms during FPW spills/releases to bodies of water may be at higher risks of exposure and hold particularly relevant toxicological interest when investigating FPW toxicity adverse outcome pathways. Fish embryos, due to their sensitive developmental stage, are an important toxicological model of exposure and are frequently used to study a range of endpoints associated to toxic contaminants (Ankley and Johnson, 2004; Embry et al., 2010). Sediment present in FPW may settle and coat the chorion of embryos following release into a body of water (He et al., 2017a). Once coated, chorion oxygen (O_2) diffusion may be restricted, limiting the amount of fresh O_2 available to the developing organism and creating a hypoxic embryonic environment. Hypoxia experienced during development has been observed to induce metabolic depression, resulting in an array of pathologies (Guppy and Withers, 1999; Storey and Storey, 2004). Additionally, fish embryonically exposed to hypoxia have significantly decreased fitness as adults (Widmer et al., 2006). Furthermore, hypoxia experienced during development has been observed to effect numerous cardiovascular physiological properties (Bagatto, 2005; Jacob et al., 2002) and morphology (Chapman et al., 2000). Zebrafish cardiogenesis occurs 5–48 hpf, with epicardium development extending to 72 hpf (Bakkers, 2011; Glickman and Yelon, 2002). This period is considered a sensitive developmental “window”, where both endogenous and exogenous disturbances may elicit highest effects on the organism.

Swimming performance as a fish fitness index is intimately associated to essential behavioural processes such as food capture/prey avoidance, reproduction and migration (Hammer, 1995; Plaut, 2001; Reidy et al., 2000). Commonly measured as critical

swimming speed (U_{crit}) (Brett, 1964), many toxicants, such as organic hydrocarbons and metals, are reported to alter U_{crit} in fish (Mager et al., 2014; Stieglitz et al., 2016; Thomas et al., 2013; Yu et al., 2015). The importance of swimming performance to overall fish health and sensitivity of this marker to toxicants has resulted in its use as a common ecological endpoint to assess sub-lethal toxicity in fish (Lee, 2003; Little and Finger, 1990; Mager et al., 2014). Despite its acceptance as a critical indicator of fish fitness, U_{crit} analyses performed on fish after embryonic exposures to chemical toxicants are relatively more rare compared to exposures on adult or juvenile fish stages (Johansen and Esbaugh, 2017; Stieglitz et al., 2016; Thomas et al., 2013). However, studies examining swim performance in fish following weathered crude oil fraction embryonic exposures and growth to later life stages have found swim performance to be significantly impacted (Hicken et al., 2011; Mager et al., 2014). Considering this and the sensitive periodicity of the embryonic stage to environmental variations (Embry et al., 2010; Incardona et al., 2013), we tested embryonically FPW exposed zebrafish during their juvenile life stage (~ 60 days post-fertilization; dpf), and measured swim performance in a U_{crit} fashion as an index of fish fitness and sub-lethal response to FPW exposure. This involved subjecting juvenile zebrafish embryonically exposed to FPW fractions to 2 types of swim challenge tests. First, a classic U_{crit} swim test was employed where fish were confronted with incremental increases in water velocity in a step-wise fashion and swam until exhaustion. The second swim performance test used was a repeat, increased exertion swim test where fish, previously swam through a U_{crit} test, were again made to swim through a similar swim challenge, but with shorter time periods between step increases in water velocity to determine if more sensitive changes to fish fitness after embryonic exposure to FPW could be determined, as has been observed in other repeat swim performance studies (Jain et al., 1998).

Swimming metabolic oxygen consumption (MO_2) is closely tied to swim performance in fish, and is often measured during U_{crit} analyses (Mager et al., 2014; Stieglitz et al., 2016; Thomas et al., 2013). Alterations to MO_2 often involve changes to metabolic capacity and respiratory efficiency and are caused by several known non-xenobiotic stressors, such as temperature and hypoxia (Schurmann and Steffensen, 1997). However, many toxicants including organic pollutants, metals, and other anthropogenic

contaminants, are also known to affect MO_2 in fish (Scott and Sloman, 2004). Changes to fish respiratory capacity measured by aerobic scope (the MO_2 difference between fish standard/maintenance routine swimming metabolic requirements [standard metabolic rate; SMR] and maximum, active metabolic requirements [active metabolic rate; AMR]) may reflect alterations to fish physiological functioning and fitness, ultimately resulting in decreased swim performance (Brett, 1964; Shingles et al., 2001; Thomas et al., 2013). In the present study, we use a modified aerobic scope measurement termed factorial real-time aerobic capacity (F-rtAC), measured as the quotient of AMR and routine metabolic rate (MO_2 during routine, free-swimming behavior; RMR), to measure changes in juvenile zebrafish aerobic potential in response to FPW exposure.

As the practice of HF continues to increase, the potentials of FPW release events to aquatic ecosystems becomes greater accordingly (Alessi et al., 2017; Entrekin et al., 2011; Goss et al., 2015). Our previous study associated embryonic cardio-respiratory toxicity to organics present in FPW (Folkerts et al., 2017a) via appearance of pericardial edema developmental deformities, reduced embryonic metabolic rate, and alterations in cardiac-related gene expression. By analyzing juvenile zebrafish fitness after acute embryonic FPW exposure, our aim was to determine if embryonic hypoxic effects are observed immediately after FPW exposure, and determine if hypoxic or other cardiovascular effects persist and affect key physiological parameters needed in later life-stage fish fitness.

6.2 MATERIALS AND METHODS

6.2.1 Animal Species

Adult zebrafish (wild-type, strain AB) housed in 30 L tanks (~ 25 fish/tank; 14 h light:10 h dark photoperiod) at the University of Alberta (U of A) zebrafish facility were bred to produce viable fertilized embryos for study. All animal use was approved by the U of A Animal Care Committee under protocol AUP00001334. Fertilized embryos were collected and incubated at 28 °C under fluorescent facility lighting with zebrafish facility water (pH: 7.4, conductivity: 168.5 ± 0.5 µS/cm, temperature: 28.5 ± 1 °C, dissolved oxygen: 7.5 ± 0.5 mg/L, general hardness: 175 mg/L as $CaCO_3$, salinity: 0 ppt) until FPW fraction exposures 24 hr later.

6.2.2 FPW Sample

A 7-day post stimulation FPW sample from a horizontally-fractured well (Duvernay formation, Fox Creek, Alberta, Canada) was used for exposures in the current study. Two treatments associated with our FPW sample were created and used for zebrafish embryonic exposures. The first, a sediment-free fraction (FPW-SF) was created by centrifuging raw FPW on an Eppendorf 5810 R centrifuge (Eppendorf, Hamburg, GER) at 3220 g for 10 min and subsequent supernatant vacuum filtration through 0.22 μm filter (Sarstedt, North Rhine-Westphalia, GER). The second termed sediment containing fraction (FPW-S), consisted of the raw FPW sample. Inorganic and organic chemical characterization details of these FPW fractions can be found in (He et al., 2017a, 2017b). A salt control (SW) matching the major ion concentrations (Na, Mg, K, Ca, and Cl; Sigma- Aldrich, MO, USA) in the raw FPW sample was also made to account for any saline influenced responses (see Table S1 [Table S3-1] of He et al., 2017a).

6.2.3 Experimental Design and Exposures

Zebrafish embryos (24 hpf) were exposed to the following treatments conditions: FPW-S, FPW-SF, SW and control (zebrafish facility water) for two different acute exposure durations of 24 and 48 hrs. Zebrafish embryos were exposed to 2.5% FPW, 5% FPW, SW and control conditions for both exposure durations. All treatments were run in parallel with each other and a paired control (zebrafish facility water) (n = 6 for each fraction exposure treatment for all assays and analyses). Fifty percent water volume replenishments were performed every 24 h. For each assay, exposures were performed at the same time, with random pairing of male and female zebrafish to obtain offspring. Subsequent selection of embryos for exposure was similarly random, with no selection criteria used besides ensuring embryos had in fact been fertilized (performed by observing under a steromicroscope).

For real-time quantitative PCR (qPCR) analyses, 10 embryos were exposed in 100 mL of fraction dilution treatment solutions in 250 mL glass beakers (10 embryos used for each RNA extraction per replicate; n = 6 for each treatment condition). Following

exposure termination, embryos were removed from initial exposure beakers, washed 3 times with clean zebrafish facility water, and snap-frozen in 1.5 mL centrifuge tubes using liquid nitrogen and stored at −80 °C for later qPCR analysis.

For juvenile swim performance and real-time aerobic capacity analyses, 5 zebrafish embryos per treatment replicate were exposed in 100 mL of fraction treatment solutions in glass beakers. Following exposure termination, 1 embryo was removed, washed 3 times with clean zebrafish facility water, and placed into individual glass beakers containing fresh facility water and grown to 120 hpf (n = 6 for each treatment condition). Once grown to 120 hpf, larval zebrafish of same embryonic exposure treatments were collectively placed into 10-L tanks containing facility water. Tanks were placed on a flow-through circuit in the U of A zebrafish facility and embryos were reared to 60 days post fertilization (dpf). Fish at this stage had an average length of 2.9 cm (± 0.2 cm) and weight of 0.149 g (± 0.05 g). At 60 dpf (± 3 d), 6 fish with no observable deformities from each treatment group were analyzed for swim performance and metabolic capacity.

6.2.4 Embryonic Quantitative Real-time PCR

Transcript abundance was quantified using quantitative real-time PCR (qPCR). Total RNA from 10 zebrafish embryos was extracted using the MasterPure RNA Purification Kit (Epicentre Biotechnologies, WI, USA). Briefly, recombinant DNase I (Ambion) and 10 × reaction buffer with $MgCl_2$ (ThermoFisher Scientific, MS, USA) were added to embryo samples prior to a 30-minute incubation period at 37 °C. Reaction was terminated with 50 mM ethylenediaminetetraacetic acid and samples were stored in nuclease-free water (non-DEPC treated, Ambion) containing SUPERase-In RNase Inhibitor at −20 °C. Total RNA was quantified using a NanoDrop ND-1000 spectrophotometer (v. 3.8.1, NanoDrop Technologies, USA). RNA integrity was assessed on a 2% formaldehyde − agarose gel with ethidium bromide. Following RNA extraction, 0.5 µg of RNA samples were subjected to same-day cDNA synthesis using the RevertAid First Strand cDNA Synthesis Kit (ThermoFisher Scientific, MS, USA) and were stored at −20 °C until further analysis.

Expression analysis was performed using an ABI 7500 Real-Time PCR system (Applied Biosystems, Foster City, CA, USA). Gene specific primers were designed against

target genes using Primer Express v3.0.1 software (Thermofisher Scientific, MS, USA). Primer sequence and target information can be found in Table 6-1. Primer efficiency validations, determined by serial dilution slope calculation after ΔCT and log input regression analyses, can be found in Figure S6-1. Individual PCR reaction mixtures contained 5 µl of SYBR Green master mix (Applied Biosystems, Foster City, CA, USA), 2.5 µl of 3.2 µM primer sets, and 2.5 µl of cDNA diluted in nuclease-free water. PCR reactions were denatured at 95 °C for 10 minutes prior to first thermal cycle initiation. A 2-step PCR thermal cycle, consisting of a denaturation step of 95 °C for 2 minutes and an annealing-extension step of 60 °C for 1 min, was employed for 40 cycles and transcript expression changes determined by ΔΔCT analysis using glyceraldehyde 3-phosphate dehydrogenase (*gadph*) as an endogenous control (Schmittgen and Livak, 2008).

6.2.5 Juvenile Zebrafish Swim Performance

Juvenile zebrafish swim performances were conducted in a 10-L, variable speed swim tunnel outfitted with a DAQ-M control device and AutoResp™ 1 software (Loligo Systems, Viborg, DEN). Unit set-up consisted of a sealed 10-L swim tunnel submerged in a 45-L buffer tank supplied with aerated 28 °C (± 0.5 °C) U of A zebrafish facility water. Juvenile zebrafish were fasted 24-h prior to swim performance and metabolic rate analyses. Fish were placed in the swim tunnel and immediately acclimated for 45 min to the swim tunnel with a 4.5 cm/s swim velocity. After 45 min, water velocity step ramp-ups and U_{crit}/U_{max} (maximum swimming speed) analyses were performed.
During U_{crit} experiments, individual fish swim trials consisted of step-wise increments in swimming velocities of 4.5 cm/s every 5 min until exhaustion. During U_{max} experiments, individual fish swim trials consisted of step-wise increments in swimming velocities of 4.5 cm/s every 1 min until exhaustion. Fish exhaustion was determined after fish rested on the swim tunnel back gate for 5 seconds and could not be stimulated to resume swimming after decreasing water velocity to 9 cm/s. Following U_{crit} experiment completion, fish were immediately placed into a respirometer (information listed in section 6.*2.6*) for active metabolic rate analyses (approximately 20 − 30 min), and then returned to the swim tunnel for U_{max} analyses. Fish were acclimated to 4.5 cm/s water velocities for 45 min prior to U_{max} experiments begun. Critical swimming speeds were calculated using the equation

described in (Brett, 1964): $U_{crit} = [U_f + (T/t) * dU]/cm$, where U_f (cm/s) represents the highest maintained swim velocity for an entire step, T (s) represents time spent at the maximum achieved swim velocity, t (s) represents the time interval of each step, and dU (cm/s) represents the swim speed increment in of each step. Fish cross-sectional area was less than 5% of swim tunnel diameter, thus solid blocking effects were not applied to U_{crit} and U_{max} values. Once swim trials and metabolic rate analyses were concluded, individual fish were euthanized using an overdose of MS-222 (1 g/L), dried via blotting on absorbent paper, weighed, and measured for length. U_{crit} and U_{max} swim speed values were subsequently corrected for standard body lengths of each individual fish, and represented as body lengths per second (BL/s). It is important to note that U_{crit}/U_{max} values can vary depending on a multitude of factors (outlined in Hammer, 1995). For purposes of this study, swim performance observations are for the majority compared solely against trends observed in similar xenobiotic exposure studies.

6.2.6 Juvenile Zebrafish O_2 Consumption and Determination of RMR, AMR, and F-rtAC

Juvenile zebrafish O_2 consumption was measured using a modified 150 mL, stand alone respirometer immersed in a 10 L, opaque buffer tank supplied with aerated 28 °C (± 0.2 °C) water. Respirometer was fitted with both a Class A pt1000 temperature probe and a Witrox II O_2 dipping probe connected to a Witrox 1 fiber optic oxygen instrument and Witrox Viewer software (v1.0.0)(Loligo Systems, Viborg, DEN). Unit set up was placed on top of a magnetic stir table, with a small stir bar slowly spinning inside the respirometer to ensure water mixing occurred during MO_2 recordings.

Prior to placement into swim tunnel and commencement of swim trials, juvenile zebrafish were placed first into the respirometer and RMR was established. Free-swimming fish RMR (mg O_2 kg^{-1} hr^{-1}) was calculated by measuring the rate of O_2 decrease inside the respirometer chamber from 30–60 min. Recordings before 30 min were not included in RMR determination to ensure fish acclimation and stable MO_2 readings occurred. Fish AMR was determined immediately following completion of U_{crit} analyses by netting fish out of the swim tunnel and swiftly placing into the respirometer chamber. AMR (mg O_2 kg^{-1} hr^{-1}) was defined as the change in O_2 following the immediate placement of fish in

the respirometer chamber, reflecting fish MO_2 at maximal sustainable speeds during the U_{crit} analysis. F-rtAC was calculated as AMR/RMR (similar to factorial aerobic scope in Shingles et al., 2001; Thomas et al., 2013). During both RMR and AMR determination, any fish MO_2 recordings below 70% air O_2 saturation levels were not included in analyses.

6.2.7 Statistical Analyses

Shapiro-Wilk tests were applied to all data to test for normality and homogeneity of variance was assessed by use of Levene's tests (SigmaPlot 13.0, Systat Software Inc., San Jose, CA, USA). When necessary, data $\log_{10}$-transformation was performed to meet assumptions of parametric tests. Non-transformed data are represented in all figures. Prism 6.0 was used for all other outlined statistical analyses (GraphPad Software Inc., San Diego, CA, USA). Transcript abundances were tested by use of a two-way ANOVA, followed by a Holm-Sidak post hoc, multiple range test with FPW fraction type and FPW dilution percentage as independent variables. Juvenile zebrafish U_{crit}/U_{max}, RMR, AMR, and F-rtAC were similarly tested by use of a two-way ANOVA, with FPW fraction type and exposure duration as independent variables. No interactions between factors were found for all two-way ANOVAs. All data are expressed as mean ± S.E.M. Differences were considered statistically significant at $p \leq 0.05$.

6.3 RESULTS

6.3.1 Transcript Abundances of Target Genes

Hypoxia-responsive gene transcript abundance was significantly affected after acute FPW fraction exposures (Figure 6-1). Acute exposures (24 h) of 5% FPW-SF and FPW-S fractions significantly increased the expression of *epoa* compared to control conditions in zebrafish embryos. Furthermore, 5% acute exposure with FPW-SF fractions (24 h) significantly increased the expression of *epoa* over SW controls (Figure 6-1B). Largest *epoa* upregulation was observed in 5%, 48 h FPW-SF and FPW-S fraction exposure embryos, where approximately 3-fold increases in expression were observed over freshwater control and SW control treatments (Figure 6-1B). Transcript abundances of both *hbbe1.1* (Figure 6-1A) and *hif1aa* (Figure 6-1C) were not significantly different among any of the treatment groups.

6.3.2 Swim Performance

Juvenile zebrafish swim performance was significantly altered after acute embryonic exposure to FPW fractions (Figures 6-2, 6-3). Juvenile U_{crit} values were reduced significantly after both 24 and 48 h embryonic exposures to 2.5% FPW-SF and FPW-S fractions compared to control conditions (Figure 6-2). Additionally, juvenile fish exposed to 2.5% S fractions for 48 h displayed a significant 19.1% reduction in U_{crit} performance compared to SW control conditions. Similarly, all juvenile U_{crit} values were significantly reduced after 24 and 48 h embryonic exposures to 5% FPW-SF and FPW-S fractions compared to control and SW control conditions, with significant U_{crit} reductions of 18.4 and 22.4% occurring in 5% FPW-SF and FPW-S exposed fish for 48 h, respectively. Greatest decreases to swimming performances were observed in U_{max} analyses, where all FPW-SF and FPW-S fraction exposures displayed significantly reduced U_{max} values compared to both control and SW control conditions (Figure 6-3).

6.3.3 RMR, AMR, and F-rtAC

Acute, embryonic exposures to FPW fractions significantly reduced metabolic capacities of juvenile zebrafish (Figures 6-4, 6-5). Regardless of embryonic exposure regiment, juvenile zebrafish RMR did not change significantly between treatment groups (Figure 6-4A). However, mirroring U_{max} results, embryonic exposures to both FPW-SF and FPW-S fractions in all treatment conditions significantly reduced AMRs in juvenile zebrafish compared to control water treatments (Figure 6-4B). Furthermore, embryos exposed to 5% FPW-S fractions for 24 h displayed a significantly reduced AMR of 48% compared to 5% SW control exposed embryos for 48 h. Consequently, F-rtAC in juvenile fish after FPW-SF and FPW-S fraction acute exposures were also significantly reduced compared to controls (Figure 6-5). Although significantly reduced F-rtAC values were observed in FPW-SF and FPW-S embryonically exposed fish at 2.5% dilutions for 48 h exposures compared to freshwater controls (44% and 41% reductions, respectively), greatest reductions of approximately 50% were observed for all FPW-SF and FPW-S fraction exposures at 5% compared to freshwater control conditions. (Figure 6-5).

6.4 DISCUSSION

The present study attributes embryonic exposure to FPW with sub-lethal physiological performance measurements in later life fish stages, as measured by swim performance and aerobic scope in juvenile zebrafish. Following our previous study which associated embryonic cardio-respiratory toxicity to organics present in FPW (Folkerts et al., 2017a), it was hypothesized that the cardiorespiratory responses observed in developing embryos (e.g. pericardial edema, reduced embryonic metabolic rate, alterations in cardiac-related gene expression) would persist and affect juvenile fish forms, thereby reducing swimming performance and aerobic capacity. Organics, such as PAHs and dioxins, are well-documented cardiovascular toxins, producing specific pathologies such as arrhythmia, altered cardiomyocyte functioning, and cardiovascular deformities (Brette et al., 2014; Incardona et al., 2009). Chemical characterization of our FPW sample (He et al., 2017a, 2017b) has identified the presence of many organic contaminants of potential toxicological concern, including many priority PAHs (such as phenanthrene, anthracene, and chrysene among others). Therefore, it is hypothesized that organics may be the primary chemical fraction responsible for much of the sub-lethal responses observed to date.

During FPW exposure, we have demonstrated a reduction in total oxygen consumption. This may either be due to a restriction in acquisition of oxygen by the embryo, an induced metabolic depression (Guppy and Withers, 1999) or a combination of the two processes. It is known that FPW has constituents, including polymers and sediments, known to associate with the chorion, potentially increasing the boundary layer and impeding oxygen acquisition (He et al., 2017a). Metabolic depression has been shown to induce a variety of pathologies in fish, including reduced growth rates, inhibited movement and activity, and cellular effects related to ion and acid-base regulation (Guppy and Withers, 1999; Storey and Storey, 2004). Similarly, hypoxia has also been shown to induce ischemia and cellular damage in an array of tissues, notably cardiovascular tissues (Chi and Karliner, 2004). As a result, organ development and functioning in mature fish after hypoxic events experienced as embryos may be altered, producing deleterious effects on metabolic capacities and fish fitness, as observed by adult zebrafish swim performances after exposure to hypoxic conditions as embryos (Widmer et al., 2006).

Of the hypoxia transcript markers analyzed in the present study, erythropoietin (EPO, subunit alpha; *epoa*) alone was significantly up-regulated in zebrafish embryos exposed to FPW fractions (Figure 6-1). EPO is a hormone primarily responsible for erythropoiesis and red blood cell (RBC) formation (Siren et al., 2001), and is often used as an index for measuring hypoxic responses in organisms (Dangre et al., 2010; El Hasnaoui-Saadani et al., 2009; Jelkmann, 1992; Lai, 2006). In the present study, both FPW-SF and FPW-S fractions induced *epoa* expression at 5% dilutions, but not the SW control, suggesting organics and other constituents in FPW may be responsible for apparent hypoxic responses. Although primarily reported in airborne exposure studies, organics and different PAHs have been shown to induce hypoxia markers, such as EPO and hypoxia-inducible factor (HIF) expression (H. J. Kim et al., 2016; Mavrofrydi et al., 2016; Song et al., 2013). However, it is important to note EPO's involvement in numerous other pathways and physiological roles, such as tissue protective functions (specifically neuro and cardioprotective roles), enhancement of immune responses, anti-inflammatory effects, and angiogenesis inductive roles (Burger et al., 2006; Katz et al., 2007; Kertesz et al., 2004; Lifshitz et al., 2010; Mitsuma et al., 2006; Wang et al., 2004). Therefore, up-regulated *epoa* expression in the absence of other hypoxia related gene markers after FPW-SF or FPW-S fraction exposure may be in response to one or more of the above-stated physiological mechanisms, and not in response to hypoxia experienced by embryos after FPW exposure. Further study is clearly needed to fully understand the dynamics and effects of EPO regulation, and delineate the interplay of EPO-mediated responses after FPW exposure.

It is theorized critical periods during development make embryos extremely susceptible to both anthropogenic and environmental disturbances relative to adult fish (Woltering, 1984). Xenobiotic exposures may potentially cause negative latent effects on fish fitness after initial acute embryonic FPW exposure. Swimming performance is a commonly used fitness index to measure organismal responses after exposure to varying environmental perturbations due to its integration of many physiological processes (Brett, 1971; MacNutt et al., 2004; Scott and Sloman, 2004; Wicks et al., 2002). Presently, we employed repeat U_{crit} analyses to observe changes in juvenile zebrafish fitness following embryonic FPW exposure. Repeat U_{crit} analyses, where two successive U_{crit} tests are

performed with a short recovery period (measuring the ability of fish to recover from exhaustive activities), are reported to be a more sensitive marker of fish fitness than single U_{crit} analyses (Jain et al., 1998; McKenzie et al., 2007). Pairing this second U_{crit}, repeat performance measure with shortened water velocity-step times can also be viewed as a U_{max} exercise test, where dynamics of fish swimming limits can be more thoroughly investigated. Unlike U_{crit} analyses where fish employ red muscle aerobic catabolism of energy (e.g. triglycerides) to power swimming behaviour, swim tests which employ shorter swim velocity-steps or induce "burst-like" swimming behaviour measure a fish's ability to promote white muscle activity to anaerobically consume glycogen reserves to power swimming (Hammer, 1995; Moyes and West, 1995). Our U_{max} performance tests, therefore, measured how embryonic FPW exposures may have influenced and affected a later life stage fish's ability to recover from strenuous activity and promote to a greater extent a larger amount of anaerobic white muscle to power swimming. PAHs and other organic compounds (dioxins, phenols, etc.) have been shown to reduce swim performance in fish exposed as both embryos/larvae and adults (Lucas et al., 2016; Mager et al., 2014; Marit and Weber, 2012, 2011; Stieglitz et al., 2016; Yu et al., 2015). Although quantitative compositional PAH analysis of our FPW sample (He et al., 2017a, 2017b) revealed a relatively low PAH exposure stress compared to other crude oil and petroleum exposure studies (Holth et al., 2014; Mager et al., 2014), juvenile zebrafish acutely exposed as embryos to 5% FPW-SF and FPW-S fractions had significantly reduced U_{crit} and U_{max} values compared to both freshwater and salt control conditions (Figures 6-2 and 6-3). These reduced swim performance observations suggest organics in our FPW sample may be responsible for decreased swim performance. Furthermore, all 2.5% FPW-SF and FPW-S fraction embryonically exposed juvenile fish displayed significantly reduced U_{max} values compared to control and SW control treatments (Figure 6-4). This differs from U_{crit} results, confirming that our repeat swim performance analyses combined with shortened swim step increments are a more sensitive swim performance assay for measuring fish fitness after exposure to xenobiotics. In the present study, U_{crit} values observed in control fish (14.7 BL/s) are greater than those observed in other studies using adult zebrafish (Thomas et al., 2013; 13.5 BL/s and Thomas and Janz, 2011; 9 BL/s). However, differences in fish age, acclimation periods, and U_{crit} protocols

could have all contributed to larger U_{crit} values observed in the present study, as differences in both fish biology/maintenance and swim test parameters have been shown to alter swimming performance in U_{crit} analyses (reviewed in Hammer, 1995).

Due to the diversity of physiological processes associated with swimming behavior, physiological and cellular mechanisms behind petroleum-related contaminant induced effects on swim performance can be cumbersome to elucidate. Fish swim performance is potentially affected by several physiological and behavioral mechanisms, such as alterations to swim O_2 handling (cardiac focused effects), recruitment of glycolytic energetic processes, and fish swim tunnel ground speed establishment (lateral line and neuronal focused effects) (Farrell, 2007; McDonnell and Chapman, 2016; Thomas et al., 2013). Previously observed developmental defects and changes to transcript expression in developing zebrafish embryos after FPW exposure (Folkerts et al., 2017a) suggested a cardio-respiratory associated toxicity to FPW exposure. Thus, it is hypothesized cardiovascular O_2 handling, measured presently as F-rtAC, may be a key organismal process affected by organic exposure in our FPW sample. Changes to aerobic capacity may be caused by a limiting stress on fish O_2 kinetics (uptake and/or delivery) to reduce AMR, or increased loading stress of metabolic processes resulting in heightened RMR (Brett, 1958; Wilson et al., 1994). FPW embryonic exposures did not significantly change RMRs in juvenile zebrafish (Figure 6-4A). However, exposure to FPW-SF and FPW-S fractions significantly reduced AMRs in juvenile zebrafish (Figure 6-4), subsequently reducing aerobic scopes in FPW-SF and FPW-S fraction exposed fish (Figure 6-5). After embryonic exposure to Alaska North Slope crude oil, PAHs were attributed to both changes in ventricular shape in developing zebrafish larvae and decreased swim performance in adult fish (Hicken et al., 2011). Although swimming respiration was not recorded in these adult fish, results from other mutational defect studies support the notion that cardiac form is tightly associated to function, with cardiac structure mutants experiencing hindered cardiac performance (Glickman and Yelon, 2002). As mentioned previously, exposure to PAHs and other oil-related contaminants has been shown to alter cardiac form and physiological functioning − pathologically manifested as arrhythmia and altered electrogenic properties of certain cells/tissue types in exposed fish (Brette et al., 2014; Edmunds et al., 2015; Incardona et al., 2009, 2004; Stieglitz et al., 2016). Correspondingly, studies exposing fish

to crude oil contaminants, in addition to other toxicants such as metals, have associated swim performance changes to either increases in SMR or decreases in AMR, ultimately reducing aerobic capacity and/or increasing cost of transport (Mager et al., 2014; Mager and Grosell, 2011; Massé et al., 2013; Stieglitz et al., 2016; Thomas et al., 2013). Our results agree with these previous studies which correlate organic contaminant exposure with reduced swimming metabolic capacities and swimming performance in exposed fish. Furthermore, significant AMR reductions in FPW-SF and FPW-S fraction embryonically exposed juveniles indicates a limiting oxygen uptake/transport stress occurs in FPW exposed fish, implicating the cardio-respiratory system as a toxicological endpoint when investigating FPW toxicity. This would agree with our previous embryonic study and other crude oil exposure studies (Folkerts et al., 2017a; Hicken et al., 2011; Incardona et al., 2015).

6.5 CONCLUSIONS

Organisms inhabiting ecosystems affected by FPW contamination may be exposed to a wide variety of chemicals and potential toxicants. The mechanisms and physiological processes in organisms affected after FPW exposure is only beginning to be explored, and research on long-term implication of FPW exposure is needed. This is the first known observation of persistent toxic effects associated to HF-FPW exposure in an aquatic model system. Embryonic/larval toxicity, in addition to persistent physiological effects, may have serious repercussions on population recruitment and structure dynamics, as seen in pink salmon (*Oncorhynchus gorbuscha*) populations after the 1989 Exxon Valdez oil release (Heintz et al., 2000; Heintz, 2007). Previous studies have identified inductive developmental morphometric deformity potentials, while subsequent gene expression analyses revealed that the cardio-respiratory system may be affected following FPW exposure in developing zebrafish (Folkerts et al., 2017a). Interestingly, observations of suppressed metabolic rates in both embryonic/larval fish immediately following FPW fraction exposure termination, and in larvae removed from FPW exposure who continued to develop (Folkerts et al., 2017a), suggest that sub-lethal toxic effects experienced by developing fish exposed to FPW may persist and potentially have chronic, lasting effects into later life stages.

Although fish overall make great cardiotox models, some fish experience greater aerobic stress and challenges during their life cycles over other fish which exhibit more sedentary and inactive behaviour. The need for a fish to be athletic and develop more robust aerobic physiology can be driven by numerous factors including predator-prey dynamics, spawning behaviours, and migration routes (Little and Finger, 1990; Scott and Sloman, 2004). It follows then that fish with heightened aerobic requirements may be more sensitive to impairments and alterations to their cardio-respiratory physiological functioning. One such familial grouping of athletic fish which comprise such aerobic lifestyles are the salmonids (*Salmonidae*) - of which the species rainbow trout (*Oncorhynchus mykiss*) is a member of. Conveniently, rainbow trout also have a historical habitat range in North America which overlaps with many regions which are experiencing intensifying hydraulic fracturing activity (Alessi et al., 2017; Kondash et al., 2018; MacCrimmon, 1971). Thus, this fish species is an ecologically relevant ecotoxicological model for investigating FPW toxicity in freshwater fish and may provide more useful results/data for regulators and policy makers governing fracturing activity and FPW management in such areas. These aspects, compounded with the fact that the rainbow trout is also a commonly used experimental species, naturally advocate for future investigations into the effects of FPW exposure on fish cardio-respiratory development and physiology.

Table 6- 1. Gene specific primer sequences used for quantitative real time PCR and analysis of transcript expression.

Target	Abbreviation	Primer Sequence (5' – 3')	Annealing Temp. (°C)	Accession #
Glyceraldehyde 3-Phosphate Dehydrogenase	*gapdh*	F: GTAGATGTGACCCCTTTGCTGTT R: CAGGCACGTGGTGCAAAC	60	NM_001115114.1
Embryonic Hemoglobin Beta 1.1	*hbbe1.1*	F: GGCCGCTTTCCAGAAATTC R: CTCTCTGTCTGTATTTAGTGGTACTGTCTTC	60	NM_198073.2
Erythropoietin alpha	*epoa*	F: CGACAGGGTGTCAGCTGATAAA R: TAATAGTCCATATCCTGCCTCCTGATA	60	NM_001038009.2
Hypoxia Inducible Factor 1aa	*hif1aa*	F: CAGAGAAAAAGGTCCGCAAAA R: ATAACCGACTTGCAACATTGGA	60	NM_001308559.1

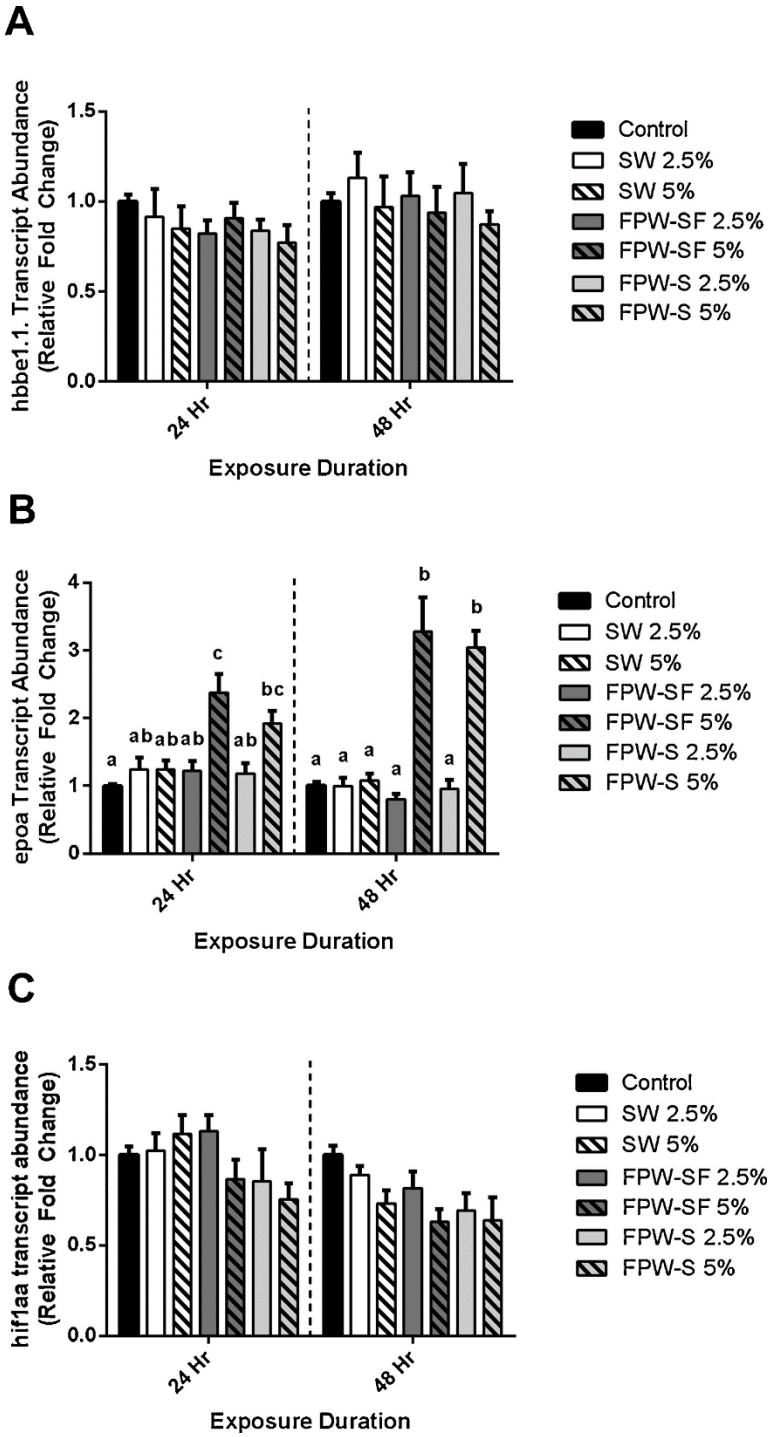


Figure 6- 1. Relative mRNA abundance of (A) embryonic hemoglobin beta 1.1 (*hbbe1.1*), (B) erythropoietin a (*epoa*), and (C) hypoxia inducible factor 1aa (*hif1aa*) in zebrafish embryos (24 hpf) acutely exposed to 24 or 48 hr control, salt control (SW), sediment-free (FPW-SF), and raw/sediment containing (FPW-S) hydraulic fracturing flowback and produced water (FPW) fractions at 2.5% or 5% dilutions. Transcript abundance was

determined by quantitative real-time PCR using the ΔΔCT method. 6 replicates of each treatment condition were performed to obtain results. Data are presented as mean ± SEM. Two-way ANOVAs using FPW fractions and FPW fraction concentration as factors at each exposure duration followed by Holm − Sidak post hoc tests were performed to determine significant changes ($p < 0.05$) of transcript abundances in zebrafish embryos. Significant differences are denoted by differing letters.

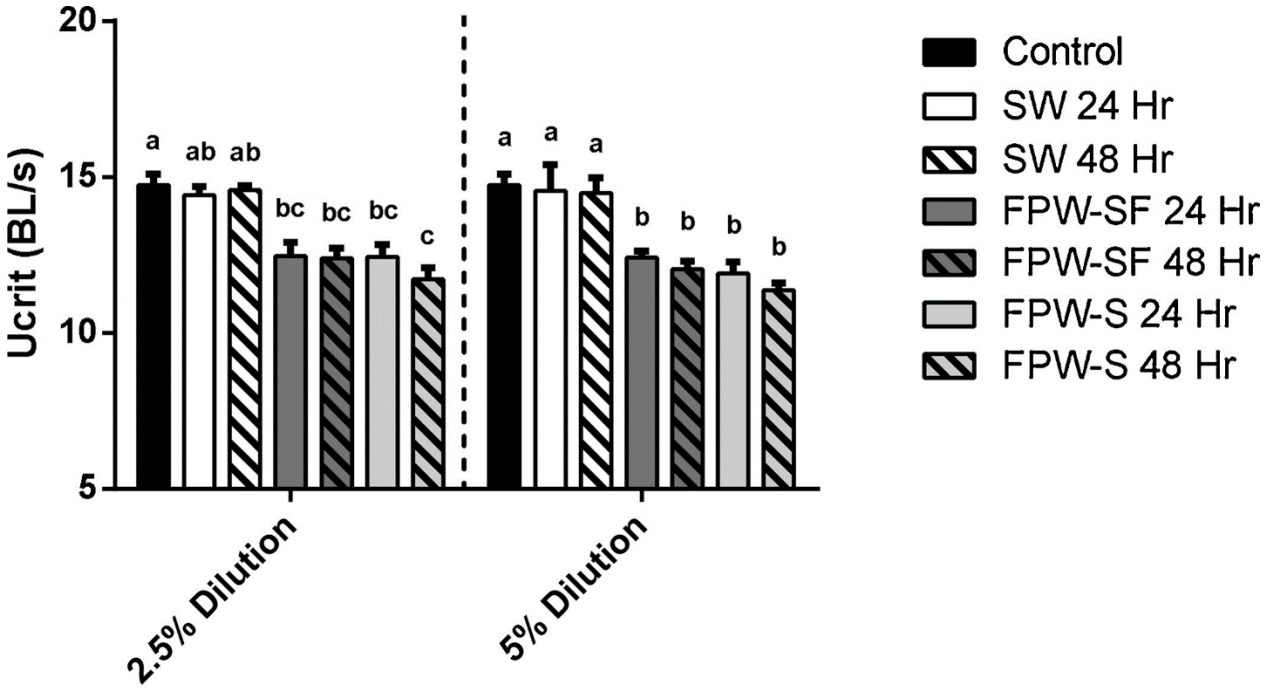


Figure 6- 2. Juvenile zebrafish (60 dpf) swimming performance measured by critical swimming speed (U_{crit}) in body lengths per second (BL/s) after 24 or 48 hr acute embryonic exposures to control, salt control (SW), sediment-free (FPW-SF), and raw/sediment containing (FPW-S) hydraulic fracturing flowback and produced water (FPW) fractions at 2.5% or 5% dilutions. Replicates of 6 fish per treatment condition were used to obtain results. Data are represented as mean ± SEM. Two-way ANOVAs using FPW fractions and exposure durations as factors followed by Holm − Sidak post hoc tests

were performed to determine significant changes ($p < 0.05$) to swimming performances of juvenile fish after embryonic FPW fraction exposures at each dilution. Significant differences are denoted by differing letters.

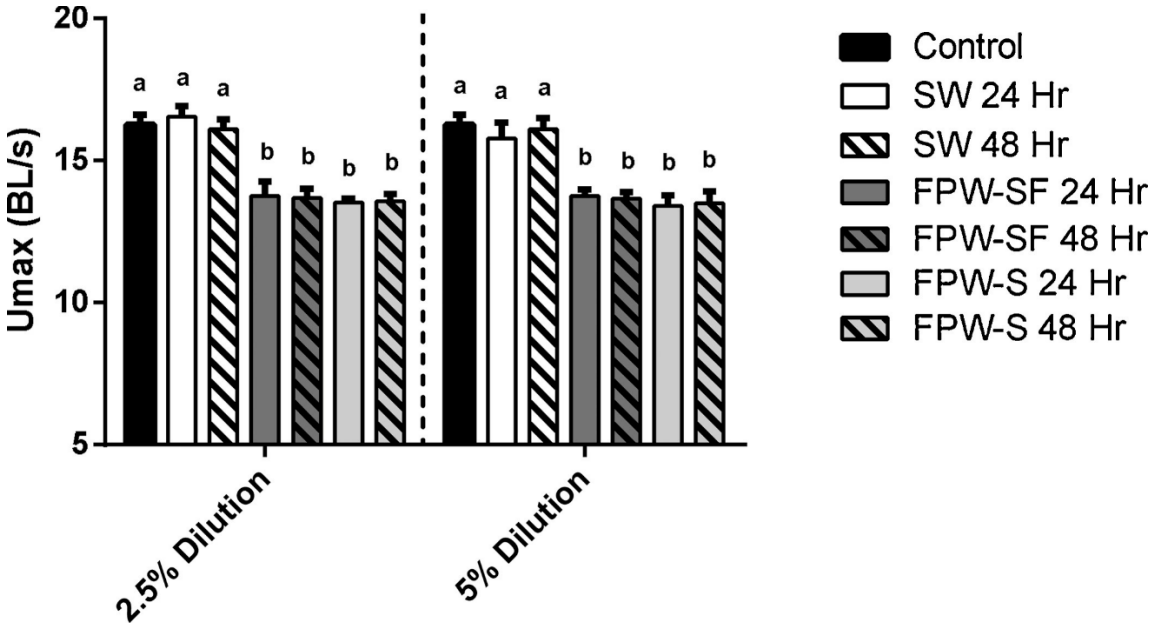


Figure 6- 3. Juvenile zebrafish (60 dpf) swimming performance measured by repeat maximal swimming speed (U_{max}) in body lengths per second (BL/s) after 24 or 48 hr acute embryonic exposures to control, salt control (SW), sediment-free (FPW-SF), and raw/sediment containing (FPW-S) hydraulic fracturing flowback and produced water (FPW) fractions at 2.5% or 5% dilutions. Replicates of 6 fish per treatment condition were used to obtain results. Data are represented as mean ± SEM. Two-way ANOVAs using FPW fractions and exposure durations as factors followed by Holm − Sidak post hoc tests were performed to determine significant changes ($p < 0.05$) to swimming performances of juvenile fish after embryonic FPW fraction exposures at each dilution. Significant differences are denoted by differing letters.

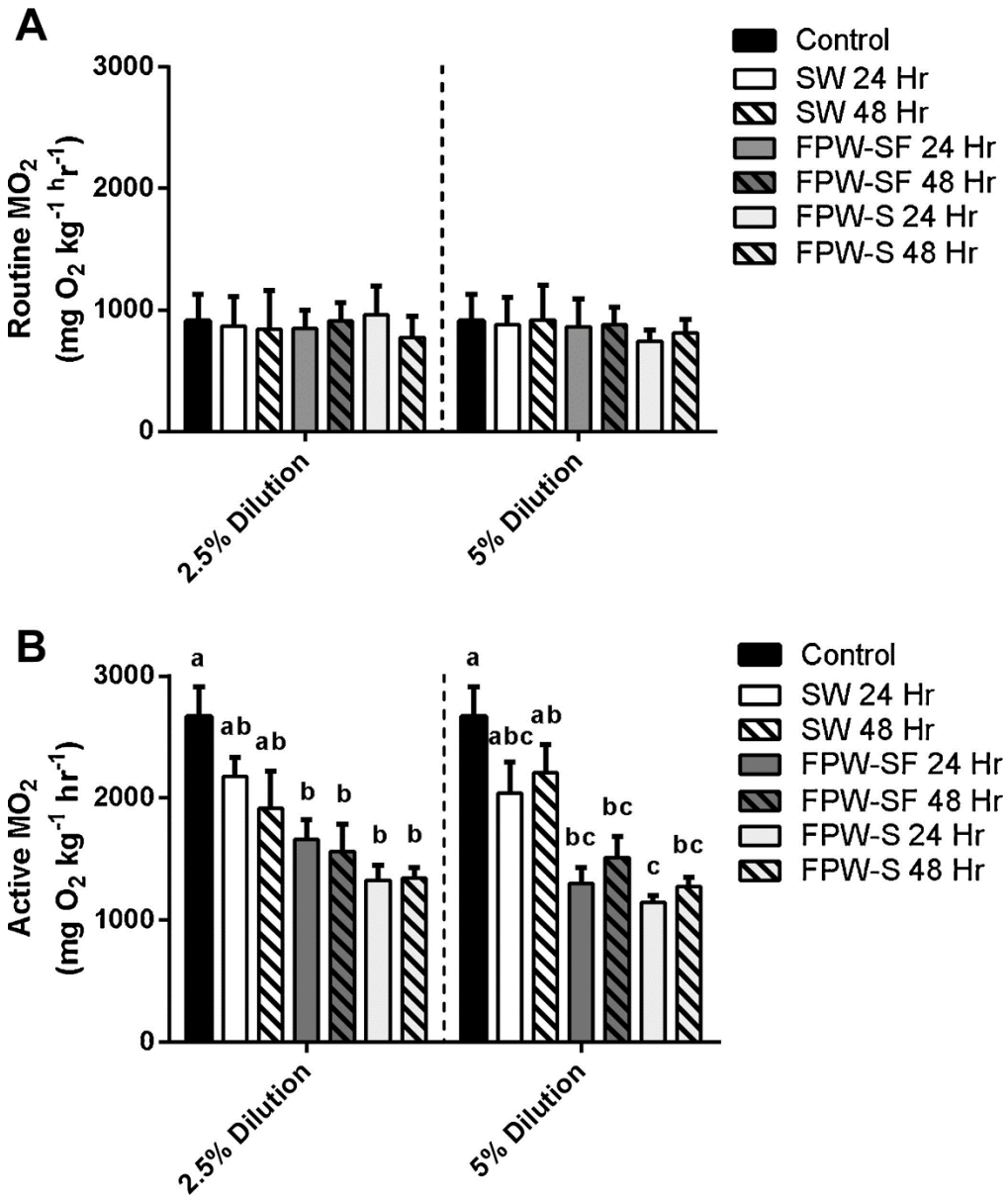


Figure 6- 4. Metabolic capacities measured as (A) routine metabolic rate (MO_2 at routine metabolic swimming) and (B) active metabolic rate (MO_2 after maximum sustainable swimming speed in U_{crit}) in juvenile zebrafish (60 dpf) after 24 or 48 hr acute embryonic exposures to control, salt control (SW), sediment-free (FPW-SF), and raw/sediment containing (FPW-S) hydraulic fracturing flowback and produced water (FPW) fractions at 2.5% or 5% dilutions. Replicates of 6 fish per treatment condition were used to obtain

results. Data are represented as mean ± SEM. Two-way ANOVAs using FPW fractions and exposure durations as factors followed by Holm − Sidak post hoc tests were performed to determine significant changes ($p < 0.05$) to swimming performances of juvenile fish after embryonic FPW fraction exposures at each dilution. Significant differences are denoted by differing letters.

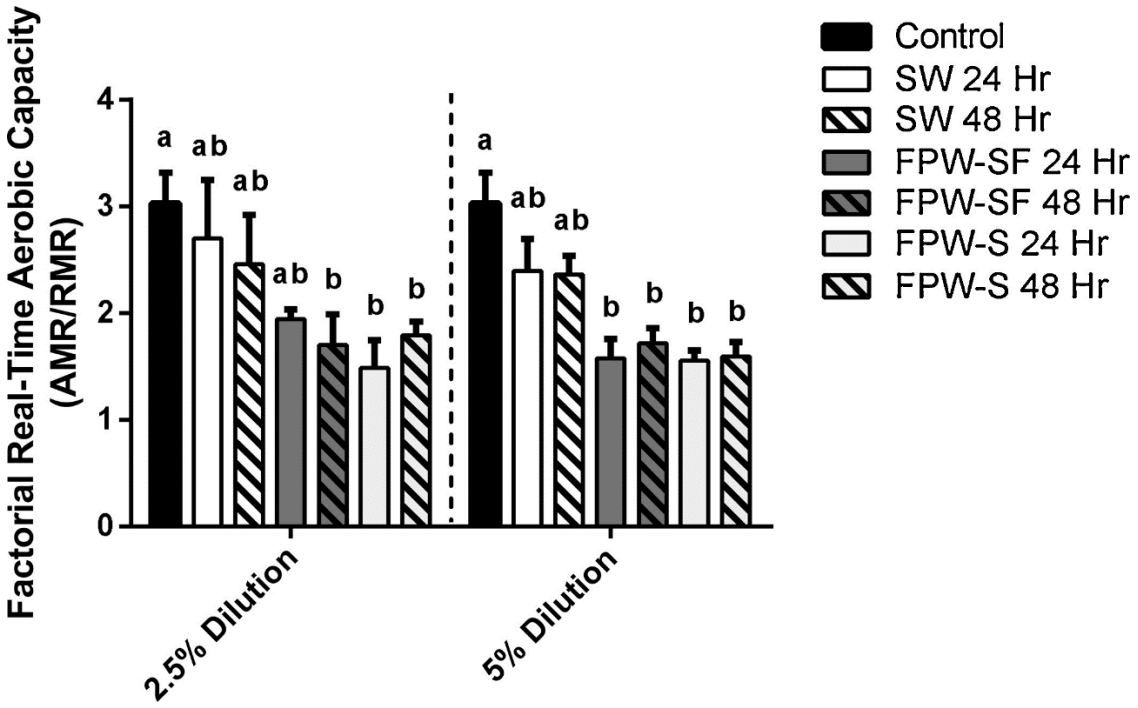


Figure 6- 5. Factorial real-time aerobic capacity (ratio of AMR to RMR) analysis of juvenile zebrafish (60 dpf) after 24 or 48 hr acute embryonic exposures to control, salt control (SW), sediment-free (FPW-SF), and raw/sediment containing (FPW-S) hydraulic fracturing flowback and produced water (FPW) fractions at 2.5% or 5% dilutions. Replicates of 6 fish per treatment condition were used to obtain results. Data are represented as mean ± SEM. Two-way ANOVAs using FPW fractions and exposure durations as factors followed by Holm − Sidak post hoc tests were performed to determine significant changes ($p < 0.05$) to swimming performances of juvenile fish after embryonic

FPW fraction exposures at each dilution. Significant differences are denoted by differing letters.

CHAPTER 7: Persistent Cardio-Respiratory Impairments in Rainbow Trout (*Oncorhynchus mykiss*) Following Embryonic Exposure to Hydraulic Fracturing Flowback and Produced Water

Folkerts, E.J., Sniher, K. N., Zhang, Y., Martin, J.W., Alessi, D.S., Goss, G.G. Persistent cardio-respiratory impairments in rainbow trout (*Oncorhynchus mykiss*) following embryonic exposure to hydraulic fracturing flowback and produced water. *To be submitted*.

7.1 INTRODUCTION

Investigations on hydraulic fracturing flowback and produced water (FPW) in aquatic organisms have recently begun to elucidate the harmful nature of this wastewater to the biota of affected ecosystems. Recent advancements in fracturing technology and improved efficiencies have led to an 8-fold increase in North American oil and gas extraction from shale reserves between the years of 2007 and 2016 (U.S. EIA, 2017). Furthermore, modelling predicts that even at low(er) oil and gas prices, increases in water usage (and subsequent generation of flowback and produced water; FPW) as a result of increased well creation modelled to increase 50- and 20-fold until the year 2030 in unconventional gas-producing and unconventional oil-producing regions of the U.S., respectively (Kondash et al., 2018). Combined with the trending global shift to "cleaner" energy use (including methane gas), other reports have natural gas production via unconventional hydraulic fracturing processes slated to increase 25% by 2035 and 45% by 2040 in Canada and the U.S., respectively (Gagnon et al., 2016). Clearly, the hydraulic fracturing process will remain a major component of global energy sectors for the foreseeable future, rendering further studies about the impacts of spilled/released FPW on the environment as vital for improved ecological sustainability.

In addition to the prominently lethal nature of these wastewaters to aquatic invertebrate and vertebrate species (Folkerts et al., 2019; Mehler et al., 2020), sublethal FPW exposure even at relatively low dilutions has been shown to elicit other negative physiological and toxicological responses, including reduced reproductive capacity (T. A. Blewett et al., 2017a), heightened endocrine disrupting activity (He et al., 2018a, 2017b; Kassotis et al., 2018b), and increased oxidative stress and xenobiotic metabolism burdens (Blewett et al., 2021; T. A. Blewett et al., 2017b; He et al., 2017b, 2017a; Weinrauch et al., 2021). Owing to its highly complex nature, determining the exact chemical components responsible for these aforementioned sub-lethal effects is difficult. Although organic chemicals (such as polycyclic aromatic hydrocarbons [PAHs] and surfactants) and certain inorganic constituents (including metals and major salt ions) are believed to contribute to overall toxicological responses observed (Folkerts et al., 2019, 2020a; C. Sun et al., 2019), until more complete characterizations of FPW are made, knowing precisely exact chemical constituents eliciting toxicological effects specific is difficult. Nevertheless, the field of

FPW toxicological study is beginning to slowly understand the toxicological extent of the biological systems and processes affected by FPW exposure in aquatic organisms. One such system recognized as significantly affected by hydrocarbon PAH and other FPW-related wastewater solution (e.g. crude oil) exposures is the cardio-respiratory system in fish (Incardona, 2017; Pasparakis et al., 2019a).

Fish are excellent cardio-respiratory toxicity models and have been shown to be sensitive to contaminants at all stages of development. However, given the presence of sediments in FPW (see Chapters 5 and 6) (Flynn et al., 2019; He et al., 2017a), and the relatively immobile nature observed in most freshwater species, fish embryos are a potentially better model for examining FPW effects in the aquatic environment, particularly in the benthic community or in a chronic exposure context (Baker et al., 1991; Entrekin et al., 2011; Hollert et al., 2000). Furthermore, wastewaters from numerous industrial applications have been shown to induce a myriad of developmental and physiological effects in a wide range of embryonic fish species (Pasparakis et al., 2019b; Philibert et al., 2016; Sfakianakis et al., 2015). Organogenesis is both a vital and sensitive developmental period within the embryonic state wherein disturbances to the process may have significant negative effects on the survival and functioning of the organism at later life stages. Primary zebrafish (*Danio rerio*) heart and related cardiovascular tissues are some of the first systems to develop, beginning at 5 and ending at 48 hours post fertilization (hpf) – although epicardium development extends well beyond this into 72 hpf (Bakkers, 2011; Glickman and Yelon, 2002). In rainbow trout (*Oncorhynchus mykiss; RT*), however, embryonic development as a whole takes significantly longer – largely in part to the required colder ambient water temperatures – and hatching does not occur until ~ 310 - 350 acclimated thermal units (ATUs) at 10 °C (31 – 35 days post fertilization [dpf], compared to zebrafish hatching out within 96 hpf at 28 °C) (Bakkers, 2011; Ignatieva, 1991; Vernier, 1969). From the limited amount of published work on embryonic cardiac development in trout, and extrapolating from zebrafish cardiac development timing, it is estimated that RT primary cardiac organogenesis initiation may begin and end anywhere from ~ 20 – 120 ATUs (2 – 12 dpf) at 10 °C (Vernier, 1969). This developmental window, therefore, may be critical for determining the extent of cardio-respiratory toxicity experienced by RT embryos following exposure to FPW.

Fish swimming performance (commonly measured as critical swimming speed, U_{crit}) and swimming respirometry are two elements of fish biology and physiology which are intimately tied to the cardio-respiratory system of these organisms. The sensitivity of swimming performance and respiration to environmental toxicants, and their importance to overall fish health and essential fish behaviors, has resulted in these endpoints being commonly used to assess and determine sub-lethal toxicities in fish (Lee, 2003; Little and Finger, 1990). Interestingly, certain contaminants have been shown to alter swimming performance and respirometry in juvenile and adult fish following exposure as embryos (Hicken et al., 2011; Mager et al., 2014), implicating that physiological processes affected during embryonic development may have latent, chronic impacts which affect later-life fish parameters. Previously, we have shown that juvenile zebrafish (*Danio rerio*) examined at 60 dpf following acute, embryonic FPW exposures as low as 2.5% display reduced U_{crit} and swimming aerobic capacity (Folkerts et al., 2017b). However, FPW effects on embryonic cardiac development and function, with provisional attention on chronic effects, have not been investigated in RT; an economically important salmonid fish species world-wide which naturally inhabits ecozones which overlap geographically with regions of intense hydraulic fracturing activities (Alessi et al., 2017; Kondash et al., 2018; MacCrimmon, 1971). Considering the close ties cardio-respiratory systems have with aerobic capacities and activities in fish, it is theorized that FPW exposure during trout embryonic stages will impact critical cardiac processes which manifest as protracted affects to fitness at later-life fish stages.

To characterize impacts to the developing cardio-respiratory system, and to test if (and the extent of) this above-mentioned developmental window being susceptible to alterations, we exposed RT embryos in a developed flow-through setup at two different developmental time points (3 dpf and 10 dpf) to two different acute (48 hr) exposures. We additionally exposed RT embryos to chronic (28-day) FPW dilutions in this same flow-through set up. Impairments to cardio-respiratory functioning and development were measured by periodically recording metabolic rates (MO_2) of developing embryos, analysis of key cardiac development and functioning genes at select developmental periods, and morphological assessment following hatching. These endpoints were used to determine the extent of induced FPW disruption to cardio-respiratory functioning and development in RT

embryos, whether exposures earlier or later within the 2 – 12 dpf developmental window cause greatest impacts, and if acute or chronic exposure regimens had differential effects. To determine if embryonic exposures (as previously detailed) produce latent, chronic impacts to fish fitness, we measured physiological performance as assessed by U_{crit} and swimming respirometry in juvenile fish reared to ~ 8 months post-fertilization. We hypothesized that FPW exposures at higher concentrations earlier within the developmental window (at 3 dpf) would elicit greatest impacts on embryonic cardio-respiratory parameters and juvenile fish swimming performance and respirometry.

7.2 MATERIALS AND METHODS

7.2.1 Hydraulic Fracturing FPW Sample

The FPW sample used in the current study was collected from a horizontal hydraulically fractured 'slickwater' well in the Devonian-aged Montney formation located in northwestern Alberta, Canada. This FPW sample was collected 4 hr-post well stimulation (4 h after the well has been brought into production), put into 20 L air-tight sealed industrial food-grade polyethylene buckets, immediately transported to the University of Alberta, and stored at room temperature until use. Inorganic characterization of the FPW sample (Table S7-1) was performed via inductively coupled plasma (ICP)-MS/MS using an Agilent 8800 Triple Quadrapole ICP-MS/MS (Agilent Technologies, USA). For FPW organic characterization, polycyclic aromatic hydrocarbon (PAH) analysis was performed via gas chromatography – MS (GC-MS) with selective ion monitoring (Table S7-3), while untargeted organic compound analyses were performed by high performance liquid chromatography/Orbitrap Mass Spectrophotometry (HPLC/Orbitrap-MS) in both positive and negative ionization modes (Figure S7-1). Details concerning both inorganic and organic FPW characterization (including analyte quantification methods, instrument details, detection limits, and precision/accuracy) can be found in the supporting information (SI; see Table S7-2, Appendices 7-1 to 7-5).

7.2.2 Animal Maintenance

RT embryos (0 hours post feralization) obtained from the Raven Trout Brood Station (Caroline, AB, CAN) were transported to the University of Alberta and incubated

in Heath embryo development trays at 9.5 ± 1.0 °C (mean ± standard deviation) with aerated flow-through dechlorinated University of Alberta facility freshwater until use (water moderately hard: $[Na^+]$=13.8 mg/L, $[Ca^{2+}]$=51.8 mg/L, $[Mg^{2+}]$=15.1 mg/L, $[K^+]$=1.9 mg/L, pH: 7.99, conductivity: 401 ± 1.5 µS/cm, dissolved oxygen: 8.1 ± 0.3 mg/L, general hardness: 175 mg/L as CaCO3, salinity: 0 ppt). Three separate batches of green, freshly fertilized hatchery embryos were collected within ~ 1 month of each other to stagger development and ensure suitable replicates at selected developmental periods. For each batch, embryos were a mix of eggs from 3 different female trout fertilized with milt mix from 5 differing male fish. Throughout rearing, temperatures were maintained at 9.5 ± 0.8 °C with constant aeration on a 14 h light:10 h dark photoperiod. Once embryo yolk sacs were absorbed, fish were fed daily satiating amounts of finely ground EWOS Vita pellets, and then fed EWOS Vita 2 mm pellets at later developmental stages (Cargill Inc., MN, USA). To accommodate fish growth relative to tank volume, rearing tank densities were occasionally reduced at equal rates across treatments. All animal use was approved by the University of Alberta Animal Care Committee (protocol #: AUP00001334).

7.2.3 Exposure and Experiment Design

At 3 days post fertilization (dpf), subsets of 150 RT embryos from each collection batch were separated and placed into respective experimental exposure trays and exposed acutely to one of the following treatments for 48 hr: control freshwater, FPW 2.5%, FPW 5%, or a saline control matching the salinity of the FPW 5% treatment (SC 5%). To investigate FPW exposure and sensitivity of differing RT development windows, subsets of 150 embryos from each collection batch were additionally exposed for 48 h at 10 dpf to the same treatments listed above as the 3 dpf-exposed embryos. To investigate the impacts of chronic FPW exposure, respective subsets of 150, 3 dpf embryos were also exposed to FPW 1% and a saline control matching the salinity of the FPW 1% (SC1%) for 28 days. All SC treatments were made by diluting with dechlorinated freshwater a stock "100%" SC solution which matched the major cation (Na^+, Ca^{2+}, K^+, and Mg^{2+}) and anion (Cl^-) constituent concentrations of the raw FPW sample. Test dilutions of FPW used for exposures were made via diluting raw, 100 FPW with dechlorinated water with chosen values based off preliminary lethal concentration causing 50% death analyses (LC_{50}),

wherein raw FPW dilutions of 5.47% were determined to cause 50% mortality (see Figure S7-2).

In total, 5 independent exposures to each respective treatment were employed (N = 5). Embryos were randomly chosen from collection batches and placed into exposure trays which had grid-like sectional dividers creating 6 defined and isolated sections per tray. In each replicate tray per treatment, one section containing 25 embryos was exclusively set apart for developmental deformity analyses. All other sections contained roughly equal densities of embryos. Daily removal of dead (white and opaque) embryos was performed.

Flow-through exposures to each respective experimental solution (control freshwater, SC or FPW) were employed by use of a calibrated and finely tuned flow-through exposure system. Briefly, embryos sitting in exposure trays were first placed into tanks pre-filled with respective exposure solutions (listed previously; created by diluting raw FPW or 100% SC with dechlorinated freshwater to desired dilutions). To maintain desired dilutions of acute exposure solutions, lines from stock raw FPW or 100% SC reservoirs were fed directly into exposure tanks in parallel with water lines feeding fresh, dechlorinated tap water at specified rates. To achieve desired dilutions, specified rates of FPW or 100% SC inflow were maintained by peristaltic pumps and were calculated against rates of freshwater inflow (controlled by 1/8" FR-4000 series acrylic tube rotameters, Muis Controls Ltd, CAN). For all treatments, total combined inflow rate (of either freshwater controls, FPW or SC) was ≥ 5.0 mL/min. For chronic embryonic exposures, exposure reservoirs of the desired FPW and SC concentrations (1%) were pre-made, with exposure solution inflow into these tanks coming from these reservoirs only (no direct freshwater input); again, flow rates were maintained by set peristaltic pump flow rates.

Regardless of embryonic exposure type, all overhead reservoirs were continually mixed via large magnetic stir plates to negate settling of reservoir sediments. Furthermore, all embryonic exposure tray-tank apparatuses were outfitted with both a recirculating pump and an air line bubbler underneath the sitting embryo exposure tray to maintain uniform distribution of inflow FPW or SC solutions and to ensure adequate oxygenation of the solution media. During acute exposures, solution temperature, dissolved oxygen levels, and pH were recorded daily. Furthermore, conductivity of exposure solutions was also measured daily and compared against raw FPW conductivity to confirm desired dilutions

of FPW or SC were being maintained (samples for Na^+ measurement via atomic absorption spectrophotometry were additionally taken to confirm sample dilutions). For chronic exposures, all previously mentioned exposure solution testing parameters were made every 5 days. Measurements of all exposure treatment solution water quality indices can be found in Table 7-1 and Figure 7-1. Following exposure termination, exposure trays with embryos not used during embryonic analyses/sampling were lifted out of exposure tanks, rinsed twice with clean dechlorinated water, and then placed into clean rearing tanks containing fresh, flowthrough dechlorinated water until hatching (~ 33 dpf). After development to yolk-sac fry (~ 5 – 7 days post hatch; dph), exposure trays were removed from rearing tanks and fish were allowed to continue to develop.

7.2.4 Developmental Deformities

After hatching, alevin trout in the specifically selected section of each treatment exposure tray were assayed for percent survival to hatch, percent pericardial (PE), percent yolk-sac edema (YSE), and percent tail/spine curvatures (TSC). Developmental deformities were determined by observing all trout fry from each specifically assigned section of each replicate treatment exposure tray under a Leica Zoom 2000 stereomicroscope (Leica Camera Co., GER). Records of malformation frequency were compared against the total number of fish to survive to hatch and expressed as a percentage.

7.2.5 Embryonic Metabolic Rates

Using a dual sensor-dish reader (SDR) system paired with PreSens-SDR_v38 software (Loligo Systems, DEN), recordings of embryonic RT O_2 consumption (MO_2) for all treatments were made immediately following 48 hr exposures, 2-, 4-, 7-, 10-, and 21-days post exposure (embryo MO_2 was additionally recorded at the end of 28-day exposures for chronic studies). Immediately following 48 hr treatment exposure, embryos randomly selected from each treatment replicate tray were gently rinsed twice with fresh, dechlorinated water and quickly observed under a stereomicroscope to confirm fertilization of selected embryos. Embryos were then individually placed into 500 µL wells filled with fresh, dechlorinated water of 24-well optical fluorescence glass sensor microplates (calibrated prior to analyses for 10 °C temperatures). Plate wells were then sealed by used

of silicon pads sandwiched between spacer plates within immersion boxes. These plate-immersion box set-ups were then connected to water inflow and outflow ports and placed onto SDRs in a stand-alone incubator devoid of light. Water was then flowed into plate-immersion box set-ups to maintain 10 °C temperatures. Embryonic MO_2 recording lasted for 30 minutes, with MO_2 calculations made between 10 – 30 min. Recordings below 70% air O_2 saturation levels were not included in analyses. MO_2 data was corrected for both well volume and embryo mass and expressed as mg O_2 g^{-1} h^{-1}. To control for background levels of respiration, averaged nominal rates of O_2 decrease from 2 – 3 'water only' wells on each microplate per trial were subtracted from embryonic MO_2 rates. These protocols were applied for all the above specified embryo MO_2 recording time points.

7.2.6 Embryonic Quantitative Real-Time PCR

Immediately following acute, 48 h treatment exposures, embryos from each replicate exposure trays at both developmental periods were randomly selected, rinsed twice in clean dechlorinated water, and viewed under a stereomicroscope to verify fertilization. Three embryos per treatment replicate were then flash frozen in liquid N_2 and stored at -80 °C. For chronic exposure studies, embryos from each treatment were sampled following the same above mention protocols at 48 h, 14 -, and 28-days (end of chronic study) exposure. Total RNA from 3 embryos was extracted and isolated using 1 mL of TRIzol according to manufacturer's protocols (Ambion Life Technologies, USA). Total RNA was then suspended in nuclease-free H_2O and run on a 2% formaldehyde-agarose gel with ethidium bromide to assess RNA integrity. RNA integrity was further assessed and quantified by running RNA samples on a Bioanalyzer 2100 (Agilent Technologies, USA). Complimentary cDNA synthesis of 0.5 µg of RNA samples was performed using SuperScriptIII Reverse Transcriptase (Invitrogen, USA) in a mix of oligo(dT_{18}) and random hexamer primers.

Quantitative real-time PCR (qPCR) was performed in 384-well plates using an ABI QuantStudio™ 6 Flex Real-Time PCR system (Applied Biosystems, USA). RT gene specific primers were designed against chosen key cardiac development and electrophysiological targets using Primer Express (v3.0.1, Thermofisher Scientific, USA) and Integrated DNA Technologies (USA) software. Primer gene targets, accession

numbers, and sequences can be found in Table S7-4. Individual PCR reaction mixtures contained 2.5 µL of sample cDNA, 2.5 µL of 3.2 µM combined forward and reverse primer sets, and 5 µL of SYBR Green master mix (Applied Biosystems, USA). PCR reactions underwent an initial 95 °C denaturation step for 10 minutes prior to thermal cycling initiation. A 2-step PCR thermal cycle, consisting of a denaturation step of 95 °C for 2 minutes and an annealing-extension step of 60 °C for 1 min, was employed for 40 cycles. A primer melt-curve analysis following each PCR thermal cycle procedure was included to ensure primers were yielding single products and suitable PCR reactions were being performed. Gene transcript expression changes were assessed *via* ΔΔCT analysis (Schmittgen and Livak, 2008) using TATA-box binding protein associated factor 12 (*tataaf12*) and ribosomal protein L7 -like 1 (*rpl7l1*) as endogenous controls. Stability and primer efficiency validations, the latter determined by using serial slope calculations after ΔCT and log $cDNA_{[dilution]}$ input regression analyses, were performed for all endogenous control genes (Figure S7-3).

7.2.7 Juvenile Swimming Performance and Respirometry

A 1.5 L Blazka-style swim tunnel respirometer (Loligo Systems, DEN) was used to assess juvenile fish critical swim speed (U_{crit}) and aerobic scope *via* automated intermittent flow respirometry. A Pt100 fiber-optic probe connected to a Witrox 1 minisensor O_2 meter (PreSens Precision Sensing, Loligo Systems, DEN) was outfitted in the swim tunnel respirometer to record MO_2 during fish swimming. Swim tunnel respirometer water velocity calibrations were performed *via* digital particle tracking velocimetry (DPTV; Loligo Systems, DEN) using green, 200 µm fluorescent polyethylene microspheres (Cospheric LLC, USA) and a UI-3240CP highspeed industrial camera (IDS Imaging Development Systems Inc., GER). A two-point calibration system was used to calibrate O_2 probes using 10 g/L Na_2SO_3 (zeroing solution) and oxygenated dechlorinated freshwater (O_2 saturating solution). Swim tunnel respirometry was performed at 10.9 °C (± 0.8 °C) and all data was collected using AutoResp v2.3.0 software (Loligo Systems, DEN).

At ~ 8 months post fertilization, juvenile RT previously exposed as embryos to experimental treatments were analyzed for swimming performance and respirometry. Fish were fasted 24 hr before swimming performance and respirometry to reduce the effects of

specific dynamic action on swimming MO_2 rates. Prior to swim tunnel respirometer introduction, juvenile fish were briefly anesthetized with low strength, 0.05 g/L MS-222 (Syndel Laboratories Ltd., CAN) buffered with $NaHCO_3$ and fish weight, length (fork and total), and body condition factor were recorded. All biometric data for juvenile fish used in swim respirometer analyses can be found in Table 7-2. Fish were then gently transferred to the swim tunnel respirometer and allowed to acclimate for ≥ 6 h at water velocities of 0.5 body lengths per second (BL/s). Once acclimation periods had finished, fish were subjected to a U_{crit} swimming protocol, wherein fish were forced to swim for 15-minute step intervals until fatigue using fixed water velocity increments of 0.75 BL/s per interval. During each 15-minute swim step (and during ≥ 6 h acclimation), intermittent respirometry recording occurred (420 s flushing, 30 s wait, 450 s MO_2 recording). Fish fatigue was determined once juvenile RT began to consistently become pinned for several seconds at a time on the back barrier of the flume despite repeated attempts to induce swimming by quickly lowering and ramping up water velocities. U_{crit} was calculated according to the formula described previously Brett, 1964: $U_{crit} = [U_f + (T/t)\, dU] / cm$, where U_f (cm/s) is the highest swim velocity maintained for a complete step interval, T (s) is the length of time spent at the final, highest swim velocity, t is the set time for each step interval (900 s = 15 min), and dU (cm/s) is the increment in swim speed of each step. All U_{crit} values were then converted to BL/s. To estimate aerobic scope - the difference between maximum metabolic rate (MMR) and standard metabolic rate (SMR) – for each fish, a scaled standardized approach was employed to account for any influences of fish size on respirometry (see Figures S7-4 and S7-5) (Mager et al., 2014; Stieglitz et al., 2016).

7.2.8 Juvenile Fish Ventricular Histology and Morphometrics

Following swimming performance commencement, fish were euthanized via MS-222 overdose. Cardiac tissue was then excised from each individual fish and fixed for 24 h in a 4% paraformaldehyde, 2.5% glutaraldehyde, 0.05 mmol L^{-1} sodium cacodylate buffer (pH = 7.4) at 4 °C until analysis. Following fixation, cardiac tissue samples were washed twice with and stored in 70% ethanol at 4 °C until further processing. Cardiac samples then underwent serial ethanol dehydration prior to embedding in paraffin wax. Tissues were trimmed and sectioned (7µm) and post-stained with haematoxylin and eosin (H&E) prior to

visualization on a Zeiss A1 light microscope outfitted with an optronic camera. All images of sectioned ventricles were then dimensionally analyzed to determine changes to ventricle size, structure, and compact myocardium thickness using ImageJ software (National Institute of Health) (as described in Blair et al., 2016; Ong et al., 2007). To reduce observer bias, a blind set-up was employed when performing analyses of images.

7.2.9 Statistics

All data was assessed for normality and equal variance via Shapiro-Wilk and Brown-Forsythe tests, respectively, wherein data transformation was performed if necessary. Data outlier analyses via Grubbs outlier tests were performed, and whole fish replicates were subsequently removed from respective analyses if deemed significant. For all statistical and plotting analyses, Prism v.6.0 software (GraphPad Software Inc., USA) or SigmaPlot 13 (Systat Software Inc., USA) were used. For developmental deformity and qPCR analyses, acute 48 hr exposure endpoint data was assessed using two-way ANOVAs with developmental state and treatment as factors. For the same analyses, chronic 28-day exposure endpoint data was assessed separately using one-way ANOVAs with treatment as a factor. For embryonic respiration analyses, both acute and chronic data was assessed using one-way ANOVAs within each time period of measurement with treatment group as a factor. For swimming performance, swimming respirometry, and juvenile heart morphology data, one-way ANOVAs or Kruskal-Wallis tests were performed to assess differences among treatment groups. Holm-Sidak *post-hoc* multiple comparisons (or Dunn's multiple comparisons for Kruskal-Wallis tests) were then performed on all acute and chronic data sets to determine significant differences between treatment groups and developmental periods of exposure. Significance was concluded when $p < 0.05$. All data are presented as mean ± S.E.M.

7.3 RESULTS

7.3.1 Developmental Deformities at Hatch

At hatch, rates of developmental deformities were significantly impacted by FPW exposure. The percent embryos surviving to hatch were significantly decreased by FPW exposure compared to corresponding freshwater or saline control conditions regardless of

FPW concentration, exposure length (acute versus chronic), and developmental time of FPW exposure ($p < 0.05$, Figure 7-2A). Embryos acutely exposed to 5% FPW at 3 dpf experienced greatest impacts to survival compared to control conditions, with a reduced survival rates (31.8%) compared to controls (74.3%). Induced rates of pericardial edema, yolk-sac edema, and tail/spine curvatures were similarly impacted by FPW exposure, with greatest rates of specified deformities occurring in acutely exposed 5% FPW embryos (24.2%, 13.3%, and 9.5%, respectively). Furthermore, acute 5% FPW treatments of both 3 and 10 dpf exposed embryos resulted in significantly increased rates of pericardial and yolk-sac edema compared to control treatments ($p < 0.05$). However, induced rates of deformities of only 3 dpf 2.5% FPW exposed embryos displayed significantly increased rates of pericardial and yolk-sac edema ($p < 0.05$). Interestingly, survivorship and rates of developmental deformities between developmental timing (3 dpf versus 10 dpf) of exposure at similar FPW dilutions were significantly different in 5% FPW exposed embryos, except for rates of tail-spine curvatures ($p < 0.05$, Figure 7-D). In 2.5% FPW exposures, there was no significant difference in deformity rates between 3 dpf and 10 dpf timed exposures ($p > 0.05$).

7.3.2 Embryo Metabolic Rates

Immediately following acute 48 h exposures, 3 dpf 2.5% FPW exposed and 5% FPW exposed embryos displayed significantly higher rates of MO_2 compared to control embryos ($p < 0.05$, Figure 7-3A). This elevated MO_2 was maintained until 15 dpf, wherein embryo MO_2 rates across treatment groups generally came together and were comparably indifferent. However, at 26 dpf, an effective 'switch' occurred, and embryos exposed to FPW displayed significantly reduced MO_2 rates compared to control embryos. Interestingly, only at select developmental points were FPW exposed embryo MO_2 rates significantly different from SC exposed embryos (see 7 and 26 dpf MO_2 rates, $p < 0.05$, Figure 7-3A). Similar to exposures at 3 dpf, embryos exposed to FPW at 10 dpf displayed significantly elevated MO_2 immediately following 48 h exposures ($p < 0.05$, Figure 7-3B). However, at all measured developmental points aside from immediately following 48 h exposure (12 dpf), embryonic MO_2 rates across treatment groups subsequently were not different from one another (except at 33 dpf, wherein FPW 5% exposed embryos singularly

displayed elevated MO_2 compared to control embryos, Figure 7-3B). In chronically exposure embryos, similar trends to those of embryos exposed acutely at 3 dpf were observed. In general, 1% FPW treatment increased MO_2 along measured developmental time points up until a certain stage – 26 dpf – where then embryonic MO_2 rates then were not different from one another (although only significant increases were observed at 15 dpf, $p < 0.05$, Figure 7-3C). Following 26 dpf, an effective 'switch' occurred, and embryos previously exposed to FPW displayed significantly reduced MO_2 compared to control embryos ($p < 0.05$, Figure 7-3B). Furthermore, much like acute 3 dpf exposures, chronic SC exposed embryonic MO_2 responses were often not significantly different from FPW exposures at measured developmental time points (save for at 15 dpf, $p < 0.05$, Figure 7-3C).

7.3.3 Embryo Cardiac Gene Expression Profiles

Similar to embryonic MO_2 rates, differential gene expression patterns were observed in embryos exposed at 3 dpf versus embryos exposed at 10 dpf. When exposed acutely at 3 dpf, embryos exposed to both dilutions of FPW significantly increased expression of the gene *atp2a2a* compared to all other treatment groups (greatest ~ 10.3 – fold change observed in 5% FPW embryos compared to control embryos, $p < 0.05$, Figure 7-4A). All other gene expression responses were relatively unchanged across treatment groups. However, in embryos acutely exposed at 10 dpf, numerous differential gene expression responses were recorded. Most notably, significant increases in expression of *nkx2.5*, *scn5lab*, and *kir2.1* for both FPW 2.5% and 5% exposed embryos compared to control and SC embryos were observed ($p < 0.05$, Figure 7-4B). Significant increases in *anp* and *vmhc* expression following exposure to 5% FPW were also observed. In chronically exposed embryos, increases in *atp2a2a* expression were observed in 1% FPW exposed embryos following 48 h exposures when compared to both control and SC embryos (~ 3.3 – fold increase in expression compared to control embryos, $p < 0.05$, Figure 7-5A). However, at 14 days into chronic exposures (17 dpf), embryo gene expression profiles more closely resembled 10 dpf, end-of-acute-exposure embryo profiles, with most notable increases in *tbx2b*, *vmhc*, and *kcnh6* expression (fold increases of ~ 4.0, ~ 1.6, and ~ 5.4, respectively, compared to control embryos, $p < 0.05$, Figure 7-5B). Increases in

expression of the genes *nkx2.5*, *anp*, *vnp*, and *kir2.1* were additionally seen in 1% FPW chronic exposed embryos compared to either control or SC. At the end of chronic exposures (28 days), no changes in gene expression responses were observed across treatment groups except for the gene *gata5*, wherein 1% FPW exposed embryos displayed significantly decreased expression compared to control embryos (~ 2.2 – fold decrease in expression, $p < 0.05$, Figure 7-5C).

7.3.4 Juvenile Fish U_{crit} and Aerobic Scope

Examination of juvenile fish swimming performance and respirometry revealed that previous exposure to FPW as embryos significantly impacted the swimming capabilities and aerobic scope of juvenile developing trout (Figure 7-6). When exposed at 3 dpf, significantly reduced U_{crit} values were observed in juvenile fish previously exposed to 2.5% and 5% FPW solutions (U_{crit} reductions of ~ 1.07 and ~ 1.14 BL/s, respectively; $p < 0.05$) compared to time-matching freshwater controls. Interestingly, embryonic exposures at 10 dpf to 5% FPW, but not 2.5% FPW, also significantly reduced juvenile swimming performance (reductions of ~ 0.84 BL/s; $p < 0.05$). However, greatest reductions to juvenile fish U_{crit} were observed in embryos chronically exposed to 1% FPW (decreases of ~ 1.23 BL/s; $p < 0.05$, Figure 7-6D). No significant changes were observed in juvenile fish SMR ($p > 0.05$), but changes to both MMR and aerobic scope mirrored trends observed for U_{crit} (Figure 7-6). Embryonic FPW exposures of 2.5% and 5% at 3 dpf diminished juvenile fish MMRs by ~ 27.2 % and ~ 25.2 %, respectively, and significantly reduced juvenile fish aerobic scopes by ~ 32.3 % and ~ 27.5%, respectively, compared to time-matching freshwater controls ($p < 0.05$). However, when exposed at 10 dpf, embryonic FPW exposures of 5% alone were responsible for reducing juvenile fish MMRs and aerobic scopes (reductions of ~ 22.9% and 29.3%, respectively; $p < 0.05$), while reductions in juvenile MMR and aerobic scope following 2.5% FPW exposures at 10 dpf were not statistically significant compared to time-matching freshwater controls ($p > 0.05$, Figure 7-6). Embryos chronically exposed to FPW 1% solutions had greatest recorded relative reductions in juvenile fish MMR and aerobic scope (decreases of ~ 30.8 % and 35.9 %, respectively; $p < 0.05$).

7.3.5 Juvenile Fish Ventricle Histology and Morphometrics

Ventricles excised from fish previously swam through U_{crit} protocols and analyzed by microscopy displayed significantly altered morphometrics as a result of embryonic FPW exposure. While there were no significant changes to juvenile fish ventricular aspect ratio or relative size between treatment groups ($p > 0.05$, Figure 7-7A,B), significant changes to ventricular compact myocardium thickness in juvenile fish previously exposed as embryos to FPW were observed (Figure 7-7C). Overall, alterations to juvenile fish compact myocardium thickness followed trends observed for juvenile swimming performance and respirometry results, although some key differences were noted. Embryonic exposures to FPW 5% solutions induced greatest relative reductions in compact myocardium thickness (9.89 µm and 7.75 µm reductions for 3dpf and 10 dpf exposures, respectively; $p < 0.05$) compared to corresponding time-matching freshwater controls, while chronic exposures also induced a relatively significant decrease in juvenile compact myocardium thickness of 9.37 µm ($p < 0.05$, Figure 7-7C). Interestingly, exposures to FPW 2.5% solutions did not induce significant changes to compact myocardium thickness in fish exposed as embryos at either 3dpf or 10 dpf.

7.4 DISCUSSION

During embryonic RT development, primary cardiac initiation and development is thought to occur between 20 – 120 ATUs at 10 °C.(Vernier, 1969; Knight, 1963) At 20 ATUs, the three germ layers – ectoderm, endoderm, and mesoderm – are established and give rise to the differentiated tissues and organs.(Knight, 1963) The heart, a vital organ for further embryonic development, is one of the first organs formed and arises from mesodermal tissue.(Incardona and Scholz, 2016; Thisse and Zon, 2002) By 100 – 120 ATUs, the first heart beat can be observed.(Knight, 1963; Vernier, 1969) The development and onset of cardiac function is a highly intricate and complex process, wherein numerous signalling molecules and development factors dictate how cell populations properly develop, move and migrate. Once established, multiple structural and contractile proteins are assembled, which then go through the process of being electrically coupled and regulated by numerous ion channels to complete physiological function. Given these multi-staged and integrated physiological processes, it stands to reason that the developing heart

is susceptible to a plethora of contaminants which may alter and affect both development and function of cardiac tissue.

7.4.1 Larval Rainbow Trout Developmental Deformities

A common manifestation of interrupted or altered development in fish following exposure to certain contaminants is the occurrence of edema in embryonic states (Incardona, 2017; Incardona et al., 2004; Incardona and Scholz, 2016). RT embryos exposed to FPW at both 2.5% and 5% displayed significantly increased occurrences of both pericardial and yolk-sac edema (Figure 7-2). Furthermore, tail/spine curvatures were increased and survivorship to hatch was significantly decreased following FPW exposure. These results follow what has previously been observed in developing zebrafish embryos exposed to FPW (Folkerts et al., 2017a). Numerous chemical species exist in FPW which have been shown to induce and cause edema (both pericardial and yolk-sac) and other deformities in developing fish species. One such exemplary class of chemicals are polycyclic aromatic compounds (PACs), or more specifically, polycyclic aromatic hydrocarbons (PAHs) (Edmunds et al., 2015; Incardona et al., 2004; Philibert et al., 2016). These compounds are known agonists of the aryl hydrocarbon receptor (AhR) - a receptor mediated detoxification system in organisms which may induce and modulate the activity of key cytochrome P450 monooxygenases (CYPs, namely CYP1A and to a lesser extent CYP3A) (Luckert et al., 2013; Naspinski et al., 2008; Tseng et al., 2005; Xu et al., 2005). The activity of these induced monooxygenases and subsequent substrate transformations have been shown to generate reactive oxygen species (ROS), whereby endothelial damage, cell apoptosis, tissue necrosis, and overall organ system compromise have been linked physiological occurrences (Cantrell et al., 1998; Incardona et al., 2004; Penning et al., 1996; Tithof et al., 2002; Zangar, 2004). However, certain PACs have been shown to also specifically target cardiac tissues and ion channels responsible for maintaining proper cardiac electrophysiological function, specifically excitation-contraction (E-C) coupling events in cardiomyocytes (Incardona et al., 2006; Brette et al., 2017; Heuer et al., 2019). It is therefore believed that PAC impacts on cardiac function during early embryonic stages result in edema of both the pericardium and yolk-sac, as early circulation is believed to play a major role in osmoregulation and other processes dependent on hydrostatic pressure

(Cherr et al., 2017; Incardona, 2017; Incardona and Scholz, 2016). Understandably, transcriptomic analyses on differing developing fish embryo species exposed to these contaminants have revealed impacts to biological pathways including cardiac function/development, ion regulation, and fluid balance homeostasis (Sørhus et al., 2017; Xu et al., 2019, 2016).

In the FPW sample used currently for embryonic exposures, the presence of numerous PAHs was identified (Table S7-3). Although measurably lower compared to other contaminant studies (such as crude oil spills) (Esbaugh et al., 2016; Incardona et al., 2015; Philibert et al., 2016), at 10. 27 ug/L, total measured PAHs in the current raw, undiluted FPW sample (subsequently diluted and used for exposures) contained concentrations of PAHs on par with what has been observed to induce edema and developmental deformities in FPW-exposed embryos of fish species other than RT (Folkerts et al., 2017a; He et al., 2017a). Additionally, owing to their decreased ability to metabolize and excrete contaminants, fish embryos have been shown to bioconcentrate PAHs, resulting in comparatively larger relative body burdens than juvenile or adult fish exposed to similar PAH exposures (Incardona, 2017; Jung et al., 2015). Furthermore, it has been theorized that surfactant-polyethylene glycol (PEG) interactions (two chemical classes present within FPW) may have intrinsic and derivative toxic effects to exposed organisms by altering the toxicokinetics of other contaminants present in FPW (Folkerts et al., 2019). Concerning PAHs, both nonionic surfactants and certain PEGs singularly have been shown to greatly increase the solubility of PAHs (through incorporation into the hydrophobic part of each compounds micelle) (Hurter and Hatton, 1992; Li and Chen, 2002; Tiehm, 1994) and have been theorized to be potential compounds used for the degradation/recovery of PAHs from contaminated aquatic environments. However, when both nonionic surfactants and PEGs concurrently exist within aqueous solutions, PEGs have been shown to increase surfactant critical micelle concentrations, reduce average micelle aggregation, and increase the polydispersity of surfactants.(Dyachuk et al., 2009; Nagarajan and Wang, 2000) Such effects would greatly increase the bioavailability of surfactants (and thus PAHs incorporated within surfactant micelles) to exposed organisms. Indeed, surfactants which incorporate PEGs directly into their chemical structure have been shown to be an effective pharmaceutical agent for drug delivery by increasing drug

absorption in tissues.(Ismailos et al., 1994; Z. Zhang et al., 2012) Collectively, FPW concentrations of PAHs, the embryonic state sensitive to bioconcentration of PAHs, and the presence of compounds theorized to alter PAH toxicokinetics all may contribute to the influence of deleterious effects observed in FPW-exposed embryos.

Another class of contaminants in FPW which may have contributed to observed developmental alterations are metals. Levels of strontium (Sr) and barium (Ba) in raw FPW were particularly high (1035.0 and 21.8 mg/L, respectively) (Table S7-1), wherein exposure to Sr specifically may have contributed to increased rates of developmental deformities observed in RT embryos. Classically considered a calcium (Ca) mimic, high doses of Sr in mammals has been shown to increase renal excretion of Ca (Pors Nielsen, 2004), while in fish, waterborne Sr has been shown to inhibit bodily and internal circulatory uptake of Ca (Chowdhury et al., 2000; Suzuki et al., 1972). In both cases, hypocalcaemia (low blood Ca) is an observed outcome. In developing fish embryos, hypocalcaemia (induced via disruptions to Ca homeostasis following metal exposure) is a physiological condition theorized to cause skeletal deformities and spinal curvatures (Cao et al., 2009; Witeska et al., 1995). Interestingly, of the known Sr water quality criteria in place to protect aquatic organisms, concentrations of Sr in our FPW exposures would have been at or above recommended Sr levels for the country of Canada (2.5 mg/L) (Environment and Climate Change Canada, 2020) and in the American states of Michigan, Ohio, and Indiana (40 - 48 mg/L) (IDEM, 2001; MDEQ, 2008; Ohio EPA, 2005).

7.4.2 FPW-Induced Alterations to Embryonic MO_2

Metabolism and respiration are two essential and linked processes for embryo survival which are sensitive to multiple environmental parameters, including temperature, oxygenation, light intensity, UV radiation, and of course, anthropogenic stressors (Barrionuevo and Burggren, 1999; Finn et al., 1995; Firat et al., 2003; Folkerts et al., 2017a; Hardy and Litvak, 2004; Hunter et al., 1979; Melendez, 2021; Ojanguren et al., 1999; Pasparakis et al., 2016). Concerning anthropogenic stressors, relatively little study has been performed on examining whole-embryo/larvae MO_2 changes – particularly for chemicals and contaminants related to FPW. Of those which have been undertaken, exposure of marine embryo/larval species to crude oil and benzene have been shown to

generally increase MO_2 responses (Pasparakis et al., 2016, 2017; Eldridge et al., 1977). In the freshwater species zebrafish (*Danio rerio*), however, acute FPW exposure has been shown to significantly decrease embryonic MO_2 during development past initial FPW exposures (Folkerts et al., 2017a). In the current study, RT embryos exposed acutely to FPW at 3 dpf displayed significantly increased MO_2 compared to freshwater control rates until 15 dpf (Figure 7-3A). At this point, MO_2 rates across treatment groups became statistically indistinct and eventually an effective 'switch' was observed wherein FPW exposed embryos at developmental time points past 15 dpf displayed significantly reduced MO_2 rates compared to freshwater controls. This general trend was also observed in embryos chronically exposed to FPW (Figure 7-3C). Despite previous research showing that salinity does not appear to overly impact MO_2 in different fish species at embryonic stages (Brown et al., 2012; Kinne and Kinne, 1962; Morgan et al., 1992), current 3 dpf exposed embryo respiratory results indicate that at certain timepoints, saline control MO_2 rates were statistically indifferent from FPW responses and even significantly different from control values. Thus, it appears salinity exposure - at least at levels used currently - does affect embryonic MO_2 to some extent at certain specific post-exposure timepoints in trout which are exposed earlier in development.

One key factor implicated in the observed differential MO_2 effects between FPW exposed zebrafish and RT embryos is the relative speed of development (~ 4 days versus ~ 35 days to hatch at typical physiological temperatures for zebrafish and RT, respectively). In our previous study analysing zebrafish embryos, termination of FPW exposures and earliest MO_2 recordings occurred at 48 hpf, a timepoint approximately midway through zebrafish development to hatch (Folkerts et al., 2017a). Correspondingly, 15 dpf is approximately midway through development to hatch for RT embryos and is also when the switch from elevated MO_2 responses to significantly depressed rates are observed. Therefore, it is theorized for these two freshwater species of fish that the relative midway point in embryonic development is when earlier FPW impacts to cardiac development may begin to manifest and negatively impact cardiac-related contributions to respiratory processes. Elevated MO_2 responses in acute FPW exposed RT embryos at timepoints prior to 15 dpf may be explained by the increased metabolic demand necessary to meet the energetic challenges associated with detoxifying and metabolizing contaminants in FPW,

as is similarly theorized in other contaminant exposure embryonic MO_2 studies (Eldridge et al., 1977; Pasparakis et al., 2016). A common response in fish embryos exposed to FPW is the induction of detoxification pathways responsible for metabolizing contaminants present within FPW, namely different CYP inductions for the metabolization of organics and PAH contaminants (CYP1a, 1b,1c, 2a, and 3a) (He et al., 2018a, 2017a). However, other energy intensive detoxification and inductive processes may also be activated in response to exposure to contaminants present within FPW (e.g. ATP-binding cassette transporter detoxification mechanisms, metallothionein induction, homeostatic management of reactive oxidative species, etc.) (Chavan and Krishnamurthy, 2012; Fabrik et al., 2009; Gourley and Kennedy, 2009; He et al., 2017b; van der Oost et al., 2003). An additional variable which may have affected observed embryonic MO_2 responses immediately post-exposure is the presence of FPW particulates on the surface of embryo chorions and associated chemical oxygen demands (CODs). Although a thorough 2-step washing protocol eliminated much of the sedimentary material attached to the chorion of embryos prior to measurement, some particulate may have still been present which could have contributed (via COD mechanisms) to increased MO_2 recordings. However, such COD effects are theorized not to have played large contributing roles to increased embryonic MO_2 and would have been eliminated by the next recording time point after the first recordings immediately made post-exposure.

7.4.3 FPW Exposure Mediated qPCR Gene Expression in Developing Embryos

Expressional changes of key cardiac developmental, structural, and electrophysiological genes revealed interesting trends which help characterize potential cardio-respiratory toxicities in RT embryos exposed to FPW. While it is probable that non-specific narcosis and oxidative stress-induced damage to cellular membranes is, in part, responsible for observed cardiac toxicities as originally postulated (Bunn et al., 2000; Meador and Nahrgang, 2019; Wezel and Opperhuizen, 1995). PAHs have been shown to specifically affect cardiac electrophysiological properties via interference with ion channels and transporters critical for proper cardiac function and action potential potentiation (Brette et al., 2017, 2014; Heuer et al., 2019). Chief among these are calcium (Ca) channels and transporters vital for Ca-mediated myofilament activation and subsequent myocyte

contraction (Brette et al., 2017, 2014; Zhang et al., 2013). Following depolarization of the sarcolemmal membrane, voltage-gated L-type Ca^{2+} channels (with contributions from reverse Na-Ca exchange through NCX1h Na/Ca exchangers) open to allow Ca^{2+} entry into the cytosol (Fabiato, 1985, 1983; Litwin et al., 1998; Shiels et al., 1999; Vornanen et al., 1994). This Ca influx triggers the release of additional Ca^{2+} from internal, sarcoplasmic reticulum (SR) stores via ryanodine channels in a Ca^{2+}-induced Ca^{2+} release process. Once the excitation-contraction E-C coupling cycle has completed, Ca^{2+} is either sequestered back into SR stores via SERCA Ca^{2+} ATPases (encoded by *atp2a2a*) or into the extracellular space via NCX1h exchangers (Bers et al., 1996; Fabiato, 1985). Impacts to any of these Ca channels involved in E-C coupling and Ca sequestration may manifest in a number of pathophysiological ways, including cardiomyocyte dysfunction, arrhythmia, and heart failure (Chen et al., 2004; Frank, 2003; Zhu et al., 2000). Immediately following 3 dpf embryo acute FPW exposures, *atp2a2a* was the only gene observed to significantly change expression (Figure 7-4A). Interestingly, while it was presently found using RT embryos that FPW exposure significantly increased *atp2a2a* expression, previous studies using zebrafish embryos have shown *atp2a2a* expression to significantly decrease following FPW exposure (Folkerts et al., 2017a). However, differences in expressional direction may be explained by the differing times of development when embryos were sampled, differential species responses, or differences in chemical makeup of the FPW solutions used. Regardless, the handling and cycling of Ca in electrogenic cardiomyocytes during an action potential (particularly sequestration of Ca back into the SR) may be affected following FPW exposure.

Counter to responses observed in 3 dpf exposed embryos, 10 dpf embryos immediately following acute FPW exposure displayed numerous changes in cardiac-related genes (Figure 7-4B). Interestingly, of the ion transporter genes involved in electrophysiological function which were investigated, *atp2a2a* was not one of the genes changed in 10 dpf embryos, but rather significant increases in expression of *scn5lab* and *kir2.1* were observed. These genes code for the main sodium (Na) channels which invoke cellular depolarization at the start of an action potential and inward-rectifying potassium (K) channels which are crucial for setting/maintaining resting membrane potential, respectively (Vornanen and Hassinen, 2016). Genes encoding for cardiac development and

differentiation were also differentially expressed in the 10 dpf exposed RT embryos. Notably, acute FPW exposure in 10 dpf embryos induced significant expression of *nkx2.5*, a homeobox-containing transcription factor required for proper cardiogenic differentiation in vertebrates and invertebrates alike (Akazawa and Komuro, 2005; Bartlett et al., 2010). Furthermore, it has been suggested that *nkx2.5* plays a significant role in developing the specialized electrosensitive cells of the rudimentary conduction system to be involved in E-C coupling for action potential potentiation (Thomas et al., 2001). Increased *nkx2.5* expression in RT embryos after FPW exposure follows what has been observed in zebrafish embryos (Folkerts et al., 2017a), although this is somewhat expected considering the observed maintenance of *nkx2.5* expression through development in other vertebrate species (Kasahara et al., 2003, 1998; Lints et al., 1993). In chronically exposed embryos at roughly the same time of development (17 dpf), gene expression profiles were comparable to 10 dpf embryos acutely exposed to FPW for 48 h (Figure 7-5B), but instead of *scn5lab*, expression of the gene encoding for a subunit of the main delayed rectifying K channel, *kcnh6*, was elevated. Delayed rectifying K currents (I_{kr}), and therefore *kcnh6*, are responsible for repolarizing the membrane of a cardiomyocyte during an action potential (Vornanen and Hassinen, 2016). Chronically FPW exposed embryos at 14 dpf also displayed elevation of another key transcription factor responsible for the development of cardiac cells, *tbx2b*. The gene *tbx2b* encodes for a T-box transcription factor, which among being involved in cardiac tissue development and differentiation processes, has also been shown to be intimately involved in regulating activity of atrial natriuretic peptide, *anp* (also known as natriuretic peptide type A). Interestingly, *anp* (a peptide hormone secreted from cardiac atrial cells to regulate atrial stretching and extracellular fluid volume which also serves as a key marker for outer myocardium development) (Auman et al., 2007) was also shown to be upregulated in both RT embryos acutely exposed to FPW at 10 dpf and in embryos exposed chronically for 14 days (17 dpf). Similarly, expression of ventricular natriuretic peptide (*vnp*, also known as natriuretic peptide type B or brain natriuretic peptide) was also increased in chronically exposed embryos at this time point. At the end of chronic FPW exposures (28 days), the only gene observed to change expression following 28 days of exposure was that of *gata5*, another key transcription factor involved in intracellular signalling pathways in developing cardiac cells, with a particularly important

role of serving as effectors for mediating hypertrophic stimulations and features (Akazawa and Komuro, 2005, 2003).

7.4.4 Juvenile Fish Swimming Performance, Respirometry, and Ventricular Morphometrics

Swimming performance is a relatively common index of fish fitness and is often studied in the context of aquatic environmental perturbations due to its incorporation of numerous physiological systems which produce such behaviors. However, due to the large diversity of systems governing swimming behavior in fish (including swim O_2 handling, glycolytic energy dynamics, and flume ground speed establishment among others) (Farrell, 2007; McDonnell and Chapman, 2016; Thomas et al., 2013), determining the cellular and physiological processes implicated in industrial contaminant-mediated impacts on swimming performance in fish can be challenging. It has, however, been extensively studied and accepted that functioning of cardio-respiratory systems in fish have appreciable impacts on aerobic capacity and activities wherein increased cardiac output and systemic O_2 delivery is required (Claireaux, 2005; Farrell et al., 2009; Steffensen and Farrell, 1998). How developmental impacts to cardiac systems during embryonic stages affect fitness - and to what extent are affects influencing critical fitness metrics such as swimming performance - in fish later life-stages is relatively less known and studied. We provide evidence that FPW-mediated impacts to embryonic cardio-respiratory development and function persist past initial FPW exposures and detrimentally affect swimming performance and respirometry in juvenile trout approximately 8 months post-exposure.

Regarding the known composition of FPW, many chemicals are known to alter fish swimming performance and respirometry immediately or quickly following exposure. These include chemical classes like PAHs (Lucas et al., 2016; Oliveira et al., 2012), metals (Mager and Grosell, 2011; Rajotte and Couture, 2002), ammonia/ammonia-containing compounds (Shingles et al., 2001; Wicks et al., 2002), and other industrially created contaminants and wastewaters like pesticides (Goulding et al., 2013; Marit and Weber, 2011), and crude oil fractions (Johansen and Esbaugh, 2017; Stieglitz et al., 2016). Fewer studies, however, have investigated embryonic exposures to these compounds/solutions and corresponding latent effects on later-stage fish swimming performance and

respirometry. Of those which have been undertaken, embryonic exposures to PAHs (and wastewaters which contain significant PAH concentrations like crude oil fractions) and pesticides have been shown to decrease swimming performance in fish at later developmental timepoints, with mixed effects on swimming respirometry and aerobic scope (Brown et al., 2017; Hicken et al., 2011; Mager et al., 2014; Marit and Weber, 2012). Presently, it was observed that acute FPW exposures of both 2.5 and 5% at 3dpf, acute FPW 5% exposures at 10 dpf, and chronic FPW exposures of 1% significantly reduced juvenile fish aerobic scope and U_{crit} (Figure 7-6). Changes to aerobic scope values following FPW exposure appear to conform with previous FPW (Folkerts et al., 2020b, 2017b) and other related wastewater (crude oil) (Johansen and Esbaugh, 2017; Lucas et al., 2016; Stieglitz et al., 2016) exposure studies depicting that limiting (not loading) stresses on fish O_2 uptake and/or delivery kinetics are primarily responsible for reduced aerobic scope and U_{crit} values in juvenile fish since MMRs (and not SMRs) were reduced in matching fashion.

Given the suppression of embryonic MO_2 past initial exposures, and previous evidence of decreased zebrafish larval heart rates (Folkerts et al., 2017a), it was suspected that juvenile trout O_2 kinetics may be affected following embryonic FPW exposure, ultimately leading to decreased swimming performance and respirometry. Indeed, it has been shown that PAHs and crude oil fractions significantly impact stroke volumes and decrease cardiac output in both larval and adult fish stages (Cox et al., 2017; Khursigara et al., 2017; Nelson et al., 2016; Y. Zhang et al., 2012b). Furthermore, PAHs and certain metals (such as barium and strontium) commonly found in FPW have been shown to directly impact and alter the electrophysiological properties of cardiac cells by targeting specific ion transporters (e.g. Ca^{2+} and K^{+}) responsible for key E-C coupling events (An et al., 2002; Brette et al., 2017; Gibor et al., 2004; Vehniäinen et al., 2019). Correspondingly, sarcomere contractile properties of isolated fish cardiomyocytes are altered and ultimately inhibited following exposure to fractions of crude oil (Heuer et al., 2019). However, overall heart morphology, and structural changes to cardiac morphological metrics, may also play a major role in dictating cardiac output, systemic O_2 delivery, and swimming performance in fish. Previous studies have showed that embryonic or chronic exposures to differing PAHs and crude oil fractions alter numerous heart morphometric indices in later-life fish

stages (Alderman et al., 2017; Brown et al., 2017; Hicken et al., 2011; Incardona et al., 2015). Presently, it was determined that ventricular compact myocardium thickness was significantly decreased in juvenile trout embryonically exposed to acute FPW 5% and chronic FPW 1% solutions (Figure 7-7C). Being the primary ventricular layer responsible for contractive forces in athletic fishes (e.g. salmonids) (Pieperhoff et al., 2009; Santer et al., 1983), this outer layer of circumferentially arranged cardiomyocytes which encase spongy myocardium is morphologically plastic in order to maintain cardiac function in response to environmental changes including temperature and O_2 availability stresses (Gamperl and Farrell, 2004; Klaiman et al., 2011). However, it is suspected that petrogenic compounds disrupt development and activity of progenitor cells of the early embryonic ventricle responsible for proper establishment of the compact myocardium in juvenile or adult fish (Incardona et al., 2015). Thus, similar events are suspected to have occurred in juvenile trout exposed to FPW during embryogenesis, producing systems in juvenile fish with suboptimal cardiac output and therefore reduced aerobic capacities and swimming performances. Although size/thickness of the compact myocardium is positively correlated with overall fish size (Farrell et al., 1988; Poupa et al., 1974), there were no statistically significant differences in fish weights and lengths between treatment groups presently Table 7-2), indicating that changes to development and growth of this tissue layer are FPW-mediated.

7.4.5 Developmental Timing of FPW Exposure

Differential responses between embryos exposed to FPW for 48 h at 3 dpf versus 10 dpf were not solely limited to cardiac gene transcriptional investigations; nearly all sub-lethal endpoints measured within the current study yielded differential responses depending on the developmental timing of FPW exposure. In our developmental deformity analyses, this was most clearly conveyed in percent pericardial edema scores (Figure 7-2B). Although exposures increased rates overall, embryos exposed to FPW at 3 dpf displayed greater significant rates of pericardial edema over embryos exposed at 10 dpf. Similar trends were observed in embryonic percent survival to hatch, percent yolk-sac edema, and percent tail/spine curvatures. When comparing embryo metabolic rates, responses between embryos exposed at 3 dpf versus 10 dpf also followed very different patterns. As

mentioned above, embryos exposed to FPW at 3 dpf had significantly higher MO_2 through development up to a certain developmental time (15 dpf), wherein a 'switch' occurred and subsequent MO_2 recordings of FPW-exposed embryos afterwards displayed significantly reduced MO_2 compared to controls. However, aside from increased MO_2 immediately following FPW exposure termination, embryos exposed at 10 dpf did not display any significant MO_2 differences from control values at any timepoint during development (Figure 7-3B). These results, in addition to the transcription differences observed, seem to suggest that trout embryos are more sensitive to perturbations and stressors at earlier developmental timepoints, and that FPW exposure earlier within the primary cardiac formation developmental window elicits more deleterious effects in developing RT embryos.

When comparing latent, long-term impacts of differing developmental exposures to FPW, differences in juvenile fish responses between 3 dpf and 10 dpf embryonic exposures were additionally recorded. Generally, juvenile fish acutely exposed to 2.5% FPW at 10 dpf did not show a significant reduction in MMR and aerobic scope, while fish exposed at 3 dpf to the same solutions did show reductions in these endpoints (acute FPW 5% exposures at both developmental time points produced significant effects) (Figure 7-6). Similar responses were observed concerning juvenile fish U_{crit} analyses. Interestingly, no such differential responses were observed when comparing juvenile fish compact myocardium thickness. In excised ventricles, juvenile fish exposed acutely to FPW 5% solutions (and chronic FPW 1%) displayed significant reductions in compact myocardium thickness, while 2.5% FPW exposures did not induce significant changes regardless of exposure developmental timepoint (Figure 7-7C). In zebrafish, progenitor cells fated for adult compact myocardium are established early during embryogenesis, with rapid proliferation occurring during juvenile stages (Gupta and Poss, 2012). It appears compact myocardium thickness is less dependent on timing of exposure and more respondent to strength of FPW exposure. Alternatively, developing precursor progenitor cells of trout embryos exposed to acute FPW 2.5% solutions may have had time to repair and/or functionally compensate physiological detriments induced from exposure, whereas 5% and chronic 1% FPW exposures generated stresses too great in strength and temporal length, respectively, producing irrevocable changes to compact myocardium thickness. Regardless,

latent effects of embryonic FPW exposure on juvenile fish fitness relatively follow responses observed during embryonic stages, wherein FPW exposures earlier during development (3 dpf) produce greater impacts on fish swimming performance and respirometry at juvenile stages.

7.5 CONCLUSION

Impacts of industrial wastewaters and whole effluents to fish embryo development is a well-studied area of toxicology. Considering the close proximity (~ 300 m) of many hydraulically fractured wells to freshwater surface bodies of waters (Entrekin et al., 2011), and frequently reported instances of FPW spills (Folkerts et al., 2020a), determining how novel contaminants and mixtures such as those found in FPW interact with and affect fish embryonic biology is critical for understanding the impacts of these contaminants on ecosystems and affected environments. Presently, we provide the first analysis of FPW-mediated impacts to RT embryonic development and cardio-respirometry physiology. Our analyses and results confirm the cardio-respiratory system as a target for FPW-mediated toxicity and indicates that specific cardiac focused developmental deformities, changes in gene expression, and impacts to embryonic MO_2 may occur. Furthermore, consequences of sub-lethal toxicities experienced during the embryonic stage appear to persist and affect fish fitness and cardiac morphology at the juvenile stage (at least ~ 8 months post exposure and possibly longer). We additionally provide evidence that embryonic exposures to FPW earlier in development may increase the severity and occurrence of said detrimental embryonic and juvenile fish sub-lethal toxicities. Considering the relative lack of unified governance over hydraulic fracturing activity, variable wastewater management practices, and variabilities in enforced spill response/remediation protocols, the results of the current study provide meaningful information to industry and regulatory agencies on the establishment of FPW risk assessment and hazard identifications.

Table 7- 1. Rainbow trout (*Oncorhynchus mykiss*) embryo acute exposure water quality parameters. N = 5 for all exposures. Data presented as average ± one standard deviation.

Acute 48 Hr Exposure	Conductivity (µS or mS)		Na^+ (mg/L)		pH		Dissolved O_2 (mg/L)		Temperature (°C)	
	START	END	START	END	START	END	START	END	START	END
3dpf Control	344.2 ± 16.2	383.2 ± 3.1	8.5 ± 3.1	10.2 ± 4.9	8.0 ± 0.1	8.0 ± 0.1	8.6 ± 0.7	8.5 ± 0.5	7.9 ± 0.4	8.0 ± 0.2
3dpf SC5%	13.1 ± 1.9	13.3 ± 2.0	2377.8 ± 268.2	2342.1 ± 416.6	8.0 ± 0.1	8.0 ± 0.1	8.5 ± 0.4	8.5 ± 0.5	7.8 ± 0.2	8.4 ± 0.6
3dpf FPW2.5%	7.5 ± 0.4	9.1 ± 0.4	1314.1 ± 155.4	1338.1 ± 194.5	7.9 ± 0.1	7.9 ± 0.0	8.4 ± 0.4	8.5 ± 0.5	7.7 ± 0.3	8.3 ± 0.4
3dpf FPW5%	12.1 ± 2.7	14.9 ± 1.2	2565.4 ± 213.4	2525.8 ± 363.6	7.8 ± 0.1	7.8 ± 0.1	8.4 ± 0.3	8.4 ± 0.4	7.8 ± 0.2	8.2 ± 0.4
10dpf Control	374.2 ± 33.8	363.4 ± 37.1	8.6 ± 2.2	9.3 ± 2.4	7.8 ± 0.3	7.9 ± 0.2	8.5 ± 0.5	8.5 ± 0.4	7.6 ± 0.4	7.5 ± 0.5
10dpf SC5%	13.0 ± 1.6	12.3 ± 2.2	2577.2 ± 82.4	2476.9 ± 215.7	7.7 ± 0.2	7.9 ± 0.1	8.4 ± 0.5	8.5 ± 0.4	7.9 ± 0.1	7.7 ± 0.4
10dpf FPW2.5%	7.7 ± 1.1	9.3 ± 0.8	1396.0 ± 272.7	1224.5 ± 58.9	7.6 ± 0.3	7.9 ± 0.1	8.5 ± 0.4	8.5 ± 0.4	7.8 ± 0.3	7.8 ± 0.4
10dpf FPW5%	11.8 ± 1.9	14.7 ± 1.5	2387.2 ± 311.1	2522.2 ± 293.3	7.6 ± 0.3	7.8 ± 0.1	8.5 ± 0.4	8.6 ± 0.5	7.6 ± 0.3	7.7 ± 0.3

Table 7- 2. Biometric data for rainbow trout (*Oncorhynchus mykiss*) analyzed for swimming performance, respirometry, and ventricle morphology.

Treatment Group	Weight (g)	Fork Length (cm)	Body Condition Factor*
Control 3dpf	5.57 ± 1.76	7.75 ± 0.81	1.33 ± 0.36
Saline Control 5% 3dpf	6.39 ± 1.16	8.06 ± 0.48	1.21 ± 0.081
FPW 2.5% 3dpf	5.59 ± 1.09	7.97 ± 0.54	1.28 ± 0.20
FPW 5% 3dpf	5.98 ± 0.94	7.95 ± 0.36	1.18 ± 0.076
Control 10 dpf	7.57 ± 1.79	8.55 ± 0.66	1.19 ± 0.10
Saline Control 5% 10dpf	5.83 ± 1.17	7.96 ± 0.52	1.14 ± 0.089
FPW 2.5% 10dpf	7.07 ± 1.93	8.35 ± 0.56	1.19 ± 0.12
FPW 5% 10dpf	7.20 ± 2.16	8.50 ± 0.74	1.15 ± 0.12
Chronic Saline Control 1%	6.41 ± 1.25	8.24 ± 0.52	1.21 ± 0.11
Chronic FPW 1%	6.21 ± 1.78	8.01 ± 0.81	1.19 ± 0.11

* Body condition factor calculated according to Ekström et al., 2019.

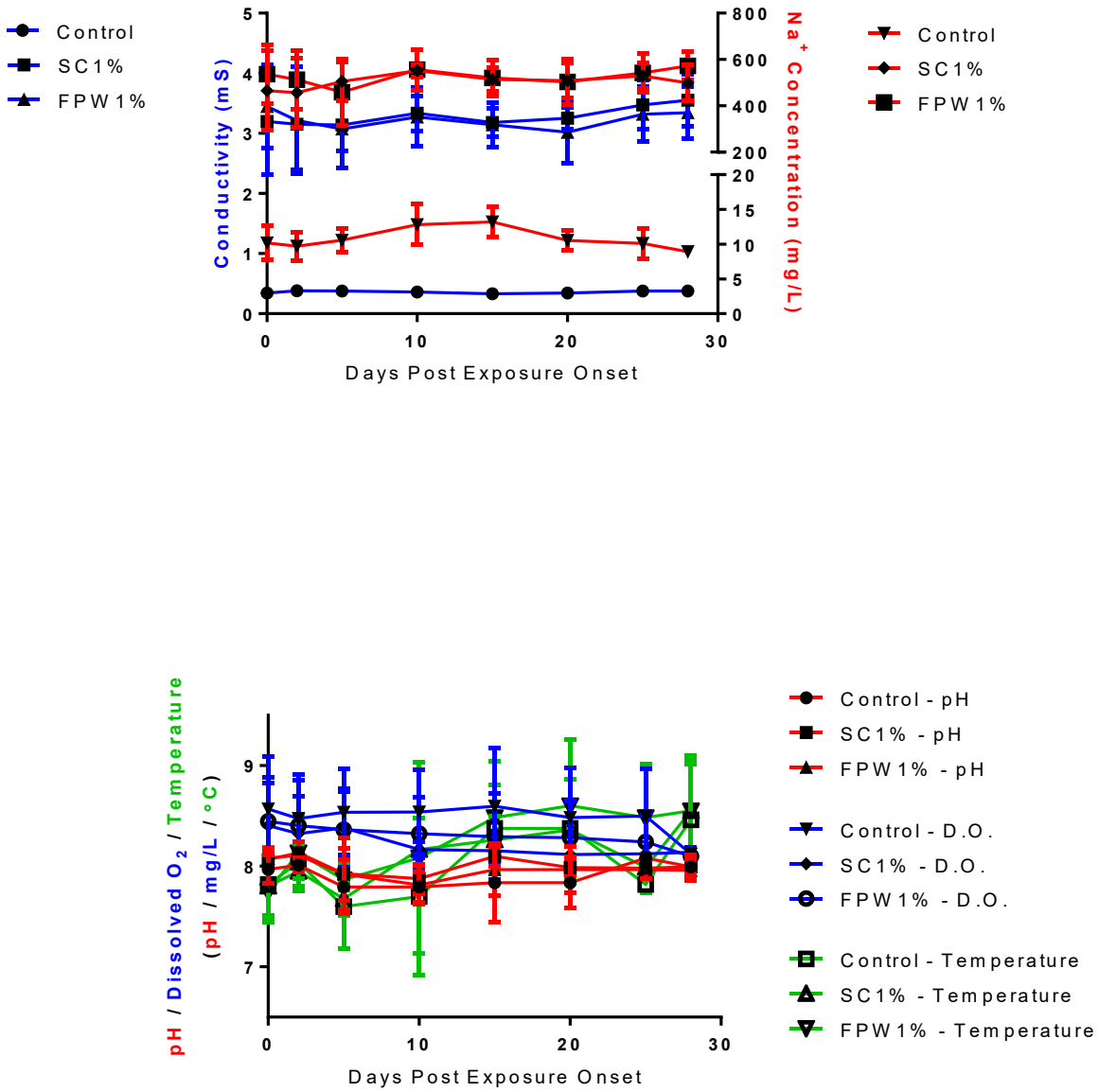


Figure 7- 1. Chronic rainbow trout (*Oncorhynchus mykiss*) embryo exposure water quality parameters. N = 5. Error bars represent ± 1 standard deviation.

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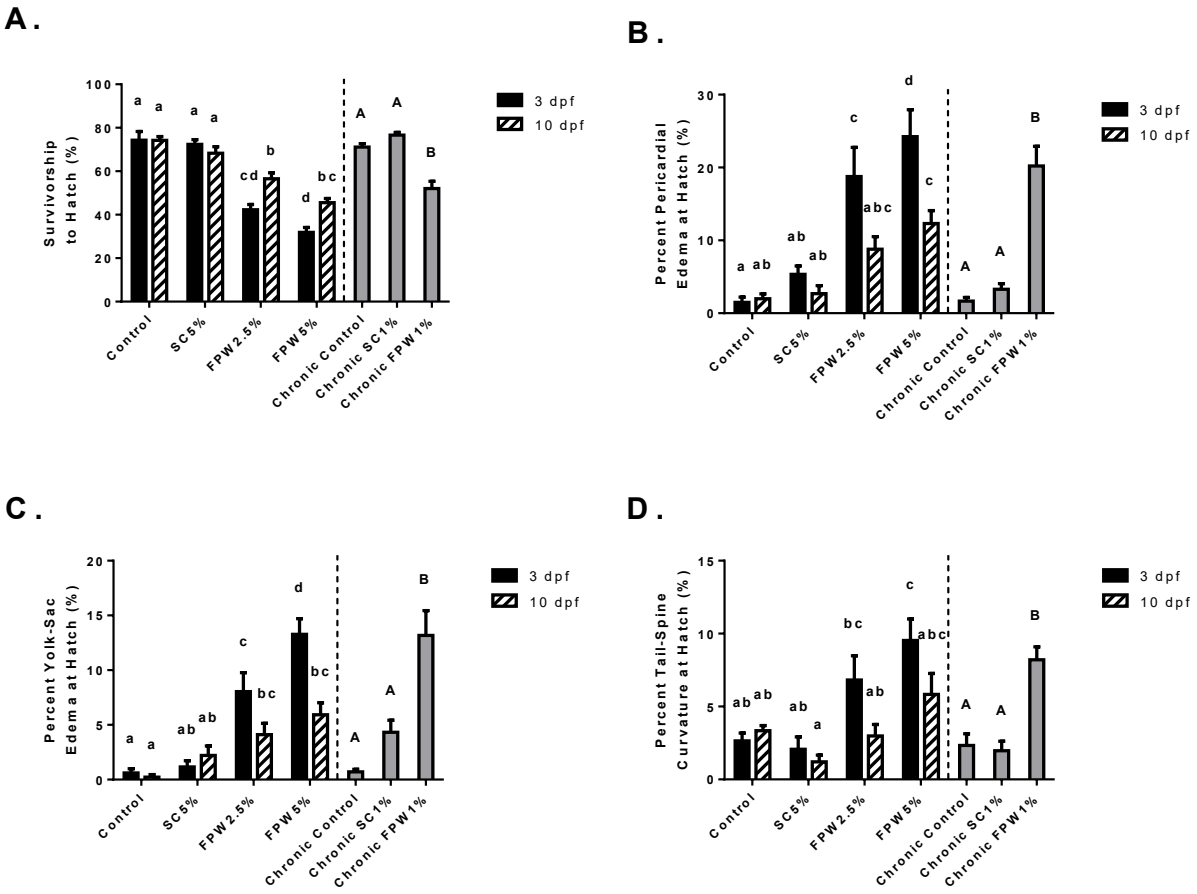


Figure 7- 2. Developmental deformities measured in rainbow trout (*Oncorhynchus mykiss*) larvae (~ 33 days post fertilization; dpf) after acute (48 h) embryonic exposures to control, saline control 5% (SC 5%), flowback and produced water 2.5% (FPW 2.5%), and FPW 5% solutions at either 3 dpf or 10 dpf developmental timepoints. FPW 5% solutions at either 3 dpf or 10 dpf developmental timepoints. Chronic 28-day exposures (D) to control, saline control 1% (SC 1%) and FPW 1% solutions also were additionally performed at 3 dpf developmental timepoints. Endpoints and developmental deformities analyzed included survivorship to hatch (A), pericardial edema (B), yolk-sac edema (C), and tail/spine curvatures (D). 5 replicates of 25 embryos per treatment tray per exposure treatment were analyzed to obtain results. Data are presented as mean ± SEM. Two-way ANOVAs utilizing treatment type and developmental timepoint as variables were performed on all acute 48 hr exposure data. For chronic, 28-day exposure data, separate one-way ANOVAs with treatment type as a variable were performed. All statistical analyses were subjected to

multiple comparison Holme-Sidak post hoc tests to determine significant differences ($p < 0.05$) between mean treatment group and developmental timepoint percentages.

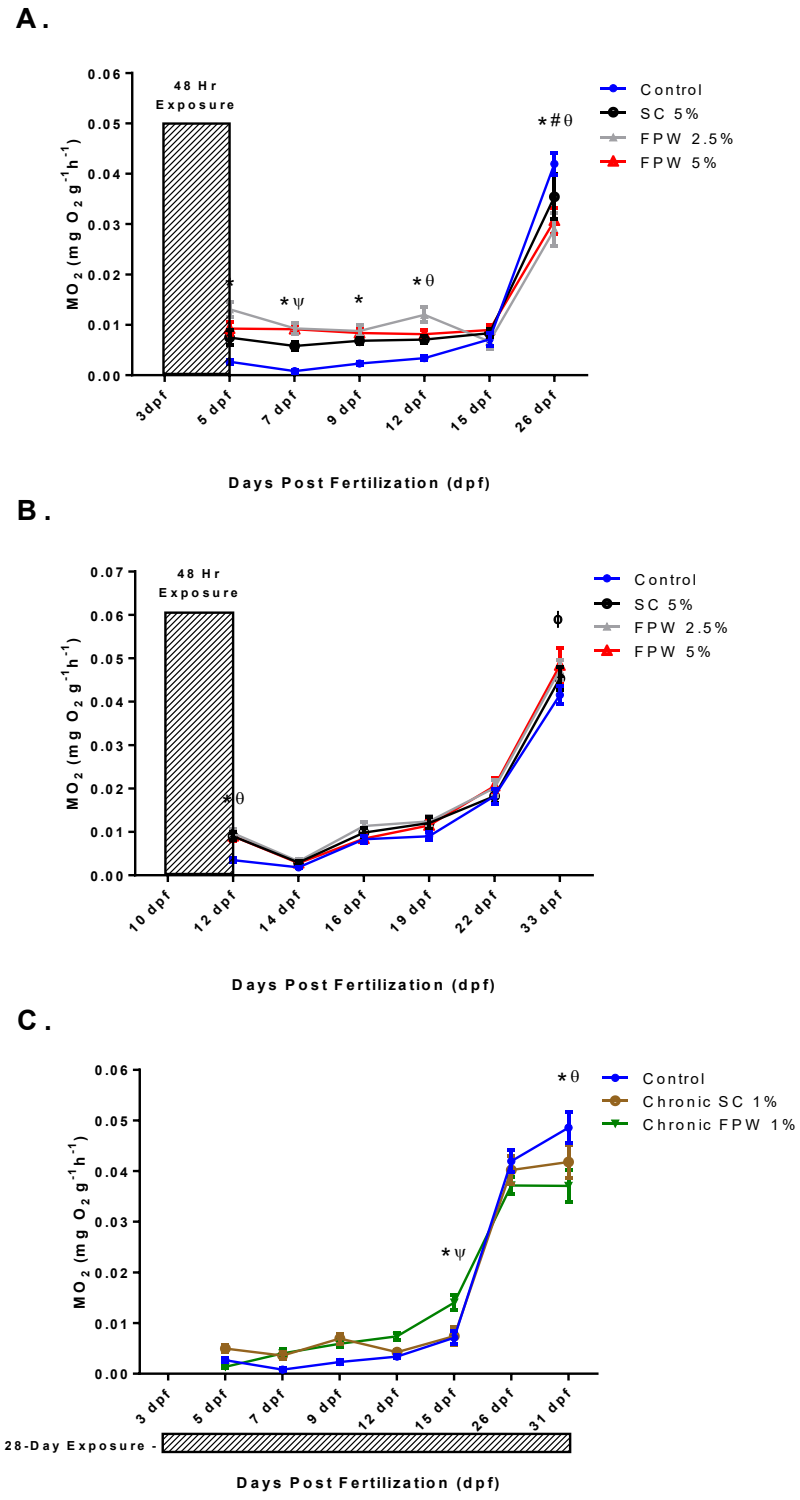


Figure 7- 3. Oxygen consumption (MO2) measurements in developing rainbow trout (*Oncorhynchus mykiss*) embryos at 10 °C after 48 h acute, embryonic exposures to control,

saline control 5% (SC 5%), flowback and produced water (FPW) 2.5%, and FPW 5% solutions at either 3 dpf or 10 dpf developmental timepoints (A and B). Chronic 28-day exposures (C) to control, saline control 1% (SC 1%) and FPW 1% solutions also were additionally performed at 3 dpf developmental timepoints. In total, 5 replicate (N = 5) exposures to each treatment condition were performed, with 8 – 15 embryos per treatment per time point measured for MO2 to obtain results. Data are presented as mean ± SEM. One-way ANOVAs for treatment at each developmental period followed by Holm – Sidak post hoc tests were performed to determine significant differences ($p < 0.05$) in O2 consumption in rainbow trout embryos after FPW exposure. In panels A and B: * denotes significant differences between control exposures and both FPW 2.5% and 5% exposures, # denotes significant differences between FPW 2.5% and SC 5% only, Ψ denotes significant differences between FPW 5% and SC 5% only, θ denotes significant differences between SC 5% and control only, and ϕ denotes significant differences between control exposures and FPW 5% only. In panel C: * denotes significant differences between control exposures and FPW 1% only, Ψ denotes significant differences between FPW 1% and SC 1% only, and θ denotes significant differences between SC 1% and control only.

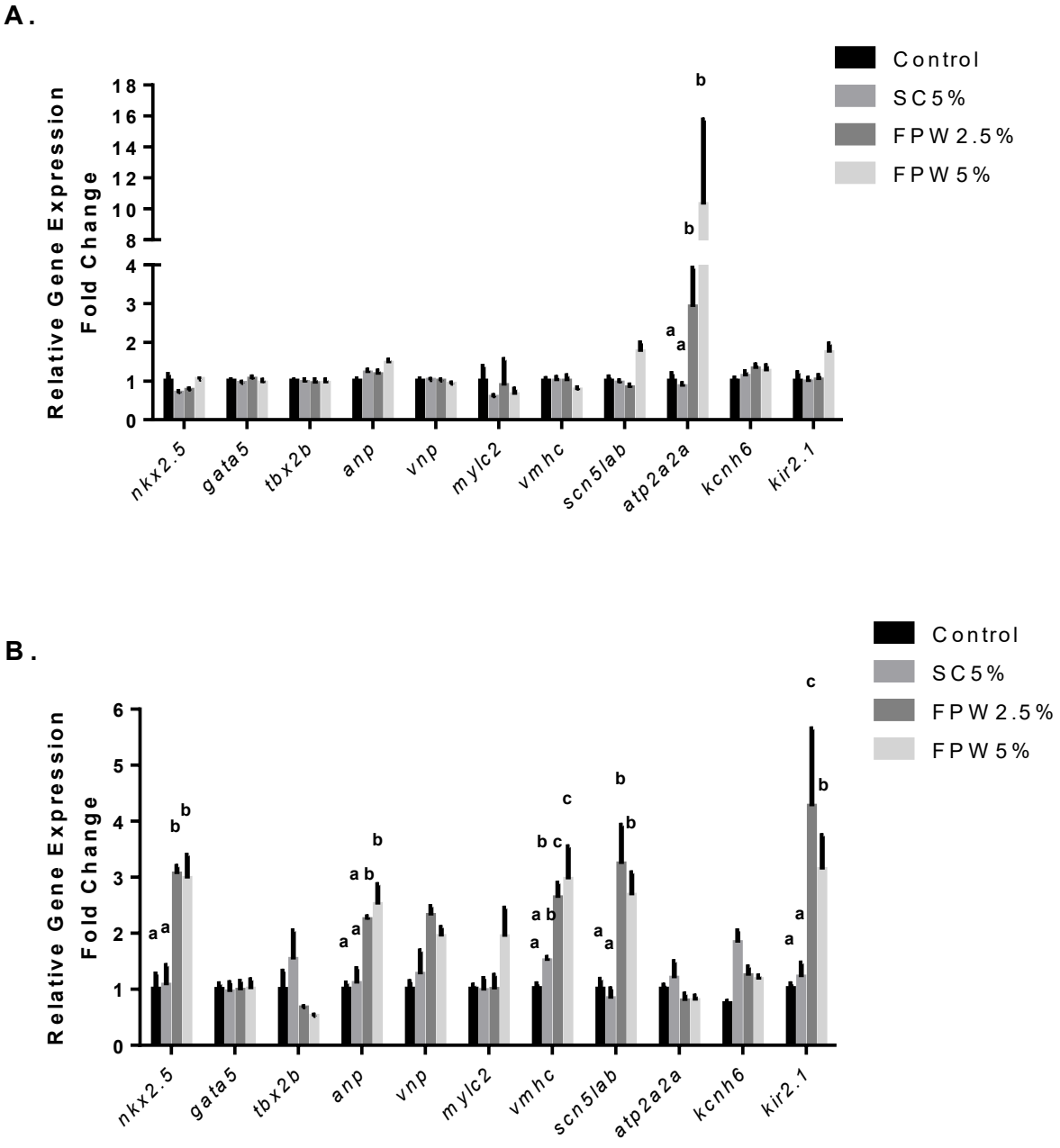


Figure 7- 4. Relative mRNA abundance of cardiac target genes homeobox protein nkx2.5 (*nkx2.5*), GATA binding protein 5 (*gata5*), T-box transcription factor 2b (*tbx2b*), atrial natriuretic peptide (*anp*), ventricular natriuretic peptide (*vnp*), myosin regulatory light chain 2 (*mylc2*), ventricular myosin heavy chain (*vmhc*), voltage gated Na+ channel, type 5ab (*snc5lab*), cardiac sarcoplasmic reticulum Ca2+ ATPase 2a2a (*atp2a2a*), voltage gated K+ channel subfamily H, member 6 (*kcnh6*), and inwardly-rectifying K+ channel 2.1

(*kir2.1*) in rainbow trout (*Oncorhynchus mykiss*) embryos following 48 h acute, embryonic exposures to control, saline control 5% (SC 5%), flowback and produced water (FPW) 2.5%, and FPW 5% solutions at either 3 dpf (A) or 10 dpf (B) developmental timepoints. Transcript abundance was determined by quantitative real-time PCR using the ΔΔCT method. Data was assessed using two-way ANOVAs with developmental state and treatment as factors. ANOVAs followed by Holm – Sidak post hoc tests were performed to determine significant changes ($p < 0.05$) of transcript abundances in zebrafish embryos. Data are presented as mean ± SEM.

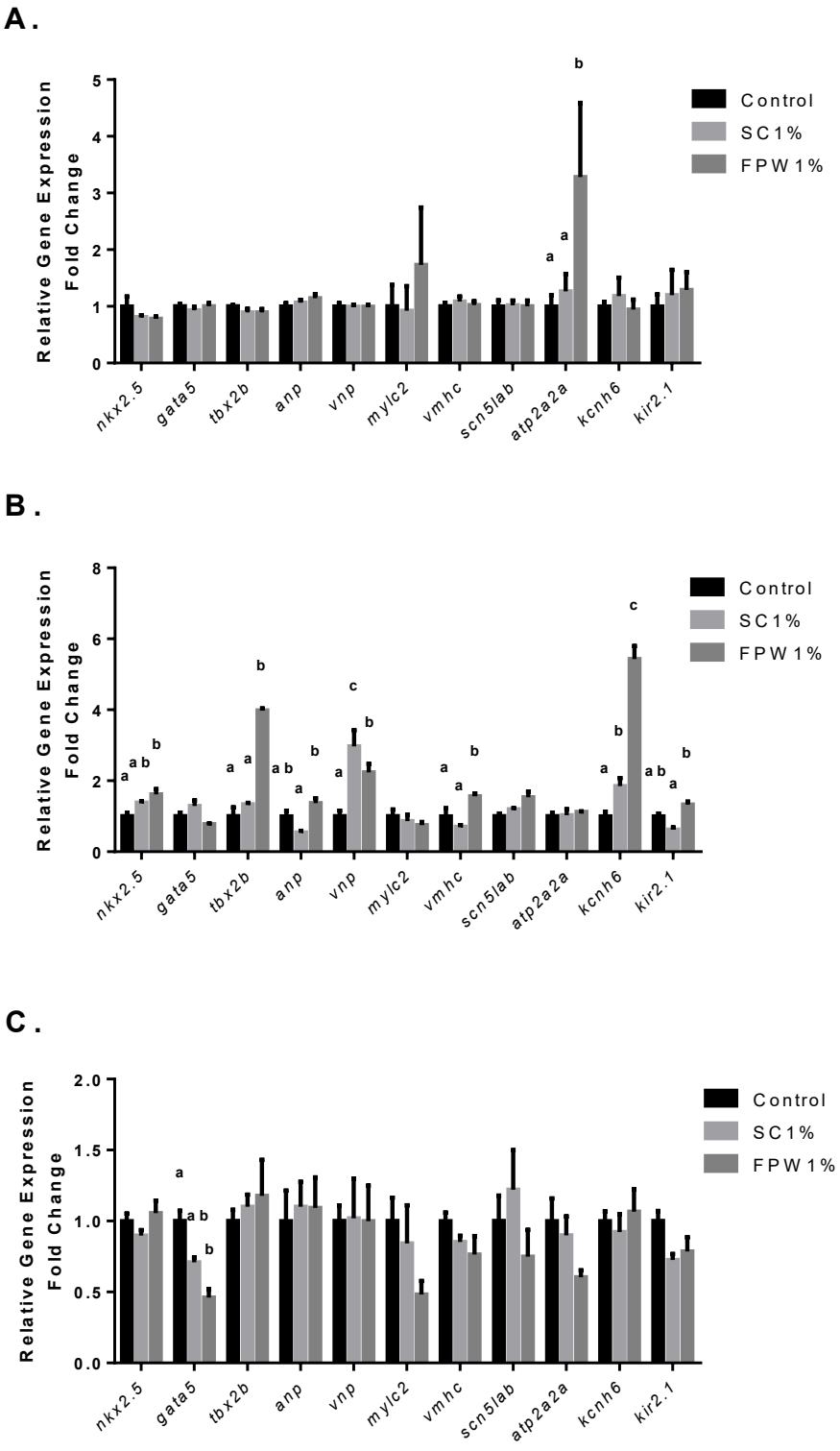


Figure 7- 5. Relative mRNA abundance of cardiac target genes homeobox protein nkx2.5 (*nkx2.5*), GATA binding protein 5 (*gata5*), T-box transcription factor 2b (*tbx2b*), atrial

natriuretic peptide (*anp*), ventricular natriuretic peptide (*vnp*), myosin regulatory light chain 2 (mylc2), ventricular myosin heavy chain (*vmhc*), voltage gated Na+ channel, type 5ab (snc5lab), cardiac sarcoplasmic reticulum Ca2+ ATPase 2a2a (*atp2a2a*), voltage gated K+ channel subfamily H, member 6 (*kcnh6*), and inwardly-rectifying K+ channel 2.1 (*kir2.1*) in rainbow trout (*Oncorhynchus mykiss*) embryos following chronic 28-day embryonic exposures to control, saline control 5% (SC 5%), flowback and produced water (FPW) 2.5%, and FPW 5% solutions. Chronic exposures started when embryos were 3 dpf. Transcript expression analyses were performed following 48 h (A), 14-days (B), and 28-days (C) of exposure to treatments. Transcript abundance was determined by quantitative real-time PCR using the ΔΔCT method. Exposure gene expression data was assessed separately using one-way ANOVAs with treatment as a factor, followed by Holm-Sidak posthoc tests to determine significant changes ($p < 0.05$) of transcript abundances in rainbow trout embryos. Data are presented as mean ± SEM.

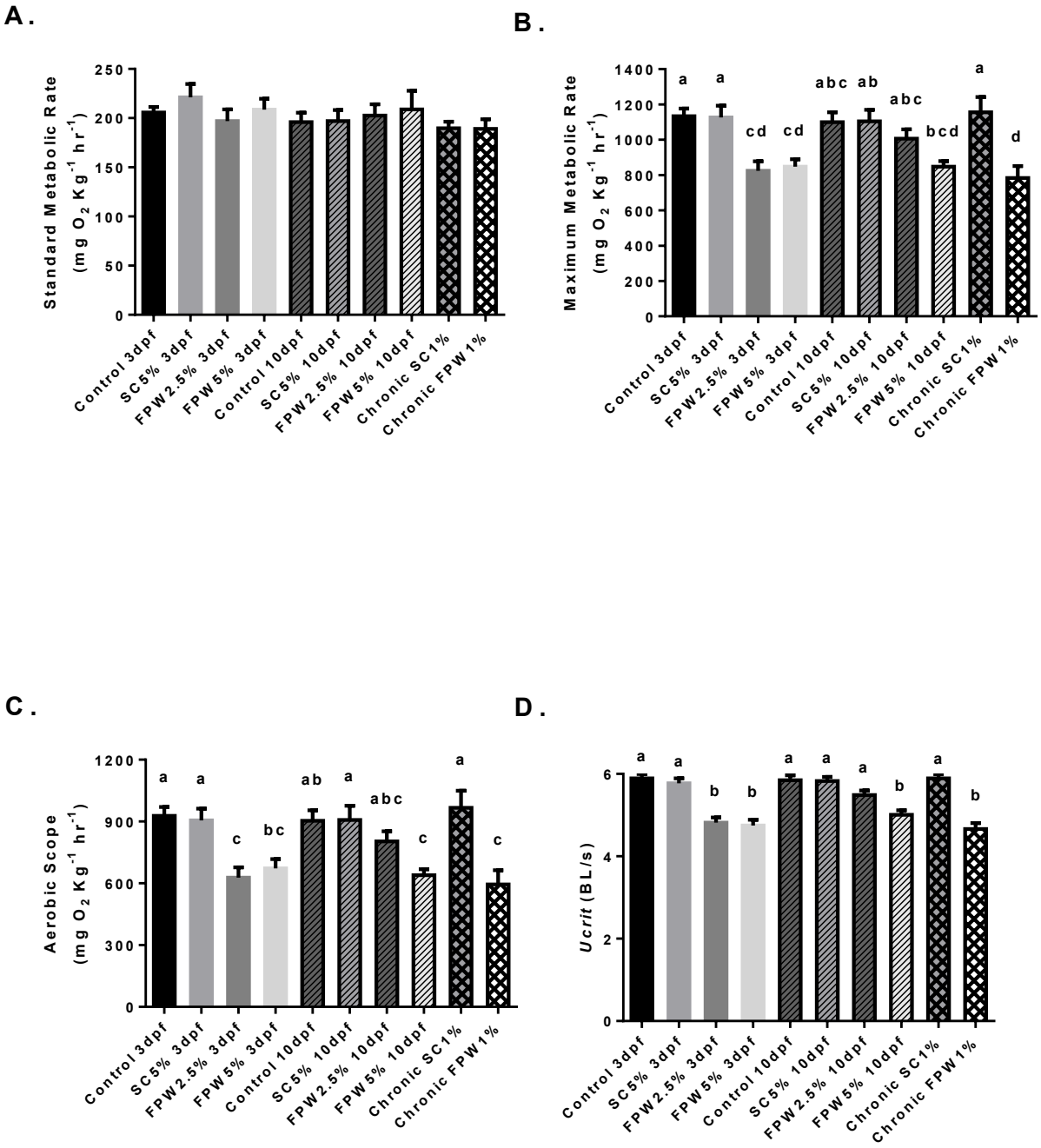


Figure 7- 6. Evaluation of (A) standard metabolic rate (SMR), (C) maximum metabolic rate (MMR), (C) aerobic scope, and (D) critical swimming speed (*Ucrit*) swimming respirometry experiments in juvenile rainbow trout (*Oncorhynchus mykiss*) following 48 h acute, embryonic exposures to control, saline control 5% (SC 5%), flowback and produced water (FPW) 2.5%, and FPW 5% solutions at either 3 dpf (*n* = 14, 13, 10, and 13,

respectively) or 10 dpf (n = 11) developmental timepoints. Chronic 28-day exposures to control, saline control 1% (SC 1%) and FPW 1% solutions (n = 14, 10, and 11, respectively) also were additionally performed at 3 dpf developmental timepoints. SMR and MMR data were normalized for body by scaling to the respective SMR and MMR values predicted for a 5 g fish (aerobic scope = normalized MMR − normalized SMR). Letters denote statistical differences $p < 0.05$ (measured via a Holm-Sidak posthoc analysis). Data are presented as means ± SEM.

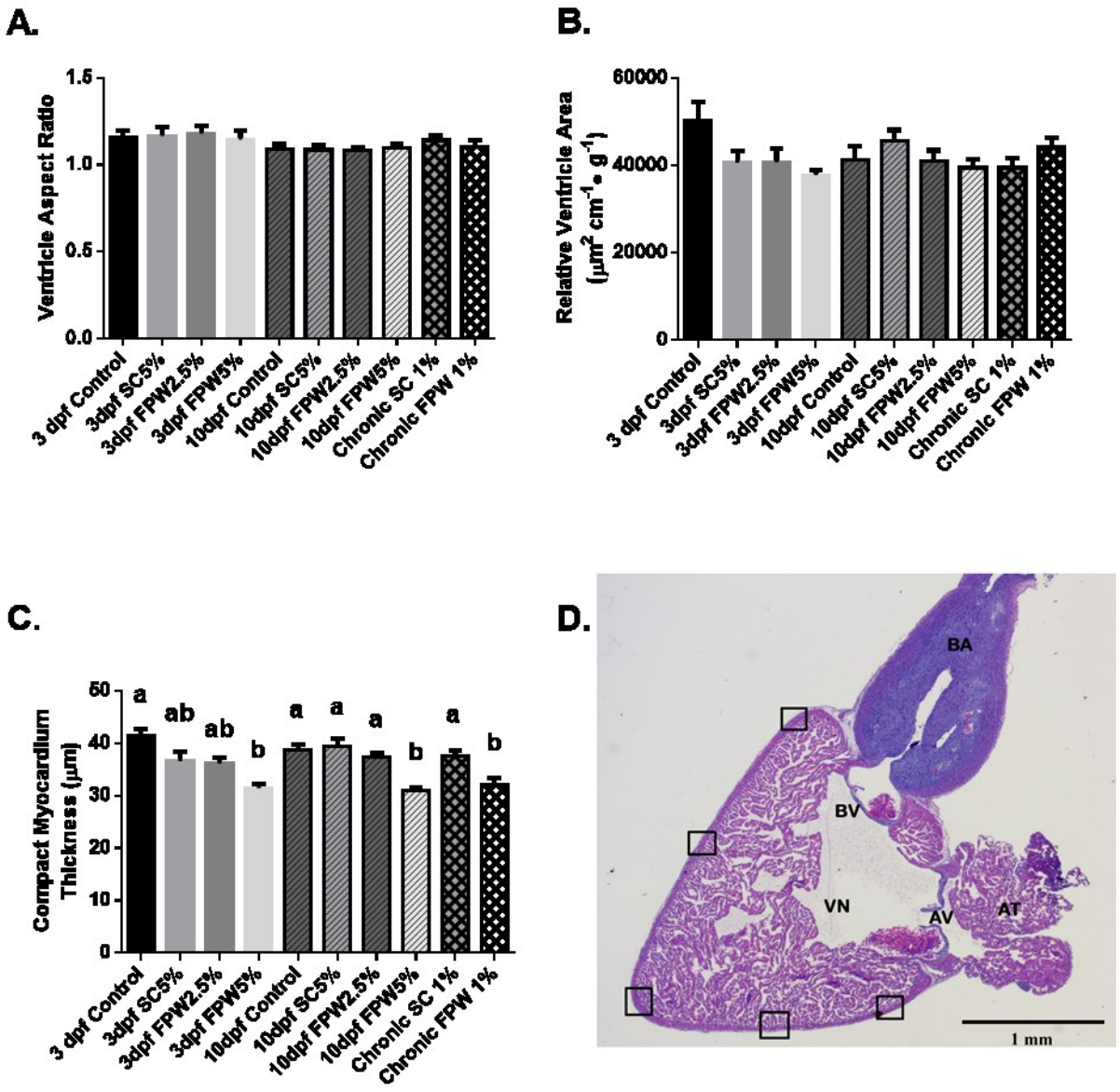
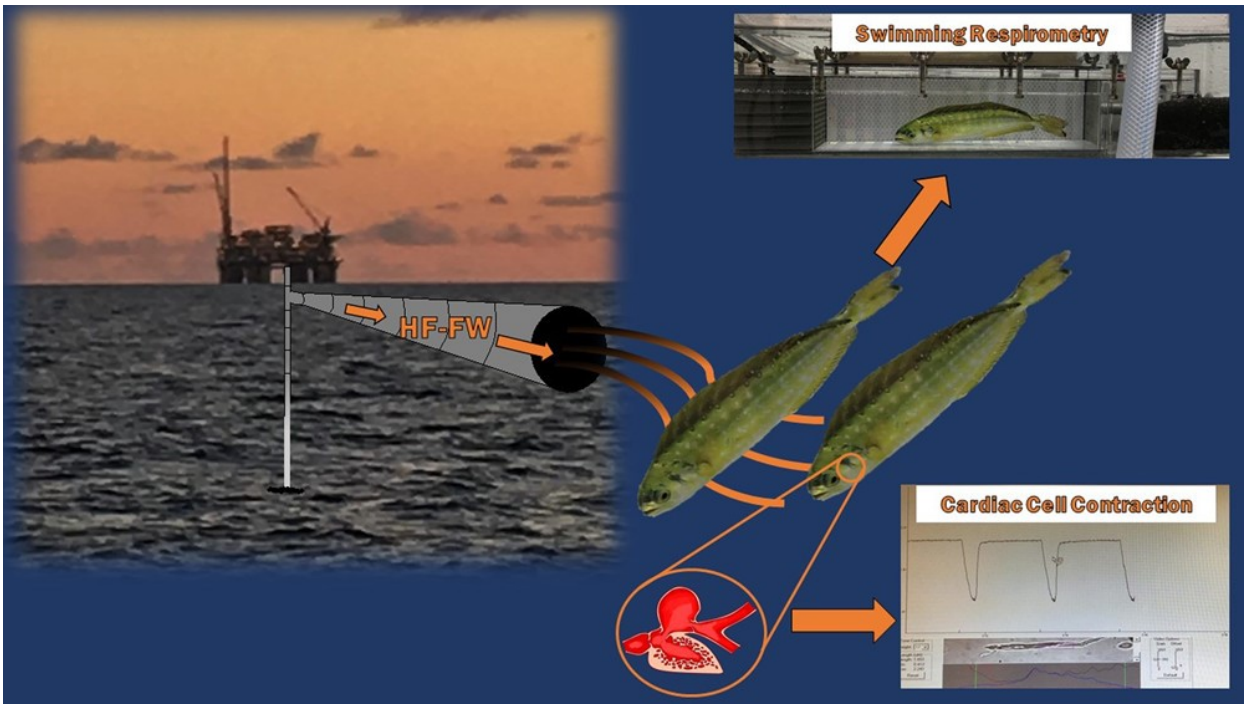


Figure 7- 7. Analysis of juvenile rainbow trout (*Oncorhynchus mykiss*) ventricular morphology and structure following 48 h acute, embryonic exposures to control, saline control 5% (SC 5%), flowback and produced water (FPW) 2.5%, and FPW 5% solutions at either 3 dpf (*n* = 14, 13, 10, and 13, respectively) or 10 dpf (*n* = 11) developmental timepoints. Chronic 28-day exposures to control, saline control 1% (SC 1%) and FPW 1% solutions (*n* = 14, 10, and 11, respectively) also were additionally performed at 3 dpf developmental timepoints. (A) ventricular aspect ratio, (B) relative ventricular area, and (C) compact myocardium thickness. Panel D depicts a representative histological ventricle

section used for performing analyses. Data was assessed using one-way ANOVAs with treatment as a factor. Letters denote statistical differences $p < 0.05$ (measured via a Holm-Sidak posthoc analysis). Data are presented as means ± SEM.

CHAPTER 8: Exposure to Hydraulic Fracturing Flowback Water Impairs *Mahi-Mahi* (*Coryphaena hippurus*) Cardiomyocyte Contractile Function and Swimming Performance



Folkerts, E.J., Heuer, R.M., Flynn, S.L., Stieglitz, J., Benetti, D.D., Alessi, D.S., Goss, G.G., and Grosell, M. (2020). Exposure to Hydraulic fracturing flowback water impairs Mahi-mahi (*Coryphaena hippurus*) cardiomyocyte contractile function and swimming performance. *Environmental Science and Technology*, **54**, 13579 – 13589. DOI: 10.1021/acs.est.0c02719.

8.1 INTRODUCTION

The use of hydraulic fracturing for unconventional oil and gas (UOG) extraction is increasing worldwide (Alessi et al., 2017; "U.S. monthly crude oil production exceeds 10 million barrels per day, highest since 1970," 2018). By 2035, ~50% of all natural gas production in the U.S. will come from horizontal hydraulic fracturing activity (a variant of the standard hydraulic fracturing process) (Gagnon et al., 2016). Despite its rise in prevalence, few studies have examined how hydraulic fracturing may impact adjacent natural environments where such activities occur. Particularly, little is known about the toxicological properties of the surface-returned wastewater of the hydraulic fracturing process: hydraulic fracturing-generated flowback (HF-FW) and produced water. Generally, flowback waters are considered as immediate-to-earlier returned waters from the wellbore and produced waters considered as later-returned waters to the surface (Alessi et al., 2017). Both waters are often highly saline and chemically complex solutions comprising original fracturing fluid components and additives (*e.g.*, biocides, scale inhibitors, surfactants, *etc.*), chemical species from the targeted geological formation (such as metals and petrogenic organic compounds), and newly transformed/created compounds from parent chemical species reacting in the high-heat, high-pressure downhole environment (DiGiulio and Jackson, 2016; He et al., 2017a; Hoelzer et al., 2016; Lester et al., 2015).

Recent studies show low dilution exposures of HF-FW and produced waters from UOG activities induce sublethal and lethal toxicities in freshwater invertebrates (as low as 0.004 and ~0.25%, respectively, in *Daphnia magna*) and vertebrates (as low as 2.5 and 2.2%, respectively, in species such as rainbow trout, *Oncorhynchus mykiss*, and zebrafish, *Danio rerio*) (T. A. Blewett et al., 2017b, 2017a; Folkerts et al., 2017a, 2017b; He et al., 2018a, 2018b, 2017b). Despite this increasing toxicological evidence, both Canada and the U.S. do not classify these wastewaters as hazardous waste (Alessi et al., 2017; Goss et al., 2015). Coupled with the regionally governed management structure of UOG activity and waste (Ralston and Kalmbach, 2018), this has resulted in highly variable systems of policy and regulation frameworks which may not effectively address the hazards UOG wastewaters pose to the environment. This is particularly true in the Gulf of Mexico (GoM), where offshore fracturing is regulated federally in the United States ("The NPDES General Permit for New and Existing Sources and New Discharges in the Offshore

Subcategory of the Oil and Gas Extraction Point Source Category for the Western Portion of the Outer Continental Shelf of the Gulf of Mexico (GMG290000)," 2017). Currently, offshore oil and gas operations are permitted to discharge wastewaters directly into GoM waters ("Fact Sheet and Supplemental Information for the Final Reissuance of the NPDES General Permit for New and Existing Sources in the Offshore Subcategory of the Oil and Gas Extraction Point Source Category for the Western Portion of the Outer Continental Shelf of the Gulf of Mexico (GMG290000)," 2017; "Final National Pollutant Discharge Elimination System (NPDES) General Permit No. GEG460000 For Offshore Oil and Gas Activities in the Eastern Gulf of Mexico," 2017; "The NPDES General Permit for New and Existing Sources and New Discharges in the Offshore Subcategory of the Oil and Gas Extraction Point Source Category for the Western Portion of the Outer Continental Shelf of the Gulf of Mexico (GMG290000)," 2017). Considering its diverse ecosystem, the potential impacts of these wastewater disposal practices on marine organisms and socioeconomic conditions could be extensively detrimental in the GoM region.

In this study, the ecologically relevant fish species mahi-mahi (*Coryphaena hippurus*; herein referred to as "mahi") was used to study the sublethal, cardio-respiratory effects of exposure to an HF-FW sample collected 2 h poststimulation from a horizontal hydraulically fractured well. Mahi are pelagic predatory fish with high metabolic demands to support their aerobic feeding and migratory lifestyles (Benetti et al., 1995b, 1995a). Impacts on cardio-respiratory systems in these fish may, therefore, have significant fitness consequences, rendering these organisms excellent cardiotoxicity models. Impacts on mahi cardio-respiratory systems and fitness have been well-documented following exposure to deepwater horizon crude oil (Pasparakis et al., 2019a), where polycyclic aromatic hydrocarbons (PAHs) have been identified as toxicants responsible for many of the observed cardiac pathologies. Although hydraulic fracturing wastewaters have been shown to contain many of the PAHs and other organic chemicals found in crude oil (although often at much lower concentrations; Folkerts et al., 2020, 2019; He et al., 2017a), these wastewaters also have unique chemical signatures derived from anthropogenic additives and downhole transformation products (Folkerts et al., 2019; He et al., 2017a). The first aim of this study was to examine how HF-FW impacts whole organism fitness, measured *via* mahi swimming performance and respirometry. After observing

impaired oxygen uptake and reduced aerobic performance of intact fish, potential cellular mechanisms underlying these reduced swimming metrics were investigated by exposing cardiomyocytes to dilutions of HF-FW and assessing contractility during baseline, standardized conditions, and by pacing cardiomyocytes over a range of stimulation frequencies representative of mahi heart rates (Heuer et al., 2019; Nelson et al., 2016). Finally, key genes associated with cardiac contractility were also examined in mahi exposed to HF-FW.

8.2 MATERIALS AND METHODS

8.2.1 Experimental Animals

Mahi (cardiomyocyte studies: 382 g, fork length = 32.9 cm, n = 24; swimming performance and respirometry: 18.9 g, fork length = 13.3 cm, n = 70) (Table 8-1) spawned from wild-caught broodstock were maintained in two 3000 L fiberglass holding tanks with flow-through seawater (26–30 °C) at the University of Miami Experimental Hatchery according to implemented standard protocols (Stieglitz et al., 2017). Mahi, depending on size, were fed either a daily combination of squid, mackerel, silversides, and pelletized feed or live planktonic feed (during larval stages), or a combination of both during juvenile fish stages. All experimental procedures and animal care practices were approved by the University of Miami's Institutional Animal Care and Use Committee (IACUC protocol 15–019).

8.2.2 HF-FW Sample, Exposures, and Chemical Analysis

The HF-FW sample used in this study was collected from a Canadian-based operator from a single horizontal hydraulically fractured well in the Duvernay formation (Alberta, Canada) at 2 h poststimulation (beginning of resource and subsequent HF-FW retrieval). The inorganic characterization of raw HF-FW was performed *via* inductively coupled plasma (ICP)–MS/MS using an Agilent 8800 Triple Quadrupole ICP–MS/MS. Specifics pertaining to analyte quantification methods, instrument specifics, detection limits, and precision/accuracy can be found in the Supporting Information (see Appendix 8-1 "Inorganic HF-FW Characterization" and Tables S8-2 – S8-4). Raw and diluted HF-FW ΣPAH analysis was performed by ALS Environmental (Kelso, WA, USA) using gas

chromatography–MS (GC–MS) with selective ion monitoring based on the USEPA method 8270D.

For swim tunnel respirometry, mahi were exposed to static HF-FW solutions similar in fashion to exposures described by Mager et al., 2014 and Stieglitz et al., 2016. Briefly, 3–4 juvenile mahi (checked for general body condition and any deformities or damage to caudal fin) were exposed in 1000 L cylindrical tanks filled with 100 L of medium for 24 h on a 16:8 h light:dark photoperiod. Mahi were randomly assigned to one of the four exposures: control seawater, 0.75% HF-FW, 2.75% HF-FW, or a salt control (SC) that matched the ionic composition in the 2.75% HF-FW treatment. FW exposures of either 0.75 or 2.75% were prepared by diluting raw HF-FW with filtered seawater. The salt control was prepared by dissolving appropriate amounts of sodium, calcium, potassium, and magnesium chloride salts in Milli-Q ddH_2O to create a salt saline control matching the raw, undiluted HF-FW cationic and chloride ion concentrations. This saline control solution was then diluted with filtered seawater to match the highest used HF-FW dilution percentage (2.75%). Filtered seawater was used for control exposures. Water samples from the beginning and end of all exposures for 0.75 and 2.75% HF-FW were sent to ALS for ΣPAH analysis. Pumps were placed inside exposure tanks to circulate the respective media. Exposure oxygen (O_2) levels and temperature were maintained at 27 °C using O_2 and compressed air lines connected to air stones and a submersible heater, respectively. All water quality and monitoring data can be found in Table S8-5. Prior to fish introduction into exposure tanks, a short mixing period (~10–15 min) established uniform dissipation of HF-FW and ensured O_2 and temperatures were at acceptable levels. Fish were then directly transferred from holding tanks to exposure tanks. Mortalities during 24 h exposures were minimal and were only observed in the HF-FW 2.75% treatment (n = 3 out of a total of 70 fish used; see Table S8-5). The number of exposures/true replicates (and total n given that multiple fish were analyzed from each replicate) for swimming performance and respirometry analyses for the control, SC, 0.75% HF-FW, and 2.75% HF-FW exposure groups were 5 (14), 4 (12), 4 (11), and 4 (10), respectively. Fish that were exposed and not included in swimming and respiratory analyses were sacrificed immediately following exposure termination, and tissues were collected for gill microscopy and ventricle quantitative real-time PCR (qPCR). Because of the rapid growth rate of juvenile mahi, the

order of all treatment exposures was interspersed to minimize confounding size factors on swimming performance and respirometry. Fish were fed the mornings prior to transfer to exposure tanks and were not fed during the 24 h exposure periods. In total, fish fasting before swimming and respirometry analyses was 32 h, a period sufficient to clear specific dynamic action effects (an observable increase in metabolism attributed to the digestion of food) (Stieglitz et al., 2018).

For cardiomyocyte exposures, raw HF-FW was first vacuum-filtered through a two-stacked 90 mm, 0.3 μm glass fiber filter to eliminate potential debris and sediment which may interfere with the IonOptix, photometric imaging system (Esbaugh et al., 2016; Heuer et al., 2019). A sample of this filtered, raw HF-FW was sent to ALS Environmental for ΣPAH analysis according to specifications listed previously above. Cardiomyocytes were exposed to either control mahi physiological extracellular saline (Table S8-6) (Galli et al., 2011; Heuer et al., 2019; Shiels et al., 2004), 1%, or 2% HF-FW, a 2% HF-FW salt control, or a 2% HF-FW osmotic control. All dilutions of HF-FW and preparation of salt and osmotic controls were made with control mahi physiological saline. The 2% HF-FW salt control (prepared in a similar fashion to salt controls used for whole-organism exposures) comprised the major cationic species and chloride concentrations matching the 2% HF-FW exposure. To control for osmotic pressure and to differentiate between ionic impacts *versus* general osmotic impacts, the osmotic control exposure matched the osmotic pressure of the 2% HF-FW exposure group by dissolving an appropriate amount of mannitol in mahi physiological saline to reach osmotic values recorded in 2% HF-FW solutions (~390 mOsm; see Table S8-7).

8.2.3 Swimming Performance and Respirometry

Three 5 L Brett-type swim tunnel respirometers (Loligo Systems, DK) were used to assess critical swim speed (U_{crit}) and aerobic scope *via* automated intermittent flow respirometry. A Pt100 fiber-optic probe connected to a Witrox 1 minisensor O_2 meter (PreSens Precision Sensing, GER) was outfitted in each swim respirometer to measure O_2 consumption (MO_2) during fish swimming. Swim tunnel respirometer water velocity calibrations were performed after every cleaning or apparatus disassembly event. Tunnel water velocity calibrations were performed by plotting motor power output (Hz) against

water flow velocities (measured using a flow meter; Höntzsch, GER). A two-point calibration system was used to calibrate O_2 probes using a 10 g/L Na_2SO_3 solution (O_2 zeroing solution) and oxygenated seawater (O_2 saturating solution). Temperatures in all swim tunnel respirometers were maintained at 27 °C (±0.5 °C). All data were collected using AutoResp 2.1.0 software (Loligo Systems).

Following mahi 24 h exposures, fish were gently transferred to and acclimated in swim respirometers overnight (~16 h) at a slow water flow of 1 body length/second (BL/s) and MO_2 recording commenced. Once acclimation periods had finished, fish were subjected to a U_{crit} swimming protocol. Fish were forced to swim for 20 min step intervals until fatigue using fixed water velocity increments of 0.5 BL/s per interval. During these 20 min step increments (and during overnight acclimation), intermittent respirometry recording occurred (530 s flushing, 30 s wait, and 600 s of MO_2). Fish fatigue was determined once mahi began to consistently rest for several seconds at a time on the back gate by supporting itself on its caudal fin despite repeated attempts to induce swimming by quickly lowering and ramping up water velocities (Mager et al., 2014). U_{crit} was calculated according to a formula described by Brett (Brett, 1964): $U_{crit} = [U_f + (T/t)dU]/cm$, where U_f (cm/s) is the highest swim velocity maintained for a complete step interval, T (s) is the length of time spent at the final, highest swim velocity, t (s) is the set time for the step interval (1200 s = 20 min), and dU (cm/s) is the increment in swim speed of each step. Fish mass and fork length were determined postswimming to minimize fish stress during swim respirometer introduction and to convert all U_{crit} data to body lengths per second (BL/s). A scaled standardized approach to estimate the aerobic scope for fish was employed to account for any influences of fish size on respirometry (see Appendix 8-3 "Fish Respirometry and Aerobic Scope Scaling and Standardization" and Figures S8-1 and S8-2 for further details) (Mager et al., 2014; Stieglitz et al., 2016).

8.2.4 Ventricle Gene Expression and Quantitative Real-Time PCR

Total RNA was extracted from mahi ventricle tissue samples, and cDNA was prepared for quantitative real-time PCR (qPCR) measurement using SYBR green master mix analyses (Applied Biosystems, USA). Further information on RNA extractions, cDNA synthesis, and qPCR reactions can be found in the Supporting Information (see Appendix

8-4 “Ventricle Gene Expression and Quantitative real-time PCR”). Gene-specific primers previously designed for mahi cardiac targets (Edmunds et al., 2015) were created using Integrated DNA Technologies (USA) online software. Primer gene targets, accession numbers, and sequences can be found in Table S8-8. Transcript expression changes were determined by ΔΔCT analysis using ribosomal protein S25 (*rps25*) as an endogenous control (Schmittgen and Livak, 2008). Endogenous control stability and primer efficiency validations, the latter determined by serial dilution slope calculation after ΔCT and log $cDNA_{[dilution]}$ input regression analyses, can be found in Figure S8-3.

8.2.5 Cardiomyocyte Sarcomere Contractility Measurements

Cardiomyocytes from mahi ventricles were isolated following protocols established in previous studies (Galli et al., 2011; Heuer et al., 2019). Details of isolation procedures can be found in the Supporting Information (see Appendix 8-5 “Ventricular Cardiomyocyte Isolation”). Mahi cardiomyocyte sarcomere contractile kinetic properties were then measured using a real-time IonOptix Myocyte Contractility system (IonOptix, MA, USA). This system was comprised of a FHDRCC1 perfusion chamber mounted on an inverted microscope (Motic AE3) with a digitizing dimensioning MyoCam-S camera attached. To stimulate myocyte contractions, the perfusion chamber was outfitted with electrodes connected to a MyoPacer stimulator. The cardiomyocyte average initial sarcomere length was determined *via* SarcLens technology within the IonWizard software (version 6.0), which uses A- and I-spacing band markers within a user-defined region of a sarcomere and performs instantaneous Fourier transformations to calculate mean sarcomere space (length) frequency. Using this system and associated software, numerous sarcomere shortening properties (used as an index of cell contractility) were analyzed. These properties included overall sarcomere contraction size (the difference between the mean relaxed and contracted sarcomere length expressed as a percent; referred to as baseline percent peak height, BL % Peak Height), contraction and relaxation velocities (d/d*t* and −d/d*t*, respectively), times to peak contraction and relaxed baseline states (*t* to Peak or BL 50, 70, and 90%), and relaxation trace exponential decay constants (tau).

Individual cell recordings (n = 12, 11, 8, 8, and 13 for pure seawater control, osmotic control, salt control, HF-FW 1%, and HF-FW 2% exposures, respectively) were

performed by recording a random, healthy cell from the perfusion chamber apparatus. After a small set of cardiomyocytes were transferred from the isolation solution storage vials to the perfusion chamber, cells were allowed to settle to the bottom of the chamber (5–10 min). Once settled, cells were gently perfused with control mahi extracellular saline, and a single viable and isolated cell was chosen for recording. If a cell did not yield an initial trace similar to that as shown in Figure 8-1, it was not used for analysis. To determine changes to sarcomere shortening in response to differing HF-FW exposures, a force-frequency stimulation protocol was employed, wherein cardiomyocytes in control mahi saline were first stimulated at a basal frequency at 0.5 Hz for 2 min prior to a perfusion chamber solution "switch" to either an identical control mahi saline or to another respective control (SC or osmotic control) or HF-FW treatment. This "switched" exposure treatment continued at a frequency of 0.5 Hz for 2 min and then cardiomyocytes entered a force-frequency protocol where progressively increasing stimulations (1.5, 2.0, 2.5, and 3.0 Hz) occurred for 12 contractions/frequency (see Figure 8-1). This range in frequency represents heart rates of *in situ* heart preparations of anesthetized mahi (~100–180 beats per minute) (Nelson et al., 2016). The middle six contractions during each frequency were then used for analysis (Heuer et al., 2019; Warren et al., 2010). Cardiomyocytes were returned to a basal, recovery frequency of 0.5 Hz to confirm cell viability and determine if changes to cell performance occurred over the course of the experiment. All stimulations irrespective of frequency were at a voltage of 6 mV. PAH characterization of raw, filtered HF-FW used for cell exposures can be found in Table S8-9.

8.2.6 Plasma Osmolarity and Gill Histology

Immediately following 24 h whole organism treatments, caudal blood samples were collected from fish and second gill arches were excised for histology. Details of blood sampling and histological procedures can be found in the Supporting Information (see Appendix 8-6 "Plasma Osmolarity and Gill Histology").

8.2.7 Statistical Analyses

All data were tested for normality by the use of the Shapiro–Wilk test and homogeneity of variance assessed by the use of the Levene's test (SigmaStat v11.0, Systat

Software Ind., USA) (datasets were transformed if necessary). All other statistical analyses and graphing functions were performed using Prism 6.0 (GraphPad Software Inc., USA). For cardiomyocyte exposures, each recorded individual contraction value at each frequency is presented as a percentage compared to the basal, pre-switched solution" condition at 0.5 Hz for that same individual cardiomyocyte. Values were assessed using a two-way repeated measure ANOVA, with frequency and treatment as factors. Holm-Sidak *posthoc* analyses were then performed to compare treatment effects on cardiomyocyte contractility measurements. For all other analyses (including swimming performance and respirometry, qPCR, and plasma osmolarity measurements), one-way ANOVAs with Holm-Sidak multiple comparisons were performed to determine significance between treatment conditions. All data are presented as mean ± SEM. Regardless of the statistical test employed, significance was concluded when $p < 0.05$.

8.3 RESULTS AND DISCUSSION

8.3.1 Swimming Performance and Respirometry

It was hypothesized that mahi swimming performance and oxygen uptake would be significantly impacted following FPW given that the cardiovascular system is intimately tied to aerobic performance in fish (Claireaux, 2005; Hicken et al., 2011). Mahi exposed to 2.75% HF-FW for 24 h displayed a 40% significantly reduced sustained critical swimming speed (U_{crit}) compared to all other control treatment groups (Figure 8-2A), while fish exposed to 0.75% HF-FW experienced less drastic and nonsignificant reductions of ~12%. These reductions in U_{crit} can be linked to the significant decrease in the maximum metabolic rate (MMR) of FPW-exposed fish (decreases of ~43, 37, and 35% in MMR of 2.75% HF-FW-exposed fish compared to control, salt control, and HF-FW 0.75% exposed fish, respectively) (Figure 8-2C). Given the lack of change in the standard metabolic rate (SMR) between treatment groups, these appreciable changes in the MMR of 2.75% exposed fish are directly responsible for the considerable HF-FW-induced decreases in observed aerobic scope (the difference between MMR and SMR), where the largest decline was observed between control and 2.75% HF-FW exposed mahi (~ 61% decline) (Figure 8-2D). Although a significant increase in plasma osmolarity was seen in salt control (409 mOsm), 0.75% (387.6 mOsm) and 2.75% HF-FW (420 mOsm) treated fish

(Figure S8-4) compared to control fish tested in pure seawater (347 mOsm), significant reductions in both U_{crit} and aerobic scope between our salt control and 2.75% HF-FW fish were observed. These results demonstrate that increased plasma osmotic pressure was not a factor contributing to the noted fish performance impairments.

PAH characterization of HF-FW dilutions used for whole organism exposures revealed Σ50 PAH concentrations (4.89 µg/L at the start of 2.75% HF-FW exposures; see Table 8-1 and Table S8-10) at lower levels compared to acute crude oil exposure studies on adult (8.4 µg/L) (Stieglitz et al., 2016) or juvenile mahi (30 µg/L) (Mager et al., 2014) previously shown to cause significant reductions in fish swimming performance. Furthermore, the distribution of PAH constituents in diluted HF-FW exposures differed from these previously mentioned crude oil studies. Characteristically, there is a dominance of 3–4 ring PAHs in weathered oil and high energy water accommodated fractions (HEWAFs) (Heuer et al., 2019; Hicken et al., 2011; Incardona et al., 2014; Mager et al., 2014; Stieglitz et al., 2016). Many of these 3–4 ring PAHs are cardiotoxic (Brette et al., 2017; Incardona et al., 2004). In the raw and diluted HF-FW solutions employed for the current study, however, there were comparatively higher proportions of two-ringed PAHs (Table 8-1 and Table S8-10), with decalin (a simple two-ringed PAH with two hydrogen atoms configured in either a cis or trans configuration with no well-studied cardiotoxic associations) being the highest in concentration. As expected, concentrations of PAHs were higher at the beginning of exposure periods compared to exposure termination (see Table 8-1), as volatilization of organics (particularly, smaller PAH molecules) would have occurred over the 24 h exposure period. Despite the differing PAH characterization profile compared to that of crude oil samples, mahi exposed to HF-FW yielded significantly reduced U_{crit} compared to all other control treatment groups.

8.3.2 qPCR Gene Expression Analysis

Of the five cardiac-related genes investigated, expression of only cardiac myosin light chain 2 (*cmlc2*) was found to be significantly altered in 2.75% HF-FW-exposed fish with ~9-fold higher expressions observed compared to all other treatment groups ($p < 0.05$) (Figure 8-3). Although there generally seemed to be overall decreases in expression of all other genes investigated in HF-FW-exposed fish, no statistically significant

reductions were determined. Molecular-level effects following exposure to petrogenic toxicants, such as PAHs, have been well-studied within the past couple of decades with many toxicant-induced transcriptional responses related to biotransformation pathways (including aryl hydrocarbon receptor activation, cytochrome P450 induction, and phase I and II genes) (Incardona, 2017; Köhle and Bock, 2007) and development/maintenance of certain tissues and organs (Xu et al., 2016). In addition, petrogenic contaminant impacts on molecular processes pertaining to cardiac function have also been well-characterized (Incardona, 2017; Incardona and Scholz, 2016).

Interestingly, our ventricular tissue analyses revealed significant changes in expression of *cmlc2* solely (Figure 8-3), while other cardiac-related genes previously implicated in reduced mahi performance following Deepwater Horizon HEWAF exposures (Edmunds et al., 2015) were unchanged. Furthermore, *cmlc2* is a gene that has previously displayed significantly reduced expression in crude oil HEWAF-exposed mahi larvae (Edmunds et al., 2015), while in our study, juvenile mahi exposed to HF-FW revealed significant increases in ventricular *cmlc2* expression (Figure 8-3). Although downregulation of a key developmental and structural gene, such as *cmlc2*, would naturally infer potential disruptions to contractile events of affected hearts, significant upregulation of such a gene could also signify an organism's transcriptional attempt to compensate and improve affected cardiac function. However, these discrepancies in gene expression may be caused by differing toxicant exposures, differing organismal life stages (larvae *vs* juvenile), whole-animal *versus* ventricular tissue sampling, differing exposure durations/regiments (and thus, times of gene expression analysis), or any combination of these elements. However, regardless of the direction of *cmlc2* transcriptional response, observations suggest that *cmlc2* may potentially be linked to the reduced cardiovascular function observed in mahi exposed to both crude oil and HF-FW.

8.3.3 Cardiomyocyte Contractile Properties

Given the significant reductions in swimming performance and aerobic scope of juvenile mahi exposed to HF-FW (and associated transcriptional changes in *cmcl2* expression), it was suspected that cardiomyocyte contractile properties might also be altered following HF-FW exposure. Cardiomyocyte sarcomeres exposed to 2% HF-FW

displayed reduced contraction sizes (BL % peak height) at higher stimulation frequencies ($p < 0.05$, Figure 8-4A), a result consistent with reduced U_{crit}, reduced MMR, and unchanged SMR in exposed fish. Furthermore, relaxation of contracted myocytes back to baseline uncontracted states was also affected by HF-FW exposure (Figure 8-4B–F), wherein 2% HF-FW treatment significantly reduced sarcomere relaxation velocities ($-d/dt$) by nearly 50% compared to all other treatment groups at all frequencies observed (Figure 8-4B). Correspondingly, tau values of 2% HF-FW exposed cells were almost doubled compared to all other treatments at all frequencies (Figure 8-4C) and contracted sarcomere relaxation times back to uncontracted, baseline length percentages (t to BL 50, 75, and 90%) were significantly larger at basal and 1.5 Hz frequencies in 2% HF-FW-exposed cells (Figure 8-4D–F) ($p < 0.05$). However, sarcomere contraction velocity (d/dt) and times to peak contraction percentages (t to Peak or BL 50, 70, and 90%) were not altered in similar patterns and were largely unchanged regardless of treatment (see Figure S8-5).

These effects were not deemed to be caused by increased salinity or osmotic pressure stresses to cardiomyocytes given that differences were found between the 2% HF-FW-exposed treatments and both the salt and osmotic control groups. These results instead suggest that electrochemically based repolarization processes in myocytes are being disrupted. The dominant repolarization event in an action potential is the opening of the rapid delayed rectifying potassium channels (I_{kr}) which are responsible for repolarizing cardiomyocyte membrane potentials (Nerbonne and Kass, 2005). Our sarcomere tau analyses further confirm a slowing of sarcomere relaxation, as larger tau values are indicative of slower relaxation velocities and longer lengths of time to reach baseline sarcomere distances. It is believed that HF-FW impacts to mahi sarcomere relaxation properties are also implicated in observed decreased contraction sizes—particularly, at higher stimulation frequencies—as cells would have less time to fully reach repolarized and relaxed states before contracting again. Changes in *cmlc2* transcription may, therefore, be acting in a compensatory manner to maintain cardiac function in these fish during HF-FW exposure. However, a modest reduction in sarcomere shortening was additionally observed at lower frequencies, suggesting that other cellular properties or ion conductance (such as Ca^{2+}) disturbances may be implicated. Similar to other fish-crude oil exposure

studies that have found impacts on cardiomyocyte I_{kr}, (Brette et al., 2014; Heuer et al., 2019) impacts on the functioning of these potassium channels and subsequent reduced contractive capacity may elicit specific pathologies observed in fish exposed to petroleum-derived toxicants, including reduced cardiac output (Nelson et al., 2016) and arrhythmia (Incardona et al., 2009).

Considering the concentration of total ΣPAHs in our undiluted raw, filtered HF-FW was 12.24 µg/L (Table S8-9), and that 2% dilutions of this solution were used for exposures, cardiomyocytes were exposed to an order of magnitude lower total ΣPAHs comparatively to previous studies using crude oil, which have recorded significant declines to cardiomyocyte sarcomere shortening and several cell electrogenic properties at total ΣPAHs as low as 2.8 (Heuer et al., 2019) and 4 µg/L (Brette et al., 2014), respectively. However, measured total ΣPAH constituents are still believed to overall have played a role in measured cardiomyocyte contractile responses following HF-FW exposure considering their presence and previously shown potent cardiotoxic pathologies (Heuer et al., 2019; Incardona et al., 2014, 2009; Mager et al., 2014). Additionally, it is important to note that despite the lower relative PAH concentrations in cardiomyocyte exposure solutions, HF-FW is a highly complex mixture with potentially thousands of different organic chemicals yet to be characterized and identified (Folkerts et al., 2019; He et al., 2017a). Thus, identified and yet to be identified organic compounds could either singularly contribute to contractile detriments observed or act in additive/synergistic manners with other characterized and uncharacterized compounds to elicit effects. More thorough future chemical characterization of HF-FW may eventually reveal other organic (and inorganic) chemical constituents which may be contributing to cardio-respiratory sublethal toxicities observed. PAHs have also been observed to bioaccumulate in multiple types of fish tissue (Meador et al., 1995). Therefore, whole-organism exposures (like those employed for swimming performance and respirometry) may result in fish cardiac systems being exposed to concentrations of PAHs at levels similar to those used in previous studies which have shown direct PAH effects on cardiomyocyte contraction and electrophysiology (Brette et al., 2014; Heuer et al., 2019).

Inorganic characterization of the HF-FW sample also revealed the presence of certain metals which may have additionally contributed to observed contractile changes.

Although classically considered, a Ca^{2+} charge "mimic" and thus eliciting pathophysiological effects *via* interference and competition with Ca^{2+} for associated ion transporters (An et al., 2002; Pors Nielsen, 2004; Spencer and Berlin, 1997), strontium (Sr) has been shown to directly shift I_{kr} voltage-dependent parameters and screen channel surface charges (Elinder et al., 1996). This behavior modifies and reduces I_{kr} channel conductance. At concentrations of ~7.2 mM (Table S8-11) in the raw, filtered HF-FW, Sr in dilutions used for cellular exposures may have impacted repolarization events by reducing potassium conductance across I_{kr} channels. Another metal present in our FPW solution known to inhibit K^+ channel conductance effectively is barium (Ba). Most readily, Ba has been shown to inhibit the gating properties and block pore conductance of *KCNQ1* or slow delayed rectifying potassium channels (I_{Ks}) (Gibor et al., 2004). However, Ba has also been shown to potently inhibit ventricular non-voltage-gated inward rectifying potassium channels (I_{kI}) (DiFrancesco et al., 1984; Quayle et al., 1997), a K^+ channel subtype more commonly associated with inward K^+ ion conductance but one which also contributes to outward K^+ conductance and myocardial repolarization (Isomoto and Kurachi, 1997; Noma, 1983) since myocardial membrane potentials (in mammals) never reach values more negative than K^+ reverse potentials (~−90 mV) (Nerbonne and Kass, 2005). At 0.13 mM (Table S8-11) in raw, filtered HF-FW, Ba in our 2% FPW solutions could have been at concentrations high enough to impact both I_{Ks} and I_{kI} conductance and contribute overall to prolonging action potentials and slowing membrane repolarization processes as seen in cardiomyocytes of other studies (DiFrancesco et al., 1984; Giles and Imaizumi, 1988; Kubo et al., 1993; Lopatin and Nichols, 2001). Given that metals (particularly Sr and Ba) are a consistent contaminant found in HF-FW and produced waters from UOG activities (Folkerts et al., 2020a; Y. Sun et al., 2019), further research investigating metal constituents and their potential role in affecting cardio-respiratory responses in fish following exposure should be made to more comprehensively understand HF-FW toxicity.

Considering the rise in UOG activities in the GoM, it is essential to understand how HF-FW exposure can affect wildlife of the Gulf ecosystem because of the increases in platform releases of oil and gas wastewaters into the ocean. Under the NPDES general permits GMG29000 ("The NPDES General Permit for New and Existing Sources and New

Discharges in the Offshore Subcategory of the Oil and Gas Extraction Point Source Category for the Western Portion of the Outer Continental Shelf of the Gulf of Mexico (GMG290000)," 2017) and GEG460000 ("Final National Pollutant Discharge Elimination System (NPDES) General Permit No. GEG460000 For Offshore Oil and Gas Activities in the Eastern Gulf of Mexico," 2017), operators are allowed to discharge wastewaters at a maximum rate of ~11.9 million liters/operator/day (75,000 barrels per day). Although toxicity testing (7-day chronic whole effluent toxicity tests using survivability and growth as endpoints for the species *Mysidopsis bahia* and *Menidia beryllina*) of wastewater samples is mandated, toxicity tests may only need to be performed 1–4 times per year (depending on discharge rate, previous operator compliance and violation records, *etc.*) ("The NPDES General Permit for New and Existing Sources and New Discharges in the Offshore Subcategory of the Oil and Gas Extraction Point Source Category for the Western Portion of the Outer Continental Shelf of the Gulf of Mexico (GMG290000)," 2017). Furthermore, passing/failure of specified toxicity tests center around created "critical dilutions" which encompass a wide range of whole effluent dilutions between 0.03 and 12.22% (values dependent on discharge rate, pipe diameter, and depth between the discharge pipe and the seafloor) ("The NPDES General Permit for New and Existing Sources and New Discharges in the Offshore Subcategory of the Oil and Gas Extraction Point Source Category for the Western Portion of the Outer Continental Shelf of the Gulf of Mexico (GMG290000)," 2017). This, along with other regulatory compliance measures, such as visual sheen tests, may inadequately address the toxicological hazards discharged HF-FW and produced water may have on the biota in the GoM.

8.4 CONCLUSIONS

Collectively, the current study, using an integrative approach spanning molecular, cell, and organismal physiological endpoints, identifies the cardio-respiratory systems of mahi as a target for HF-FW sublethal toxicity. Considering our swimming performance, sarcomere contraction, and ventricle qPCR results, in addition to the lack of gross morphological alterations identified in H&E stained gill tissue sections following HF-FW exposure (Figure S8-6), as well as previous studies identifying cardiac abnormalities in HF-FW exposed freshwater fish (Folkerts et al., 2017a; He et al., 2018a), it is thought that whole organism reductions in U_{crit}, and aerobic scope can largely be attributed to

deleterious impacts to the cardiac system, although complete elimination of impacts to gill or other biological inputs (including sensory systems and musculature metabolic processes) cannot be excluded. However, additional gene and cellular investigations on the toxic effect of HF-FW on mahi cardio-respiratory systems are needed to better understand and appreciate the impact of HF-FW exposure. As recently reviewed by Meador and Nahrgang, (2019), other organic contaminants besides PAHs (and other nonspecific modes of toxicity) may be playing additional causative roles in overall toxicological responses observed and thus warrant future consideration and investigation concerning HF-FW toxicological work as well. Regardless, this study is the first to examine HF-FW impacts to mahi and is one of the first published toxicological investigations regarding the potential effects of hydraulic fracturing activities on marine organisms in general. Despite using an HF-FW sample not from a GoM formation, previous work has shown shared classes of chemical compounds and elements commonly found across HF-FW and produced waters irrespective of formation exploited by UOG activities, including numerous PAHs, other organics, and metals (such as those discussed in this study) (Folkerts et al., 2020a; Luek and Gonsior, 2017; Y. Sun et al., 2019). Accordingly, it is suspected that many of the cardiotoxic chemical compounds identified and discussed in this study are also present in GoM HF-FW samples and are released into offshore Gulf waters during UOG activities. However, performing a comparative chemical characterization of a GoM HF-FW sample against inland, onshore produced HF-FW samples would conclusively corroborate this theory. Obviously, a publicly obtainable chemical characterization profile of a GoM HF-FW sample is a pressing avenue for future inquiry. Presently, our results may better inform regulators of potential hazards to the environment and biota of habitats concerning current UOG wastewater disposal and management strategies (both in the GoM and other offshore regions). As UOG activities (both on and offshore) are only expected to increase in prevalence, understanding how HF-FW affects the environment is increasingly important and will aid in the development of better management strategies.

Table 8- 1. Amounts and Proportions of PAHs in HF-FW Samples and Treatments Exposed to Cardiomyocytes or Whole Organism Juvenile *Mahi-Mahi*[a]

	Σ50 PAH (µg/L ± S.D.)	**ΣTOTAL PAH (µg/L ± S.D.)**	**ΣTOTAL 2 - Ring PAH (%)**	**ΣTOTAL 3 and 4 - Ring PAH (%)**	**ΣTOTAL ≥ 5 - Ring PAH (%)**
Cardiomyocyte Exposures					
Raw, Filtered HF-FW[b]	0.93	12.24	98.07	1.93	0.00
Swimming Respirometry Exposures					
Raw HF-FW*	141.97	1508.10	93.84	6.04	0.12
0.75% HF-FW Start[c]	1.29 ± 0.20	12.97 ± 1.0	94.11	5.82	0.070
0.75% HF-FW End[c]	0.11 ± 0.027	3.51 ± 0.18	98.88	1.04	0.21
2.75% HF-FW Start[c]	4.89 ± 1.08	45.66 ± 6.39	92.95	6.92	0.15
2.75% HF-FW End[c]	0.70 ± 0.45	20.03 ± 5.05	97.28	2.43	0.26

[a]Values are represented as means ± one standard deviation (S.D.) where applicable.

[b]No associated standard deviation because only one measurement was taken.

[c]Sample size of four.

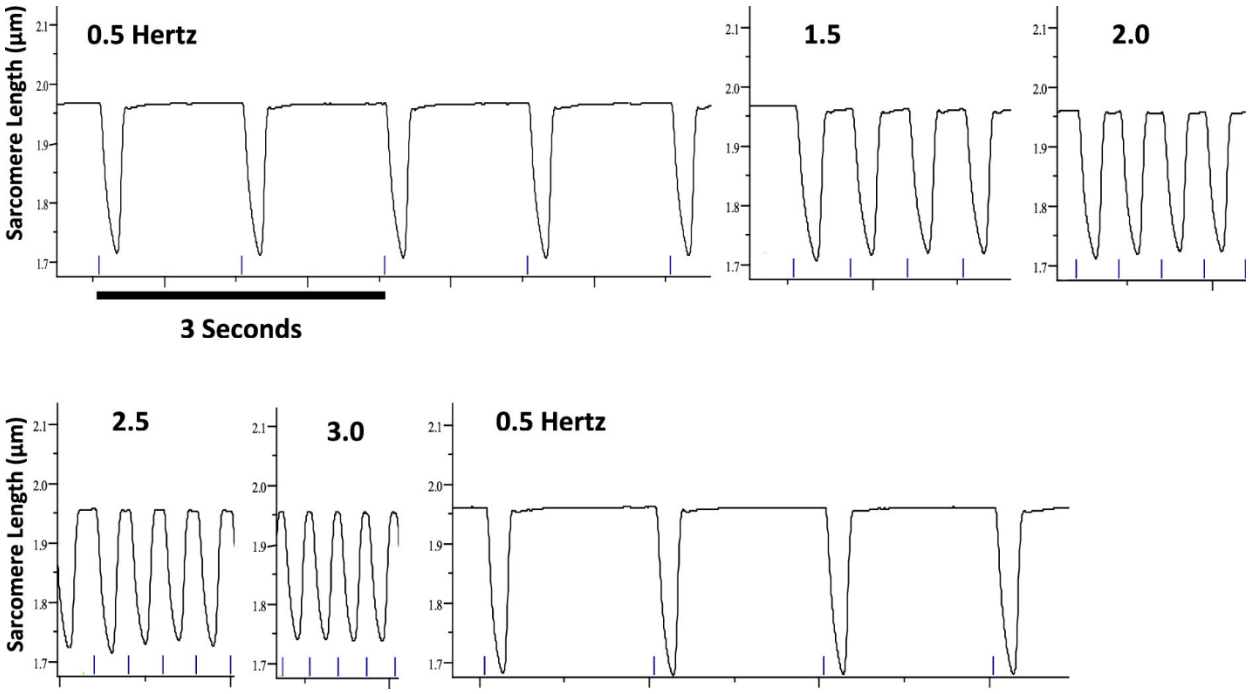


Figure 8- 1. Representative trace of a control, isolated cardiomyocyte going through a force-frequency (FF) stimulation procedure. Only 4 contraction events at each frequency are shown (12 total contractions per frequency were performed with the middle 6 contractions used for analyses). Data were analyzed by comparing the respective treated cell contractile indices at each frequency of stimulation to basal, pre-exposed contractile indices of the same cell. Cells which did not complete the entire FF procedure were not included in analyses.

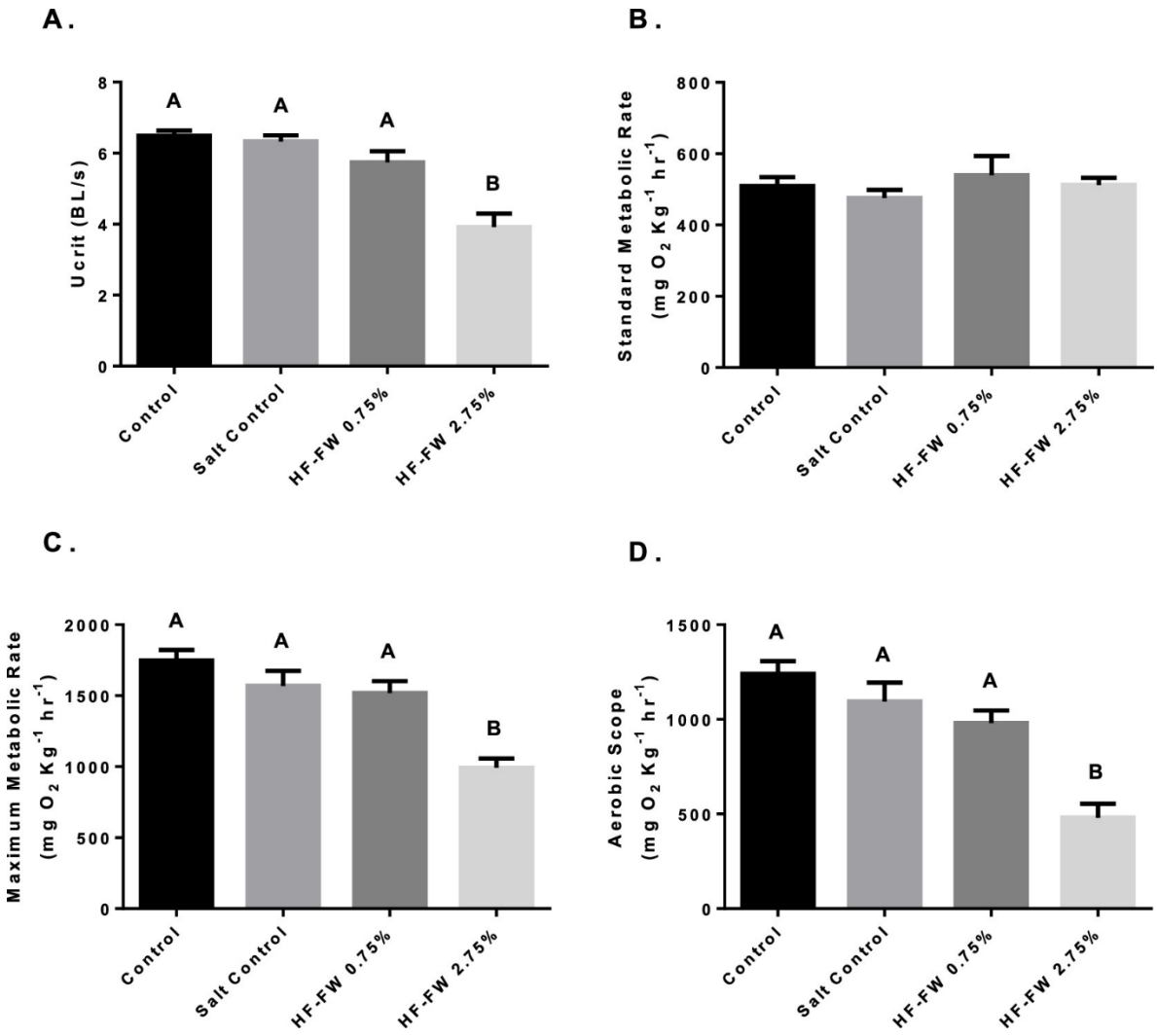


Figure 8- 2. Evaluation of (A) critical swimming speed (U_{crit}), (B) standard metabolic rate (SMR), (C) MMR, and (D) aerobic scope swimming respirometry experiments from juvenile *mahi-mahi* exposed to control, salt control, HF-FW 0.75%, or HF-FW 2.75% treatments (n = 14, 12, 11, and 10, respectively) for 24 h prior to swimming. SMR and MMR data were normalized for body by scaling to the respective SMR and MMR values predicted for a 20 g fish (aerobic scope = normalized MMR–normalized SMR). Letters denote statistical differences $p < 0.05$ (measured via a Holm-Sidak posthoc analysis). Data are presented as means ± SEM.

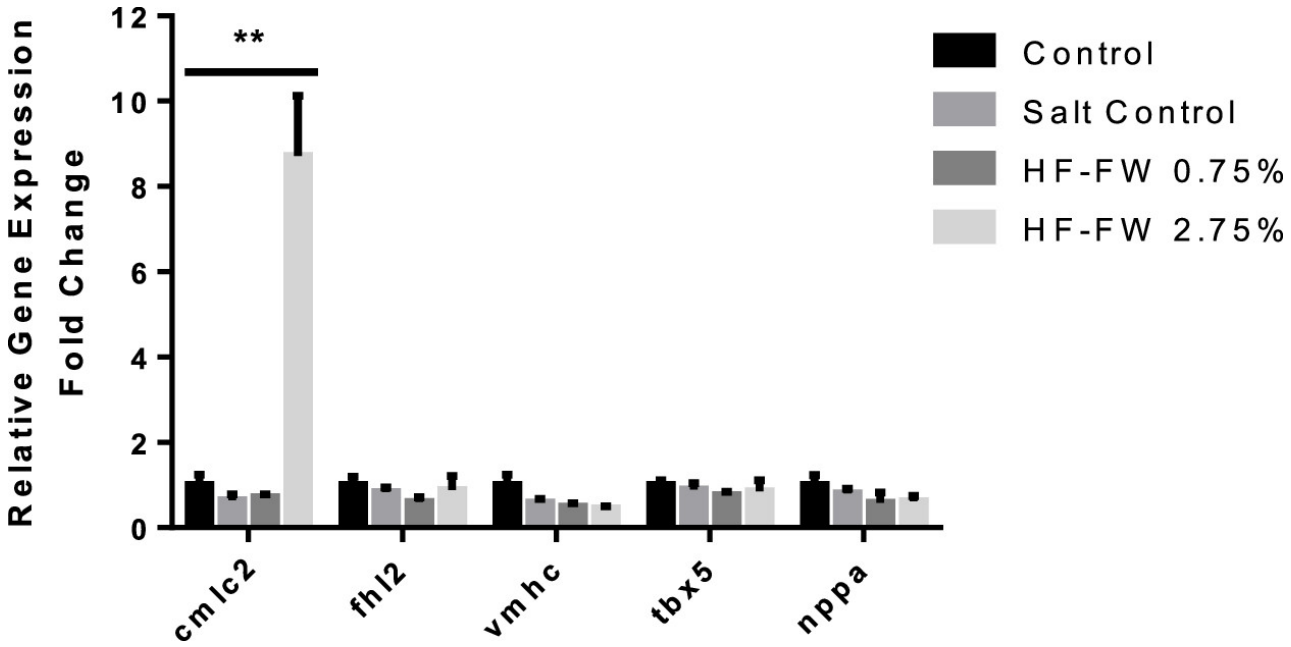


Figure 8- 3. Relative mRNA abundance of cardiac target genes cardiac myosin light chain (*cmlc2*), four and a half LIM domain 2 (*fhl2*), ventricular myosin heavy chain (*vmhc*), T-box transcription factor 5 (*tbx5*), and natriuretic peptide a (*nppa*) in ventricle tissue from juvenile *mahi-mahi* exposed to 24 h control, salt control, 0.75% HF-FW, or 2.75% HF-FW (n = 4–6 for all treatments). Transcript abundance was determined by quantitative real-time PCR using the ΔΔCT method. Data are presented as mean ± SEM. Holm-Sidak posthoc tests were performed to determine significant changes ($p < 0.05$) of transcript abundances in mahi-mahi ventricle tissue.

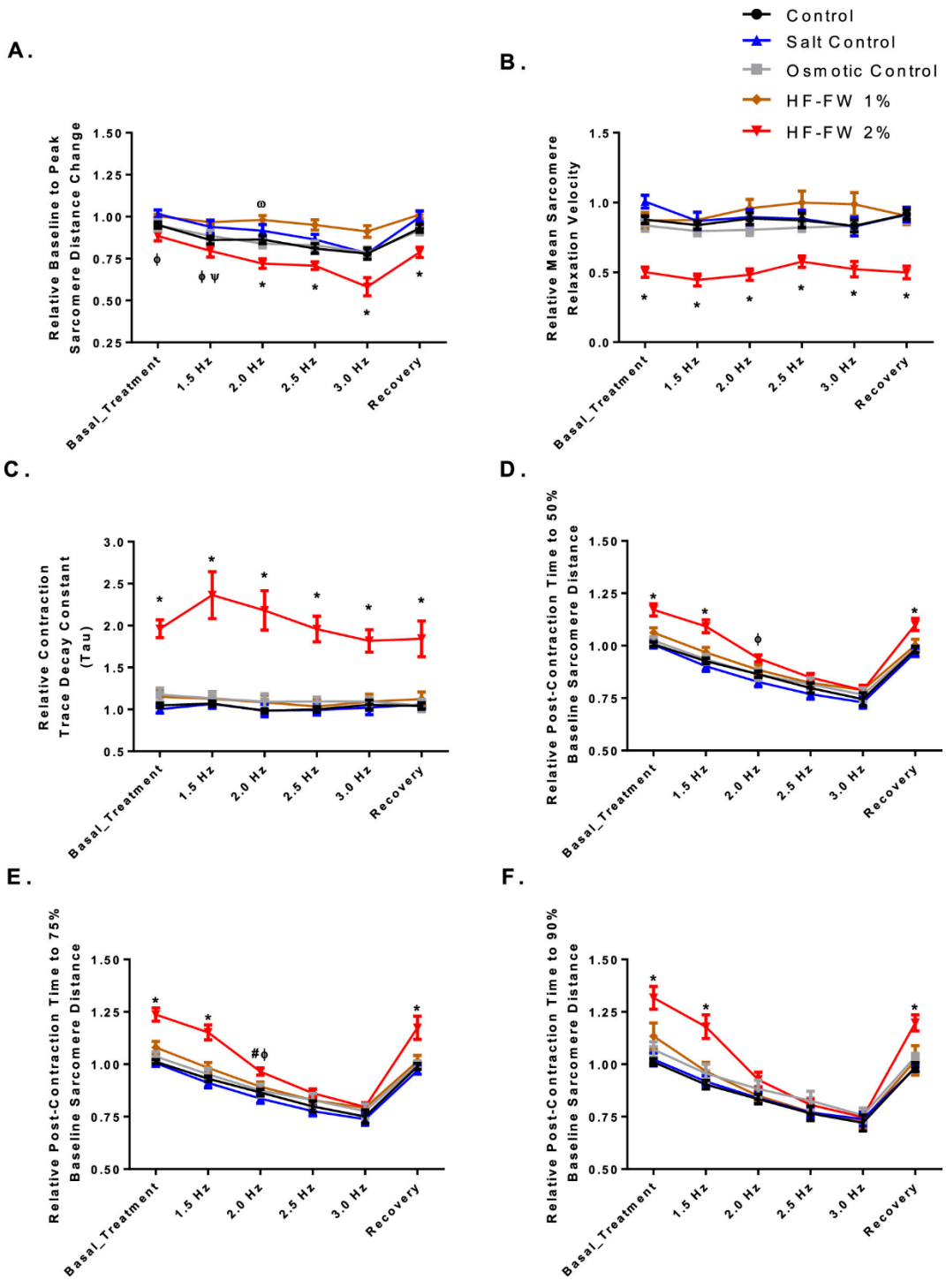


Figure 8- 4. Effects on mahi-mahi cardiomyocyte sarcomere contractility properties following HF-FW exposure. Data points represent contractile indices at specified

frequencies and treatments compared to basal frequencies (0.5 Hz) of stimulation under control conditions for the same cell prior to solution exposure switches. (A) Relative size of contraction events measured as the distance between sarcomeres at baseline, uncontracted states to distances between sarcomeres at peak contraction states. (B) Relative speed of sarcomere relaxation from peak-contracted states to relaxed baseline states. (C) Relative sarcomere relaxation trace decay constants (tau) from peak-contracted states to relaxed baseline states. (D–F) Relative times for sarcomeres to go from peak-contracted states to 50, 75, and 95% baseline-relaxed states, respectively. * indicates significant differences between HF-FW 2% exposures and all other treatment groups. # indicates significant differences between HF-FW 2% and control treatment groups only. Φ indicates significant differences between HF-FW 2% and salt control (SC) treatment groups only. ψ indicates significant differences between HF-FW 2% and HF-FW 1% treatment groups only. ω indicates significant differences between HF-FW 1% and osmotic control treatment groups only. Significant differences were determined following a Holm-Sidak posthoc analysis ($p < 0.05$). Data are presented as means ± SEM (n = 12, 11, 8, 8, and 13 cells for control, osmotic control, salt control, HF-FW 1%, and HF-FW 2% treatments, respectively).

CHAPTER 9: Conclusions and Perspectives

My doctoral thesis embodies work aimed at characterizing mechanisms underlying FPW toxicity in a range of aquatic organisms. While general toxicity was first investigated to understand the sensitivities and extent of FPW toxic potential (Chapter 2), experimental focus eventually shifted to examining more classically defined biomarkers and physiological systems tied to anthropogenic disturbances (e.g. biotransformative processes, oxidative stressors, and organismal energy dynamics) (Chapters 3 and 4). However, ultimately my main experimental motivations settled on characterizing cardio-respiratory responses in fish following both embryonic and juvenile FPW exposures (Chapters 5 – 8). The current chapter serves as a general summary wherein I recap major findings of the previous chapters, draw and make inferences on how findings may be connected, and suggest potential future streams of research relating to FPW toxicological study.

9.1 GENERL FPW TOXICITY

Understanding the chemical composition of a mixture can often give clues as to how toxic the solution will be and which physiological systems will be affected in exposed organisms. Earlier studies revealed that solutions coming up from the well-bore of a hydraulically fractured well are highly saline in nature (Lester et al., 2015; Ziemkiewicz et al., 2014), and therefore, osmotic stresses were immediately suspected to be of primary toxicological concern in my experiments using freshwater organisms. Indeed, preliminary characterizations and first studies to come from our group confirmed that FPW samples from the Duvernay and Montney shale formations located in central-west Alberta and north-west Alberta, respectively, were highly saline and became more saline (eventually plateauing in salinity) as the well continued to gnerate produced water, plus oil and gas in varying amounts (He et al., 2017a). These characteristics were similarly observed in the study comprising Chapter 2 (Folkerts et al., 2019). Although saline control solutions (matching the major cationic and anion elements of the most saline FPW sample tested) generally held lowest toxicity amongst the species tested, appreciably low dilutions of the saline control solutions still induced toxicity in all organisms, with LC_{50} values of 2.81% and 2.11% dilutions recorded for *Daphnia* and zebrafish embryos, respectively (Table 2-3) (Folkerts et al., 2019). Similarly low dilutions of FPW-matching saline controls causing toxicity have been observed in other studies using *Daphnia* neonates (T. A. Blewett et al., 2017a). Other effects associated to the high saline content of FPW have been observed in

exposed juvenile rainbow trout, wherein one of the main responses is drastic gill morphological remodelling in the form of significantly reduced interlamellar cell mass (T. A. Blewett et al., 2017b; Delompré et al., 2019). This may just be a temporary response, however, as rainbow trout exposed to saline waters have been shown to compensate and return gill morphology to normal modelling even after exposure to 96 hr of 50% strength seawater (Blair et al., 2016). However, other less saline-tolerant fish which inhabit ecosystems affected by FPW activity (e.g. Arctic grayling; *Thymallus arcticus*) may be unable to tolerate such osmotic stresses during FPW spill/leak scenarios, as evidenced by a lack of gill morphological acclimatory and ionoregulatory transcriptional responses (Blair et al., 2016).

However, results from Chapter 2 also indicate that other FPW components aside from salinity are responsible for inducing further toxicity in exposed organisms. While toxicological responses to the series of temporally differing FPW samples were mixed and species dependent, a prevailing theme was that for the majority of the species, FPW samples - regardless of timepoint - were significantly more toxic than the control saline solution. Chemical characterization of the 3 temporally differing FPW samples performed in Chapter 2 (and from characterizations made in other earlier studies; He et al., 2017a; Lauer et al., 2016; Rosenblum et al., 2017b, 2017a), it is evident that organics and other inorganic species (e.g. metals) are major constituent classes which comprise FPW, and which all inevitably contribute to overall toxicity and induce specific sub-lethal responses in exposed organisms.

9.3 SPECIFIC FPW-INDUCED SUB-LETHAL RESPONSES

Given the considerable chemical complexity of FPW, and how operational variables and formation locations can influence the chemical make-up of the solutions, numerous specific physiological processes are theorized to be affected by FPW exposure in organisms. This creates a scenario where many sub-lethal toxicological endpoints could and are relevant for study when investigating sub-lethal FPW toxicity. In Chapters 3 and 4, the endpoints chosen for study were xenobiotic biotransformative processes, oxidative stress responses, nutrient handling dynamics, and to a lesser extent, potential endocrine disruption events (He et al., 2017b; Weinrauch et al., 2021).

My results demonstrate that liver tissue excised from rainbow trout acutely exposed to FPW display significant increases in both *cyp1a* and EROD transcript expression and enzyme activity levels, respectively. Excised gill filaments also displayed significantly increased EROD activity. Induction of *cyp1a*, a gene which plays major phase I xenobiotic metabolism roles (Incardona, 2017; Incardona et al., 2006), and EROD activity, a biomarker for CYP1a induction (Sarkar et al., 2006; Whyte et al., 2000), are heavily mediated through AhR agonistic binding events from certain organic chemical classes – one important class being PAHs. The strong responses observed in hepatic tissue from trout exposed to FPW suggest that despite the relatively lower concentrations of PAHs compared to other industrial/anthropogenic waste exposures (e.g. crude oil spills), PAH-induced biotransformative processes and toxicities are still experienced to a high degree in FPW exposed fish. These results corroborate other FPW studies finding increased *cyp1a* expression and EROD activity in other tissues and organisms (T. A. Blewett et al., 2017b; He et al., 2018a, 2017a). Evidence of increased phase II gene expression (*udpgt* and *gst*) further suggests that FPW exposed fish undergo a xenobiotic metabolism stress following FPW exposure. These results are important as the presence of PAHs, and induced biotransformative pathways, may generate further sub-lethal toxicological responses and affect other physiological processes.

PAHs (and subsequent metabolic pathways creating substrate intermediates) have been shown to increase ROSs and contribute to oxidative stress (Dalton et al., 2002; Zangar, 2004). In natural settings, potential inductive oxidative damage associated with FPW exposures may additionally be heightened in the presence of UV light (photoactivation of PAHs and other compounds) (Lampi et al., 2006; Spehar et al., 1999; Yu, 2002). Indeed, it was shown that FPW solutions intrinsically hold an inherent ROS potential (Chapter 3). In trout liver samples, acute FPW exposure increased transcriptional expression of key genes which combat physiological oxidative stress (*sod* and *gpx*), while increased TBARS formation (a bioindicator of lipid peroxidation) was observed in liver, gill, and kidney tissue. It is acknowledged that other contaminants in FPW presumably also contribute to ROS production and oxidative stress in exposed trout (including metals species such as Fe, Sr, and Ba). However, it is evident that overall, oxidative stress is a sub-lethal form of toxicity experienced by exposed organisms. This, along with xenobiotic

metabolism stresses, may increase energetic demands in exposed organisms, and therefore alter energy homeostatic conditions.

I also demonstrate that hepatic nutrient handling and energy homeostatic processes are altered in trout acutely exposed to sub-lethal dilutions of FPW. While osmotic and xenobiotic biotransformation stresses were observed, along with gross hepatic histological alterations and significantly increased glucose uptake in isolated hepatocytes, overall plasma glucose levels were unaffected in fish following FPW exposure. Furthermore, transcriptional expression and activity of key genes and enzymes, respectively, related to hepatic metabolic pathways (e.g. glycolysis, gluconeogenesis, aerobic metabolism, and amino acid catabolism) displayed varied responses without clear connections to an overall energetic response. However, certain altered enzymatic activities (namely PEPCK and GDH), along with increased glucose uptake in isolated hepatocytes, indicates that an immediate shift in nutrient handling processes does occur during/following FPW exposure, where energy homeostatic conditions may have become altered and dysregulated. However, following 3-weeks of recovery, all indices and metrics of energy homeostatic dynamics returned to control levels, indicating that nutrient handling and organismal energetics are processes acutely affected by FPW exposure without lasting and persistent effects, provided that adequate times for recovery are presented.

Although not as heavily investigated in my thesis experiments, FPW exposure is also associated with endocrine disruption in rainbow trout. After acute FPW exposure, excised trout liver tissue displayed elevated expression of vitellogenin (*vtg*) and estrogen receptor 2-α (*era2*), indicating that impacts to the endocrine system occur due to FPW exposure. Further work on this topic in other studies has identified additional expression changes in other key genes tied to endocrine mediated processes (e.g. aromatase, androgen receptor, and estrogen receptor 2-β) following FPW-extract exposures in zebrafish embryos (He et al., 2018a), while cell-line reporter assays have identified certain organic FPW fractions to directly stimulate estrogenic and anti/pro-androgenic responses (He et al., 2018b). Thus, the endocrine system and related processes appear to be another sub-lethal form of toxicity experienced by FPW-exposed organisms.

9.4 FPW-INDUCED CARDIO-RESPIRATORY RESPONSES

9.4.1 Embryonic Development and Cardiac Structure

In the latter parts of my thesis, my investigative focus and experimental efforts shifted to examination and characterization of the impacts of FPW exposure on the development and function of cardio-respiratory systems in fish, with additional interest in how these impacts may alter critical performance metrics such as swimming performance and aerobic capacity. Although fish have been shown to be great cardiotoxicological models at all stages of development, the sedentary nature, complex developmental processes, and limited metabolic and excretory ability (Incardona, 2017; Jung et al., 2015) of the embryonic stage renders the fish embryo as a highly desirable developmental model for investigation of cardio-respiratory toxicity in fish. In my thesis, FPW exposure is observed to increase, among other developmental deformities, rates of pericardial edema in two different freshwater fish species (zebrafish and rainbow trout) (Folkerts et al., 2017a). Furthermore, zebrafish embryos display reduced heart rates following acute FPW exposures. Given the key role circulation is believed to have in early development osmoregulation (Cherr et al., 2017; Incardona, 2017; Incardona and Scholz, 2016), and the fact many cardiotoxic contaminants (e.g PAHs) have been shown to exist in FPW, it is perhaps not surprising that edema is a readily observable developmental deformity in fish embryos exposed to FPW. However, what was more surprising was the occurrence (and degree) of decreased compact myocardium thickness in juvenile rainbow trout ventricles ~ 8 months post-embryonic FPW exposure. Despite previous studies on embryonic crude oil or PAH exposures also showing differential effects on heart morphological metrics in later-stage fish (Brown et al., 2017; Incardona et al., 2015; Lee, 2003), the comparatively low dilutions used for embryonic FPW exposures in this thesis highlight the sensitivity of embryonic fish and the high toxicological potential of FPW for inducing developmental deformities and altering heart morphology in fish at later developmental timepoints.

9.4.2 Cardiac Gene Expression

When investigating specific genes in freshwater fish embryos responsible for cardiac development, electrochemical processes, and structural elements, it became

apparent that certain cardiac physiological processes were being affected and were reflected in the transcriptional responses mounted in exposed organisms. In both zebrafish and rainbow trout embryos following exposure, the gene *nkx2.5*, a critical homeobox transcription factor required for proper cardiogenic differentiation in both invertebrates and vertebrates (Akazawa and Komuro, 2005; Bartlett et al., 2010), which has also been suggested to have a major role in developing early specialized electrosensitive cells of rudimentary E-C coupling mechanisms (Thomas et al., 2001), displayed significantly elevated expression levels. Although not examined in zebrafish embryos, another key cardiac tissue development and differentiation gene, T-box transcription factor *tbx2b*, was also significantly elevated in rainbow trout embryos during chronic exposures to FPW. Interestingly, *tbx2b* is suggested to be involved in regulating activity of atrial natriuretic peptide, *anp*; a peptide hormone secreted from cardiac atrial cells to regulate atrial stretching and extracellular fluid volume (Auman et al., 2007). Correspondingly, *anp* was also observed to have significantly elevated expression in rainbow trout embryos following FPW exposure.

Curiously, *atp2a2a*, a gene encoding for the main cardiac Ca^{2+} ATPase responsible for sequestering Ca back into the SR following an E-C coupling event, was observed to significantly decrease in expression in zebrafish embryos following FPW exposure but significantly increase in expression in rainbow trout embryos. However, these differential responses may be explained by the differing developmental timepoints when embryos were sampled, differential species responses, or differences in chemical make-up of the FPW solutions used in each respective study. Regardless, E-C Ca cycling during an action potential appears to be a electrochemical process affected following FPW exposure, a result in agreement with other electrophysiological studies examining impacts of crude oil or PAH exposures in isolated cardiomyocytes (Brette et al., 2017, 2014; Vehniäinen et al., 2019). Other electrochemically related genes affected by FPW exposure in embryonic rainbow trout were *scn5lab* (coding for a subunit of the main Na channel responsible for invoking cellular depolarization during an action potential), *kcnh6* (coding for the subunit of the main delayed rectifying potassium channel responsible for repolarizing membrane potentials during an action potential), and *kir2.1* (coding for a subunit of an inwardly-rectifying potassium channel crucial for setting and maintaining resting membrane

potential) (Vornanen and Hassinen, 2016). Again, these gene expression results agree with electrophysiological observations of previous studies exposing isolated fish cardiomyocytes to crude oil fractions or differing PAHs (Brette et al., 2017; Heuer et al., 2019; Vehniäinen et al., 2019).

Examination of genes specifically related to structural and functional indices of developing hearts in embryonic fish showed varied gene transcriptional expression in zebrafish embryos, with the only structural gene displaying altered expression being *tnnt2a* (cardiac muscle troponin T 2a), a filament regulatory complex conferring Ca-sensitivity to myocytes deemed essential for myosin-actin activity, sarcomere assembly, and cardiomyocyte contractility (Kaplan and Hamroun, 2013; Nesan and Vijayan, 2012; Sehnert et al., 2002). However, decreased expression patterns of *tnnt2a* in zebrafish was determined to be associated with the saline osmotic challenges of FPW and not to other chemical components of the solution. In rainbow trout embryos, the primary structural gene observed to change expression following FPW exposure was *vmhc*, ventricular myosin heavy chain. Interestingly, in acutely FPW-exposed *mahi mahi*, ventricle tissue displayed significantly increased expression of *cmlc2*, another gene crucial for cardiac development and function in organisms (Chapter 8). These genes indicate, although to lesser degrees, that structural and functional genes of the developing fish heart may also be impacted and responding to changes induced by FPW exposure.

9.4.3 Embryonic Respiration/Metabolism

In my thesis experiments, I also examined whole embryo/larvae metabolic responses immediately post-FPW exposure and throughout continued development in freshwater following initial FPW exposures. Upon finding increased rates of cardiac deformities, reduced heart rates, and altered expression patterns of numerous genes tied to cardiac health, function, and development, it was theorized that FPW would significantly reduce oxygen consumption in developing embryonic fish. Zebrafish embryos exposed to higher initial FPW concentrations for longer periods of time significantly reduced their metabolic rates to greatest degress at all timepoints measured during development. Rainbow trout embryos exposed to FPW at 3 dpf, however, conversely displayed significantly elevated metabolic rates immediately following exposure and through

development until 15 dpf. At this timepoint, rates of O_2 consumption seemed to become equal before a 'switch' occurred, wherein embryos exposed to FPW then displayed significantly reduced metabolic rates at timepoints past 15 dpf, a trend similar to that observed in my previous zebrafish analyses. Chronically FPW exposed rainbow trout embryos also followed very similar trends to those seen in 3 dpf exposed embryos.

It is suspected that the relative speed of development between zebrafish and rainbow trout (and therefore timing of developmental metabolic analyses) is the key variable which dictated differential responses observed between the two studies analyzing embryos of different species of fish and their metabolic responses to FPW exposure. Furthermore, as other metabolic analyses performed on embryos of marine fish species have shown, the occurrence of increased O_2 consumption following other related contaminant exposures (primarily crude oil) has been previously observed (Eldridge et al., 1977; Pasparakis et al., 2017, 2016). Increased embryonic metabolic rates following FPW exposure is therefore theorized to be in response to costs associated with induced energy intensive metabolizing processes for chemical components of FPW, including CYPs, ATP-binding cassette transporters, metallothionein induction, and homeostatic management of ROS (Chavan and Krishnamurthy, 2012; Fabrik et al., 2009; Gourley and Kennedy, 2009; He et al., 2018a, 2017a; van der Oost et al., 2003). At timepoints past 15 dpf, however, rainbow trout embryos are approximately at the same developmental stage as the zebrafish embryos from Chapter 5, where it is believed that earlier FPW impacts on cardiac development may begin manifesting and deleteriously impacting cardiac-related contributions to overall embryonic/larval respiratory and metabolic processes.

9.4.4 Swimming Performance and Aerobic Capacity

The functioning and performance of fish cardio-respiratory systems are metrics intimately tied to aerobic capacity and execution of activities which require increased cardiac output and systemic O_2 delivery (Claireaux, 2005; Farrell et al., 2009; Steffensen and Farrell, 1998). Regarding FPW, many chemical species constituting the solution are known to alter fish swimming performance and respirometry, including PAHs (Johansen and Esbaugh, 2017; Lucas et al., 2016; Oliveira et al., 2012; Stieglitz et al., 2016) and metals (Mager and Grosell, 2011; Rajotte and Couture, 2002). Thus, after acutely exposing

juvenile *mahi mahi*, a marine pelagic fish which has a highly aerobic lifestyle dependent on fast swimming speeds and considerable cardiac output, it was not surprising that FPW exposure decreased swimming performance (U_{crit}) in selected fish, with corresponding decreases in MMR and aerobic scope (Chapter 8) (Folkerts et al., 2020b). However, what was more surprising was the severity of decreases to these indices in *mahi mahi* and the relatively low dilutions of FPW which caused them (2.75%).

To compliment this acute FPW exposure study in *mahi mahi,* I investigated swimming performance and respirometry in juvenile zebrafish and rainbow trout which were previously exposed to FPW dilutions during embryonic stages in an effort to determine if cardio-respiratory detriments experienced during development persisted to affect later-stage fish performance and fitness. A limited number of other studies have investigated latent effects of chemical and waste solution embryonic exposures on later-stage fish swimming performance (Brown et al., 2017; Mager et al., 2014; Marit and Weber, 2012). Juvenile zebrafish and rainbow trout that were embryonically exposed for only 48 h to dilute FPW displayed significantly reduced U_{crit}. However, while other studies investigating persistent effects of embryonic chemical and waste solution exposures on fish swimming performance and respirometry have had mixed results concerning swimming O_2 consumption and aerobic scope, juvenile zebrafish and rainbow trout previously exposed as embryos to FPW matched trends observed in *mahi mahi* exposures (Chapter 8) and displayed significantly reduced MMR and aerobic scope. Collectively, I show that swimming performance and respirometry are excellent fitness metrics that are highly susceptible to change during FPW exposures in fish. Futhermore, these studies suggested potential cardio-respirometry alterations experienced during embryonic stages as a result of FPW exposures persist and latently affect the ability of a fish to swim and effectively mobilize systemic O_2 during aerobic activity.

9.4.5 Cardiomyocyte Contractile Properties

Having investigated tissue and whole organism level responses in my thesis research undertakings, I aimed to have my thesis serve as an endeavor in concentrating focus into exploring cellular events of the heart to gain a more rounded and whole picture view of FPW-induced cardio-respiratory sub-lethal toxicity in fish. Isolated *mahi mahi*

cardiomyocytes exposed to filtered 2% FPW dilutions exhibited numerous altered changes to traces of sarcomere contractile properties, including reduced contraction sizes and inhibited sarcomere relaxation metrics (Folkerts et al., 2020b). These affected properties in turn also led to observable increases in returned timing to relaxed, baseline sarcomere distances from peak contracted states at stimulation frequencies up to 2 Hz. Furthermore, these cellular responses were determined to be separate of saline and osmotic influence, implying that chemicals in the filtered FPW sample - namely organics such as PAHs or inorganics such as metals – are driving changes in cardiomyocyte contraction physiological processes. Correspondingly, filtered FPW characterization identified numerous 2 and 3-ring PAHs and metal species (e.g. Sr and Ba) which have been previously shown to impact electrochemical properties and cellular ion transport mechanisms in isolated myocytes of other experimental species (Brette et al., 2017; DiFrancesco et al., 1984; Elinder et al., 1996; Gibor et al., 2004; Vehniäinen et al., 2019). Taken together, impacts at the cellular level following FPW exposure agree with tissue and whole organism fitness effects previously observed. While behaviours such as swimming performance in fish are governed by numerous complex sensory and physiological systems which are intimately connected and intertwined with each other, my results indicate that electrochemical processes impacted by FPW exposure at the level of the cell may contribute significantly to limiting cardiac function and output, and thereby reduce aerobic capacity and swimming ability in fish.

9.4.6 Developmental Timing of Exposures and Differential Effects

Among the many investigative purposes the study served as, Chapter 7 also examined if FPW exposures at timepoints earlier or later in the primary embryonic cardiac developmental "window" (20 – 120 ATUs at 10 °C) (Knight, 1963; Vernier, 1969) differentially affected cardio-respiratory parameters in developing and juvenile rainbow trout. By employing two temporally different exposure regimes wherein embryos were exposed to differing dilutions of FPW for 48 h at 3dpf or 10 dpf, I was able to determine that embryos exposed at 3 dpf generally had increased rates of developmental deformities and significantly altered embryonic metabolism over their 10 dpf exposed counterparts. Interestingly, gene transcription analyses revealed differential expression patterns of many

key cardiac-focused genes between embryos acutely exposed to FPW at 3 dpf versus those exposed at 10 dpf. In general, 3 dpf exposed embryos displayed a lower number of significantly altered gene expressions (only *atp2a2a*), while 10 dpf FPW exposures resulted in many significantly changed cardiac-related gene expression levels. Latent affect analyses revealed that 3 dpf embryonic exposures resulted in juvenile fish with reduced U_{crit} and aerobic scope at all FPW dilutions, while juvenile fish exposed at 10 dpf to 2.5% FPW did not show such reductions in these endpoints. Collectively, these results suggest that FPW exposures earlier within the cardiac developmental window evoke more nocuous impacts to developing rainbow trout embryos which then have lasting, more detrimental impacts on juvenile fish swimming and aerobic capacity parameters.

9.5 FUTURE DIRECTIONS

Technological advancements in the oil and gas sectors have led to companies being able to exploit formations that were previously thought to be of limited potential for hydrocarbon extraction. The rapid rise of hydraulic fracturing methodologies, and variations of these UOG methods for collecting resources from geological formations (such as horizontal hydraulic fracturing), has resulted in an expansion of reserve development. However, it has also increased concern about risks to the environment. These concerns include water sourcing availability, increased seismic activity hazards, wastewater storage and disposal conundrums, and ground aquifer and surface water contamination events. While the body of work of my thesis provides novel information on the potential toxicological hazards associated with spills of FPW to surface waters and impacts on aquatic organisms, with a greater focus on cardio-respiratory implications in fish, there are still many unknowns and potential avenues of future research which would greatly aid in our understanding of toxicological hazards posed to the biota of affected ecosystems. Presented below are some key areas that future studies should address.

9.5.1 Greater FPW Characterization

Understanding the chemical constituents present when investigating the toxicity of a solution is paramount for any toxicological endeavor. Without such information, hazard identifications and risk assessment analyses are severely limited in their scope and breadth. To date, many insights into the inorganic and organic chemical composition of FPW have

been made. Inorganic analyses have identified the highly saline nature of FPW owing to large salt-related ion concentrations present, the existence of different metal species (of which Sr and Ba have been identified as metals at high enough concentrations for toxicological concern), and how high FPW concentrations of $Fe_{(II)}$ may oxidize to form additional $Fe_{(III)}$ oxyhydroxides and serve as a potential treatment technique for removing organic contaminants from FPW via aeration since organic compounds are observed to sorb unto solids present in FPW (Flynn et al., 2019; Folkerts et al., 2019; He et al., 2017a, 2017b). Organic characterizations have revealed FPW to contain many similar PAHs to those also found in other waste exposure solutions, including crude oil. Furthermore, untargeted Orbitrap HPLC-MS in both positive and negative ionization modes has identified numerous possible compounds related to polyethylene glycols, quaternary ammonium compounds, amine oxides, organophosphorus compounds (biocides), phthalate diesters, hydroxyquinoline, and series of alkyl ethoxylate carboxylate surfactants which are suspected to be proprietary additives (Folkerts et al., 2019; C. Sun et al., 2019). However, given the extreme chemical complexity and variability associated with FPW, many analytical pitfalls still exist. In particular, organic chemical profiling of FPW is still in a fairly untargeted and non-quantified state, with many exact organic chemical species still unknown and at unknown concentrations. Furthermore, PAH analyses have been generally restricted to a select number of commonly measured species and their alkylated forms. More thorough and complete PAH analyses (as made in Chapter 8; Folkerts et al., 2020) would greatly improve understanding of the potential PAH risks associated with FPW exposure to organisms inhabiting surface water environments.

9.5.2 FPW Toxicity Identification Evaluation (TIE) Studies

Whole effluent toxicity (WET) studies are a necessary and valuable tool for evaluating toxicological concerns associated with novel wastewaters or toxic solutions and are what have been primarily employed in most FPW toxicological studies to date. However, as different components of FPW chemical characterization become more known, it follows that complex solutions should begin to be separated and studied in a more deconstructed manner to further tease apart specific fraction or chemical influences towards mediating toxicity. It has been observed that following activated charcoal

treatment and filtration of FPW (therefore removing much of the PAHs, and in theory, metals from solution) (He et al., 2017b), many sub-lethal toxicological responses are either dampened or not seen in exposed organisms (T. A. Blewett et al., 2017a; Folkerts et al., 2017b, 2017a; He et al., 2017b). Furthermore, exposures of the organic fraction of FPW (created via solid phase extraction methods) have determined the developmental toxicity and androgenic/estrogenic responses associated with compounds contained within this fraction (He et al., 2018a, 2018b). However, such organic fraction exposure studies previously performed employed concentrated amounts of the FPW organic fraction, and therefore the relationship between laboratory and expected results in natural settings is less clear. Furthermore, attention to FPW trace metal toxicity has been virtually non-existent. TIE studies which focus on different FPW fractions (e.g. solids and organics) and trace metal components would significantly improve understanding of overall toxicological hazards associated with FPW exposures.

9.5.3 Latent Effects of FPW Exposure on Cardiomyocyte Electrophysiological Properties

To date, studies on contaminant impacts to cardiomyocyte electrophysiology have been generally performed by exposing respective cells to contaminants directly (Brette et al., 2017, 2014; Heuer et al., 2019; Vehniäinen et al., 2019). However, surmising chemicals and concentrations in a recording chamber which accurately reflect internal physiological conditions in an organism being exposed can be difficult and likely not possible. Furthermore, by isolating cells from one another and performing individual exposures, the relevancy of what's happening in the recording chamber is one step further removed from how that cell might actually behave in the organism when its being exposed to the specified contaminant. This experimental setup can also only investigate immediate impacts of contaminants on cellular physiology, which while valuable, fail to capture how cardiomyocytes may be affected following exposure. Thus, while direct exposure studies should be performed to determine if similar impacts to action potential and ion (e.g. Ca, K, and Na) current densities and amplitudes are observed following FPW exposure as seen in other contaminant exposure studies (Brette et al., 2017; Heuer et al., 2019; Vehniäinen et al., 2019), these same electrophysiological properties could be recorded in cells in normal

physiological solutions in the recording chamber which were isolated from organisms which have been previously exposed to FPW. By doing so, one could determine if and the degree to which cardiac cells are impacted from organismal FPW exposures and if any latent effects are observed. Studies of this manner would also allow researchers the ability to gain an understanding as to how long affects from organismal FPW exposures persist to affect cardiomyocyte electrophysiological properties.

9.5.4 Other Excitable Cell FPW Exposure Studies

A pathophysiological condition commonly observed in fish exposed to crude oil and other related petrogenic contaminants is severely affected eye development and detriments to other sensory capabilities (Magnuson et al., 2020; Philibert et al., 2016; Xu et al., 2019). Given the presence of many similar petrogenic contaminants found in FPW to those in crude oil and related wastewater solutions, studies defining the occurrence and degree of FPW impacts on eye and other sensory structure development and function would be of great merit. However, considering the neuronal and nervous system basis of many sensory systems in vertebrates, a targeted approach to understanding the cellular electrophysiological implications of FPW exposure to these systems would greatly improve overall understanding of FPW toxicity to excitable cells. Following an experimental process as defined in *section 9.5.3*, exposures of FPW to neuronally-derived cells would confirm whether channels and ion currents affected in cardiomyocytes are also altered in neuronal cells. Then, whole organism exposures and subsequent neuronal cell isolations paired with electrophysiological analyses would confirm whether neuronal cells experience perturbations within the organism and if potential immediate effects are persistent.

9.5.5 FPW Behavioural Studies

A combined and thorough analysis at all levels of biological organization is the ultimate goal of any researcher or agency when investigating a contaminant or toxic solution of interest. For FPW, much attention has been given to elucidating the sub-lethal toxic responses of exposed organisms at the tissue level, with more limited analyses at the cellular and whole organism level. However, most (if not all) of the analyses made to date have been genetic, biochemical, or physiological in nature. FPW studies which accounted

for endpoints linking these previously mentioned elements are lacking. Although swimming performance can be considered a behavioural trait, other behavioural analyses associated with FPW exposure to organisms are nonexistent. Anthropogenically derived contaminants and similar wastewater solutions to FPW (e.g. crude oil fractions and oil sands process-affected waters; OSPW) have been shown to damage fish olfactory sensory responses to natural stimulants/odorants (Lari et al., 2015; Reichert et al., 2017; Tierney et al., 2008), and thereby alter avoidance/attraction behaviours when exposed to contaminants in an arena (Reichert et al., 2017; Schlenker et al., 2019b, 2019a). It is unknown whether FPW is sensed in fish and evokes any avoidance/attraction behaviour, and whether these responses are altered following exposure to FPW when subjected to a natural odorant cue. These behavioural studies, paired with electro-olfactogram (EOG) neurophysiological analyses, would answer whether fish sense FPW in the environment, react positively or adversely to the solutions presence in water, and if a fish's ability to sense natural odorants is altered following previous FPW exposure. Gaining a better understanding of such behavioural-based queries can be crucial for determining the extent of damage to populations of fish in regions experiencing hydraulic fracturing activity.

9.6 SUMMARY

Technological advancements in the oil and gas sectors have led to companies being able to exploit formations that were previously thought to be of limited potential for hydrocarbon extraction. Unfortunately, the wastewaters produced from UOG activities (FPW) have the potential to be the next great industrial wastewater management conundrum given the extensive volume produced per well, the vast number of producible reserves worldwide, and the expectation of continued UOG expansion. Overall, my thesis demonstrates that FPW is relatively highly toxic to aquatic organisms and induces a myriad of sub-lethal responses in organisms exposed to the wastewater at sub-lethal and environmentally relevant concentrations, including oxidative stresses responses, xenobiotic metabolism burdens, impacts to nutrient handling and organismal energy dynamics, and endocrine disrupting activities. Focusing on cardio-respiratory characteristics, I have also determined that FPW exposure in fish embryos significantly affects cardiac development, differentially induces an array of expressional trends in numerous cardiac-focused gene transcripts, and negatively impacts embryonic/larval O_2 consumption (an effect which

persists past initial FPW exposures). These embryonic FPW exposures also are shown to have latent impacts on juvenile fish swimming performance and respirometry, thereby highlighting the potential of FPW to affect organism physiological and behavioural attributes past initial exposures. FPW exposure was also shown to directly impact sarcomere contractile properties of isolated cardiomyocytes, a response suspected to be involved in hindered swimming performance and decreased aerobic scope in acutely exposed juvenile fish. My results may serve to identify certain key points required for effective FPW risk assessment development and improve release identification, assessment, and remediation protocols.

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Appendices

Supplemental Tables

Table S1- 1. Radioactive species (pCi/L) present in hydraulic fracturing flowback and produced water samples. (N= number of collective samples used to establish range).

Chemical	$\bar{X}$ Conc.	Median Conc.	Range	N
Gross Alpha	14091.94	9593	54.6 - 123000	40
Gross Beta	2861.58	1723.5	0 - 21300	40
^{226}Ra	5761.12	4164.5	0 - 26600	44
^{228}Ra	697.67	611	0.029 - 1900	38
^{234}U	0.83	0.38	0 – 3.82	13
^{235}U	0.21	0.11	0 – 1.17	13
^{238}U	0.22	0.077	0 – 0.77	13
^{40}K	462.86	368	31 - 2630	22

Table S2- 1. Complete inorganic characterization of flowback and produced water samples collected from a horizontally hydraulic fractured well 1.33 hr, 72 hr, and 228 hr post-well stimulation. Measurement error was quantified as the deviation across four replicate measurements for each sample for ICP-MS/MS.

		FPW Sample Characterizations (mg/L)		
Chemical	**Detection Limit Mg/L**	**1 hr, 20 min**	**3 - Day**	**9.5 – Day**
Total Dissolved Solids (TDS)		144597.7	164063.7	175688.5
Total Nitrogen (TN)		334.3	287.8	351.5
Total Organic Carbon (TOC)		4712.2	218.8	191.4
Cl	0.4	83882.4	81424.4	103053.3
Na (23)	0.33	47382.3 ± 716	50248.6 ± 406	54121.9 ± 396
Li (7)	0.00066	34.0 ± 0.19	38.8 ± 0.08	42.1± 0.19

B (11)	0.011	66.9 ± 2.2	76.0 ± 1.5	84.8 ± 1.4
Mg (24)	0.23	614.4 ± 1.3	744.4 ± 4.3	799.9 ± 6.5
Al (27)	0.0016	BDL	BDL	BDL
Si (28)	0.22	BDL	BDL	22.2 ± 0.27
S (32 – 48)	0.17	87.3 ± 3.9	101.9 ± 3.9	61.7 ± 0.38
K (39)	0.23	1574.1 ± 11.4	1734.7 ± 10.9	1940.7 ± 33.1
Ca (40)	0.33	6759.1 ± 51.0	7718.4 ± 107	9057.7 ± 84.0
Mn (55)	0.0015	6.6 ± 0.04	4.7 ± 0.04	4.6 ± 0.02
Fe (56)	0.093	185.0 ± 0.61	54.3 ± 0.39	68.2 ± 0.46
Fe He (56)	0.0059	165.9 ± 1.22	52.7 ± 0.34	75.5 ± 0.93
Ni (60)	0.005	BDL	BDL	BDL
Cu (63)	0.0063	BDL	BDL	BDL
Zn (66)	0.0054	BDL	BDL	BDL
As (75 – 91)	0.0026	BDL	BDL	BDL
Br (79)	0.062	228.3 ± 1.12	270.3 ± 1.84	230.9 ± 2.6
Sr (88)	0.019	672.9 ± 3.23	826.7 ± 6.19	880.8 ± 2.1
Cd (114)	0.00017	0.01 ± 0.005	BDL	BDL
Ba (137)	0.00021	15.0 ± 0.19	24.7 ± 0.32	11.5 ± 0.03
Pb (208)	0.00021	0.03 ± 0.002	0.02 ± 0.001	BDL

BDL – Below Detection Limit

Table S2- 2. ICP-MS/MS operation parameters from the analysis of flowback and produced water samples collected from a horizontal hydraulic fractured well. The accuracy and precision of analysis was determined through the analysis of a midrange standard (concentration listed in below) at the beginning, middle and end of each run (*n*=3).

Element	M/Z	Gas Mode	Linear Range Examined	Target Concentration (ppm)	Precision (%) (n=3)	Average Recovery (%)

			(ppm)			
Na*	23	No Gas	10-100	50	±1.3	98.7
Li	7	No Gas	0.0099-1.84	0.1	±0.16	94.9
B	11	No Gas	0.049-1.77	0.1	±32	162
Mg	24	No Gas	0.65-110	70	±3.6	105
Al	27	No Gas	0.0048-0.49	0.1	±4.4	101
Si	28	He	0.0097-0.49	0.1	±2.1	89.3
S	32→ 48	O_2	0.47-27	10	±2.9	98.5
K	39	No Gas	0.96-110	70	±7.4	112
Ca	40	H_2	0.88-108	70	±1.3	100
Mn	55	No Gas	0.0048-1.8	0.1	±2.3	104
Fe	56	No Gas	0.0098-1.83	0.1	±3.1	103
Fe	56	He	0.0098-1.83	0.1	±2.8	107
Ni	60	No Gas	0.00031-0.5	0.1	±0.61	98.3
Cu	63	No Gas	0.00031-0.5	0.1	±1.73	101
Zn	66	He	0.0048-0.49	0.1	±0.91	101
As	75→ 91	O_2	0.0048-0.5	0.1	±3.72	95
Br	79	No Gas	0.97-27	10	±0.55	101
Sr	88	No Gas	0.48-27	10	±1.1	102
Cd	114	No Gas	0.0047-1.8	0.1	±0.34	101
Ba	137	No Gas	0.0047-1.8	0.1	±0.47	101
Pb	208	No Gas	0.0047-0.5	0.1	±0.76	102

*The Na standard was composed only of Na and run solely during the run for Na analysis.

Table S2- 3. Total carbon and nitrogen analysis, and ion chromatography operation parameters, and accuracy from the analysis of flowback and produced water samples collected from a horizontal hydraulic fractured well.

Element	Linear Range Examined (ppm)	Target Concentration (ppm)	Precision (%) (n=4)	Average Recovery (%)
Total Nitrogen (TN)	0.5-50	10		97.6

Total Organic Carbon (TOC)	0.0099-1.84	9.73		102
Cl	2-100	20	±3.8	101

Table S2- 4. Summary of the standards used to prepare the standards for ICP-MS/MS analysis.

Element	Concentration (mg/L)	Manufacturer	Lot Number
Na	1001±5	SpexCertiPrep	CL9-8NAY
Li	1001±5	SpexCertiPrep	19-217LIY
B	1002±5	SpexCertiPrep	20-145BY
Mg	1001±5	SpexCertiPrep	CL8-172MGY
Al	999±5	SpexCertiPrep	CL7-82ALY
Si	1000±5	RICCA	4504605
S	1000±5	RICCA	4712C36
K	1001±5	SpexCertiPrep	CL8-121Y
Ca	1000±5	FisherSci	KSC5265455
Mn	1001±5	SpexCertiPrep	CL7-45MNY
Fe	1001±5	SpexCertiPrep	22-136FEY
Ni	999±5	SpexCertiPrep	CL7-140NIY
Cu	1001±5	SpexCertiPrep	CL7-98CUY
Zn	1002±5	SpexCertiPrep	CL7-46ZNY
As	1002±5	SpexCertiPrep	CL8-04ASY
Br	1003±5	SpexCertiPrep	3-122BR-2Y
Sr	1002±5	SpexCertiPrep	20-81SRT
Cd	998±5	SpexCertiPrep	22-116CDT
Ba	999±5	SpexCertiPrep	20-183BAY
Pb	999±5	SpexCertiPrep	CL7-81PBY

Table S2- 5. Confirmation measurements of LC_{50} dilutions used during analyses. Data presented within represent dilutions used for most organisms collectively. Dilution

percentages were determined by measuring conductivity (mS/cm) of exposure mediums, then comparing to original FPW sample (1.33 hr, 72 hr, and 228 hr) and salt control conductivities.

Treatment	Dilutions Used for LC_{50} Analyses (%)	Actual Measured Dilutions (% Average)	Difference (+/- %)
Salt Control	0.5	0.488	- 0.012
	1	1.121	+ 0.121
	2	1.968	- 0.032
	3	3.331	+ 0.331
	4	3.925	- 0.075
	5	5.279	+ 0.279
	7	7.359	+ 0.359
	7.5	7.428	- 0.072
	9	9.399	+ 0.399
	10	9.523	- 0.477
	11	11.613	+ 0.613
	13	12.476	- 0.524
	15	14.579	- 0.421
	20	19.546	- 0.454
	25	24.582	- 0.418
FPW 1.33 Hr	0.5	0.521	+ 0.021
	1	1.255	+ 0.255
	2	2.151	+ 0.151
	2.5	2.559	+ 0.059
	3	3.101	+ 0.101
	3.5	3.642	+ 0.142
	5	5.144	+ 0.144
	7	7.203	+ 0.203
	7.5	7.474	- 0.026
	9	9.317	+ 0.317
	10	9.672	- 0.328
	11	11.477	+ 0.477
	13	12.526	- 0.474
	15	15.043	+ 0.043
	20	19.654	- 0.346
FPW 72 Hr	0.5	0.476	- 0.024
	1	1.202	+ 0.202
	2	1.960	- 0.040
	2.5	2.568	+ 0.068
	3	3.325	+ 0.325
	3.5	3.624	+ 0.124
	5	5.220	+ 0.220
	7	7.314	+ 0.314
	7.5	7.358	- 0.142
	9	9.499	- 0.001
	10	9.946	- 0.054
	11	11.407	+ 0.407
	13	13.481	+ 0.481
	15	14.882	- 0.118
	20	19.458	- 0.515
FPW 228 Hr	0.5	0.485	- 0.015
	1	1.238	+ 0.238

2	1.935	- 0.065
2.5	2.513	+ 0.013
3	3.152	+ 0.152
4	4.007	+ 0.007
5	5.247	+ 0.247
7	7.398	+ 0.398
7.5	7.501	+ 0.001
9	9.502	+ 0.502
10	9.757	- 0.243
11	11.572	+ 0.572
13	13.521	+ 0.521
15	15.725	+ 0.725
20	20.438	+ 0.438

Table S3- 1. Target ions, qualifier ions, internal standards used for each analyte, and method detection limit (MDL) in GC-MS analysis.

Target analytes	Target Ions (m/z)	Qualifier Ions (m/z)	Internal Standards	MDL for Liquid-Liquid Method (ng/L)
Naphthalene	128	102	**Naphthalene-d8**	3.6
Acenaphthylene	152	76	**Acenaphthene-d10**	0.14
Acenaphthene	153	76	**Acenaphthene-d10**	2.3
Fluorene	165	139	**Fluorene-d10**	2.6
Phenanthrene	178	152	**Phenanthrene-d10**	4.8
Anthracene	178	152	**Anthracene-d10**	0.58
Fluoranthene	202	101	**Fluoranthene-d10**	1.2
Pyrene	202	101	**Pyrene-d10**	1.5
Benz[a]anthracene	228	113	**Benz[a]anthracene-d12**	0.052
Chrysene	228	113	**Chrysene-d12**	0.072
Benzo[b]fluoranthene	252	126	**Benzo[b]fluoranthene-d12**	1.5
Benzo[k+j]fluoranthene	252	126	**Benzo[k]fluoranthene-d12**	0.17
Benzo[a]pyrene	252	126	**Benzo[a]pyrene-d12**	0.17
Indeno[1,2,3-cd]pyrene	276	138	**Indeno[1,2,3-cd]pyrene-d12**	0.060
Benzo[g,h,i]perylene	276	138	**Benzo[g,h,i]perylene-12**	0.061
Dibenz[a,h]anthracene	278	138	**Dibenz[a,h]anthracene-d14**	0.094
Dibenzothiophene	184	139	**Dibenzothiophene-d8**	0.22
Retene	219	234	**Benz[a]anthracene-d12**	0.30
1-Methylfluorene	165	180	**Fluorene-d10**	0.78

1-Methylphenanthrene	192	95	**Phenanthrene-d10**	0.11
3,6-Dimethylphenanthrene	206	191	**Phenanthrene-d10**	0.070
1-Methylpyrene	216	189	**Pyrene-d10**	0.050

Table S3- 2. Nucleotide sequence and efficiencies of primer pairs used in qPCR. All primers were designed by authors based on sequence information obtained from GeneBank.

Abb.	Gene	Category	Sequence 5' – 3'	Eff.	GenBank Accession #
elf1a	Elongation Factor 1 Alpha	Endogenous	F: CTGTTGCCTTTGTGCCCATC R: CATCCCTTGAACCAGCCCAT	2.02	AF498320
cyp1a	Cytochrome P450 1A (1A1, 1A2, 1A3)	Phase I Biotransformation	F: CCGCACCATGAAGAGGTTTA R: GTCAATGAGGGAGTCAGTGATG	2.05	U62796, U62797, NM_001124754
cyp3a	Cytochrome P450 3A45	Phase I Biotransformation	F: GTGTCCTCTCACCTTCCTTTAC R: GGTCTTTATCTGCCTGCTTCT	2.08	AF267126
udpgt	UDP-Glucoronosyl Transferase	Phase II Biotransformation	F: GCTGAGGTATGACTACTCCTTTG R: CGCTGGAAGTGGAGCTATTT	2.00	CB497940.1
gst	Glutathione Transferase Omega-1	Phase II Biotransformation/ Oxidative Stress	F: GGTGATCTACGAGTCACCAATC R: TTCTGTTGGGCCTTCTGAAA	1.87	BT074035
gpx	Glutathione Peroxidase	Oxidative Stress	F: TCCTGAGGTCCCTGAAGTATATC R: CCTTCCCATTCACATCCATCTT	2.11	AF281338
sod	Superoxide Dismutase	Oxidative Stress	F: CGATGATGTCAGGCACATAGG R: GGTCCAGTGAGAGTCAGTATCT	1.83	NM_001160614
mtb	Metallothionien Beta	Metal	F: GAAAAGTTGCTGCCCCTGC R: TATCGCAGGTCTTGCCCTTG	2.01	X59394
vtg	Vitellogenin	Endocrine Disruption	F: ACTCTGTGGAAAGGCTGATG R: AAGGAAGCACCCAGGAATG	2.03	CA0516331
erα1	Estrogen Receptor Alpha 1	Endocrine Disruption	F: CCCTGCTGGTGACAGAGAGAA R: ATCCTCCACCACCATTGAGACT	2.00	AJ242740

erα2	Estrogen Receptor Alpha 2	Endocrine Disruption	F: ATGGAAGGAACGAGGTAGGA R: TTACGCTGAAGAGGTTTGGG	2.06	DQ177438
erβ1	Estrogen Receptor Beta 1	Endocrine Disruption	F: CCCAAGCGGGTCCTAGCT R: TCCTCATGTCCTTCTGGAGGAA	2.09	DQ177439
erβ2	Estrogen Receptor Beta 2	Endocrine Disruption	F: CATCCAAGGCCACAATGATTAC R: CTTCATCATGCCCACTTCATAAC	2.10	DQ248229

Table S3- 3. The 16 EPA PAHs and 6 alkyl PAHs in the water samples.

	HF-FPW-AC (ng/L)				HF-FPW-SF (ng/L)				HF-FPW-S (ng/L)			
	24 h		48 h		24 h		48 h		24 h		48 h	
Species	**2.5%**	**7.5%**	**2.5%**	**7.5%**	**2.5%**	**7.5%**	**2.5%**	**7.5%**	**2.5%**	**7.5%**	**2.5%**	**7.5%**
Naphthalene*	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
Acenaphthlene*	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
Acenaphthene*	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
Fluorene*	N.D.	N.D.	N.D.	N.D.	1.8	1.8	1.8	1.8	29	63	17	55
Phenanthrene*	N.D.	N.D.	N.D.	N.D.	15	17	8.1	7.5	31	87	20	42
Anthracene*	N.D.	N.D.	N.D.	N.D.	0.33	0.57	0.23	0.073	1.8	6.1	1.5	3.6
Fluoranthene*	N.D.	N.D.	N.D.	N.D.	6.5	6.3	4.7	4.6	9.2	14	8.3	11
Pyrene*	N.D.	N.D.	N.D.	N.D.	5.4	4.6	3.7	2.9	9.1	20	8.4	21
Benz[a]anthracene*	N.D.	N.D.	N.D.	N.D.	0.28	0.37	0.19	0.29	1.1	4.7	1.4	4.7
Chrysene*	N.D.	N.D.	N.D.	N.D.	0.51	0.85	0.28	0.22	8.4	22	7.9	19
Benzo[b]fluoranthene*	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
Benzo[k+j]fluoranthene*	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
Benzo[a]pyrene*	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
Indeno[1,2,3-cd]pyrene*	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
Benzo[g,h,i]perylene*	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
Dibenz[a,h]anthracene*	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
Dibenzothiophene**	N.D.	N.D.	N.D.	N.D.	0.34	0.43	0.16	0.16	0.34	1.7	0.16	1.7
Retene**	N.D.	N.D.	N.D.	N.D.	2.7	2.3	0.48	0.48	2.8	8.4	1.7	7.2
1-Methylfluorene**	N.D.	N.D.	N.D.	N.D.	0.55	3.9	0.55	0.55	31	78	27	52
1-Methylphenanthrene**	N.D.	N.D.	N.D.	N.D.	0.53	0.43	0.074	0.15	13	44	9.3	39
3,6-Dimethylephenanthrene**	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	13	42	9.7	43
1-Methylpyrene**	N.D.	N.D.	N.D.	N.D.	0.059	0.035	0.035	0.093	0.77	4.2	0.68	3.6
16 EPA PAHs	N.D.	N.D.	N.D.	N.D.	30	31	19	17	89	220	64	160
Total PAHs (with alkylated)	N.D.	N.D.	N.D.	N.D.	34	39	20	19	150	390	110	300

*EPA PAHs; **alkyl PAHs; N.D., not detected**.**

Table S4- 1. Inorganic characterization of raw 5 h post-stimulated flowback and produced water (FPW) sample collected from a horizontal hydraulically fractured well.

Chemical	Detection Limit (mg/L)	5 h FPW Sample (mg/L)
Total Dissolved Solids (TDS)		145109.9

Total Nitrogen (TN)		270.7
Total Organic Carbon (TOC)		481.8
Cl	0.40	70757.5
Na (23)	0.33	46198.7
Li (7)	0.00038	33.6
B (11)	0.021	72.3
Mg (24)	0.089	628.8
Al (27)	0.0022	BDL
Si (28)	0.37	BDL
S (32 – 48)	0.17	100.1
K (39)	0.23	1536.1
Ca (40)	0.33	6684.7
Mn (55)	0.00089	6.2
Fe (56)	0.093	166.6
Fe He (56)	0.0052	153.0
Ni (60)	0.0055	BDL
Cu (63)	0.01	BDL
Zn (66)	0.018	BDL
As (75 – 91)	0.0050	BDL
Br (79)	0.062	236.0
Sr (88)	0.049	684.7
Cd (114)	0.00013	BDL
Ba (137)	0.00022	21.3
Pb (208)	0.00014	BDL

BDL = Below Detection Limits

Table S4- 2. Polycyclic aromatic hydrocarbon (PAH) analysis of 5 h flowback and produced water (FPW). All measurements are in µg/L. Total PAH content of a specific compound determined via summation of both aqueous (A) and sediment (S) phase measurements of the specified compound in the respective FPW sample. Total summation of US EPA 16 PAHs (shaded compounds) and all PAHs analyzed (shaded + un-shaded compounds) for respective FPW samples identified as **Σ16 PAHs** and **Σ22 PAHs**, respectively.

PAHs	Aqueous	Sediment	Total
Naphthalene	N.D.	N.D.	N.D.
Acenaphthlene	N.D.	N.D.	N.D.
Acenaphthene	N.D.	N.D.	N.D.
Fluorene	0.26	2.48	2.74
Phenanthrene	0.19	4.46	4.66
Anthracene	0.0038	0.34	0.35
Fluoranthene	0.0053	0.088	0.094
Pyrene	0.037	2.40	2.44
Benz[a]anthracene	0.0023	0.42	0.42
Chrysene	0.024	0.97	0.99
Benzo[b]fluoranthene	N.D.	0.16	0.16
Benzo[k+j]fluoranthene	N.D.	N.D.	N.D.
Benzo[a]pyrene	N.D.	N.D.	N.D.
Indeno[1,2,3-cd]pyrene	N.D.	N.D.	N.D.
Benzo[g,h,i]perylene	N.D.	N.D.	N.D.
Dibenz[a,h]anthracene	N.D.	N.D.	N.D.
Dibenzothiophene	0.0080	0.27	0.28
Retene	0.11	0.29	0.30
1-Methylfluorene	0.25	3.60	3.85
1-Methylphenanthrene	0.25	8.36	8.60
3,6-Dimethylphenanthrene	0.17	7.00	7.18
1-Methylpyrene	0.0097	3.76	3.77
		Σ16 PAHs	**11.85**
		Σ22 PAHs	**35.82**

Table S4- 3. Hydraulic fracturing-generated flowback water (HF-FW) sample operational ICP-MS/MS parameters. Accuracy and precision were determined through the analysis of a

midrange standard (concentration listed below) at the beginning, middle and end of each run (n=3).

Element	M/Z	Gas Mode	Linear Range Examined (ppm)	Target Concentration (ppm)	Precision (%) (n=3)	Average Recovery (%)
Na*	23	No Gas	10-100	50	±1.3	98.7
Li	7	No Gas	0.0099-1.84	0.1	±0.16	94.9
B	11	No Gas	0.049-1.77	0.1	±32	162
Mg	24	No Gas	0.65-110	70	±3.6	105
Al	27	No Gas	0.0048-0.49	0.1	±4.4	101
Si	28	He	0.0097-0.49	0.1	±2.1	89.3
S	32→ 48	O_2	0.47-27	10	±2.9	98.5
K	39	No Gas	0.96-110	70	±7.4	112
Ca	40	H_2	0.88-108	70	±1.3	100
Mn	55	No Gas	0.0048-1.8	0.1	±2.3	104
Fe	56	No Gas	0.0098-1.83	0.1	±3.1	103
Fe	56	He	0.0098-1.83	0.1	±2.8	107
Ni	60	No Gas	0.00031-0.5	0.1	±0.61	98.3
Cu	63	No Gas	0.00031-0.5	0.1	±1.73	101
Zn	66	He	0.0048-0.49	0.1	±0.91	101
As	75→ 91	O_2	0.0048-0.5	0.1	±3.72	95
Br	79	No Gas	0.97-27	10	±0.55	101
Sr	88	No Gas	0.48-27	10	±1.1	102
Cd	114	No Gas	0.0047-1.8	0.1	±0.34	101
Ba	137	No Gas	0.0047-1.8	0.1	±0.47	101
Pb	208	No Gas	0.0047-0.5	0.1	±0.76	102

*The Na standard was composed only of Na and run solely during the run for Na analysis.

Table S4- 4. Operational parameters and accuracy for total carbon, total nitrogen, and ion chromatography operation for analysis of a hydraulic fracturing-generated flowback water sample collected from a horizontal hydraulically fractured well.

Element	Linear Range Examined (ppm)	Target Concentration (ppm)	Precision (%) (n=4)	Average Recovery (%)

Total Nitrogen (TN)	0.5-50	10		97.6
Total Organic Carbon (TOC)	0.0099-1.84	9.73		102
Cl	2-100	20	±3.8	101

Table S4- 5. Summary standard preparation for ICP-MS/MS analysis.

Element	Concentration (mg/L)	Manufacturer	Lot Number
Na	1001±5	SpexCertiPrep	CL9-8NAY
Li	1001±5	SpexCertiPrep	19-217LIY
B	1002±5	SpexCertiPrep	20-145BY
Mg	1001±5	SpexCertiPrep	CL8-172MGY
Al	999±5	SpexCertiPrep	CL7-82ALY
Si	1000±5	RICCA	4504605
S	1000±5	RICCA	4712C36
K	1001±5	SpexCertiPrep	CL8-121Y
Ca	1000±5	FisherSci	KSC5265455
Mn	1001±5	SpexCertiPrep	CL7-45MNY
Fe	1001±5	SpexCertiPrep	22-136FEY
Ni	999±5	SpexCertiPrep	CL7-140NIY
Cu	1001±5	SpexCertiPrep	CL7-98CUY
Zn	1002±5	SpexCertiPrep	CL7-46ZNY
As	1002±5	SpexCertiPrep	CL8-04ASY
Br	1003±5	SpexCertiPrep	3-122BR-2Y
Sr	1002±5	SpexCertiPrep	20-81SRT
Cd	998±5	SpexCertiPrep	22-116CDT
Ba	999±5	SpexCertiPrep	20-183BAY
Pb	999±5	SpexCertiPrep	CL7-81PBY

Table S4- 6. Water quality data for employed rainbow trout (*Oncorhynchus mykiss*) flowback and produced water (FPW) exposures. Data are presented as mean ± standard deviation. Parentheses indicate fish deaths at the end of 48 h exposures.

Treatment	pH	D.O. (mg/L)	Conductivity (mS)
Flux Analyses			
Control (0)	7.97 ± 0.08	8.06 ± 0.94	0.44 ± 0.025
Saline Control (1)	7.93 ± 0.12	8.16 ± 1.17	19.97 ± 1.85
FPW 2.5% (0)	7.85 ± 0.08	8.01 ± 1.06	6.98 ± 0.94
FPW 7.5% (2)	7.79 ± 0.15	7.92 ± 1.04	21.48 ± 2.43
Enzyme Analyses			
Control (0)	8.15 ± 0.11	8.22 ± 0.62	0.42 ± 0.037
Saline Control (2)	8.08 ± 0.09	8.13 ± 0.97	19.71 ± 0.49
FPW 2.5% (1)	7.95 ± 0.17	8.09 ± 1.05	7.15 ± 1.20
FPW 7.5% (3)	7.86 ± 0.13	8.01 ± 0.82	20.63 ± 3.18

Table S4- 7. Quantitative real-time PCR gene names, primer sequences, and accession numbers.

Gene Name	Gene Target	Primer Sequence (5' – 3')	GenBank Accession #
cyp1a	Cytochrome P450 1A	F - GAACTGAAGGAAAAGGTGGGAAT R - GGCTTCCAGCAGAGGTAAGTTG	AF015660.1
cyp1b1	Cytochrome P450 1B1	F - CCGGGAGTTCAGTAAATTTATCGT R - CTGTCATGTCCCGGATTGTG	NM001174149.1
pk	Pyruvate Kinase	F - TCGTCGCGGTAACAAGAAGTC R - GGAACAGCACTGGGAACACA	AF246146.1
pepck	Phosphoenolpyruvate Carboxykinase	F - GCTGAGTACAAAGGCAAGGTTATCA R - ACCGAAGTTGTAGCCGAAGAAG	NM001124275.1
ldh	Lactate Dehydrogenase D	F - TGCAAGGCCTACTCCACAGA R - GCATCAGGTCCTCCTTGGTTT	XM021568708.1
gdh	Glutamate Dehydrogenase	F - CATCAGCCAGGGCGGTAT R - GAAGTTCTCAATGCCATGGAACA	AJ419570.1
tata12	TATA-Box Binding Associated Factor 12	F - TCAGATGAGATTCGGCCTTATAAGA R - TTGGCTGTTTTGCGGATCA	NM001160631.1

Table S7- 1. Inorganic characterization of a raw 4 h post-stimulated flowback and produced water (FPW) sample collected from a horizontal hydraulically fractured well.

Chemical	Detection Limit (mg/L)	5 h FPW Sample (mg/L)
Total Dissolved Solids (TDS)		172954.6
Cl	0.40	55202.5
Na (23)	0.33	49988.8
Li (7)	0.00038	39.7
B (11)	0.021	BDL
Mg (24)	0.089	1386.8
Al (27)	0.0022	BDL
Si (28)	0.37	BDL
S (32 – 48)	0.17	310.5
K (39)	0.23	1871.1
Ca (40)	0.33	13604.3
Mn (55)	0.00089	3.9
Fe He (56)	0.0052	6.44
Ni (60)	0.0055	BDL
Cu (63)	0.01	BDL
Zn (66)	0.018	BDL
As (75 – 91)	0.0050	BDL
Br (79)	0.062	202.7
Sr (88)	0.049	1035.0
Cd (114)	0.00013	BDL
Ba (137)	0.00022	21.8
Pb (208)	0.00014	BDL

Table S7- 2. Hydraulic fracturing-generated flowback and produced water (FPW) sample operational ICP-MS/MS parameters. Accuracy and precision were determined through the analysis of a midrange standard (concentration listed below) at the beginning, middle and end of each run (*n*=3).

Element	M/Z	Gas Mode	Linear Range Examined (ppm)	Target Concentration (ppm)	Precision (%) (n=3)	Average Recovery (%)
Na*	23	No Gas	10-100	50	±1.3	98.7
Li	7	No Gas	0.0099-1.84	0.1	±0.16	94.9
B	11	No Gas	0.049-1.77	0.1	±32	162
Mg	24	No Gas	0.65-110	70	±3.6	105
Al	27	No Gas	0.0048-0.49	0.1	±4.4	101
Si	28	He	0.0097-0.49	0.1	±2.1	89.3
S	32→ 48	O_2	0.47-27	10	±2.9	98.5
K	39	No Gas	0.96-110	70	±7.4	112
Ca	40	H_2	0.88-108	70	±1.3	100
Mn	55	No Gas	0.0048-1.8	0.1	±2.3	104
Fe	56	No Gas	0.0098-1.83	0.1	±3.1	103
Fe	56	He	0.0098-1.83	0.1	±2.8	107
Ni	60	No Gas	0.00031-0.5	0.1	±0.61	98.3
Cu	63	No Gas	0.00031-0.5	0.1	±1.73	101
Zn	66	He	0.0048-0.49	0.1	±0.91	101
As	75→ 91	O_2	0.0048-0.5	0.1	±3.72	95
Br	79	No Gas	0.97-27	10	±0.55	101
Sr	88	No Gas	0.48-27	10	±1.1	102
Cd	114	No Gas	0.0047-1.8	0.1	±0.34	101
Ba	137	No Gas	0.0047-1.8	0.1	±0.47	101
Pb	208	No Gas	0.0047-0.5	0.1	±0.76	102

Table S7- 3. Polycyclic aromatic hydrocarbon (PAH) analysis of 4 h flowback and produced water (FPW). All measurements are in µg/L. Total PAH content of a specific compound determined via summation of both aqueous (A) and sediment (S) phase measurements of the specified compound in the respective FPW sample. Total summation of US EPA 16 PAHs (shaded compounds) and all PAHs analyzed (shaded + un-shaded compounds) for respective FPW samples identified as **Σ16 PAHs** and **Σ33 PAHs**, respectively.

PAHs	Aqueous	Sediment	Total
Naphthalene	N.D.	N.D.	N.D.
Acenaphthlene	N.D.	N.D.	N.D.
Acenaphthene	N.D.	N.D.	N.D.
Fluorene	0.75	0.64	1.39
Phenanthrene	0.68	0.59	1.27
Anthracene	N.D.	N.D.	N.D.
Fluoranthene	0.012	0.054	0.066
Pyrene	0.081	0.36	0.44
Benz[a]anthracene	0.0057	0.019	0.025
Chrysene	0.062	0.30	0.36
Benzo[b]fluoranthene	0.0040	0.020	0.024
Benzo[k+j]fluoranthene	N.D.	N.D.	N.D.
Benzo[a]pyrene	N.D.	N.D.	N.D.
Indeno[1,2,3-cd]pyrene	N.D.	N.D.	N.D.
Benzo[g,h,i]perylene	0.0029	0.010	0.013
Dibenz[a,h]anthracene	N.D.	N.D.	N.D.
Retene	0.023	0.14	0.16
1-Methylnaphthalene	N.D.	N.D.	N.D.
1-Ethylnaphthalene	N.D.	N.D.	N.D.
2-n-Propylnaphthalene	N.D.	N.D.	N.D.
9-Ethylfluorene	N.D.	N.D.	N.D.
9-n-Butylfluorene	N.D.	N.D.	N.D.
9-n-Butylphenanthrene	N.D.	N.D.	N.D.
1-Ethylpyrene	N.D.	N.D.	N.D.
1-n-Butylpyrene	N.D.	N.D.	N.D.
1-Methylbenz[a]anthracene	N.D.	N.D.	N.D.
6-Ethylchrysene	N.D.	N.D.	N.D.
6-n-Butylchrysene	N.D.	N.D.	N.D.
5-Methylchrysene	N.D.	N.D.	N.D.
1-Methylfluorene	1.28	0.69	1.97
1-Methylphenanthrene	0.78	1.51	2.29
3,6-Dimethylphenanthrene	0.48	1.61	2.09
1-Methylpyrene	0.033	0.14	0.17
		Σ16 PAHs	**3.59**

Table S7- 4. Quantitative real-time PCR gene names, targets, primer sequences, and associated accession numbers.

Gene Name	Gene Target	Primers (5' – 3')	GenBank
nkx2.5	Homeobox Protein nkx2.5	F - GCTCAAACTGACCCCCACACA R - CTCTGGTCCTGTCGCTGTCTCT	XM_021561683.2
gata5	GATA Binding Protein 5	F - CTAAGATGCCCAAGTCCAAACC R - CGTAGATGCATGTTCCGAGACA	XM_021615971.2
tbx2b	T-box Transcription Factor 2b	F - AATGGCCCAGAGCACAAGTCTA R - GCTGCCCGATTTGGAAGA	XM_021587818.2
anp	Atrial Natriuretic Peptide	F - GGTAATGCCACATGCGGTAAA R - GGGAGGCGAAATCTGAAAATG	NM_001124211.1
vnp	Ventricular Natriuretic Peptide	F - AGAAGAGAATATTTGGGAACTGAAGAA R - TGGAGATTTCCACAGAGGTCAGA	NM_001124212.2
mylc2	Myosin Regulatory Light Chain 2	F - CCATCCTCGCAGCCTTCA R - TTCATCAATAATCGTCTAAACTCATCCT	XM_021622041.1
vmhc	Ventricular Myosin Heavy Chain	F - TGATGAGGACCGCAAGAATATG R - ATCTCTTGTAGGCTTTCACTTTCAGTT	AY009126.1
scn5lab	Voltage Gated Na^+ Channel, Type 5ab	F - CCCATGGTGAGCGGTGATA R - CTGCTGTTTGAGAGCATCCATCT	XM_021562572.2
atp2a2a	Cardiac Sarcoplasmic Reticulum Ca^{2+} ATPase 2a2a	F - GTGCAATGCCCTTAACAGCCT R - ACGGGCAGTGGCTCCACATA	XM_036979656.1*
kcnh6	Voltage Gated K^+ Channel Subfamily H, Member 6	F - CTCCCAGCCGATGGTCAA R - GGTTCCCGGGCAACGA	XM_021567337.2
kir2.1	Inwardly-rectifying K^+ Channel 2.1	F - TCCTGGTGTCCCCCATCA R - CTCCAGCTGTTGCTTGCTCAT	DQ435675.1
tataaf12	TATA-Box Binding Associated Factor 12	F - TCAGATGAGATTCGGCCTTATAAGA R - TTGGCTGTTTTGCGGATCA	NM_001160631.1

rpl7l1	Ribosomal Protein L7-like 1	F - GGTCGCTCTCACAGACAACA R - TTATGTCCGTCCCTCTGGGT	NM_001160672.2**

* Korajoki and Vornanen, 2012. *Journal of Experimental Biology* 215, 1162 – 1169.

** Santos et al., 2019. *Ecotoxicology and Environmental Safety* 170, 778 – 788.

Table S8- 1. *Mahi-mahi* biometric data for respective physiological analyses. Data is presented as average ± standard deviation.

Experimental Analyses	Body Mass (g)	Heart Mass (g)	Fork Length (cm)	Number of Fish
Cardiomyocyte Contractility	382.73 ± 97.83	1.00 ± 0.31	32.92 ± 3.03	24
Swimming Performance and Respirometry	18.91 ± 6.47	N/A	13.31 ± 1.53	70

Table S8- 2. Hydraulic fracturing-generated flowback water (HF-FW) sample operational ICP-MS/MS parameters. Accuracy and precision were determined through the analysis of a midrange standard (concentration listed below) at the beginning, middle and end of each run (*n*=3).

Element	M/Z	Gas Mode	Linear Range Examined (ppm)	Target Concentration (ppm)	Precision (%) (n=3)	Average Recovery (%)
Na*	23	No Gas	10-100	50	±1.3	98.7
Li	7	No Gas	0.0099-1.84	0.1	±0.16	94.9
B	11	No Gas	0.049-1.77	0.1	±32	162
Mg	24	No Gas	0.65-110	70	±3.6	105
Al	27	No Gas	0.0048-0.49	0.1	±4.4	101
Si	28	He	0.0097-0.49	0.1	±2.1	89.3
S	32→ 48	O_2	0.47-27	10	±2.9	98.5
K	39	No Gas	0.96-110	70	±7.4	112
Ca	40	H_2	0.88-108	70	±1.3	100
Mn	55	No Gas	0.0048-1.8	0.1	±2.3	104
Fe	56	No Gas	0.0098-1.83	0.1	±3.1	103
Fe	56	He	0.0098-1.83	0.1	±2.8	107

Ni	60	No Gas	0.00031-0.5	0.1	±0.61	98.3
Cu	63	No Gas	0.00031-0.5	0.1	±1.73	101
Zn	66	He	0.0048-0.49	0.1	±0.91	101
As	75→ 91	O_2	0.0048-0.5	0.1	±3.72	95
Br	79	No Gas	0.97-27	10	±0.55	101
Sr	88	No Gas	0.48-27	10	±1.1	102
Cd	114	No Gas	0.0047-1.8	0.1	±0.34	101
Ba	137	No Gas	0.0047-1.8	0.1	±0.47	101
Pb	208	No Gas	0.0047-0.5	0.1	±0.76	102

Table S8- 3. Operational parameters and accuracy for total carbon, total nitrogen, and ion chromatography operation for analysis of a hydraulic fracturing-generated flowback water sample collected from a horizontal hydraulically fractured well.

Element	Linear Range Examined (ppm)	Target Concentration (ppm)	Precision (%) (n=4)	Average Recovery (%)
Total Nitrogen (TN)	0.5-50	10		97.6
Total Organic Carbon (TOC)	0.0099-1.84	9.73		102
Cl	2-100	20	±3.8	101

Table S8- 4. Summary standard preparation for ICP-MS/MS analysis.

Element	Concentration (mg/L)	Manufacturer	Lot Number
Na	1001±5	SpexCertiPrep	CL9-8NAY
Li	1001±5	SpexCertiPrep	19-217LIY
B	1002±5	SpexCertiPrep	20-145BY
Mg	1001±5	SpexCertiPrep	CL8-172MGY
Al	999±5	SpexCertiPrep	CL7-82ALY

Si	1000±5	RICCA	4504605
S	1000±5	RICCA	4712C36
K	1001±5	SpexCertiPrep	CL8-121Y
Ca	1000±5	FisherSci	KSC5265455
Mn	1001±5	SpexCertiPrep	CL7-45MNY
Fe	1001±5	SpexCertiPrep	22-136FEY
Ni	999±5	SpexCertiPrep	CL7-140NIY
Cu	1001±5	SpexCertiPrep	CL7-98CUY
Zn	1002±5	SpexCertiPrep	CL7-46ZNY
As	1002±5	SpexCertiPrep	CL8-04ASY
Br	1003±5	SpexCertiPrep	3-122BR-2Y
Sr	1002±5	SpexCertiPrep	20-81SRT
Cd	998±5	SpexCertiPrep	22-116CDT
Ba	999±5	SpexCertiPrep	20-183BAY
Pb	999±5	SpexCertiPrep	CL7-81PBY

Table S8- 5. Water quality data for *mahi-mahi* whole organism exposures performed for swimming analyses. Water quality values were obtained from exposure start and end collected measurements, except for ammonia (final only). Data are presented as mean ± standard deviation. Parentheses indicate fish deaths at the end of 24 h exposures.

Treatment	Water Temp. (°C)	pH	D.O. (mg/L)	Salinity (PPT)	Ammonia (µM)
Control (0)	27.06 ± 0.13	7.96 ± 0.05	9.32 ± 1.80	39.63 ± 0.74	10.16 ± 5.75
Salt Control (0)	27.1 ± 0.09	7.95 ± 0.1	8.93 ± 1.31	42 ± 1.31	13.81 ± 6.49
HF-FW 0.75% (0)	27.1 ± 0.17	7.94 ± 0.04	8.87 ± 1.12	39.38 ± 1.19	31.72 ± 3.14
HF-FW 2.75% (3)	27.23 ± 0.15	7.84 ± 0.08	9.08 ± 1.67	42.5 ± 1.2	50.60 ± 5.83

D.O. = dissolved oxygen

PPT = parts per thousand

Table S8- 6. Cardiomyocyte isolation solution and extracellular saline composition. Isolation solution was used for retrograde perfusions of mahi hearts, and extracellular saline was used for contractility measurements.

Mahi Heart Isolation Solution

Chemical	Concentration (mM)
NaCl	100
KCl	10
KH_2PO_4	1.2
$MgSO_4$	4
Taurine	50
Glucose	20
HEPES	20
pH	6.9

Mahi HEPES Extracellular Saline

Chemical	Concentration (mM)
NaCl	150
KCl	5.4
$MgCl_2$	1.5
$CaCl_2$	3.2
Glucose	10
HEPES	10
pH	7.7

Table S8- 7. *Mahi-mahi* cardiomyocyte exposure solution osmolarities. Replicate numbers are in parentheses.

Treatment	Solution Osmolarity (mOsm)
Control (12)	318.46 ± 3.15
Salt Control (8)	390.92 ± 2.81
Osmotic Control (11)	389.08 ± 7.71
HF-FW 1% (8)	355.42 ± 2.19
HF-FW 2% (13)	390.47 ± 4.10

Table S8- 8. Quantitative real-time PCR gene primer sequence, accession number, and species of origin information. See Edmunds et al., 2015* for more information on primer sequencing and BLAST techniques employed.

Gene	Primers (5' – 3')	GenBank	Species

cmlc2	F - GACCAAGACAGAGACGGTGTTA R - GCATCTCATCCAACTCCTCGT	NM_001145995	*Oryzias latipes*
fhl2	F - GCTTCAAGTGCTTCCAGTGC R - GCAGGTGAAGCAGGTCTCAT	DQ225183	*Sparus aurata*
nppa	F - CAGCAGGAGGACTTTGAAGA R - ATCAGGGGTTCTTGATTTCC	AB162777	*Cottus kazika*
tbx5	F - GGTCACAGGCCTCAATCCAA R - AGAGTCCGGGTGAACGTAGA	NM_001201500	*Oryzias latipes*
vmhc	F - CCAAAACCATCAGGAATGAC R - TCAGCTCTTCAGCATCATTG	NM_001112733	*Danio rerio*
rps25	F - GACAAGGCGACCTACGACAA R - CCACGGATCTTCAGCCTCTC	AB291578	*Solea senegalensis*

* Edmunds, R. C.; Gill, J. A.; Baldwin, D. H.; Linbo, T. L.; French, B. L.; Brown, T. L.; Esbaugh, A. J.; Mager, E. M.; Stieglitz, J.; Hoenig, R.; Benetti, D.; Grosell, M.; Scholz, N. L.; Incardona, J. P. Corresponding Morphological and Molecular Indicators of Crude Oil Toxicity to the Developing Hearts of Mahi Mahi. *Sci. Rep.* **2015**, *5* (1), 17326. https://doi.org/10.1038/srep17326.

Table S8- 9. Polycyclic aromatic hydrocarbon (PAH) characterization of filtered raw, undiluted hydraulic fracturing-generated flowback water (HF-FW) used for cardiomyocyte exposures. HF-FW sample was vacuum filtered through a two-stacked 0.3 µm, 90 mm circumference glass fiber filter prior to being diluted for cell exposures. Shaded rows indicate included Σ50 PAHs.

PAH	Concentration (µg/L)
cis/trans-Decalin	11.2
C1-Decalins	N.D.
C2-Decalins	N.D.
C3-Decalins	N.D.
C4-Decalins	N.D.
Benzo(b)thiophene	N.D.
C1-Benzothiophenes	N.D.
C2-Benzothiophenes	N.D.
C3-Benzothiophenes	N.D.
C4-Benzothiophenes	N.D.
Naphthalene	0.0194
C1-Naphthalenes	ND
C2-Naphthalenes	0.439
C3-Naphthalenes	0.237

C4-Naphthalenes	ND
Biphenyl	0.0380
Dibenzofuran	0.0262
Acenaphthylene	0.0751
Acenaphthene	0.0234
Fluorene	ND
C1-Fluorenes	ND
C2-Fluorenes	ND
C3-Fluorenes	ND
Anthracene	0.0201
Phenanthrene	0.0380
C1-Phenanthrenes/Anthracenes	N.D.
C2-Phenanthrenes/Anthracenes	N.D.
C3-Phenanthrenes/Anthracenes	N.D.
C4-Phenanthrenes/Anthracenes	N.D.
Retene	N.D.
Dibenzothiophene	N.D.
C1-Dibenzothiophenes	N.D.
C2-Dibenzothiophenes	N.D.
C3-Dibenzothiophenes	N.D.
C4-Dibenzothiophenes	N.D.
Benzo(b)fluorene	N.D.
Fluoranthene	N.D.
Pyrene	N.D.
C1-Fluoranthenes/Pyrenes	N.D.
C2-Fluoranthenes/Pyrenes	N.D.
C3-Fluoranthenes/Pyrenes	N.D.
C4-Fluoranthenes/Pyrenes	N.D.
Naphthobenzothiophene	N.D.
C1-Naphthobenzothiophenes	N.D.
C2-Naphthobenzothiophenes	N.D.
C3-Naphthobenzothiophenes	N.D.
C4-Naphthobenzothiophenes	N.D.
Benz(a)anthracene	0.0130
Chrysene	N.D.
C1-Chrysenes	N.D.
C2-Chrysenes	N.D.
C3-Chrysenes	N.D.
C4-Chrysenes	N.D.
Benzo(b)fluoranthene	N.D.
Benzo(k)fluoranthene	N.D.
Benzo(a)fluoranthene	N.D.
Benzo(e)pyrene	N.D.
C30-Hopane	N.D.
Benzo(a)pyrene	N.D.

Perylene	N.D.
Indeno(1,2,3-cd)pyrene	N.D.
Dibenz(a,h)anthracene	N.D.
Benzo(g,h,i)perylene	N.D.
4-Methyldibenzothiophene	N.D.
2-Methyldibenzothiophene	N.D.
1-Methyldibenzothiophene	N.D.
3-Methylphenanthrene	N.D.
2-Methylphenanthrene	N.D.
2-Methylanthracene	N.D.
9-Methylphenanthrene	N.D.
1-Methylphenanthrene	N.D.
2-Methylnaphthalene	N.D.
1-Methylnaphthalene	0.0191
2,6-Dimethylnaphthalene	0.0209
2,3,5-Trimethylnaphthalene	0.0293
Carbazole	0.0399
ΣTOTAL PAH	**12.24**
Σ50 PAH	**0.93**

N.D. = Not Detected

Table S8- 10. Polycyclic aromatic hydrocarbon (PAH) characterization of raw, undiluted hydraulic fracturing-generated flowback water (HF-FW) and of water exposures used during swimming analyses. All concentrations are in ug/L. Parentheses indicate one standard deviation in averaged PAH concentrations where applicable. Shaded rows indicate included Σ50 PAHs.

	Raw HF-FW Concentration	**0.75% HF-FW Start** Concentration	**0.75% HF-FW End** Concentration	**2.75% HF-FW Start** Concentration	**2.75% HF-FW End** Concentration
Decahydronaphthalene (Total)	66.9	0.61 (0.08)	0.098 (0.019)	2.06 (0.39)	0.20 (0.23)
C1 - Decalins	137	1.31 (0.09)	0.14 (0.019)	4.32 (0.70)	1.05 (0.29)
C2 - Decalins	323	3.29 (0.22)	0.79 (0.019)	10.70 (1.74)	4.78 (1.16)
C3 - Decalins	403	3.55 (0.17)	1.24 (0.075)	12.066 (1.30)	6.56 (1.53)
C4 - Decalins	420	2.77 (0.29)	1.12 (0.081)	11.043 (1.11)	6.65 (1.45)
Benzo(b)thiophene	0.11	N.D.	N.D.	N.D.	N.D.
C1 - Benzothiophenes	2.01	N.D.	N.D.	0.14 (0.017)	N.D.
C2 - Benzothiophenes	2.39	N.D.	N.D.	N.D.	N.D.
C3 - Benzothiophenes	1.02	N.D.	N.D.	N.D.	N.D.
C4 - Benzothiophenes	N.D.	N.D.	N.D.	N.D.	N.D.
Naphthalene	N.D.	0.0054	0.012 (0.0001)	N.D.	N.D.

C1 - Naphthalenes	1.42	0.025 (0.0029)	0.021 (0.001)	0.049 (0.01)	0.033 (0.0033)
C2 - Naphthalenes	7.54	0.090 (0.0065)	0.027	0.28 (0.061)	0.11
C3 - Naphthalenes	18.1	0.25 (0.0083)	N.D.	0.73 (0.17)	0.13
C4 - Naphthalenes	25.7	0.29 (0.016)	N.D.	1.027 (0.21)	0.40
Biphenyl	0.408	0.023 (0.0017)	0.034 (0.014)	0.036 (0.0038)	0.029 (0.0019)
Dibenzofuran	0.264	N.D.	N.D.	0.018 (0.015)	0.0077
Acenaphthylene	N.D.	N.D.	N.D.	N.D.	N.D.
Acenaphthene	N.D.	0.024	N.D.	0.049 (0.0044)	0.038
Fluorene	N.D.	0.013 (0.0062)	0.0069 (0.0005)	0.030 (0.64)	N.D.
C1 - Fluorenes	3.92	0.028	N.D.	0.10 (0.0260	N.D.
C2 - Fluorenes	10.3	0.088 (0.001)	N.D.	0.38 (0.11)	N.D.
C3 - Fluorenes	13.1	0.14 (0.014)	N.D.	0.57 (0.059)	N.D.
Anthracene	N.D.	0.0052	N.D.	0.016	0.011
Phenanthrene	0.19	0.0082 (0.007)	0.011 (0.0008)	0.012 (0.0018)	0.012 (0.002)
C1 - Phenanthrenes/Anthracenes	2.58	0.054 (0.0064)	N.D.	0.16 (0.051)	0.032 (0.009)
C2 - Phenanthrenes/Anthracenes	9.83	0.14 (0.014)	N.D.	0.44 (0.1)	0.076 (0.01)
C3 - Phenanthrenes/Anthracenes	12	0.14 (0.017)	N.D.	0.47 (0.076)	0.18 (0.036)
C4 - Phenanthrenes/Anthracenes	7.46	N.D.	N.D.	0.21 (0.0049)	N.D.
Retene	0.194	N.D.	N.D.	N.D.	N.D.
Dibenzothiophene	0.0842	N.D.	N.D.	N.D.	N.D.
C1 - Dibenzothiophenes	N.D.	N.D.	N.D.	N.D.	N.D.
C2 - Dibenzothiophenes	1.79	N.D.	N.D.	N.D.	N.D.
C3 - Dibenzothiophenes	2.69	N.D.	N.D.	N.D.	N.D.
C4 - Dibenzothiophenes	3.37	N.D.	N.D.	N.D.	N.D.
Benzo(b)fluorene	0.0279	N.D.	N.D.	N.D.	N.D.
Fluoranthene	0.0292	0.0029	0.0037	N.D.	0.0036
Pyrene	0.0804	0.0041 (0.004)	N.D.	0.0071 (0.0019)	0.0036
C1 - Fluoranthenes/Pyrenes	1.04	N.D.	N.D.	0.050 (0.0091)	N.D.
C2 - Fluoranthenes/Pyrenes	1.88	N.D.	N.D.	0.079 (0.0076)	N.D.
C3 - Fluoranthenes/Pyrenes	2.94	N.D.	N.D.	0.17	N.D.
C4 - Fluoranthenes/Pyrenes	2.79	N.D.	N.D.	N.D.	N.D.
Naphthobenzothiophene	N.D.	N.D.	N.D.	N.D.	N.D.
C1 - Naphthobenzothiophenes	0.586	N.D.	N.D.	N.D.	N.D.
C2- Naphthobenzothiophenes	0.629	N.D.	N.D.	N.D.	N.D.
C3 - Naphthobenzothiophenes	N.D.	N.D.	N.D.	N.D.	N.D.
C4 - Naphthobenzothiophenes	N.D.	N.D.	N.D.	N.D.	N.D.
Benz(a)anthracene	0.030	0.0097 (0.0008)	0.0096 (0.001)	0.012 (0.0013)	0.011 (0.0019)
Chrysene	0.279	N.D.	0.0053	0.010 (0.0018)	N.D.
C1 - Chrysenes	1.8	0.022 (0.0028)	N.D.	0.067 (0.0079)	0.033 (0.0064)

C2 - Chrysenes	3.66	0.036 (0.0015)	N.D.	0.12 (0.013)	0.080 (0.014)
C3 - Chrysenes	2.8	N.D.	N.D.	0.068	N.D.
C4 - Chrysenes	2.36	N.D.	N.D.	N.D.	N.D.
Benzo(b)fluoranthene	0.050	0.0091 (0.0027)	0.0075 (0.0001)	0.010 (0.0049)	0.010 (0.00530
Benzo(k)fluoranthene	N.D.	N.D.	N.D.	N.D.	N.D.
Benzo(a)fluoranthene	N.D.	N.D.	N.D.	N.D.	N.D.
Benzo(e)pyrene	0.159	N.D.	N.D.	0.0071	N.D.
C30-Hopane	1.44	N.D.	N.D.	0.050 (0.0019)	0.044 (0.0037)
Benzo(a)pyrene	0.087	N.D.	N.D.	N.D.	N.D.
Perylene	N.D.	N.D.	N.D.	N.D.	N.D.
Indeno(1,2,3-cd)pyrene	N.D.	N.D.	N.D.	N.D.	N.D.
Dibenz(a,h)anthracene	N.D.	N.D.	N.D.	N.D.	N.D.
Benzo(g,h,i)perylene	N.D.	N.D.	N.D.	N.D.	N.D.
4-Methyldibenzothiophene	N.D.	N.D.	N.D.	N.D.	N.D.
2-Methyldibenzothiophene	N.D.	N.D.	N.D.	N.D.	N.D.
1-Methyldibenzothiophene	N.D.	N.D.	N.D.	N.D.	N.D.
3-Methylphenanthrene	0.59	0.013 (0.0011)	N.D.	0.038 (0.012)	0.0046 (0.0007)
2-Methylphenanthrene	0.69	0.016 (0.002)	N.D.	0.045 (0.012)	0.0050 (0.001)
2-Methylanthracene	N.D.	N.D.	N.D.	N.D.	N.D.
9-Methylphenanthrene	0.35	0.0080 (0.0012)	N.D.	0.023 (0.0062)	0.0062 (0.0004)
1-Methylphenanthrene	0.53	0.011 (0.0028)	N.D.	0.034 (0.0098)	0.0070 (0.0022)
2-Methylnaphthalene	0.892	0.013 (0.0004)	0.016 (0.0011)	0.031 (0.0013)	0.023 (0.0002)
1-Methylnaphthalene	0.469	0.0098 (0.0006)	0.011 (0.0004)	0.022 (0.0012)	0.013 (0.0011)
2,6-Dimethylnaphthalene	2.96	0.035 (0.0002)	0.0091 (0.11)	0.11 (0.0280	0.012
2,3,5-Trimethylnaphthalene	2.41	0.034 (0.0013)	N.D.	0.11 (0.031)	0.0056
Carbazole	0.17	N.D.	N.D.	N.D.	N.D.
Fluorene-d10	81	81.33 (1.53)	80 (1.73)	81 (1.0)	82.67 (2.89)
Fluoranthene-d10	105	102.33 (2.082)	102 (1.73)	103.33 (5.03)	103 (1.73)
Terphenyl-d14	87	99.33 (1.53)	101 (6.93)	97 (3.0)	99.33 (5.86)
ΣTOTAL PAH	1508.10	12.97 (1.0)	3.51 (0.18)	45.66 (6.39)	20.03 (5.05)
Σ50 PAH	141.97	1.29 (0.20)	0.11 (0.027)	4.89 (1.08)	0.70 (0.45)

Table S8- 11. Inorganic characterization of raw, undiluted and filtered 2 h post-stimulated hydraulic fracturing-generated flowback water (HF-FW) sample collected from a horizontal hydraulically fractured well. Measurement error was quantified as the deviation across four replicate measurements for each sample for ICP-MS/MS.

Chemical	**Detection Limit**	**2 hr HF-FW Sample**

	mM	mg/L	mM
Total Dissolved Solids (TDS)		142533	
Total Nitrogen (TN)		315.8	
Total Organic Carbon (TOC)		1346.6	
Cl	0.0011	81964.4	2311.9
Na (23)	0.014	44180.7	1921.7
Li (7)	9.5×10^{-5}	31.91	4.6
B (11)	0.0010	68.92	6.4
Mg (24)	0.0095	578.55	23.8
Al (27)	5.9×10^{-5}	BDL	BDL
Si (28)	0.0078	BDL	BDL
S (32 – 48)	0.0053	83.59	2.6
K (39)	0.0059	1469.3	37.6
Ca (40)	0.0082	6209.5	154.9
Mn (55)	2.7×10^{-5}	5.49	0.09
Fe (56)	0.0017	153.93	2.8
Fe He (56)	9.9×10^{-5}	140.71	2.4
Ni (60)	8.5×10^{-5}	BDL	BDL
Cu (63)	9.9×10^{-5}	BDL	BDL
Zn (66)	8.3×10^{-5}	BDL	BDL
As (75 – 91)	3.5×10^{-5}	BDL	BDL
Br (79)	7.8×10^{-4}	220.16	2.8
Sr (88)	2.2×10^{-4}	631.99	7.2
Cd (114)	1.5×10^{-6}	0.0104	9.3×10^{-5}
Ba (137)	1.5×10^{-6}	17.50	0.13
Pb (208)	1.0×10^{-6}	0.025	1.2×10^{-4}

Supplemental Figures

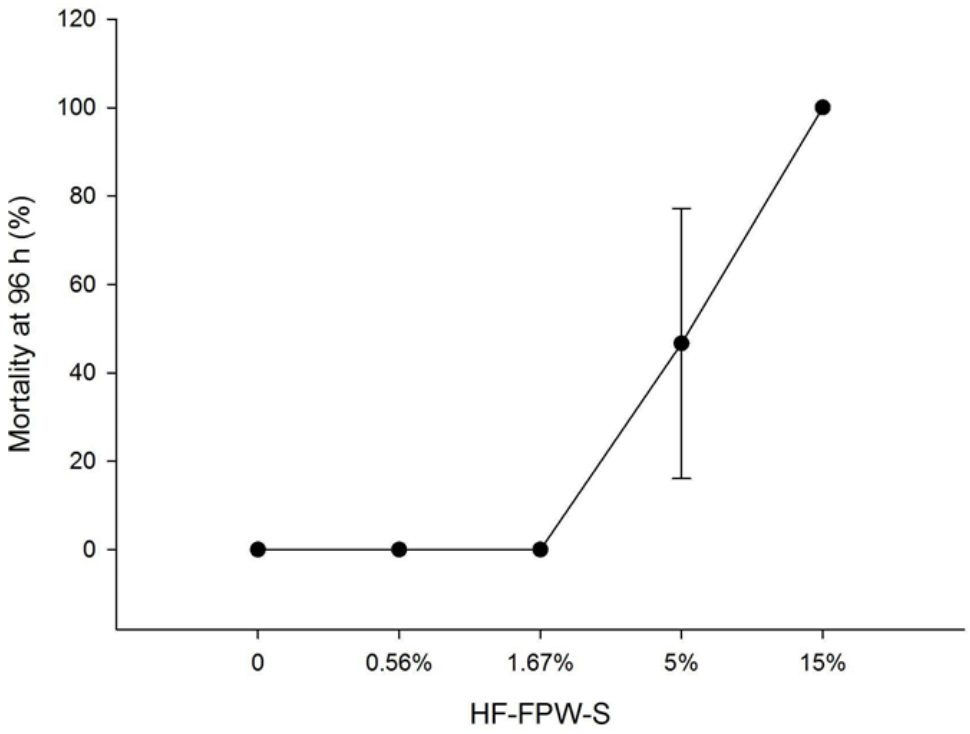

Figure S3- 1. Preliminary range finding test of HF-FPW-S using finger-length juvenile rainbow trout (96 h exposure). Each exposure concentration contained 3 groups with 5 individuals in each group. All fish in 15% groups were dead by 48 h. All fish in 5% groups were observed to be dead at the end of exposure (96 h), and no death was observed between 0 to 72 h in 5% group.

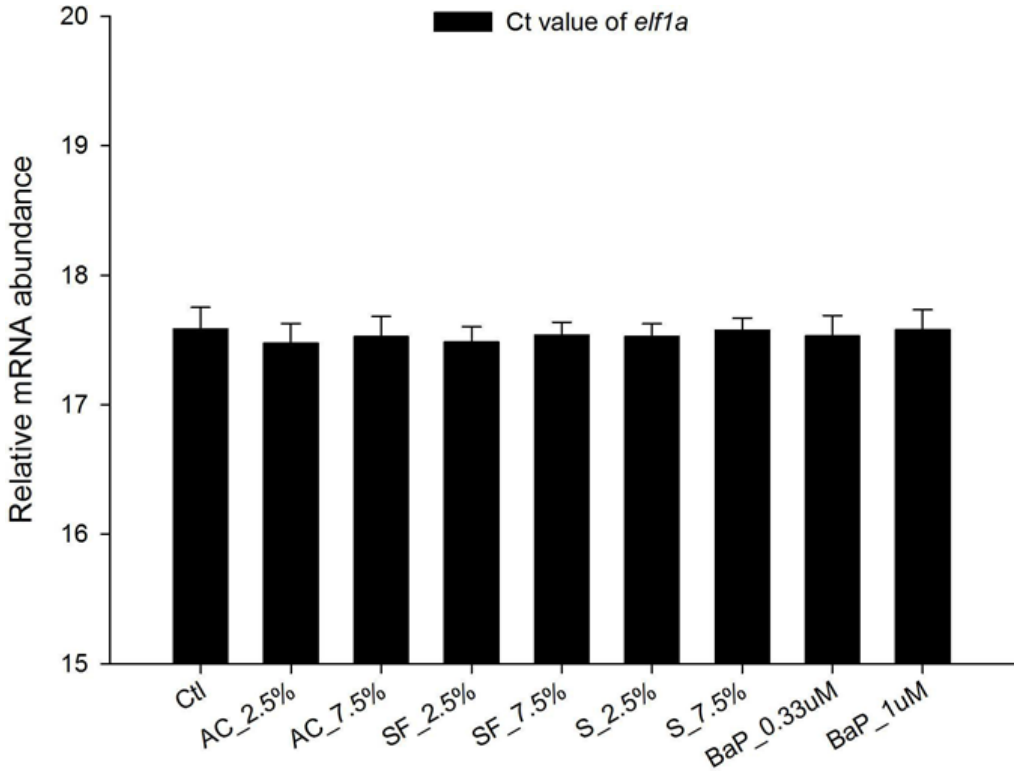

Figure S3- 2. The Ct values of *elf1a* in all control/treatment groups after 48 h exposure (*n* = 6). There was no significant difference among all the exposure groups, indicating *elf1a* is an excellent housekeeping gene in the current study.

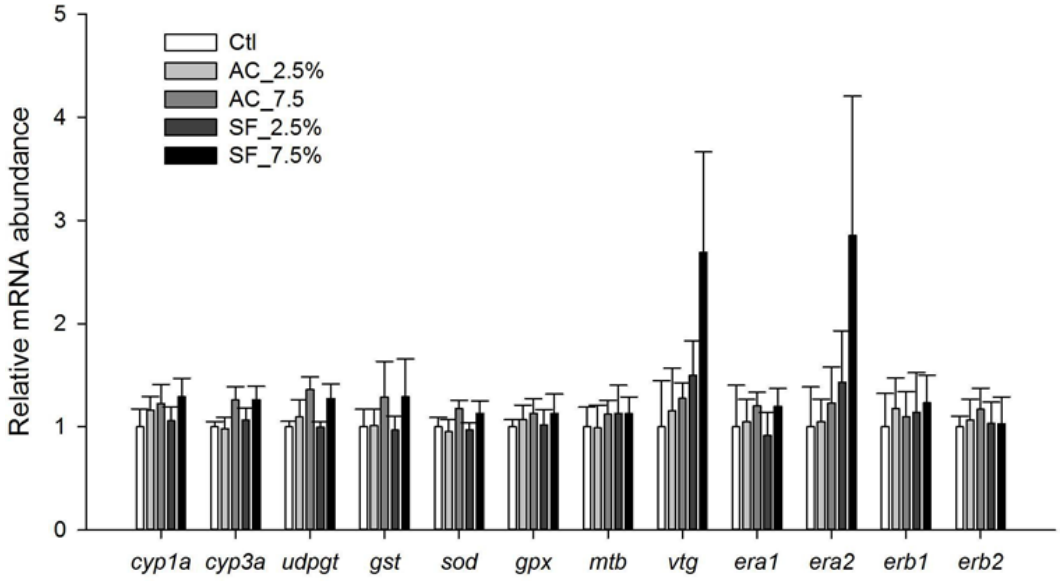

Figure S3- 3. The relative changes of hepatic mRNA expression of 11 genes after 48 h exposure of AC and SF (2.5% and 7.5%). There were no significant changes of mRNA abundance in all tested genes (n = 6, $\rho < 0.05$). Gene information can be found in Table S3-2.

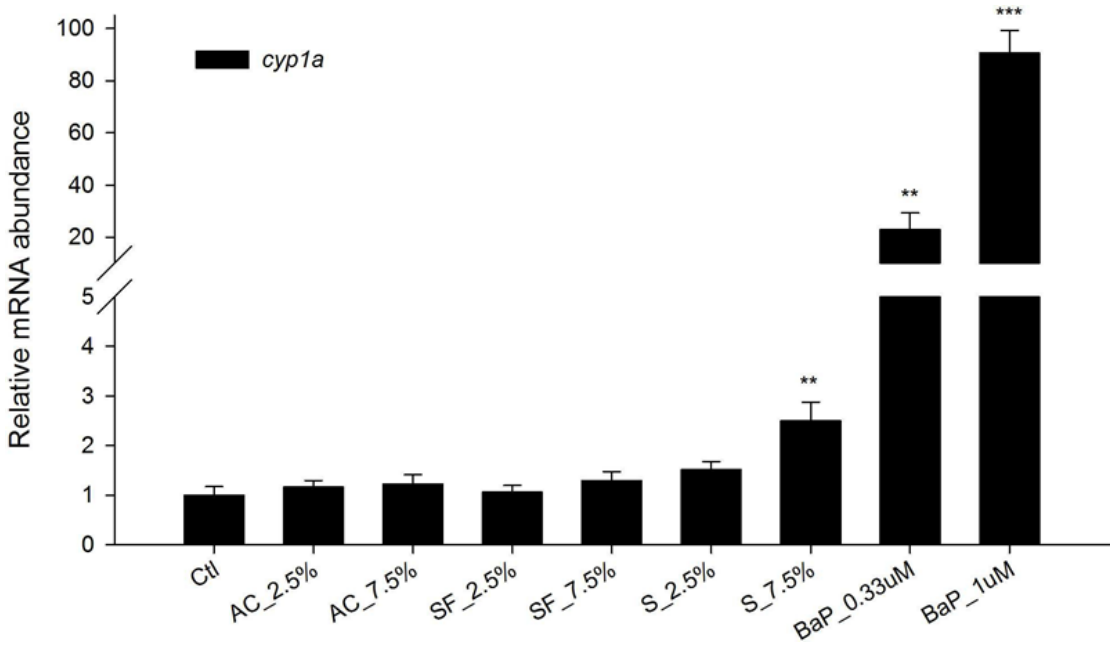

Figure S3- 4. The relative changes of hepatic mRNA expression of cyp1a with BaP (0.33 and 1 μM) used as positive control, resulting in 22.95±6.32 and 90.63±8.59-fold increase compared to the control, respectively. Asterisks (* $\rho < 0.05$; ** $\rho < 0.01$; *** $\rho < 0.001$; $n = 6$) indicate significant differences from control.

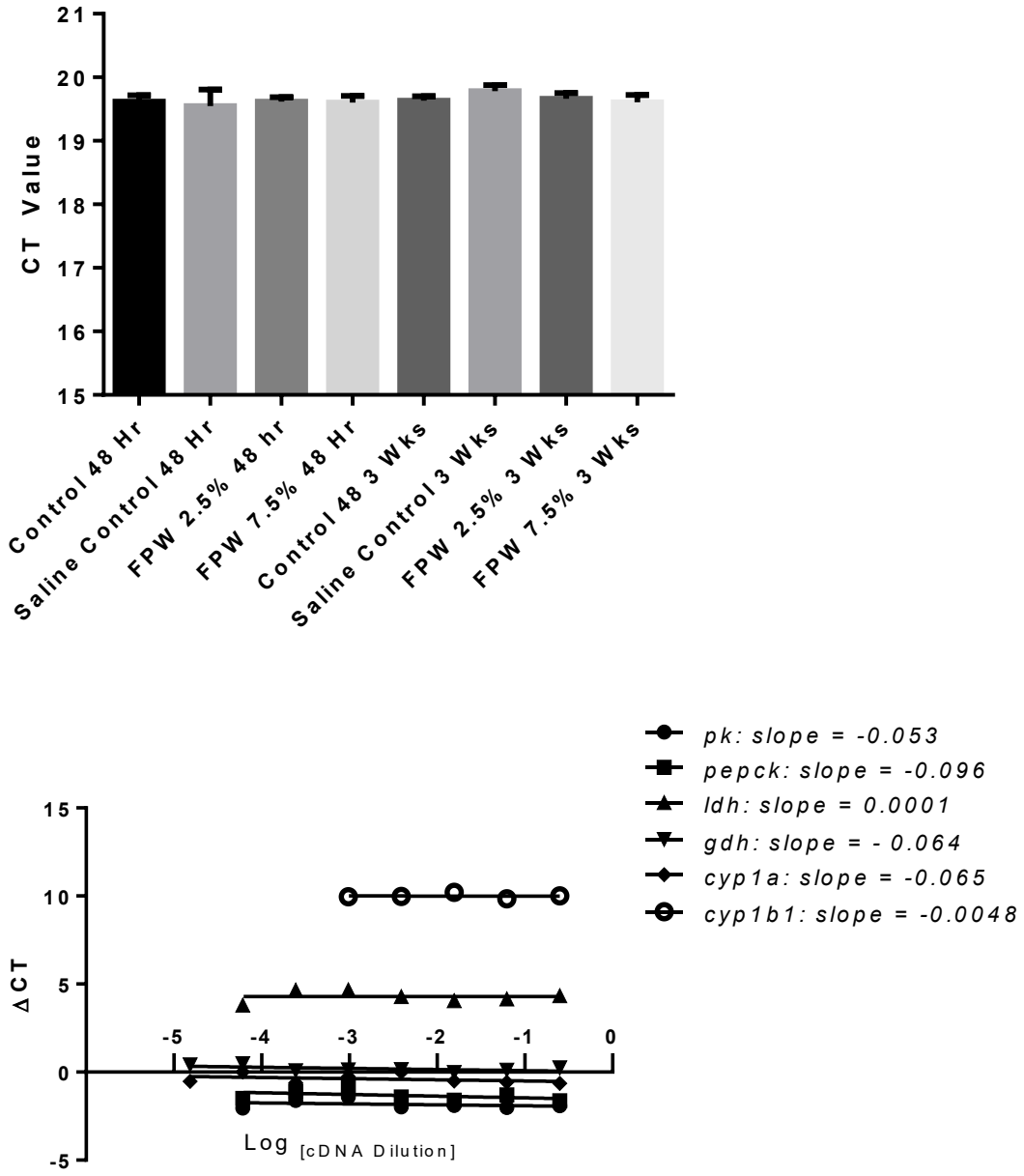

Figure S4- 1. Endogenous control gene (*tata12*) stability assay and primer efficiency validations for quantitative real-time PCR. A.) Endogenous control primer *tata12* CT values were determined in liver tissue from rainbow trout (Oncorhynchus mykiss) exposed to every treatment condition. If CT values between 2 or more treatment groups varied by more than an average of 0.5 CT, endogenous control gene was considered unsuitable for analysis. B.) Gene target primer set CT values were normalized to endogenous control primer set (*tata12*) CT values (ΔCT) across control rainbow trout liver cDNA dilutions.

Equivalent target and endogenous control primer efficiencies were determined by linear regression slope analysis, where slopes of 0 ± 0.1 were determined equally efficient and suitable for ΔΔCT analysis. Primer set efficiencies for cardiotoxic responsive genes *cyp1a*, *cyp1b1*, *pk, tbx5*, and *nppa*.

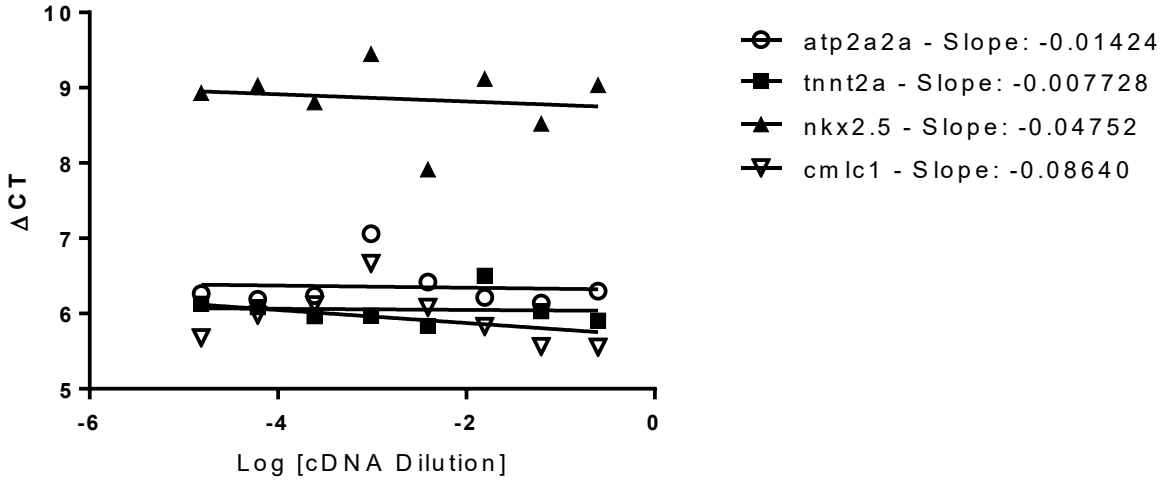

Figure S5- 1. Quantitative real-time PCR primer validations. Gene target primer set CT values were normalized to endogenous control primer set (*gapdh*) CT values (ΔCT) across control zebrafish cDNA dilutions of 5 orders of magnitude. Equivalent target and endogenous control primer efficiencies were determined by linear regression slope analysis, where slopes of 0 ± 0.1 were determined equally efficient and suitable for ΔΔCT analysis. Primer set efficiencies for cardiotoxic responsive genes *atp2a2a*, *tnnt2a*, *nkx2.5*, and *cmlc1*.

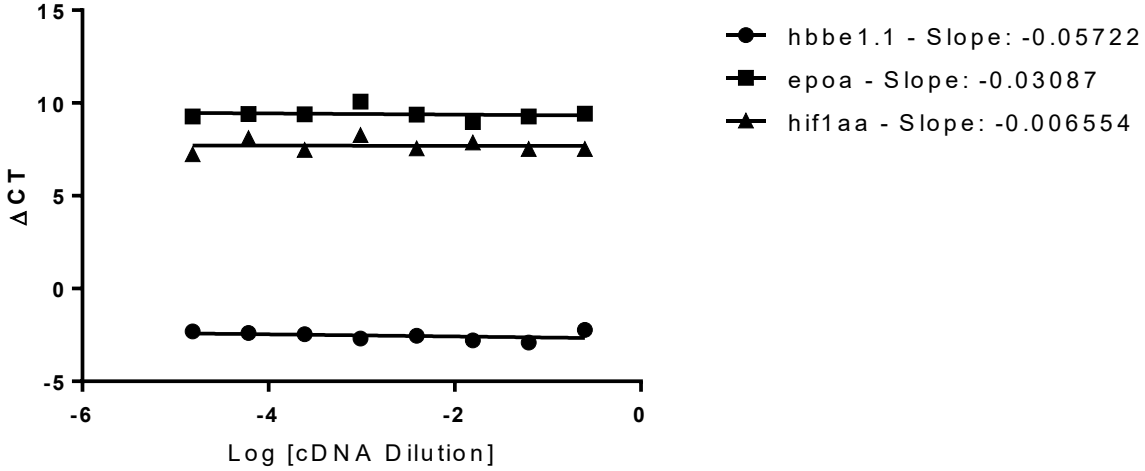

Figure S6- 1. Quantitative real-time PCR primer validations. Gene target primer set CT values were normalized to endogenous control primer set (*gapdh*) CT values (ΔCT) across control zebrafish cDNA dilutions of 5 orders of magnitude. Equivalent target and endogenous control primer efficiencies were determined by linear regression slope analysis, where slopes of 0 ± 0.1 were determined equally efficient and suitable for ΔΔCT analysis. Primer set efficiencies for hypoxia responsive genes *hbb1.1*, *epoa*, and *hif1aa*.

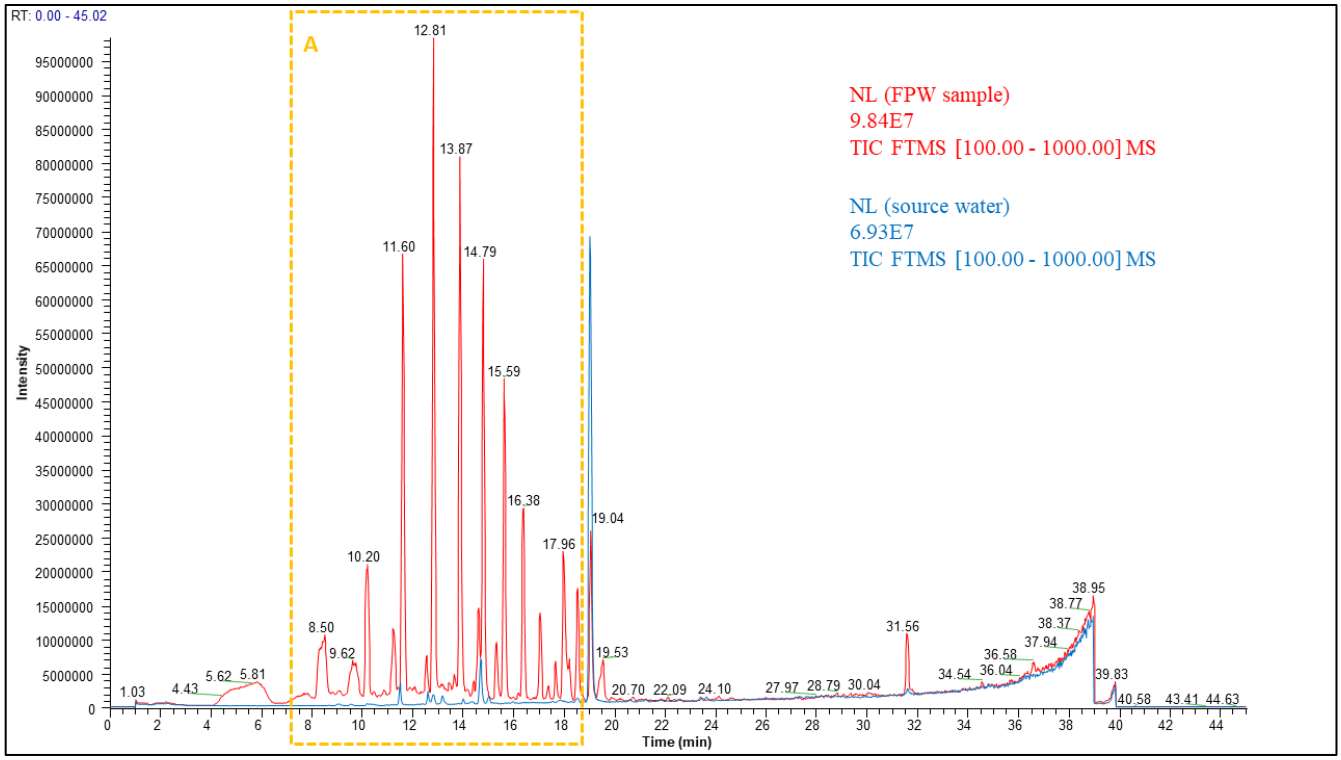

Figure S7- 1. HPLC-Orbitrap MS total ion chromatograms of a 4 h FPW sample and a corresponding source water. NL is the normalized total ion abundance. Box A covers a group of polyethylene glycols (PEGs).

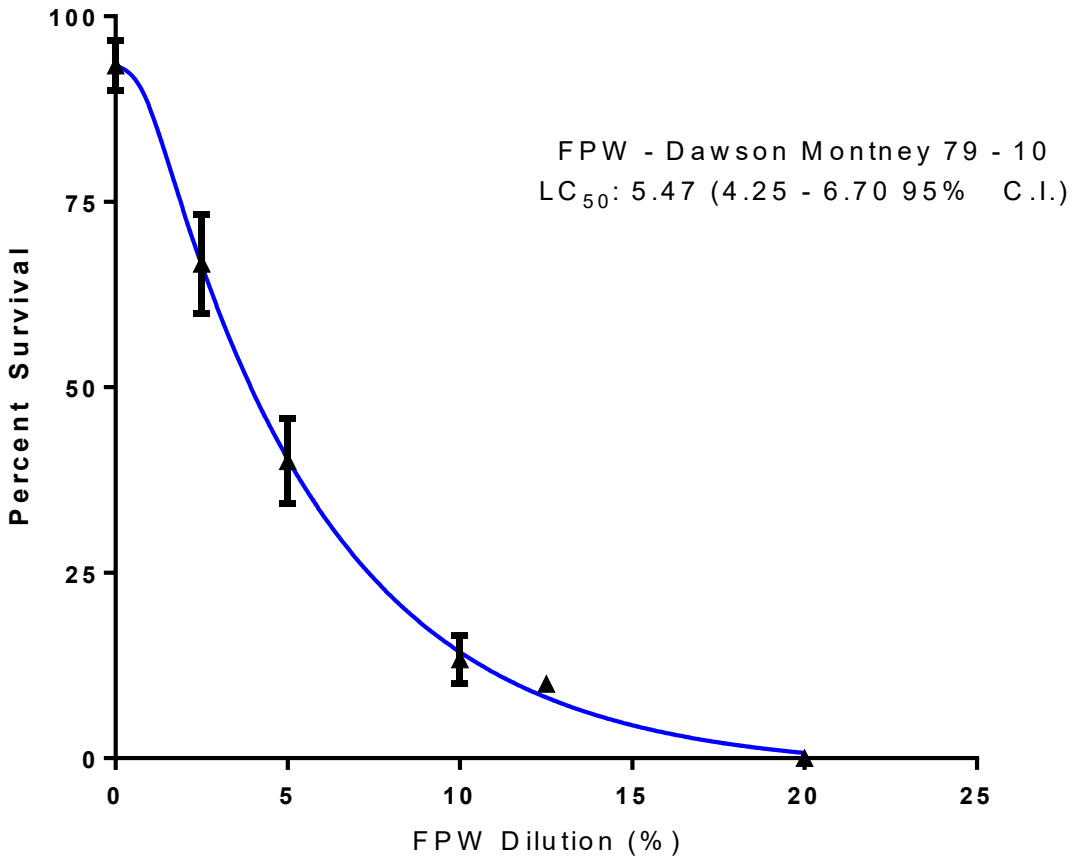

Figure S7- 2. Preliminary range finding test of a 4 h FPW sample (ID: 79 – 10) using ~ 5 dpf rainbow trout embryos (96 h exposure). Each exposure concentration contained 3 groups (n = 3) with 10 individuals in each group. All fish in 20% groups were dead by 48 h. Analysis was made using a three-parameter probit model (TRAP; Toxicity Relationship Analysis Program, v1.30a, U.S. EPA).

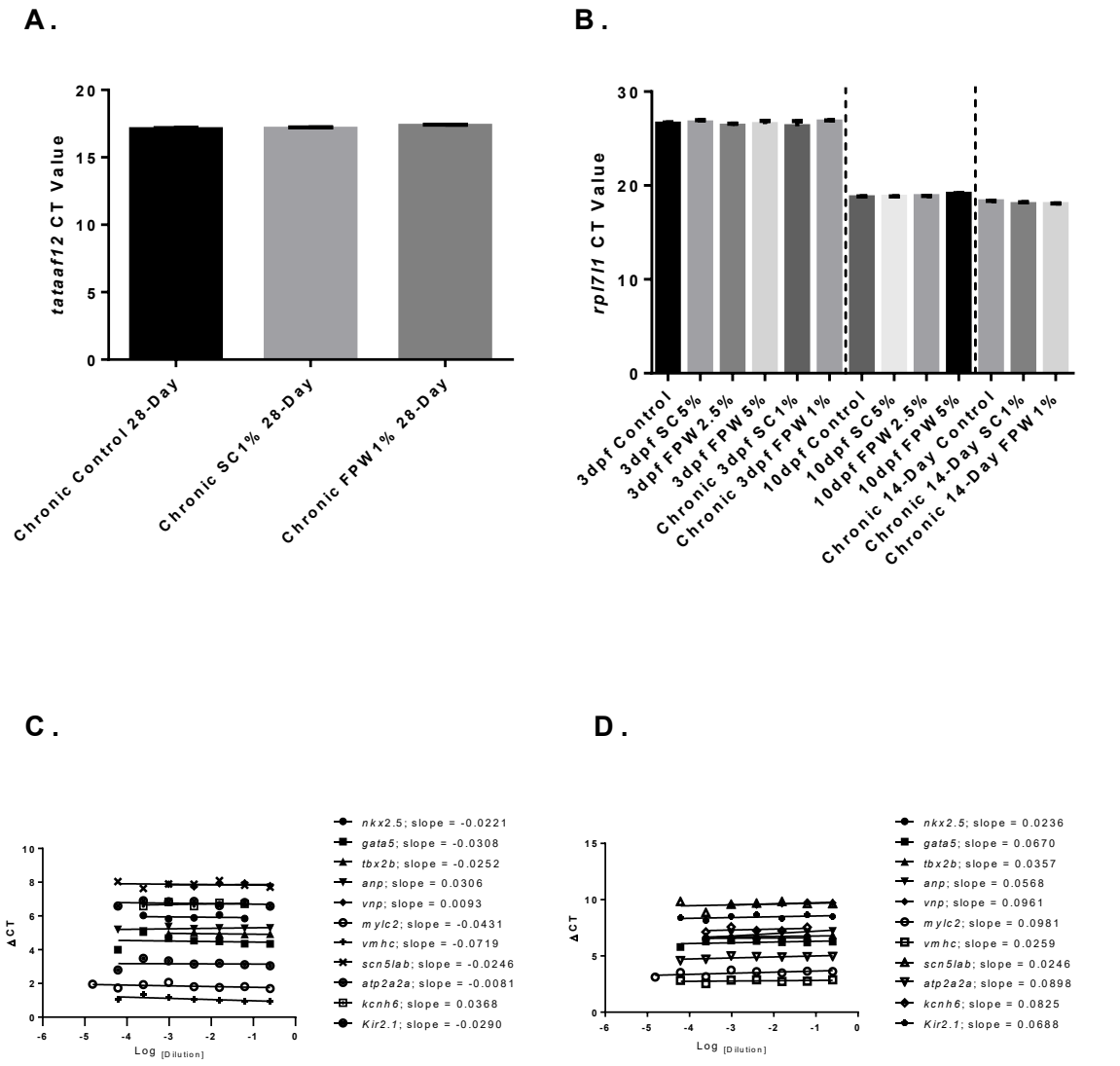

Figure S7- 3. Endogenous control gene stability assay and primer efficiency validations for quantitative real-time PCR. A.) Endogenous control primer *tataaf12* CT values were determined in rainbow trout (*Oncorhynchus mykiss*) embryos exposed to 28-daychronic treatment conditions. B.) Endogenous control primer *rpl7l1* CT values were determined in rainbow trout (*Oncorhynchus mykiss*) embryos exposed to every treatment condition except 28-day chronic exposures. If CT values between 2 or more treatment groups varied by more that 0.5 CT, specific endogenous control gene was considered unsuitable for analysis. C.) Gene target primer set CT values were normalized to endogenous control primer set (*tataaf1212* – panel C; *rpl7l1* – panel D) CT values (ΔCT) across control rainbow trout embryo cDNA dilutions of 5 orders of magnitude. Equivalent target and endogenous

control primer efficiencies were determined by linear regression slope analysis, where slopes of 0 ± 0.1 were determined equally efficient and suitable for ΔΔCT analysis. Primer set efficiencies for cardiotoxic responsive genes *nkx2.5*, *gata5*, *tbx2b*, *anp*, *vnp*, *mylc2*, *vmhc*, *scn6lab*, *atp2a2a*, *kcnh6*, and *kir2.1*.

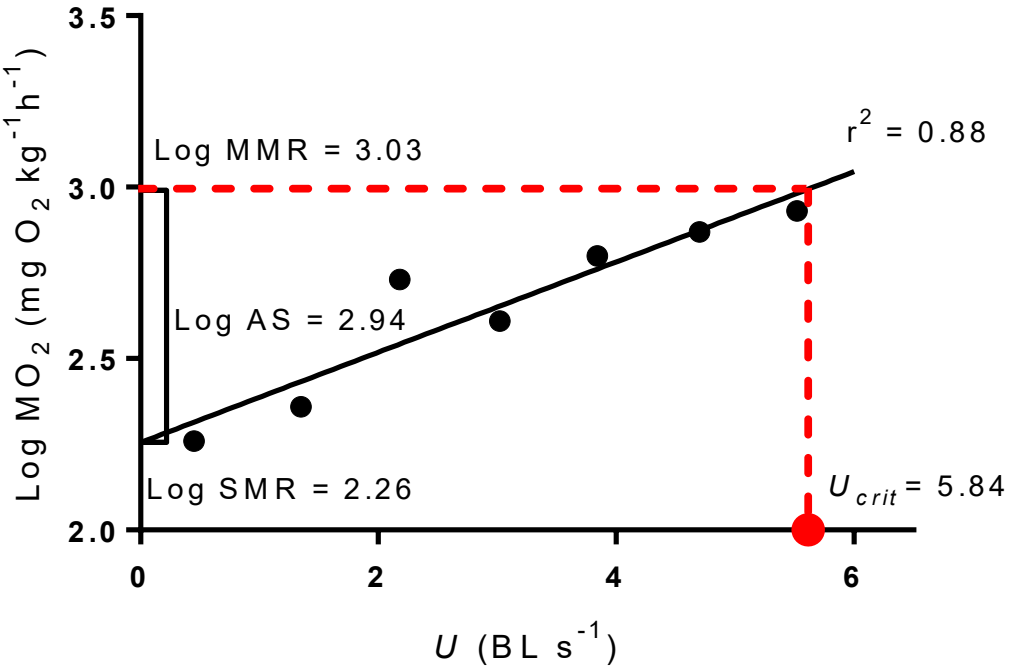

Figure S7- 4. An example least-squares linear regression analysis of metabolic rate versus swimming speed (*U*) from an individual juvenile rainbow trout (*Oncorhynchus mykiss*) during a swimming respirometry trial. SMR (y-intercept), MMR (extrapolated at *Ucrit*), and aerobic scope (AS; AS = MMR-SMR) were determined from the resulting linear regression equation. Regressions from fish yielding r^2 values ≤ 0.7 were not used for analyses. Resulting metabolic values were then normalized and scaled to a constant mass of 20 g using scaling factors derived in Figure S7-5.

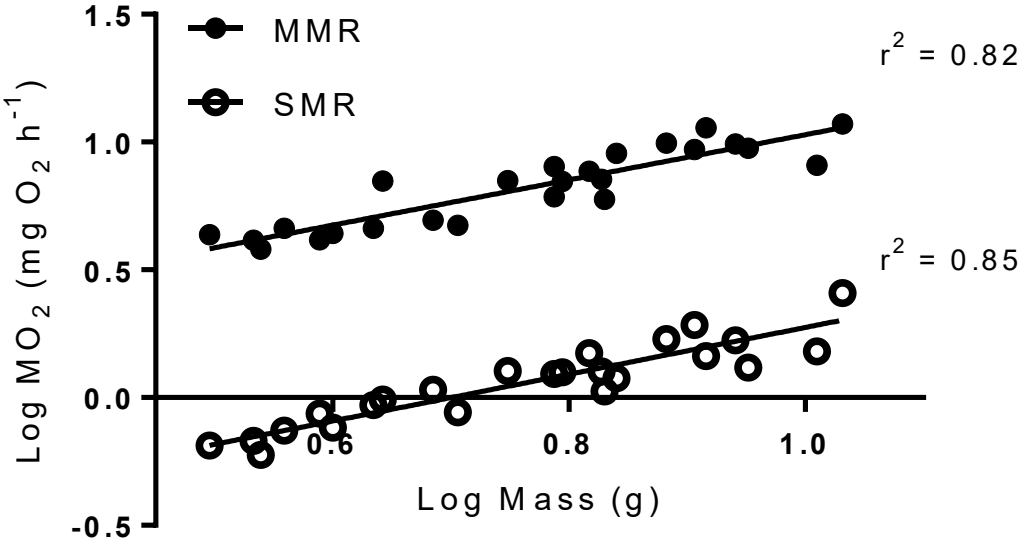

Figure S7- 5. Scaling factor derivation for juvenile rainbow trout (*Oncorhynchus mykiss*) SMR and MMR values. Metabolic rates of fish were normalized to a 5 g fish. Plotted log SMRs (open circles) and log MMRs (closed circles) against log fish mass (g) were used to calculate linear equations to determine SMR and MMR values. Corresponding SMR and MMR values for each individual were then calculated by first determining the SMR and MMR values for that specific fish' mass according to the calculated linear regressions and then adding the residual differences from each regression to the predicted SMR or MMR value for a 5 g representative fish. Only control fish were included in scaling determinations to eliminate exposure-type influences.

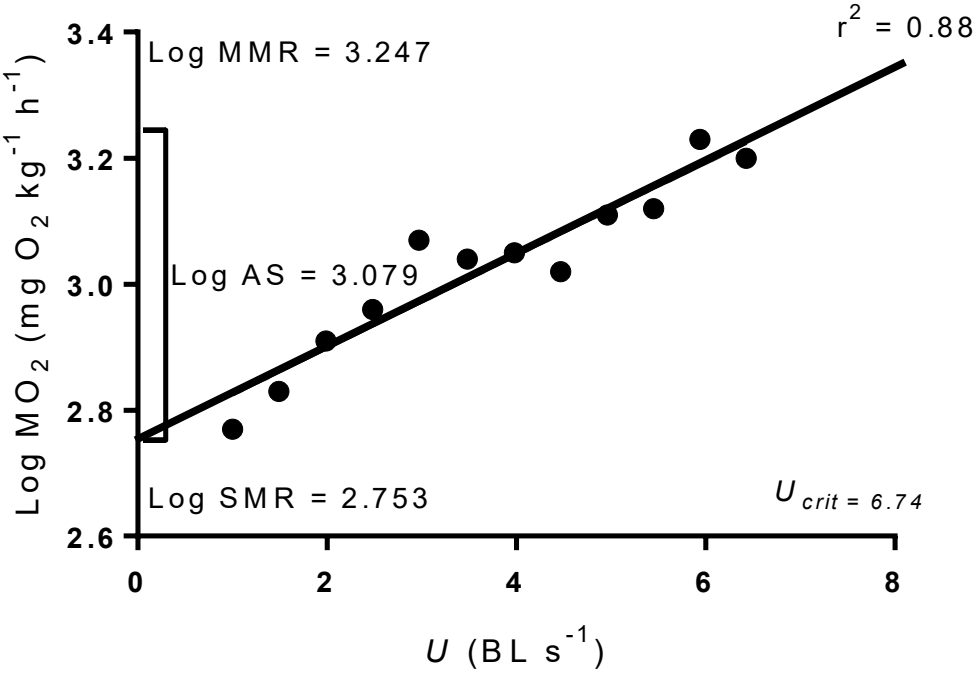

Figure S8- 1. An example least-squares linear regression analysis of metabolic rate versus swimming speed (*U*) from an individual juvenile mahi-mahi (*Coryphaena hippurus*) during a swimming respirometry trial. SMR (y-intercept), MMR (extrapolated at *Ucrit*), and aerobic scope (AS; AS = MMR-SMR) were determined from the resulting linear regression equation. Regressions from fish yielding r^2 values ≤ 0.7 were not used for analyses. Resulting metabolic values were then normalized and scaled to a constant mass of 20 g using scaling factors derived in Figure S8-2.

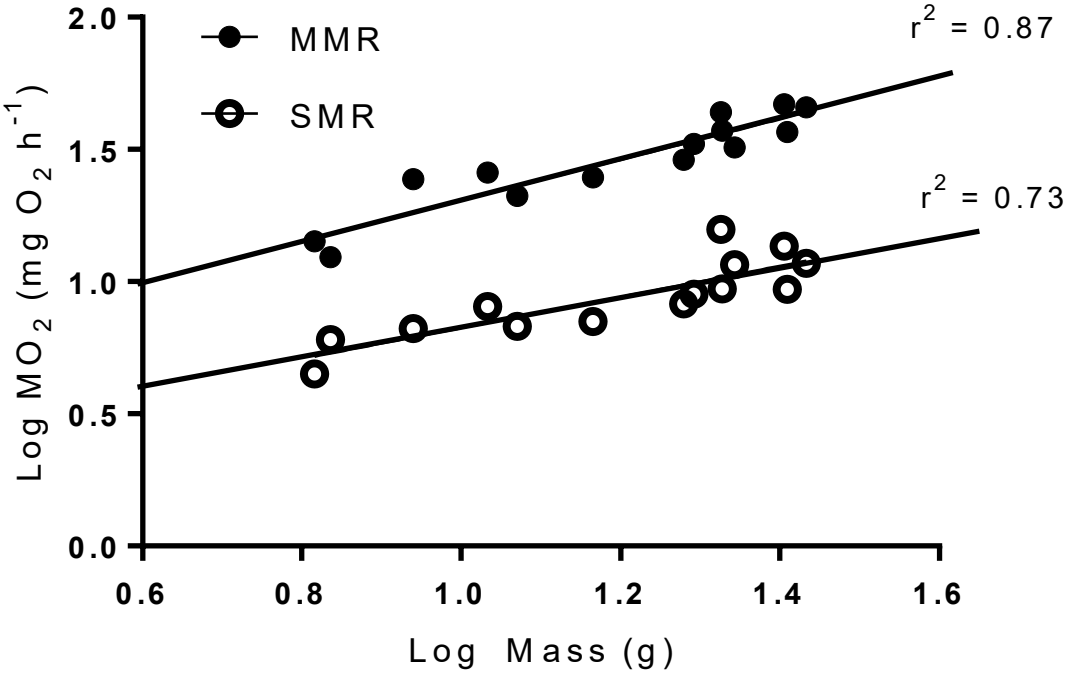

Figure S8- 2. Scaling factor derivation for juvenile mahi-mahi (*Coryphaena hippurus*) SMR and MMR values. Metabolic rates of fish were normalized to a 20 g fish. Plotted log SMRs (open circles) and log MMRs (closed circles) against log fish mass (g) were used to calculate linear equations to determine SMR and MMR values. Corresponding SMR and MMR values for each individual were then calculated by first determining the SMR and MMR values for that specific fish' mass according to the calculated linear regressions and then adding the residual differences from each regression to the predicted SMR or MMR value for a 20 g representative fish. Only control fish were included in scaling determinations to eliminate exposure-type influences.

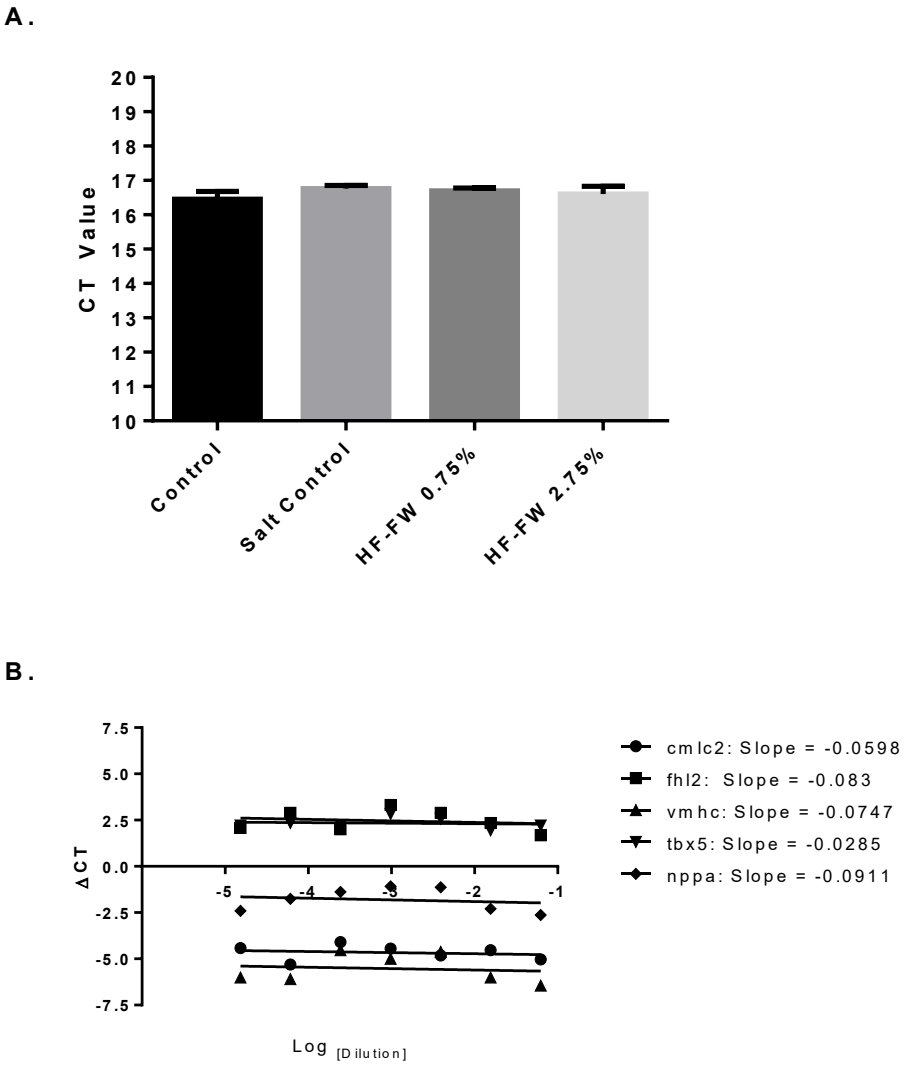

Figure S8- 3. Endogenous control gene (*rps25*) stability assay and primer efficiency validations for quantitative real-time PCR. A.) Endogenous control primer *rps25* CT values were determined in ventricle tissue from mahi exposed to every treatment condition. If CT values between 2 or more treatment groups varied by more that 0.5 CT, endogenous control gene was considered unsuitable for analysis. B.) Gene target primer set CT values were normalized to endogenous control primer set (*rps25*) CT values (ΔCT) across control mahi ventricle cDNA dilutions of 5 orders of magnitude. Equivalent target and endogenous control primer efficiencies were determined by linear regression slope analysis, where slopes of 0 ± 0.1 were determined equally efficient and suitable for ΔΔCT analysis. Primer set efficiencies for cardiotoxic responsive genes *cmlc2*, *fhl2*, *vmhc*, *tbx5*, and *nppa*.

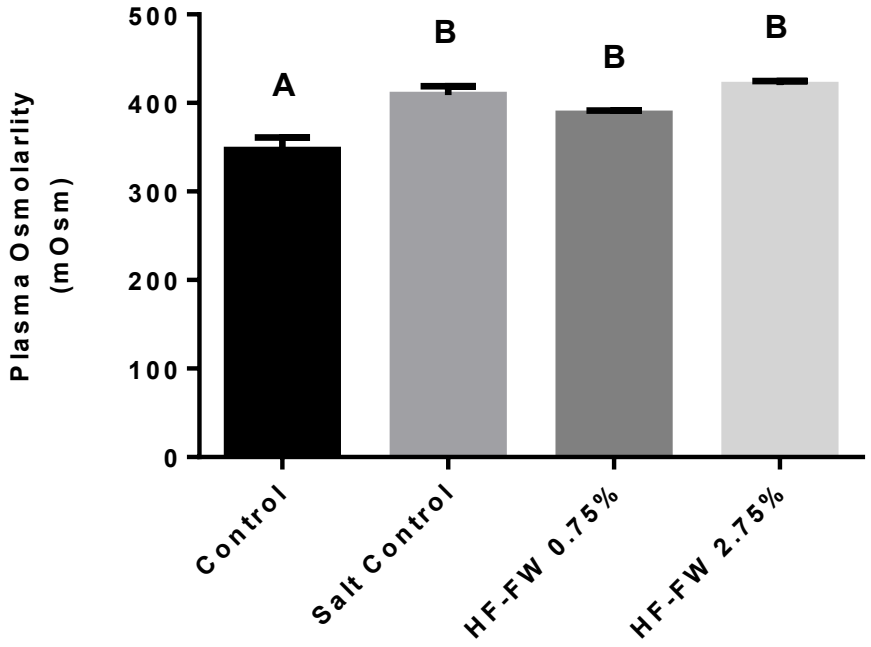

Figure S8- 4. Mahi plasma osmolarity measurements following 24 hr exposures to control, salt control, HF-FW 0.75%, and HF-FW 2.75% (n = 4 - 5). Data presented as means ± SEM. Significant differences determined by a 1-way ANOVA followed by a Holm-Sidak post-hoc analysis ($p < 0.05$).

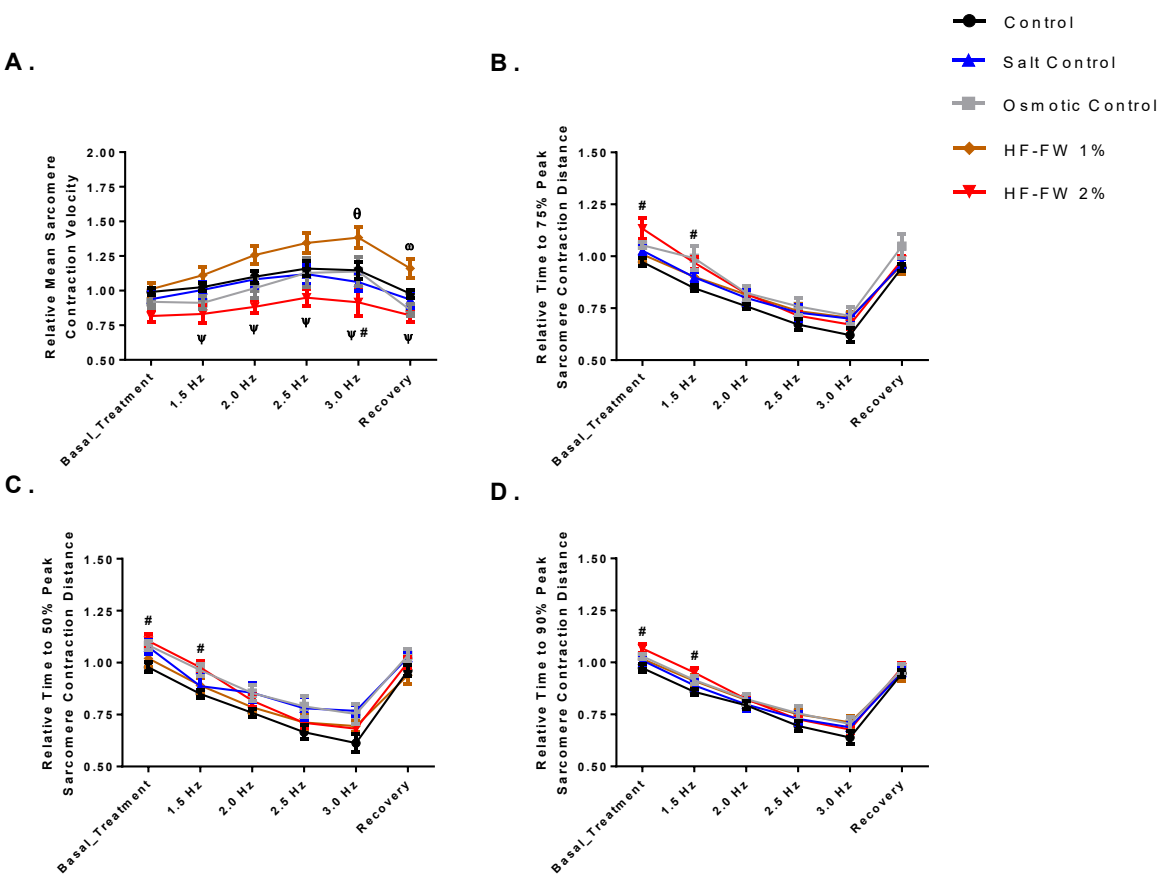

Figure S8- 5. Impacts to *mahi-mahi* cardiomyocyte sarcomere contractility properties following HF-FW exposure. Data points represent contractile indices at specified frequencies and treatments compared to basal frequencies (0.5 Hz) of stimulation under control conditions for the same cell prior to solution exposure switches. A.) Relative speed of sarcomere contraction from relaxed baseline states to peak contracted states. B - D.) Relative times for sarcomeres to go from relaxed baseline states to 50%, 75%, and 95% peak contracted states, respectively. # - indicates significant differences between HF-FW 2% and control treatment groups only. ψ - indicates significant differences between HF-FW 2% and HF-FW 1% treatment groups only. ω - indicates significant differences between HF-FW 1% and osmotic control treatment groups only. θ – indicates significant differences between HF-FW 1% and salt control (SC) treatment groups only. Significant differences determined following a Holm-Sidak post-hoc analysis ($p < 0.05$). Data are presented as mean ± SEM (n = 12, 8, 12, 8, and 13 cells for control, salt control, osmotic control, HF-FW 1%, and HF-FW 2% exposures, respectively).

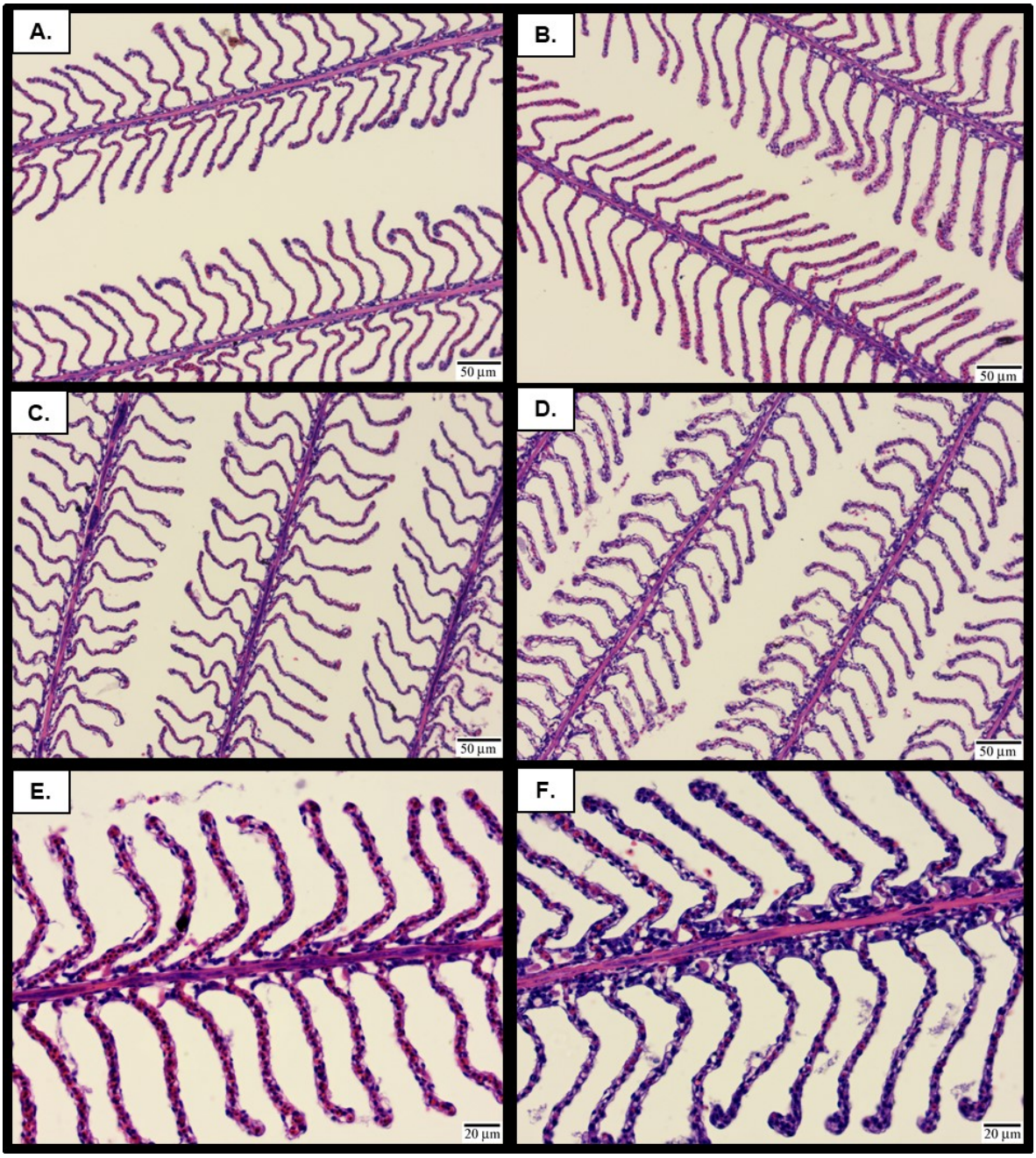

Figure S8- 6. Hematoxylin and eosin (H&E) gill histological sections at 20x magnification from juvenile *mahi-mahi* exposed to control (A.), salt control (B.), HF-FW 0.75% (C.), or HF-FW 2.75% (D.) treatments for 24 hrs. Gill sections at 40x magnification for control (E.) and HF-FW 2.75% (F.) are also shown to further support the notion that gross gill morphology across treatment groups does not appear to be affected.

Supplemental Text

Appendix 3-1. Chemicals

1,1,3,3 – tetraethoxypropane, 2-deoxy-d-ribose, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 7-ethoxyresorufin (7-ER), benzo(a)pyrene (BaP), butylated hydroxytoluene (BHT), $CaCl_2 \cdot 2H_2O$, dicumarol, dimethyl sulfoxide (DMSO), ethylenediaminetetraacetic acid (EDTA), glucose, $MgSO_4 \cdot 7H_2O$, Nicotinamide adenine dinucleotide phosphate (NADPH), KCl, KH_2PO_4, NaCl, $NaHPO_4 \cdot H_2O$, sodium salt of resorufin, thiobarbituric acid, trichloroacetic acid, and Tris-HCl were all obtained from Sigma-Aldrich (USA). BCA Protein Assay Kit was obtained from Thermo Scientific (USA). Twelve-well tissue culture plates were obtained from Corning (Corning, NY), and 96-well viewplates were obtained from Nunc (USA). BaP, 7-ER, sodium salt of resorufin, and dicumarol were dissolved in DMSO with proper concentrations. The concentration of DMSO in any treatment/assay solution was never above 0.1%.

Appendix 3-2. PAHs Analysis

HF-FPW subsamples (500 mL) were analyzed for polycyclic aromatic compounds by a liquid-liquid extraction method. Analytes included parent polycyclic aromatic hydrocarbons (PAHs), and various alkyl-PAHs, including dibenzothiophene (DBT) and retene. An internal standard mixture (10 ng of each) was spiked into each sample, and shaken for 3 minutes; then 50 mL dichloromethane was added and shaken for 3 minutes. The dichloromethane extract was removed and another 50 mL dichloromethane was added to extract again. The dichloromethane extracts were combined (100 mL) and concentrated to about 5 mL by nitrogen gas evaporation. Afterward, precleaned copper powder and anhydrous sodium sulfate were vortexed with the extracts, and the extracts are transferred into a new tube, and continued to concentrate to about 1 mL. Silica solid phase extraction cartridges (Waters, 1g/6cc) were pre-conditioned with 5 mL hexane, then the 1 mL extract was loaded, washed with 4 mL hexane. PAHs were eluted with 5 mL of hexane/dichloromethane 4:1 (v/v), concentrated to 0.2 mL, taken up in 3 mL hexane and concentrated to 0.2 mL which was transferred to vials with glass inserts. These vials were centrifuged and 50 µL of supernatant was transferred into new glass inserts for GC-MS

analysis. GC-MS analysis was by splitless injection (1 μL) and chromatographic separation was performed on DB-5ms (Agilent; 20 m × 0.18 mm × 0.18 μm) with a helium flow of 1.8 mL/min. Targeted analytes were analyzed in single ion monitoring mode. The method detection limit (MDL) was defined as the concentration with a signal-to-noise ratio of 3 if the specific PAH was not detected in the blanks. For analytes detected in blanks, MDL was defined as the mean blank concentration plus three times the standard deviation of the blank. The information on target ions, qualifier ions and internal standards used for each analyte, and MDLs are shown in the Table S3-1.

Appendix 3-3. Hepatic EROD assay

Hepatic EROD activity assay was initiated by homogenizing fish liver tissue (approximately 0.05 g) in ice-cold KCl-HEPES buffer (0.15 M KCl, 0.02 M HEPES, pH 7.5) using a Potter-Elvehjem homogenizer. The homogenate was centrifuged at 10,000 g (4 °C) for 20 min, and the supernatant was collected as S-9 fraction which was directly used in EROD assay. 40 μL cold S-9 hepatic fractions were added to a 96-well plate, followed by 200 μL cold EROD buffer (0.1 M Tris-HCl, 1 mM EDTA, 2.5 μM 7-ethoxyresorufin (7-ER), pH 7.4). Reaction initiation by addition of 10 μL fresh NADPH solution (5 mM, dissolved in nanopure water) occurred after all samples and a resorufin standard series (0-1000 pM) were loaded. Incubation occurred at room temperature in the dark with gentle shaking for 20 min. Following incubation, fluorescence was measured at 535 and 590 nm (excitation and emission, respectively) using Victor3V 1420 Multilabel Counter (Perkin Elmer, MA). EROD activity was normalized to S-9 fraction protein content using a BCA protein assay kit (Thermoscientific, USA) and signal activity read as picomole of resorufin per mg protein per minute. Values are expressed as relative fold change to control.

Appendix 3-4. Branchial EROD assay

The gill-filament-based EROD assay was performed by incubation of the tips of fish gill filament in 7-ER reaction buffer. Briefly, gill arches were excised, gently washed and immersed in ice-cold HEPES-Cortland (HC) buffer (0.38 g KCl, 7.74 g NaCl, 0.23 g $MgSO4 \cdot 7H2O$, 0.23 g $CaCl2 \cdot 2H2O$, 0.41 g $NaHPO4 \cdot H2O$, 1.43 g HEPES, 1 g glucose per 1 L nanopure water, pH 7.7). Tips of filaments (2 mm) were carefully cut and selected by

comparing with a standard. Duplicate groups of 10 filament tips were transferred using a Pasteur pipet to a 12-well tissue culture plate (Corning, NY) containing HC buffer. The HC buffer was replaced with 500 µL reaction buffer (HC buffer supplemented with 1 µM 7-ER and 10 µM dicumarol). After 10 min pre-incubation at room temperature, 700 µL fresh reaction buffer was substituted. After 30 min of incubation, 200 µL aliquots from each well were transferred to a 96-well viewplate for fluorescence measurement as described above.

Appendix 3-5. TBARS assay

TBARS assay using fish tissues was initiated by homogenizing tissue samples in phosphate buffer followed by centrifuging at 1,000 g for 1 min. 130 µL homogenate supernatant was diluted with 455 µL phosphate buffer. 32.5 µL BHT (1 mM) was added to each diluted sample to prevent further oxidation of samples, followed by 162.5 µL TCA (50%) to precipitate out protein. Samples were centrifuged at 13,000 g for 2 min and 120 µL supernatant transferred to a 96-well microplate, followed by 75 µL thiobarbituric acid (1.3%, dissolved in 0.3% NaOH). The microplate was incubated at 80°C for 60 min and fluorescence was recorded at 531 and 572 nm (excitation and emission respectively). A standard curve was generated using 1,1,3,3 – tetraethoxypropane (0 - 25 µM) on the same plate (Sigma, USA). Total protein concentration present in the sample homogenate supernatants were determined by BCA Protein Assay Kit using the manufacturer's protocol (Thermo Scientific, USA). Dilutions of sample homogenate supernatants were made accordingly to yield approximately equal protein content (± 1000 µg/mL) amongst samples prior to phosphate buffer dilution step.

Intrinsic oxidative potentials of HF-FPW fractions (water samples alone) were also determined via direct incubation of substrate with HF-FPW fractions. Briefly, a final concentration of 4 mM 2-deoxy-d-ribose (2-DR) was spiked in 5mL HF-FPW fractions at low (2.5%) and high (7.5%) dilutions in a 12-well plate (Corning, New York, USA). After 1 hour incubations at 25°C under ambient room light, 130 µL samples were analyzed for TBARS formation using the protocol detailed earlier.

Appendix 3-6. Quantitative Real-Time PCR assay

Total RNA was extracted from liver samples by use of NucleoSpin RNA Mini Kit according to the manufacturer's protocol (Macheret-Nagel, Düren, Germany). Purified RNA was quantified by use of a NanoDrop ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE). First-strand cDNA synthesized from 600 ng of total RNA using Thermo Scientific RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, ON, Canada) according to the manufacturer's instructions were stored at −80 °C until further analysis.

Quantitative Real-time PCR (Q-RT-PCR) was performed on an ABI 7500 Real-Time PCR System in 96-well PCR plates (Applied Biosystems, CA). Single PCR reaction mixtures contained 5 µL SYBR Green master mix (Applied Biosystems), 2.5 µL sense/antisense gene- specific primers (Integrated DNA Technologies, IA), and 2.5 µL cDNA diluted in RNase-free water (Qiagen, Venlo, Netherlands). PCR reactions were denatured at 95 °C for 2 min followed by 40 thermal cycles characterized by 15 s at 95 °C and annealing and extension for 1 min at 60 °C. Dissociation curves analyses were performed after amplification to ensure single product outcomes. Efficiency, uniformity, and linear dynamic range of each qPCR assay were assessed by construction of standard curves by use of serially diluted cDNA standards. Eleven genes representing Phase I and II biotransformation, oxidative stress, and endocrine disruption in rainbow trout were selected for screening. Changes in abundances of transcripts of target genes were quantified by normalizing to elongation factor 1a (*elf1a*). There was no difference in the expression of elf1a among all the exposure groups (Figure S3-2). Gene name, abbreviation, sequences of primers, efficiency, and GeneBank reference number are listed in Table S3-2.

Appendix 4-1. Inductively Coupled Plasma (ICP) -MS/MS Analysis

ICP-MS/MS analysis was used to quantify the concentration of cations, bromide, and total sulfur in raw, filtered HF-FW (SI Table 1). Before analysis, all samples were filtered through a 0.2 µm nylon filter membrane and diluted by a factor of 850 for Na analysis and 85 for all other elements with 18 MΩ·cm ultrapure water. Diluted 10 ml samples were then acidified at a rate of 12 µL using 15.698 N trace metal grade nitric acid per 10 mL of sample. For analysis, an Agilent 8800 Triple Quadrupole ICP-MS/MS (ICP-

MS/MS) was used with a RF power of 1550 W, a RF reflected power of 18 W, a microMist nebulizer and nickel/copper cones. Samples and external standards were analysed in high matrix mode, in which samples were diluted inline with 8 mL/min argon. Additionally, analysis was performed in MS/MS mode for greater mass resolution and a collision gas reaction cell was used with He gas (3 mL/min), O_2 gas (10% max flow) or H_2 (5 mL/min) gas to overcome matrix interferences (Table S4-3). Instrumental drift was accounted for using 0.5 ppm solution of indium, which was added to each sample using an inline internal standard addition system. For quality assurance and control, a standard solution was run at the start, middle, and end of each run (every 10 sample for $n = 3$) to quantify the run precision and percent recovery for each element (Table S4-3).

Appendix 4-2. Anion, Total Organic Carbon and Total Nitrogen Analysis

Ion chromatography was used to determine the concentration of chloride (Table S4-1). Before analysis, samples were filtered through a 0.2 µm nylon filter membrane and diluted by a factor of 2000 using 18 MΩ·cm ultrapure water. Analysis was then performed on a Dionex Ion chromatography DX 600 with a 4mm analytical column (AS9-HC), guard column (AG9-HC), and a 4 mm ASRS Ultra suppressor. For total non-purgeable organic carbon (TOC) and total nitrogen (TN) a subsample of the filter sample was diluted by a factor of five and analyzed using on a TOC analyser (Shimadzu model TOC-V-CHS/CSN). For quality assurance and control for IC a control standard solution was run every five samples (n=4) to quantify the run precision and percent recovery for each element (Table S4-4).

Appendix 4-3. Extraction of Organics

For the FPW sample, 100 mL was vacuum filtered through a glass fiber membrane (90 mm diameter, pore size: 0.4 µm) and spiked with an internal standard mix (10 ng of each, Wellington Laboratories, ON, CAN). Aqueous filtrates were then freeze-dried for 48 h and reconstituted in 3 mL hexane for further cleanup.

Accelerated solvent extraction (ASE) was used for PAH extraction from particulates in the freeze-dried glass filters sediments. A different, separate glass fiber filter was added at the bottom of the extraction cell, and ~ 2-3 g of florisil (pre-cleaned by DCM)

was added, followed by dried sediment filters, to which the internal standard mix was then spiked (10 ng of each). ASE cells were filled with solvent (hexane/DCM 4:1 v:v; Optima™, Fisher Scientific, NH, USA), pressurized to 14 MPa, and heated to 80 °C within 6 min. Pressure and temperature were held for 5 min (static extraction), followed by rinsing with cold solvent (50% of the cell volume) and purging with nitrogen gas for 90 s. This extraction cycle was repeated once more. Approximately 40 mL of total extract was gently concentrated by nitrogen gas evaporation and reconstituted in 3 mL of hexane for further cleanup.

Appendix 4-4. Polycyclic Aromatic Hydrocarbon (PAH) Analysis

In order to characterize and measure FPW sample PAHs, copper powder and anhydrous sodium sulfate (pre-cleaned with DCM) were added into the 3 mL extracts (Appendix 4-3) and vortexed. Solid phase extraction was used for cleanup of all the sample extracts. A Sep-Pak Silica 6 cc Vac cartridge (1 g; Waters, MA, USA) was conditioned with 5 mL solvent (hexane/DCM 7:3 v:v; Optima™, Fisher Scientific, NH, USA), followed by 5 mL of hexane. Extracts (3 mL) were loaded into cartridges and washed with 4 mL of hexane. PAHs were finally eluted with 5 mL of hexane/DCM 7:3 (v/v) which was subsequently concentrated to 200 μL for gas chromatography-mass spectrometry (GC-MS) analysis. Details of PAH standards, internal standard corrected recoveries of the detectable PAHs based on accelerated solvent extraction method (range from 60.0–114%) and liquid-liquid extraction (range from 82.9–179%), and the GC–MS instrumental method have been described in previous work (Zhang et al., 2016). Similarly, details of PAH analyte detection limits can be found in a previous study (He et al., 2018a).

Appendix 4-5. Hepatic Enzyme Assays

For all enzyme assays (unless otherwise noted), frozen liver tissue was pulverized under liquid nitrogen (Mommsen et al., 1980). Pulverized samples were homogenized in ice-cold homogenization buffer (50 mM imidazole, pH 7.4) or for the LDH assay, extraction buffer (20 mM HEPES, 1 mM EDTA, 0.1% Triton X-100, pH 7.0) and centrifuged (12, 000 *x* g, 4°C, 5 min). Aliquots (5 or 10 μL) were then plated in triplicate into 200 μL of assay buffer (see specifics below). Kinetic assays measuring the conversion

of NADH to NAD^+ were measured using a microplate spectrophotometer (Spectromax 190, Molecular Devices, Sunnyvale, CA, USA) in clear, flat-bottom 96-well microplates. Values are expressed as relative fold-change to control. All enzymatic activity was normalized to each respective sample's homogenate protein content using Bradford reagent as per manufacturer's instructions (MilliporeSigma, USA). Specifics pertaining to each kinetic assay procedure are listed below.

For lactate dehydrogenase (EC 1.1.1.27), homogenized sample aliquots were combined with 200 µL of 0.15 mM NADH and 0.2 mM sodium pyruvate prepared in LDH homogenization buffer (see above). Glutamate dehydrogenase (EC 1.4.1.3) activity was measured by adding sample to the following: 0.1 mM EDTA, 0.12 mM NADH, 1 mM ADP, 14 mM α-ketoglutarate, prepared in 250 mM ammonium acetate (pH 7.4). Negative control samples were run in tandem and consisted of the same cocktail without α-ketoglutarate. Pyruvate kinase (EC 2.7.1.40) activity was measured by combining a homogenized sample with 10 mM Mg Cl_2, 2.5 mM ADP, 0.15 mM NADH, 1U LDH, 10 mM phosphoenolpyruvate, dissolved in homogenization buffer. Kinetic assays were compared to negative controls that omit the substrate, phosphoenolpyruvate. Phosphoenolpyruvate carboxykinase (EC 4.1.1.32) activity rates were measured by combining homogenized sample with the following: 20 mM $NaHCO_3$, 1 mM $MnCl_2$, 1.1 mM NADH, 0.5 mM phophoenolpyruvic acid, 10U malate dehydrogenase, 0.2 mM deoxyguanosine diphosphate, prepared in homogenization buffer. Activity rates were compared to negative controls that did not contain deoxyguanosine diphosphate.

Hepatic EROD activity was measured *via* a modified protocol from (Hodson et al., 1991). Briefly, frozen trout liver tissue (0.25 mg) was homogenized in 1 mL of ice-cold homogenizing buffer (0.1 M $Na_2HPO_4 \cdot H_2O$, 0.15 M KCl, 1mM EDTA, 1mM dithothretiol, 10% (v/v) glycerol, pH 7.4) over ice. Crude homogenates were then centrifuged at 12,000 *x* g for 20 min at 4 °C to recover S9 fraction supernatants. Supernatants were collected carefully as to avoid disturbing pellets and floating lipid layers and then added to wells of a 96-black well, clear bottom plate in 40 µl volumes. Wells were then loaded with 200 µl of an EROD reaction solution (2.5 µM 7-ethoxyresorufin, 100 mM Tris-HCl, 1mM EDTA, pH 7.4) and background fluorescence was read at 535 nm excitation/590 nm emission using a Victor3V Multilabel Counter (Perkin Elmer, USA). Reaction initiation was

performed by adding 10 µl of fresh NADPH solution to wells (5 mM NADPH dissolved in MiliQ H_2O) after a resorufin standard series (0 – 1 nM) was loaded. The loaded plate was then incubated at room temperature in the dark for 20 min with gentle shaking. Following incubation, fluorescence was again read at 535 nm excitation/590 nm emission. EROD activity was normalized to S9 supernatant fraction protein content using Bradford reagent as per manufacturer's instructions (Sigma-Aldrich, USA), and signal activity was read as nanomoles of resorufin per mg protein per minute. Values are expressed as relative fold change to control.

Appendix 4-6. Quantitative Real-Time PCR

Total RNA was extracted and isolated from trout liver tissue (~ 0.5 mg) using 1mL of TRIzol according to manufacturer's protocols (Ambion Life Technologies, USA). Total RNA was then suspended in nuclease-free H_2O and quantified using a NanoDrop ND-1000 spectrophotometer (v. 3.8.1, NanoDrop Technologies, USA). To assess RNA integrity, quantified RNA samples were run on a 2% formaldehyde-agarose gel with ethidium bromide. Complimentary cDNA synthesis of 0.5 µg RNA samples was performed using SuperScriptIII Reverse Transcriptase (Invitrogen, USA) with a mix of olido(dT) and random hexamer primers. RNA isolation, quantification, integrity assessment, and cDNA synthesis all occurred on the same day to optimize both RNA and cDNA quality prior to freezing and storage at -80 °C.

Quantitative real-time PCR (qPCR) was performed in 384-well plates using an ABI QuantStudio™ 6 Flex Real-Time PCR system (Applied Biosystems, USA). Gene specific primers were designed against chosen trout liver detoxification and nutrient/energy regulating enzymes using Primer Express (v. 3.0.1, Thermofisher Scientific, USA) and Integrated DNA Technologies (USA) software. Primer gene targets, accession numbers, and sequences can be found in Table S4-7. Individual PCR reaction mixtures contained 2.5 µl of cDNA, 2.5 µl of 3.2 µM combined reverse and forward primer sets, and 5 µl of SYBR Green master mix (Applied Biosystems, USA). PCR reactions first underwent a 95 °C, 10 min denaturation step prior to thermal cycling initiation. A 2-step PCR thermal cycle, consisting of a denaturation step of 95 °C for 2 min and an annealing-extension step of 60 °C for 1 min, was employed for 40 cycles. Gene transcript expression changes were

determined by ΔΔCT analysis (Schmittgen and Livak, 2008) using TATA-box binding protein associated factor 12 (*tata12*) as an endogenous control. Endogenous control stability and primer efficiency validations, the latter determined by using serial slope calculations after ΔCT and log $cDNA_{[dilution]}$ input regression analyses, can be found in Figure S4-1.

Appendix 7-1. Inductively Coupled Plasma (ICP) – MS/MS Analysis

ICP-MS/MS analysis was used to quantify the concentration of cations, bromide, and total sulfur in raw, filtered HF-FW (Table S7-1). All samples for analysis were first were filtered through a 0.2 µm nylon filter membrane and diluted by a factor of 850 for Na analysis and 85 for all other elements with 18 MΩ·cm ultrapure 18 MΩ·cm water. 10 ml of each diluted sample was then acidified with 12 µL using 15.698 N trace metal grade nitric acid. For analysis, an Agilent 8800 Triple Quadrupole ICP-MS/MS (ICP-MS/MS) was used with a RF power of 1550 W, a RF reflected power of 18 W, a microMist nebulizer and nickel/copper cones. Under high matrix mode, samples and external standards were then diluted inline with 8 mL/min argon and analyzed. Analysis was performed in MS/MS mode for greater mass resolution while utilizing a collision gas reaction cell using He gas (3 mL/min), O_2 gas (10% max flow) or H_2 (5 mL/min) gas to overcome matrix interferences (Table S7-2.). Indium (0.5 ppm) was added to each sample using an inline internal standard addition system to account for instrument drift. Furthermore, a standard solution was run at the start, middle, and end of each run (every 10 sample for n = 3) to quantify the run precision and percent recovery for each element, and for quality assurance and control purposes (Table S7-2).

Appendix 7-2. Anion (Cl) Analysis

Ion chromatography was used to determine the concentration of chloride (Table S7-1). Before analysis, a 0.2 µm nylon filter membrane was used to filter samples, which were then diluted by a factor of 2000 using 18 MΩ·cm ultrapure water. A Dionex Ion chromatography DX 600 with a 4mm analytical column (AS9-HC), guard column (AG9-HC), and a 4 mm ASRS Ultra suppressor was then used for analyses. For quality assurance

and control for IC, a control standard solution was run every five samples (n=4) to quantify the run precision and percent recovery for each element (Table S7-2).

Appendix 7-3. Extraction of Organics

100 mL of each FPW was vacuum filtered through a 0.4 μm, 90 mm diameter glass fiber membrane and spiked with an 10 ng internal standard mix (Wellington Laboratories, ON, CAN). Aqueous filtrates were then freeze-dried for 48 h and subsequently reconstituted in 3 mL hexane for further cleanup. For freeze-dried glass filter sediment particulates, accelerated solvent extractions (ASE) were used for PAH extractions. Briefly, a different, separate glass fiber filter was added at the bottom of the ASE extraction cell, and ~ 2-3 g of DCM pre-cleaned florisil was added and followed by dried sediment filters. 10 ng of the internal standard mix was then spiked to each prepared ASE cell. Solvent (hexane/DCM 4:1 v:v; Optima™, Fisher Scientific, NH, USA), pressurized to 14 MPa, and heated to 80 °C within 6 min was then added to each ASE cell. 2 cycles of pressure and temperature were holding for 5 min (static extraction), followed by rinsing with cold solvent (50% of the cell volume) and purging with nitrogen gas for 90 s was applied to each ASE cell. Approximately 40 mL of total extract was gently concentrated by nitrogen gas evaporation and reconstituted in 3 mL of hexane for further cleanup.

Appendix 7-4. Polycyclic Aromatic Hydrocarbon (PAH) Analysis

To characterize and measure FPW sample PAHs, DCM pre-cleaned copper powder and anhydrous sodium sulfate were added into the 3 mL extracts (Appendix 7-3) and vortexed. Solid phase extraction (SPE) was used for cleanup of all the sample extracts. Conditioning of Sep-Pak Silica 6 cc Vac cartridges (1 g; Waters, MA, USA) were performed by loading cartridges with 5 mL solvent (hexane/DCM 7:3 v:v; Optima™, Fisher Scientific, NH, USA), followed by 5 mL of hexane. 3 mL extracts were then loaded into conditioned cartridges and washed with 4 mL of hexane. PAHs were then eluted with 5 mL of hexane/DCM 7:3 (v/v) which was subsequently concentrated to 200 μL for gas chromatography-mass spectrometry (GC-MS) analysis. Details of PAH standards, internal standard corrected recoveries of the detectable PAHs based on accelerated solvent extraction methods (range from 60.0–114%) and liquid-liquid extraction (range from 82.9–

179%), and the GC–MS instrumental method have been described in previous work (Zhang et al., 2016). Similarly, details of PAH analyte detection limits can be found in a previous study (He et al., 2018a).

Appendix 7-5. Non-Target Orbitrap HPLC-MS Organic Analyses

For non-target orbitrap analyses, 20 mL of the FPW sample and a source water sample from the location of the well were filtered through a 0.4 μm, 90 mm diameter glass fiber membrane (Glass Fiber Store). Oasis-HLB cartridges (Waters, 150 mg/6 mL) were used for organic compound extraction and desalination. Briefly, conditioning with 2 mL of methanol followed by 2 mL of 1% ammonium hydroxide, 2 mL of 0.1M formic acid, and then 2 mL of pure water (LC-MS grade) was applied to each cartridge. Following cartridge conditioning, 7 mL of filtrate was loaded per cartridge and subsequently washed with 7 mL of pure water and 2 mL of 0.1 M formic acid. Cartridges were then vacuum dried and organic compounds eluted using 2 mL of methanol followed by 2 mL of 0.2% ammonium hydroxide in methanol. Eluate evaporation to near-dryness was then performed under a gentle stream of high-purity nitrogen at 40 °C. 300 μL of methanol and 200 μL of pure water were then used to reconstitute the sample for analysis by HPLC-Orbitrap-MS. Organic extracts (10 μL; equivalent to 140 μL of the original FPW) was injected for analysis. HPLC instrumentation utilized a C18 analytical column (Poroshell 120 EC-C18, 2.1 × 100 mm, particle size 2.7 μm, Agilent Technologies) with a flow rate of 250 μl/min. Elution gradients followed a protocol of 99% A (LC-MS grade water with ammonium formate) and 1% B (methanol) at start, which was held for 1 min, ramped to 100% B by 36 min, held until 39 min, and returned to initial conditions by 42 min. Positive electrospray ionization was used for mass spectrometer instrumentation analysis, acquiring in full scan mode (m/z 100 to 1000) at 2.3 Hz with nominal resolving power of 120,000 at m/z 400.

Appendix 8-1. Inductively Coupled Plasma (ICP) -MS/MS Analysis

ICP-MS/MS analysis was used to quantify the concentration of cations, bromide, and total sulfur in raw, filtered HF-FW (Table S8-11). Before analysis, all samples were filtered through a 0.2 μm nylon filter membrane and diluted by a factor of 850 for Na analysis and 85 for all other elements with 18 MΩ·cm ultrapure water. Diluted 10 ml

samples were then acidified at a rate of 12 µL using 15.698 N trace metal grade nitric acid per 10 mL of sample. For analysis, an Agilent 8800 Triple Quadrupole ICP-MS/MS (ICP-MS/MS) was used with a RF power of 1550 W, a RF reflected power of 18 W, a microMist nebulizer and nickel/copper cones. Samples and external standards were analysed in high matrix mode, in which samples were diluted inline with 8 mL/min argon. Additionally, analysis was performed in MS/MS mode for greater mass resolution and a collision gas reaction cell was used with He gas (3 mL/min), O_2 gas (10% max flow) or H_2 (5 mL/min) gas to overcome matrix interferences (Table S8-2.). Instrumental drift was accounted for using 0.5 ppm solution of indium, which was added to each sample using an inline internal standard addition system. For quality assurance and control, a standard solution was run at the start, middle, and end of each run (every 10 sample for $n = 3$) to quantify the run precision and percent recovery for each element (Table S8-2).

Appendix 8-2. Anion, Total Organic Carbon and Total Nitrogen Analysis

Ion chromatography was used to determine the concentration of chloride (Table S8-11). Before analysis, samples were filtered through a 0.2 µm nylon filter membrane and diluted by a factor of 2000 using 18 MΩ·cm ultrapure water. Analysis was then performed on a Dionex Ion chromatography DX 600 with a 4mm analytical column (AS9-HC), guard column (AG9-HC), and a 4 mm ASRS Ultra suppressor. For total non-purgeable organic carbon (TOC) and total nitrogen (TN) a subsample of the filter sample was diluted by a factor of five and analyzed using on a TOC analyser (Shimadzu model TOC-V-CHS/CSN). For quality assurance and control for IC a control standard solution was run every five samples (n=4) to quantify the run precision and percent recovery for each element (Table S8-3).

Appendix 8-3. Fish Respirometry and Aerobic Scope Scaling and Standardization

Briefly, a linear regression analysis using the logarithm of MO_2 plotted against swimming speed (BL/s) was first performed to estimate standard metabolic rate (SMR; y intercept) and maximum metabolic rate (MMR; extrapolated at U_{crit}) according to derived linear equations. Only individuals yielding a regression with a $r^2 \geq 0.7$ were considered for analysis. Similarly, SMR and MMR were then scaled to a standard mass (20 g in the case

of the current experiment) before calculation of aerobic scope (MMR – SMR) (Mager et al., 2014; Stieglitz et al., 2016). See figures S8-1 and S8-2 for more information.

Appendix 8-4. Ventricle Gene Expression and Quantitative Real-Time PCR

Total RNA was extracted and isolated from excised mahi ventricles using ~ 1 mL of TRIzol according to the manufacturer's protocols (Ambion, Life Technologies, USA). Total RNA was suspended in nuclease-free water and quantified using a NanoDrop ND-1000 spectrophotometer (v. 3.8.1, NanoDrop Technologies, USA). Once quantified, RNA integrity was assessed on a 2% formaldehyde - agarose gel with ethidium bromide. Following RNA extraction, RNA samples underwent same-day complimentary DNA (cDNA) synthesis using SuperScript III Reverse Transcriptase (Invitrogen, USA) with a mix of oligo(dT) and random hexamer primers.

Quantitative real-time PCR (qPCR) was performed in 96-well plates using an ABI 7500 Real-Time PCR system (Applied Biosystems, USA). Gene specific primers previously designed for mahi cardiac targets (Edmunds et al., 2015) were created using Integrated DNA Technologies (USA) online software. Primer gene targets, accession numbers, and sequences can be found in Table S8-8. Individual PCR reaction mixtures contained 5 ml of SYBR Green master mix (Applied Biosystems, USA), 2.5 ml of respective 3.2 mM primer sets, and 2.5 ml of cDNA diluted in nuclease-free water. PCR reactions were denatured at 95 °C for 10 min prior to first thermal cycle initiation. A 2-step PCR thermal cycle, consisting of a denaturation step of 95 °C for 2 min and an annealing-extension step of 60 °C for 1 min, was employed for 40 cycles and transcript expression changes determined by ΔΔCT analysis using ribosomal protein S25 (rps25) as an endogenous control (Schmittgen and Livak, 2008). Endogenous control stability and primer efficiency validations, the latter determined by serial dilution slope calculation after ΔCT and log cDNA $_{[dilution]}$ input regression analyses, can be found in Figure S8-3.

Appendix 8-5. Ventricular Cardiomyocyte Isolation

Cardiomyocytes from mahi ventricles were isolated following protocols established in previous studies (Galli et al., 2011; Heuer et al., 2019). Briefly, following collection from holding tanks and euthanasia via pithing, mahi hearts were extracted and immediately

retrograde perfused with an isolation solution (Table S8-6) for 10 – 15 min. Isolation solution was switched out for a proteolytic isolation solution containing Trypsin (Type 1X, 0.5 mg/L, Sigma-Aldrich), Collagenase (Type 1A, ~0.4 mg/L, Sigma-Aldrich), and fatty-acid free bovine serum albumin (0.75 mg/L, Sigma-Aldrich), and mahi heart retrograde perfusion continued for another 10 – 15 min. Following perfusion termination, ventricles were separated and transferred into fresh isolation solution and lightly triturated to free cardiomyocytes. Cardiomyocytes were stored in fresh isolation solution for periods no longer than 8 h following initial isolation procedures.

Appendix 8-6. Plasma Osmolarity and Gill Histology

Immediately following 24 hr whole organism treatments, caudal blood samples were collected using a heparinized-rinsed syringe and spun at 13000 rpm to separate hematocrit from plasma. Plasma was then flash frozen in liquid nitrogen for osmolarity readings using a vapor pressure osmometer (Vapro 5520, Wescor Inc., FRA). Second gill arches excised from four randomly chosen mahi at each whole-organism exposure treatment were fixed overnight in a buffered zinc formalin solution (Z-Fix; Anatech Ltd., USA). Following fixation, gills underwent ethanol rehydration prior to paraffin wax embedding. Tissues were then trimmed and sectioned (7 μm) prior to being stained with haematoxylin and eosin (H&E). Visualization occurred on a Zeiss A1 microscope (Zeiss Microscopy, GER) and image capture via an optronic camera. Gross gill morphology and condition was assessed, with qualitative abnormalities identified. Representative digital images at 20x and 40x objective lens magnification were used to present findings (Figure S8-6).