Using Biomimetic Extraction-Solid Phase Microextraction (BE-SPME) for Evaluating the Chronic Toxicity of Oil Sands Process-Affected Water in Early Life Stage Rainbow Trout (*Oncorhynchus Mykiss*)

by

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Declaration of Committee

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Degree:	**Master of Resource Management**
Title:	**Using Biomimetic Extraction-Solid Phase Microextraction (BE-SPME) for Evaluating the Chronic Toxicity of Oil Sands Process-Affected Water in Early Life Stage Rainbow Trout (*Oncorhynchus Mykiss*)**
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Ethics Statement

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or

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Abstract

Early life-stage rainbow trout (*Oncorhynchus mykiss*) were exposed for ~30 days in 2021 and 2022 to whole OSPW series dilutions and treated OSPW to evaluate the toxicity of OSPW. Biomimetic extraction-solid phase microextraction (BE-SPME) quantified OSPW's complex bioavailable organic fraction to develop a rapid animal-free, cost-effective hazard assessment tool. Significant differences in endpoints including mortality; a 3-day hatching delay; craniofacial, skeletal, and edemas deformities; incomplete yolk sac absorption; and length and weight reductions were mainly observed in dilutions 10.0% to 100% OSPW. OSPW treatment significantly reduced toxic effects. Non-specific narcosis, cardiovascular problems, and endocrine disruption could have affected embryonic development and larval stages. The LC50 is 20.8 µmol/mL PDMS in 2021 and 10.1 µmol/mL PDMS in 2022. The EC50 for total deformities is 20.2 µmol/mL PDMS in 2021 and 10.3 µmol/mL PDMS in 2022. Compositional changes in the organic fraction of treated OSPW could have overestimated toxicity predictions using BE-SPME.

Keywords: Chronic Toxicity; Oil Sand Process-Affected Water (OSPW); Rainbow Trout; Early Life-Stage; Dissolved Organic Contaminants; SPME-based Passive Samplers

Dedicated to my dear parents for their love and support.

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Table of Contents

List of Tables

List of Figures

List of Acronyms

2,3-DMN	2,3-dimethylnaphthalene
ANOVA	Analysis of variance
BE	Biomimetic extraction
DO	Dissolved oxygen
DOC	Dissolved organic carbon
DPF	Days Post Fertilization
EMBSI	ExxonMobil Biomedical Sciences Inc.
FET	Fish embryo toxicity
GC-FID	Gas Chromatograph with Flame Ionization Detector
KT	Kearl Treatment
LAC	Library and Archives Canada
LC_{50}	Lethal concentration, 50%
LOEC	Lowest observed effect concentration
NAs	Naphthenic acids
NAFC	Naphthenic acid fraction components
NOEC	No observed effects concentration
OMS	Orbitrap Mass Spectrometry
OSPW	Oil sands process-affected water
PDMS	Polydimethylsiloxane
RBT	Rainbow trout
SFU	Simon Fraser University
SPME	Solid phase microextraction
TOC	Total organic carbon
TSS	Total suspended solids
WQG	Water quality guideline

Chapter 1. Introduction

1.1. Alberta Oil Sands

Northern Alberta, Canada is home to the third largest reserve of natural oil sands deposits in the world, covering a total area of 142,200 km^2 with proven oil reserves of 168 billion barrels of which only 3% is minable (Alberta Government, 2012). Alberta's oil reserves contribute to the local economy by supplying energy to Canada and the U.S. This oil sands area contains bitumen, a black viscous form of crude oil composed of complex hydrocarbons. In 2022, Alberta produced approximately 3.3 million barrels of crude bitumen per day, accounting for three-quarters of Albertas's crude oil production (AER, 2023a). Crude bitumen production is forecasted to increase to 4.0 million barrels per day by 2032 (AER, 2023b).

1.1.1. Oil Sands Extraction

The oil sands extraction process involves the surface mining of oil sands deposits and the separation of bitumen from sand, silt, and clay in a separation vessel with 7 to 10 m^3 of hot water per 1 m^3 of bitumen (GOC, 2015). Water is mainly obtained from the Athabasca River, the longest river in Alberta which flows near the minable oil sands area. Water-based gravity pulls sand, silt, and clay to the bottom, and a bitumen froth forms on the surface (GOC, 2019). The recovered bitumen froth is viscous and thick and requires further upgrading or dilution to produce synthetic crude oil (GOC, 2019). The remaining water in the separation vessel that is transferred to tailing ponds for solids to settle is considered as oil sands process-affected water (OSPW). Approximately 75% of OSPW is recycled for further bitumen extraction operations, minimizing the use of freshwater from the Athabasca River and other natural water resources (CAPP, n.d.a; Imperial Oil Limited, n.d.). OSPW contains clay and sand particles, organic compounds from bitumen such as naphthenic acids (NAs) and polycyclic aromatic hydrocarbons (PAHs), metals, salts (Li et al., 2017), and other constituents. OSPW is thus considered hazardous to nearby ecosystems and watersheds (Vander Meulen et al., 2023; Thienpont et al., 2021; Herbert et al., 2011).

1.1.2. Policy and Management

Although ~75% of OSPW is recycled, new water collected from the Athabasca River, precipitation, underground brackish aquifers, and on-site drainage is still required for more bitumen extraction (GOC, 2016). Through the Water Act and the Athabasca River Water Management Framework, the Government of Alberta sets strict volume limits of new freshwater withdrawal (GOC, 2016; GOA, 2015a). This framework manages, monitors, and adjusts weekly withdrawals according to real-time Athabasca River flow, seasonal variability, and modelled future conditions to maintain natural river flow levels, ensure the ecological integrity of the river, and encourage OSPW recycling (GOC, 2016; GOA, 2015a).

Regulated by the Environmental Protection and Enhancement Act, OSPW is stored in large on-site tailing ponds as part of a zero-discharge policy to prevent its release to the Athabasca River or other natural water sources and to promote water recycling. But the volume of stored OSPW increases with time with more bitumen extraction, posing a risk to the environment and human health and a liability to producers, operators, and the Province. For these reasons, the Environmental Protection and Enhancement Act and Oil Sands Conservation Act regulate the Tailings Management Framework (TMF) for the Mineable Athabasca Oil Sands released in 2015, to set limits for the cumulative volume of tailing ponds for oil sands operations (GOA, 2015b). TMF ensures that oil sands tailing ponds are treated, monitored, and managed during the life of a project and are in a ready-to-reclaim stage within 10 years after the life of the mine ends (i.e., when bitumen mining is completed) (GOA, 2015b; AER, n.d.). Directive 085 was introduced in 2016 by the AER and replaced Directive 074 to enforce the TMF and establish requirements for the reduction of existing (legacy) and new fluid tailings growth in addition to their reclamation with innovative treatment technology (AER, n.d.). Producers and operators have to constantly monitor surface water and groundwater and submit Environmental Impact Assessments that describe cumulative environmental effects and plans to mitigate these effects (CAPP, n.d.b).

Eventually, releasing treated OSPW into the environment is a potential management option to avoid water accumulation in retention sites and ensure reclamation of tailing ponds. However, freshwater quality guidelines for the protection of aquatic life do not exist yet for NAs and all PAH congeners. It is therefore necessary to

first understand the potential toxic effects of OSPW on aquatic organisms before considering the release of OSPW to the environment as an option. It is also important to develop a rapid hazard assessment method that measures toxicity to aquatic organisms attributed to the organic fraction of OSPW. Results from the hazard assessment method used in the present study could contribute to the future development of water quality guidelines for OSPW and the evaluation of OSPW treatment as an effective management method that complies with government regulations.

1.2. Oil Sands Process-Affected Water (OSPW)

1.2.1. Chemistry and Toxicity of OSPW

OSPW is mainly composed of complex organic compounds including polycyclic aromatic hydrocarbons (PAHs) and naphthenic acids (NAs), the latter considered as the primary cause of acute toxicity of OSPW (Hughes et al., 2017). Classical NAs are a mixture of cyclopentyl and cyclohexyl carboxylic acids with a general formula of $C_nH_{2n+z}O_2$, where n represents the carbon number and z the hydrogen deficiency resulting from the formation of rings or double bonds (Li et al., 2014). NAs have other types of structures. Oxidized NAs, referred as oxy-NAs, contain 3 or more oxygen atoms; heteroatom NAs contain nitrogen or sulfur atoms; aromatic NAs with aromatic rings; diamondoid carboxylic acids; estrogen-like steroidal acids; and many more structures (Li et al., 2014; Hughes et al., 2017). As previously mentioned, OSPW also contains an inorganic fraction composed of a variety of metals and salts. Overall, the organic and inorganic composition of OSPW will vary depending on the oil sands mine location, the bitumen extraction process, seasonality, and the retention time and types of microorganisms in the tailing ponds.

The toxicity of the organic extractable fraction of OSPW mainly depends on the composition and structure of NAs. Acute toxicity and recalcitrance are associated with increasing carbon number or molecular weight of classical NAs, specifically carbon number ≥17 (Hughes et al., 2017). However, increased carboxylic acid content and number of rings reduces hydrophobicity, recalcitrance, and acute toxicity of high molecular weight NAs (Frank et al., 2009; Frank et al., 2010). It is suggested that the relationship between hydrophobicity and acute toxicity is mainly attributed to narcosis or baseline toxicity, a non-specific mode of action that disrupts the integrity of cell

membranes. NAs act as surfactants with a hydrophobic (non-polar alkyl groups) and hydrophilic (carboxylic groups) end, concentrating at aqueous and non-aqueous interfaces (Kannel and Gan, 2012). Because of the surfactant-like structure of NAs, the hydrophobic alkyl group of NAs penetrates the lipid bilayer of cell membranes, disrupting the fluidity, surface tension, and thickness of the membrane and inducing osmotic stress that results in cell death (Quagraine et al., 2005; Frank et al., 2009). pH also plays a role in the toxicity of NAs. At high pH, NAs exist mainly in their ionized form at the water-lipid interface and concentrate as micelles in water. At low pH, NAs are un-ionized and are more likely to diffuse across cell membranes (Havre et al., 2003). Thus, the hydrophobicity and toxicity of NAs decreases with increasing pH. It is possible that NAs also have specific modes of action. Besides narcosis, NAs has been observed to have endocrine and immunological disrupting effects and to induce oxidative stress in several aquatic organisms mediated by the interaction of NAs with specific receptor sites in cells (Kannel and Gan, 2012; Li et al., 2014).

The current study utilizes whole OSPW samples from the Athabasca Oil Sands region for chronic toxicity tests compared to most studies that use extractions of NAs from OSPW. Using extractions of NAs to measure the toxicity of OPSW excludes the potential toxic impacts from other organic and inorganic compounds also found in OPSW, resulting in an underestimation of toxicity. Also, most studies have focused on conducting acute toxicity tests on fish that only observe the embryonic stage. The present study considers the toxic impact of the entire composition of treated and untreated OSPW by exposing rainbow trout to whole OPSW samples. It also contributes results of chronic toxicity bioassays that include embryonic and larval stages.

1.2.2. Treatment Pilot Studies

The treatment of OSPW supports the objectives of the TMF to decrease the accumulation of stored OSPW, progressively reclaim tailing ponds, conserve ecosystems and the Athabasca River watershed, minimize liability to the Government of Alberta, and the safe release of treated OSPW to the environment as a potential management option. The Province and the industry support and encourage the application of innovative treatment technology methods. Nature-based solutions such as constructed wetlands offer promising cost-effective and ecologically sustainable options for OSPW treatment by harnessing ecosystem services and functions to remove

pollutants from contaminated water (Thorslund et al., 2017). Currently, two pilot studies located in Alberta are investigating the effectiveness of nature-based treatment methods to remediate OSPW: the Kearl treatment (KT) wetland developed by Imperial Oil Resources Ltd. and an aquatic mesocosm pond treatment by InnoTech Alberta. OSPW treated by these two pilot studies are also used in the current study for toxicity tests and the application of BE-SPME as a management option.

Kearl Treatment (KT) Wetland

All the following details on the design and operation of the KT wetland are obtained from Cancelli and Gobas (2020; 2022) and Cancelli et al. (2022). The KT wetland is a constructed free water surface-flow wetland consisting of three shallow cells (aera 1, 2, and 3; 0.4 m each) and three deep cells (forebay area, deep pool 1, and final deep pool; 1.7 m each) which are separated in series by interior overflow banks. All cells contain submerged and emergent plant species local to the Athabasca oil sands region such as Common cattail (*Typha latifolia*), hardstem bulrush (*Schoenoplectus acutus*), and small-fruited bulrush (*Scirpus microcarpus*). The rooting medium of the wetland consists of 300 mm of non-compacted peat soil on top of 200 mm of compacted peat soil obtained from a muskeg near the area. The KT wetland has a slope of 0.014% that allows the hydrologic flow of water through all six cells during high water flood periods. It only operates seasonally during warm weather, usually from May to September. OSPW is first detained in an overburden disposal basin next to the KT wetland for initial settlement of suspended sediments. Then OSPW is pumped during a single pump event each year into the forebay area of the wetland at 5 L/s (430 m^3/day), with a hydraulic retention time of 14 days and a total volume of ~6,000 m^3 of water. It operates as a closed-circuit system, so the water is recycled back to the overburden disposal basin. Influent OSPW is considered as Day 0 water and effluent as Day 15 water.

Aquatic Mesocosm Pond

Below-ground aquatic mesocosms are open-topped ponds (15,000L) contained and embedded in an outer tank that provides thermal conductance and secondary support in case of water overflow due to precipitation (InnoTech Alberta, n.d.). Mesocosms represent a balance between controlled laboratory-based studies and realistic field studies such as wetlands (WUR, n.d.). The mesocosm pond contains floating plants in cylindrical mesh socks or on tall shelving units and submergent plants

in mesh socks or on medium-sized shelving units with soil or tailings sands at the bottom of the pond (Davies, 2018; Melnichuk, 2020). Vegetation local to the Athabasca Oil Sands Region includes cattail, water sedge (*Carex aquatilis*), hornwort (*Ceratophyllum demersum*), and Northern milfoil (*Myriophyllum sibiricum*) (Davies, 2018; Melnichuk, 2020). The system also contains a community of microorganisms and invertebrates (WUR, n.d.). OSPW is loaded into the mesocosm pond and is allowed to age for long periods. The treated OSPW used in the current study was left to age for two years. An advantage of using aquatic mesocosms at InnoTech Alberta is that treatment water can overwinter without damaging the pond or freezing at the bottom allowing submergent plants and macroinvertebrates to survive during harsh weather environments (InnoTech Alberta, n.d.; Melnichuk, 2020).

1.3. Biomimetic Extraction- Solid Phase Microextraction (BE-SPME)

The complex nature of the organic fraction of OSPW makes it difficult to assess and quantify its toxicity. Passive samplers used for biomimetic extraction via solid phase microextraction (BE-SPME) offer a convenient, cost-effective hazard assessment approach that accounts for the complexity of hydrocarbon mixtures and directly quantifies the bioavailability of all freely dissolved organic contaminants in OSPW (Redman et al., 2018a,b). Studies demonstrate that the toxicity of complex hydrocarbon mixtures in aquatic organisms can be explained by a partitioning model, the target lipid model (TLM). TLM assumes that toxicity of organic chemicals in aquatic organisms is governed by their octanol-water partition coefficient (K_{ow}), that a target lipid is the site of action, and that narcosis is the mode of action (Di Toro et al., 2000; Redman et al., 2018a,b). The TLM has been used to demonstrate that narcotic toxicity of hydrocarbon mixtures in aquatic organisms occurs when the total mixture of hydrocarbons accumulated in the lipids of an organism after equilibrium has reached exceeds a toxicological threshold (Di Toro et al., 2000; Di Toro and McGrath, 2000)

SPME passive samplers contain a fiber coated with polydimethylsiloxane (PDMS), an organic phase surrogate for organism lipids to which OSPW hydrocarbons partition according to the substance partitioning coefficient and abundance in the exposure media (Parkerton et al., 2000). NAs have a low potential of bioconcentrating in phospholipid bilayer membranes due to NAs mainly existing in their ionized form under

the pH of OSPW (pH 8.4) (Zhang et al., 2016). Ionized NAs are predominantly charged and thus cannot easily partition into amphipathic biological membranes. Acidifying OSPW converts NAs into their un-ionized form, which could be a better method to predict the partitioning potential of the organic fraction to cell membranes (Redman et al., 2018b). The contaminants absorbed in the SPME fibers after equilibrium has reached under acidic conditions can thus be considered as a biomimetic extraction of dissolved organic substances, analogous to the bioconcentration of a chemical in cell membranes of an aquatic organism.

Previous studies have used passive samplers to measure the toxicity of individual organic compounds from OSPW, commercial mixtures of NAs, and acid extractable organics derived from OSPW (Redman et al., 2018a,b; Swigert et al., 2015). However, the partition coefficient of individual organic compounds varies according to their composition, commercial mixtures of NAs are more biodegradable than NAs in oil sand tailing ponds (Scott et al., 2005), and acid extractable organics do not account for the toxic impacts of inorganic compounds and other constituents in OSPW. The current study presents an alternative approach that applies BE-SPME to whole OSPW samples instead of individual or extracted NAs. Also, the application of BE-SPME is a novel hazard assessment method that has been used mainly in the United States while the current study is one of the few studies that has applied this method in Canada using OSPW from Canadian tailing ponds.

1.4. Rainbow Trout

Rainbow trout (*O. mykiss*) is the recommended study salmonid species for chronic toxicity tests because of their well-characterized early developmental stages, their sensitivity to contaminants, and available well-studied standardized Canadian research methodologies for salmonids (Environment Canada (EC), 1998).

RBT is a cold-acclimated freshwater fish species that belongs to the salmonid family and is native to North America, ranging from Alaska to Baja California and including interior regions of British Columbia (Ward, 2014). Native RBT is found in the upper watersheds of the Athabasca River system and tributaries in western Alberta but generally inhabit streams between 900-1500 m above sea level (Ward, 2014). Thus, RBT is an ecological significant species that has been widely used as a model research

organism for toxicological studies. Also, toxicology results from RBT exposure to OSPW could be used to extrapolate to other sensitive fish species.

1.5. Research Objectives

The objectives of this study are:

- To assess the chronic toxicity of raw and treated oil sands process-affected water (OSPW) in early life stage rainbow trout (*Oncorhynchus mykiss*) to determine mortality, deformities, and developmental endpoints.

- To demonstrate the efficacy of BE-SPME for robust, cost-effective hazard assessment of OSPW.

If successful, BE-SPME could be used to predict or gauge chronic effects of OSPW-derived bioavailable organic compounds on rainbow trout. BE-SPME would be an alternative approach to animal-based testing. Considering that most studies focus mainly on acute toxicity tests and there is limited information on naphthenic acids concentrations available in the literature, this study provides new information of chronic toxicity on rainbow trout and naphthenic acids concentrations from whole OSPW. This novel information could contribute to the development of water quality guidelines and the management and remediation of oil sands tailing ponds.

Chapter 2. Materials and Methods

2.1. Sources of treatment water

During the spring of 2021 and 2022, 5 types of water samples were collected in the Athabasca Oil Sands region by Imperial Oil Resources Ltd. In 2021, 300L of Athabasca River water was sampled downstream of the Muskeg River confluence near Fort McKay and of several oil sands company operations. In 2022, 300L of 2-year aged OSPW was sampled from a small pond mesocosm pilot study at InnoTech Alberta. In both years, 1000L of OSPW was collected from a tailing pond from the Imperial Oil's Kearl Oil Sands Project. Also, constructed wetland water was collected from the Imperial Oil's Kearl Treatment (KT) wetland pilot study. The free water surface-flow KT wetland was designed to investigate on-site treatment of OSPW. 40L of OSPW influent was collected on day 0 of being loaded into forebay area of the KT wetland and 40L of OSPW effluent from the deep final pool on day 15 of treatment. During the experiment, all water samples were stored in 20L buckets and chilled in water baths at 8-10 ºC in Alcan Aquatic Research Centre, SFU, Burnaby. Upon arrival, samples of each type of water sample were collected for analysis.

2.2. Control water

Dechlorinated Burnaby municipal water was used as the main control water for all treatment water samples. Due to a delay in sample shipments from Imperial Oil's warehouse to the Alcan centre, water samples arrived at different time intervals. Therefore, toxicity experiments were initiated at different times, and each type of water sample had its own dechlorinated control water to avoid delay in toxicity experiments. Dechlorinated water was also used to prepare OSPW dilutions.

In 2022, a "hard" water treatment was prepared in addition to the dechlorinated control water by following the preparation method of reconstituted water of a desired hardness from Environment Canada's biological test method: Test of Reproduction and Survival Using the Cladoceran *Ceriodaphnia dubia* (2007). The "hard" water was prepared by adding $NaHCO_3$, $CaSO_4 \cdot 2H_20$, $MgSO_4$, and KCl to dechlorinated water to attain a hardness of approximately 160 mg/L to match the hardness of the aged

mesocosm OSPW (Environment Canada, 2007). Another toxicity experiment with wood frogs (*Lithobates sylvaticus*) exposure to hard water treatment resulted in increased snout-vent length and wet weight at metamorphosis compared to exposure to dechlorinated water (Stenner, 2023). For this reason, the “hard” water treatment was included in the 2022 RBT experiment to evaluate whether water hardness had different effects on fish mortality, hatching, development, and deformities.

2.3. Rainbow Trout

Triploid female eyed-stage rainbow trout embryos (~25 days post fertilization (DPF)) were commercially obtained from Troutlodge (Bonney Lake, WA, USA). The embryos were acclimatized to the test protocol temperature of 14±1 ºC by carefully transferring them to a 10L glass tank containing dechlorinated water at 8 ºC with gentle aeration and slowly increasing the temperature by 2 ºC per day until reaching the test protocol temperature. The dechlorinated water was 50% renewed every 24 hr. Embryos were kept in the dark and were inspected for damage, size, and colour.

2.4. OSPW Toxicity test

2.4.1. Study Design

The toxicity test closely followed methods described in Environment Canada’s biological test method for toxicity tests using early life stages of salmonid fish (rainbow trout), specifically the static-renewal fish embryo toxicity (FET) test (EPS 1/MR/13) (EC, 1998). Protocols were approved by Simon Fraser University’s Animal Care Committee [protocol #1296B-19].

The FET test starts at the onset of eyed-stage rainbow trout embryos, followed by the alevin larval stage with yolk sac, and terminates prior to the swim-up fry stage when the yolk sac is fully absorbed, in approximately 30 days (~55 DPF). No feeding is required as the yolk sac serves this purpose. Before the addition of embryos to the test vessels, water quality parameters for pH, dissolved oxygen (mg/L), dissolved oxygen saturation (%), temperature (ºC), and conductivity (µS/cm) were observed and adjusted if necessary to fall within the range recommended by Environment Canada (1998) for

rainbow trout survival. The FET tests were conducted during the spring of 2021 and 2022 at the Alcan Research Centre.

2021 Toxicity Tests

Toxicity tests included exposure of embryos to OPSW from the tailing pond, Athabasca River water, and constructed wetland water influent (Day 0) and effluent (Day 15).

The OSPW was diluted to six dechlorinated water/OSPW series dilutions of 100, 46.0, 22.0, 10.0, 4.60, and 2.20% OPSPW, plus a control with 100% dechlorinated water and 0% OSPW, in 20L buckets. Water was measured with a 2.0L ± 0.020 L borosilicate graduated glass. All RB exposures were conducted in quadruplicate (n=4) in 6L glass tanks (Figure 1). The tanks containing each exposure dilution and control were randomly placed into water baths chilled at 14±1 ºC with EcoPlus 1/10 HP water chillers. Tanks were individually aerated to the recommended dissolved oxygen concentration of 10-11mg/L with aeration manifolds and filtered compressed air coming out from the research facility. Samples of 20 viable RBT embryos were randomly selected from the acclimatized embryo batch and randomly exposed to the 4 replicates of each exposure solution. All exposure solutions were 80% renewed every 48 hours. During the experiment, pH level, dissolved oxygen (mg/L), dissolved oxygen saturation (%), temperature (ºC), and conductivity (µS/m) were measured and collected from one tank replicate of each exposure solution every 24 hr with a Hach HQ40d multi meter probe. Mortality, deformities, and health development checks were conducted every 24 hr. Embryos were incubated in the dark with red lighting until one week after all embryos in the control replicates hatched. For the remainder of the experiment, the laboratory facility was maintained to a light intensity of 100-500 lux and a photoperiod of 16±1 h light: 8±1 h dark. Exposure terminated when approximately 50% of alevins in the control replicates reached swim-up fry behaviour, after ~30 days. Remaining alive fish were euthanized with 0.5 g/L of tricaine methane sulphonate (MS-222) buffered to pH = 7 with $NaHCO_3$ for subsequent analysis of deformities and health development.

Due to less sample volume available, the study design for exposures to Athabasca River water and constructed mesocosm water was slightly altered. RBT exposure to Athabasca River water followed the same study design as the OSPW dilutions (Figure 1), except that the Athabasca River water was not diluted and included

its own control dechlorinated water. Similarly, Day 0 and Day 15 (constructed wetland) water were not diluted and included their own control water. However, only 5 embryos were exposed to the constructed wetland water and their control in 1L Mason jars, 4 replicates each, to avoid overcrowding (Figure 2). The Mason jars were chilled in smaller water baths. All samples, including dilutions, were first collected in assigned buckets before water renewal.

During the experiment, the pH of 46.0% and 100% OSPW dilutions and constructed wetland water drifted above the recommended pH level after 24 hrs of water renewal. Parallel testing was conducted by adjusting the pH of these two dilutions to 7.0 with 1N hydrochloric acid (HCl) (4mL of HCl/L for 46.0% OPSW and 4.7 mL of HCl/L for 100% OSPW) and diluting with 10% unflavored Perrier carbonated water (USEPA, 2006; Scroggins, 2018). Day 0 and Day 15 water were adjusted with 1.0 mL of HCl/L and diluted with 5% Perrier water, each. pH-adjusted and unadjusted treatments were prepared the day before water renewal and aerated and chilled for approximately 24 hrs (USEPA, 2006). Parallel testing was used to evaluate differences in toxic effects related to changes in pH and ammonia levels.

Multiple samples of control and treatment water were collected from buckets before water renewal and tanks after 48 hr exposure to measure total ammonia (mg/L) with a Hach ammonia kit test. Ammonia testing followed the Hach Salicylate Method (#8155) and used the Agilent BioTek Gen5 2.06 microplate reader software. A nitrogen-ammonia (NH_3-N) standard curve was created with dilutions of 1.00 mg/L NH_3-N standard solution to calculate the NH_3-N concentration (mg/L) in each sample. The un-ionized ammonia (µg/L) fraction of the NH_3-N concentration was obtained using multiplication factors that depend on the collected pH and temperature of each sample. Results of total nitrogen-ammonia (mg/L) and un-ionized ammonia (mg/L and µg/L) are presented as mean ± SE of samples.

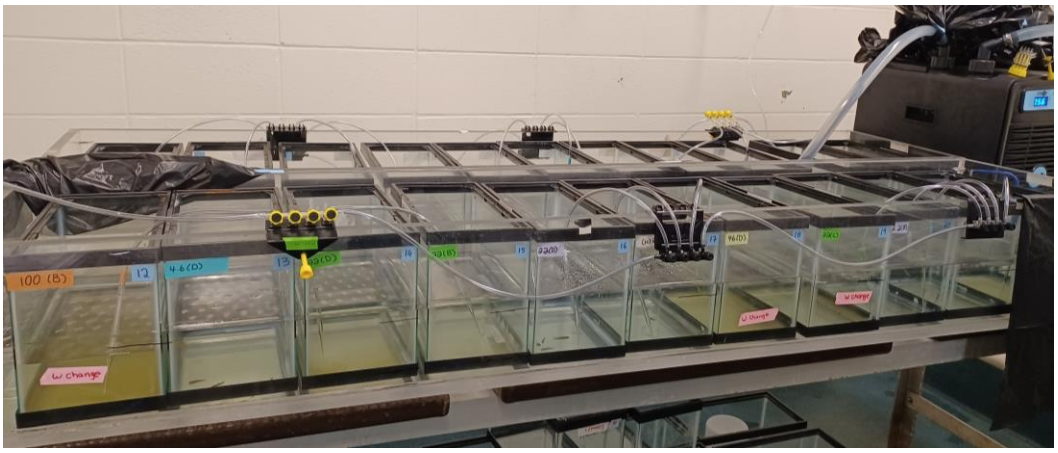

Figure 1. **RBT exposure experiments to OSPW dilutions and Athabasca River water with control dechlorinated water in 6L glass tanks. Water baths contain random allocation of four replicates of each treatment water, with 20 RBT embryos per tank. Each treatment water was individually aerated with compressed air and aeration manifolds. Water was chilled at 14 ± 1 ºC and circulated with chillers in two sets of water baths.**

Photo: Valeria Vega

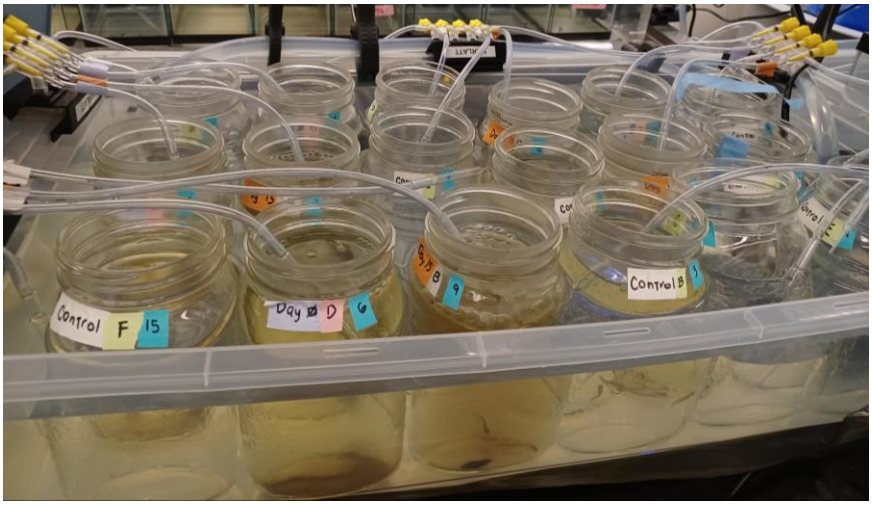

Figure 2. **RBT exposure experiments to constructed wetland water (Day 0 and 15) and aged OSPW with control dechlorinated water in 1L Mason jars. Water baths contain random allocation of four replicates of each treatment water, with 5 RBT embryos per jar. Each treatment water was individually aerated with compressed air and aeration manifolds. Water was chilled 14 ± 1 ºC and circulated with a chiller in one water bath.**

Photo: Valeria Vega

2022 Toxicity Tests

Exposure experiments in 2022 followed the same study design as in 2021 but with slight adjustments. The OSPW was diluted to seven dechlorinated water/OSPW series dilutions of 100, 46.0, 22.0, 10.0, 4.60, 2.20, and 1.00% OPSPW, plus a control dechlorinated water. A “hard” water treatment was included to evaluate impacts on RBT development due to differences in water hardness. 20 embryos were exposed to OSPW dilutions in 6L glass tanks, with 4 replicates (Figure 1). Aged OSPW and constructed wetland water were not diluted, each treatment water containing its own control dechlorinated water. 5 embryos were randomly allocated among 4 replicates of 1L Mason jars containing the aged OSPW and constructed wetland water (Figure 2).

2.4.2. Hatching Success and Mortality

Hatching observations were collected daily by counting the number of embryos that hatched into alevins. Incomplete hatches were counted as dead. Hatching success (%) is considered as the percentage ratio of hatched embryos to sample size (n_s=20 or 5) per tank or jar per day. Because approximately 90% of embryos hatched before the start of the 2022 exposure tests, hatching success was only determined in the 2021 toxicity tests. Mortality counts were reported daily from the beginning of the exposure tests until termination. Embryos were considered dead when they had an opaque or cloudy appearance and alevins or swim-up fry when they lacked heartbeat. Dead individuals were gently removed from tanks or jars with a 10 mL Turkey Baster and inspected under a dissecting microscope for deformities and development. Accidental removals were not included in mortality and hatching counts. Mortality percentage (%) is considered as the percentage ratio of dead individuals to sample size (n_s=20 or 5) per tank or jar. Estimated mortality endpoints include LC_{50}, LOEC, and NOEC.

2.4.3. Deformities and Development

Throughout the experiment and at termination, each dead individual was inspected under the microscope to observe and count for deformities (skeletal, craniofacial, finfold, and edemas) (Holm et al., 2005; Rudolph, 2006) and development indicators (yolk sac absorption, total length, and wet weight) (Hegeman and Marlatt, 2021).

Skeletal deformities include scoliosis (sideways curvature of the spine), kyphosis (inward curvature of the spine), and lordosis (outward curvature of the spine). Craniofacial deformities include bump(s) on head, short snout, short or large lower jaw, missing eye(s), and enlarged or reduced eye size. Finfold deformities comprise missing, reduced, or malformed fins. Edemas include fluid accumulation in pericardial cavity, yolk sac, head, or eyes. Deformity severity was assessed following a scoring index strategy described in Holm et al., (2005), where 0 indicates no deformity, 1 indicates slight severity, 2 indicates moderate severity, and 3 indicates extreme severity. Deformity percentage (%) for each deformity category is considered as the percentage ratio of the total count of individuals with deformities to the sample size (n_s=20 or 5) per tank or jar. Total deformity percentage (%) was also obtained by considering individuals with at least one category of deformity in each replicate and treatment. Therefore, it represents the average percentage (%) of RBT with deformities in each treatment. Estimated total deformity endpoints include EC_{50}, LOEC, and NOEC.

Yolk sac absorption was scored as 1, 2 or 3, where 1 represents a completely absorbed yolk sac with a fully connected abdominal epidermis indicating full development; 2 represents some visible yolk sac with a separated epidermis; and 3 represents a protruding yolk sac indicating the least development (Hegeman and Marlatt, 2021). Total length (mm) of dead and euthanized individuals was measured with a 150mm ± 0.02mm digital vernier caliper. Total length was measured from the tip of the snout to the end of the tail fin. Wet weight (g) was measured with a 120.0g ± 0.001g portable digital balance. Developmental endpoints include LOEC and NOEC for yolk sac absorption score 1 as an indicator for full development.

2.5. Water Quality Monitoring

Samples of each exposure water were collected upon arrival and were sent to ALS Environmental (Burnaby, BC) for initial analysis of hardness, total organic carbon (TOC), dissolved organic carbon (DOC), total suspended solids (TSS), ammonia, total and dissolved metals, and other physical water characteristics. Total and dissolved metals were compared to the British Columbia Ministry of Environment and Climate Change Strategy Ambient Water Quality Guidelines and the Canadian Council of Ministers of Environment (CCME) Environmental Quality Guidelines to analyze if the exposure solutions exceeded available recommended guidelines.

2.5.1. BE-SPME and Total Naphthenic Acids (NA) Measurements

Throughout the exposure tests in 2021 and 2022, samples of treatment solutions were periodically collected in amber glass containers from buckets and tanks, were stored at approximately 5 ºC, and were analyzed at three different laboratories: InnoTech Alberta (Edmonton, Alberta), ExxonMobil Biomedical Sciences Inc. (EMBSI) (Annandale, New Jersey), and SFU Toxicology Research Group laboratory (Burnaby, BC).

Samples in 2021 and 2022 sent to InnoTech Alberta (Edmonton, Alberta) for automated BE-SPME analysis were quantified via Gas Chromatograph with Flame Ionization Detector (GC-FID) following methods described in Redman et al. (2018a). BE-SPME concentrations extracted from InnoTech Alberta were used to present all endpoints, critical endpoints (i.e., LC_{50}, EC_{50}, LOEC, and NOEC), and toxicity predictions using BE-SPME passive samplers. Because Redman et al. (2018a) analyzed OSPW at ExxonMobil Biomedical Sciences Inc. (EMBSI) and obtained different BE-SPME concentrations than InnoTech Alberta, SFU, and other laboratories, samples were also sent to EMBSI in 2021 and 2022 for automated analysis. However, EMBSI was only able to analyze whole OSPW. The BE-SPME concentrations extracted from each OSPW dilution by InnoTech Alberta were used to estimate the BE-SPME concentrations from EMBSI for each dilution utilizing the maximum concentration (Cmax) of 100% OSPW. The observed percentage of Cmax for each OSPW dilution was calculated by dividing the BE-SPME concentration of the dilution (extracted from InnoTech Alberta) by the Cmax. The observed percentages of Cmax were then multiplied by the 100% OSPW derived BE-SPME concentration from EMBSI to estimate the concentrations of the other dilutions. Results from EMBSI were used to compare LC_{50} values from the present study to LC_{50} values reported by Redman et al. (2018a). Only samples from the 2022 exposure tests were analysed for BE-SPME at SFU using a SPME autosampler. SFU-derived concentrations were only used to observe for inter-laboratory variability of BE-SPME concentrations within the three analysis facilities.

The following BE-SPME extraction process follows methods described by Redman et al. (2018a,b) Samples are transferred to 20 mL glass vials sealed with Teflon faced septum caps and are acidified to a pH of ~2.4 with 50 µL of phosphoric acid. A 30 µm PDMS SPME fibre is thermally cleaned in the GC injection port at 280 ºC until a

clean baseline is observed for ≥3 minutes. The SPME autosampler injects the cleaned fibre into the vials containing the samples for 100 minutes at 22 ºC with orbital agitation (~250 rpm). The GC oven temperature is programed at 40 ºC for three minutes and is increased up to 300 ºC at 45 ºC/min. The FID and GC inlet temperature are 300 ºC and 280 ºC, respectively. A helium carrier gas is set at a constant flow rate of 17 mL/min. After equilibration, the autosampler injects the fibre into the GC injection port to thermally desorb for 3 minutes the extracted organic components of OSPW that partitioned into the PDMS.

Total integrated peak areas from the GC-FID responses are calibrated and converted to nanomole concentrations of 2,3-dimethylnaphthalene (2,3-DMN) using a standard curve. The standard curve is made with 0.5 µL injections of 2,3-DMN dissolved in dichloromethane at concentrations of 20, 100, and 200 µg/L. The BE results are normalized to the PDMS volume (0.132 µL) of the fibre and reported as units of µmol/mL PDMS. Results are background corrected with deionized water as a blank GC chromatographic run to remove residuals in the SPME fibre.

Total naphthenic acids (NAs) was only analyzed by InnoTech Alberta in 2021 and 2022. Samples are acidified to a pH of ~2.0 with formic acid. Naphthenic acids are extracted using Bond Elut PPE solid-phase extraction (SPE) cartridges that are conditioned with 5 mL of LC-MS grade methanol and 10 mL of acidified water purified with a Milli-Q Gradient A10 System. NAs are eluted with 5 mL of LC-MS grade methanol. 250 µL of extracted samples are then transferred into vials for semi-quantification using a HPLC Orbitrap Elite mass spectrometer (OEMS). Semi-quantification of classical NAs is conducted with a calibration curve made with Merichem oil. InnoTech Alberta reports results as total NAs with units of µg/L and estimates percentage of naphthenic acid congeners.

BE-SPME and total NAs results are presented with the non-linear Langmuir adsorption model in the liquid phase, which describes the adsorption capacity of a liquid adsorbate (the organic fraction of OSPW) onto a solid homogeneous surface or adsorbent (the surface of fish tanks, jars, or buckets) (Islam et al., 2021). It is described with the following Langmuir equation:

$$q_e = \frac{Q_m \, x \, K_L \, x \, D}{1 + (K_L \, x \, D)} \quad (1)$$

where q_e is the adsorption density of the liquid adsorbate at equilibrium (µmol/mL PDMS). It describes how many molecules are adsorbed per unit volume of adsorbent. Q_m is the maximum adsorption capacity of the adsorbent (µmol/mL); K_L is the Langmuir equilibrium constant (mL/µmol); and D is the dilution of OSPW (%) (Islam et al., 2021; Fernández-Andrade et al., 2021).

2.6. Statistical Analysis

Mortality, deformities and development results and graphs are reported as mean ± standard error (SE) of the mean per treatment using Microsoft Excel (Version 16.0.1). Hypothesis testing was performed with IBM SPSS Statistics (version 27). Shapiro-Wilk test was used for normal distribution and Levene's test for homogeneity of variance. One-way ANOVA followed by a Dunnett's Post Hoc test were used to determine significant differences in OSPW dilutions and constructed wetland water treatments compared to their corresponding control dechlorinated water. Non-parametric Kruskal-Wallis test followed by pairwise comparisons were used when parametric assumptions were not met. The pairwise comparisons were also used to estimate no-observed-effects concentrations (NOECs) and lowest-observed-effects concentrations (LOECs) for mortality, total deformities, and development. RStudio (version 2023.03.0) was used to produce concentration-response graphs for mortality, total deformities, and development; calculate the LC_{50}, EC_{50}, LOEC, and NOEC critical endpoints with standard deviations and 95% confidence intervals; and compare LC_{50} values of the present study with other toxicity studies. Independent-samples t-test was used to compare the Athabasca River and aged mesocosm water treatments to their corresponding control treatments. A non-parametric independent samples Mann-Whitney U Test was used when parametric assumptions were not met. A significance level of $p < 0.05$ was used for all analyses.

Chapter 3. Results and Discussions

3.1. Water Quality Analysis

3.1.1. Laboratory Water Quality Parameters

In general, water quality parameters monitored in the lab met the recommended water quality guidelines from EC's biological test method for salmonid fish (EC, 1998). In 2021 and 2022, pH and conductivity increased with decreasing OSPW dilution (Figure 3 and 5). pH values for the dechlorinated control, hard water, Athabasca River water, and 1.00 – 22.0% OSPW dilutions met the pH test requirement of 6.5 - 8.5. Day 0 and Day 15 water, 46.0% and 100% OSPW dilutions, and aged OSPW had on average pH values above 8.5 and up to 8.74 (Table 1 and 2). Despite attempts of adjusting the pH values down to 7.0 with 1N hydrochloric acid (HCl) and aeration before fish exposure, the pH values of the most concentrated treatments eventually increased again above 8.5. Similar high pH levels have been reported in raw OSPW from different tailing ponds (Mahaffey and Dubé, 2017) and in Athabasca River (Hebben, 2009). Dissolved oxygen (DO) was stable in adjusted and unadjusted OSPW dilutions form 2021 and within the recommended DO range of 10 - 11 mg/L, expect for 4.60% OSPW dilution (Figure 3b). OSPW dilutions in 2022 had on average lower DO concentrations than in 2021 but close to 10 mg/L (Table 2). Treated OSPW and Athabasca River water also met recommended DO guidelines.

The mean water temperature varied in 2021 OSPW dilutions, treated OSPW, and Athabasca River water but met the recommended temperature of 14 ± 1 ºC. Higher water temperatures were observed for some dilutions in 2022, beyond the recommended guideline (Table 2). In the last 4 days of the 2022 experiment, the chiller of the Alcan facility that cooled down the water used in the water baths broke down, affecting water temperature. The EcoPlus 1/10 HP chillers were adjusted to the lowest temperature setting, but the temperature in the water baths remained high due to the warm temperature inside the facility. However, the differences in mean temperature between control and treatments were <1 ºC (Table 2).

Table 1. Water quality parameters of pH, dissolved oxygen (mg/L), dissolved oxygen saturation (%), conductivity (µS/cm), and temperature (ºC) measured from tanks in 2021 from OSPW dilutions (n=28), pH-adjusted OSPW dilutions (n=21), constructed wetland water (Day 0 and 15 water) (n=24 each), and Athabasca River water (n=25). Values are presented as mean ± SD.

Parameter	pH	Dissolved oxygen	Dissolved oxygen saturation	Conductivity	Temperature
Unit		mg/L	%	µS/cm	ºC
Control	8.06 ± 0.12	10.31 ± 0.22	103.91 ± 0.19	61.92 ± 15.84	13.92 ± 0.28
2.20% OSPW	7.97 ± 0.14	10.39 ± 0.09	104.59 ± 0.99	84.84 ± 2.90	13.94 ± 0.23
4.60% OSPW	7.96 ± 0.12	13.77 ± 17.94	104.55 ± 0.80	115.48 ± 4.78	13.93 ± 0.28
10.0% OSPW	8.04 ± 0.12	10.37 ± 0.44	104.12 ± 04.45	185.67 ± 3.23	13.81 ± 0.29
22.0% OSPW	8.29 ± 0.08	10.51 ± 0.09	105.37 ± 0.58	327.74 ± 48.06	13.75 ± 0.27
46.0% OSPW	8.49 ± 0.11	10.49 ± 0.11	105.26 ± 0.94	586.78 ± 159.21	13.84 ± 0.23
100% OSPW	8.67 ± 0.13	10.36 ± 0.23	103.62 ± 2.40	1186.04 ± 25.19	13.71 ± 0.21
Adjusted 46.0% OSPW	8.49 ± 0.11	10.55 ± 0.11	105.50 ± 0.85	702.24 ± 122.29	13.62 ± 0.22
Adjusted 100% OSPW	8.62 ± 0.11	10.51 ± 0.14	105.15 ± 1.06	1194.33 ± 34.41	13.77 ± 0.24
Control	8.14 ± 0.22	10.40 ± 0.28	105.28 ± 2.70	111.44 ± 92.98	14.10 ± 0.28
Day 0	8.69 ± 0.19	10.52 ± 0.08	106.11 ± 0.50	1212.00 ± 36.95	14.03 ± 0.21
Day 15	8.74 ± 0.19	10.32 ± 0.67	104.08 ± 6.70	1164.67 ± 35.26	14.05 ± 0.17
Control	7.99 ± 0.40	10.14 ± 0.31	102.68 ± 1.73	338.17 ± 182.71	14.02 ± 0.94
Athabasca River	7.97 ± 0.49	10.07 ± 0.24	102.29 ± 1.20	330.69 ± 353.13	13.89 ± 1.08

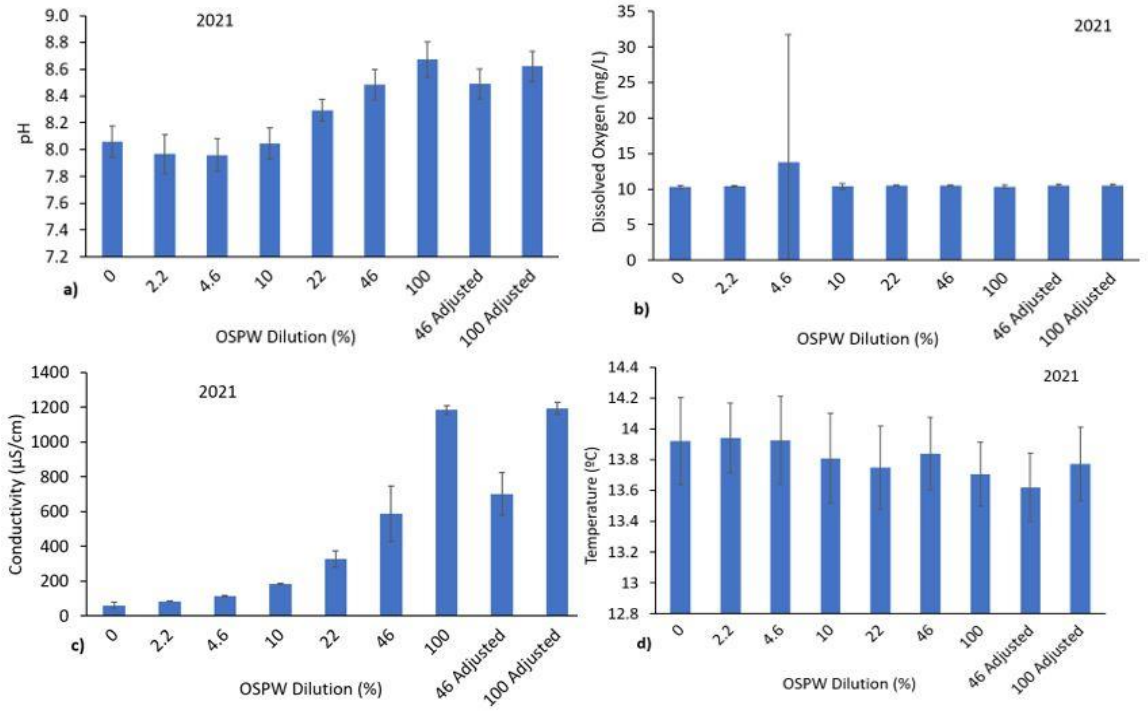

Figure 3. **Water quality parameters as a function of OSPW dilutions (%) (n=28) and pH-adjusted OSPW dilutions (%) (n=21) in 2021. Bar graphs represent a) mean pH ± SD, b) mean dissolved oxygen ± SD (mg/L), c) mean conductivity ± SD (µS/cm), d) mean temperature ± SD (ºC).**

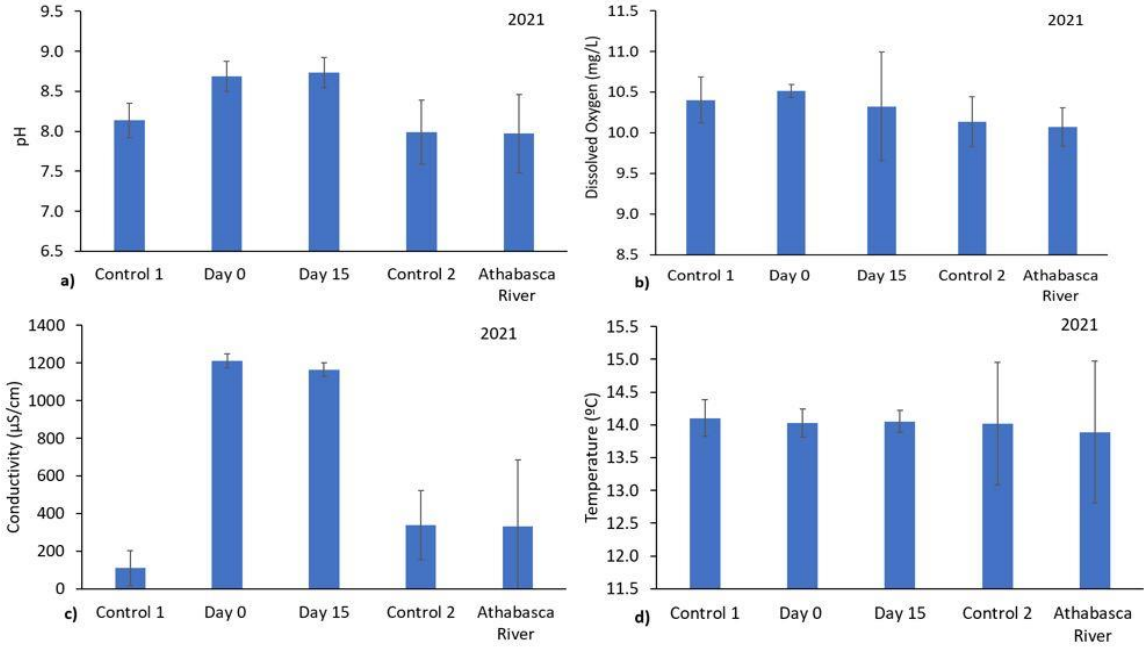

Figure 4. **Water quality parameters as a function of constructed wetland water (n=24) and Athabasca River water (n=25) in 2021. Bar graphs represent a) mean pH ± SD, b) mean dissolved oxygen ± SD (mg/L), c) mean conductivity ± SD (µS/cm), d) mean temperature ± SD (ºC).**

Table 2. **Water quality parameters of pH, dissolved oxygen (mg/L), dissolved oxygen saturation (%), conductivity (µS/cm), and temperature (ºC) measured from tanks in 2022 from OSPW dilutions and hard water (n=23), constructed wetland water (Day 0 and 15 water) (n=19 each), and aged OSPW (n=24). Values are presented as mean ± SD.**

Parameter	pH	Dissolved oxygen	Dissolved oxygen saturation	Conductivity	Temperature
Unit		mg/L	%	µS/cm	ºC
Control	7.36 ± 0.25	9.79 ± 0.44	101.03 ± 3.70	53.23 ± 3.26	15.15 ± 1.18
Hard water	7.86 ± 0.15	9.96 ± 0.27	101.51 ± 1.51	451.10 ± 387.24	14.59 ± 0.86
1.00% OSPW	7.37 ± 0.16	9.95 ± 0.29	101.78 ± 1.32	83.92 ± 75.30	14.80 ± 0.94
2.20% OSPW	7.20± 0.14	9.93 ± 0.33	101.12 ± 2.71	85.86 ± 4.54	14.60 ± 1.22
4.60% OSPW	7.22 ± 0.09	9.94 ± 0.36	102.41 ± 5.07	118.02 ± 8.22	14.72 ± 1.01
10.0% OSPW	7.43 ± 0.13	9.85 ± 0.43	96.83 ± 18.50	202.63 ± 36.35	14.82 ± 1.37
22.0% OSPW	7.74 ± 0.13	9.90 ± 0.41	101.35 ± 2.23	356.95 ± 9.88	14.90 ± 1.45
46.0% OSPW	8.22 ± 0.13	10.03 ± 0.33	102.13 ± 1.58	641.75 ± 9.11	14.61 ± 1.36
100% OSPW	8.49 ± 0.12	9.99 ± 0.28	93.35 ± 27.43	1143.24 ± 265.59	14.72 ± 0.96
Control	7.27 ± 0.30	9.98 ± 0.29	101.80 ± 1.77	53.20 ± 1.59	14.45 ± 0.54
Day 0	8.58 ± 0.40	10.19 ± 0.14	102.64 ± 0.76	1252.38 ± 290.23	13.92 ± 0.78
Day 15	8.69 ± 0.20	10.15 ± 0.24	149.28 ± 206.26	1420.63 ± 11.77	13.91 ± 0.57
Control	7.22 ± 0.23	10.13 ± 0.43	101.88 ± 3.95	54.32 ± 1.77	13.94 ± 0.66
Aged OSPW	8.74 ± 0.10	10.20 ± 0.21	103.53 ± 4.59	947.05 ± 363.14	13.83 ± 0.52

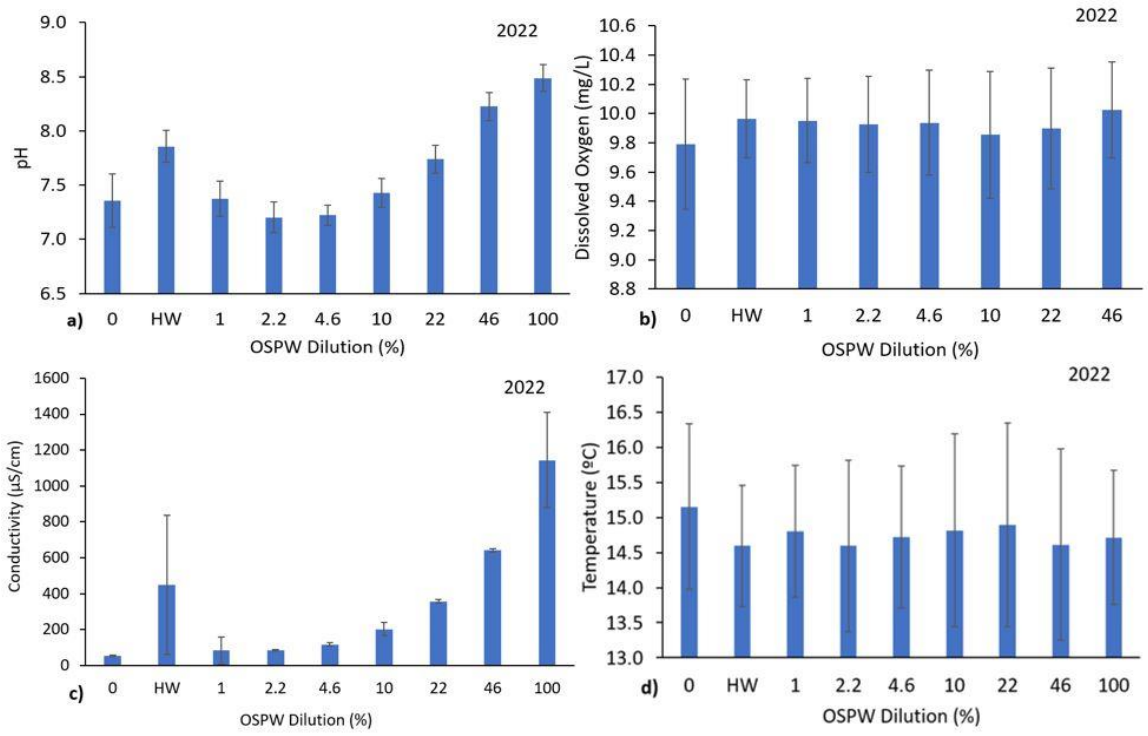

Figure 5. **Water quality parameters as a function of OSPW dilutions (%) and hard water (HW) (n=23) in 2022. Bar graphs represent a) mean pH ± SD, b) mean dissolved oxygen ± SD (mg/L), c) mean conductivity ± SD (µS/cm), d) mean temperature ± SD (ºC).**

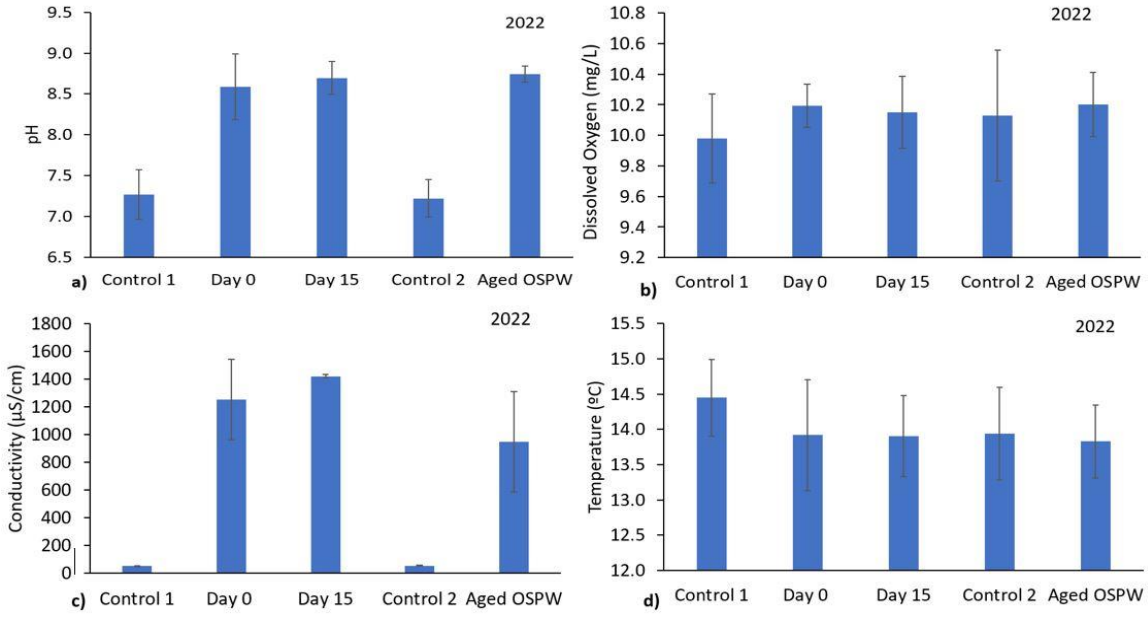

Figure 6. **Water quality parameters as a function of constructed wetland water (n=19) and aged OSPW (n=24) in 2022. Bar graphs represent a) mean pH ± SD, b) mean dissolved oxygen ± SD (mg/L), c) mean conductivity ± SD (µS/cm), d) mean temperature ± SD (ºC).**

Ammonia concentrations were high in the 2021 experiment. Ammonia concentration was lower in samples collected from preparation buckets than in tanks/jars, indicating ammonia levels elevated after 48 hrs of exposure (Table B.1, Figure 7, Figure 8). Control dechlorinated water, Athabasca River water, and Day 15 water samples collected from buckets in 2021 met EC's water quality guideline (EC WQG) for un-ionized ammonia of <5.00 µg/L at pH ≤ 6.5 (EC, 1998). Athabasca River water collected from tanks had lower un-ionized ammonia (10.2 ± 6.33 µg/L) than Day 0 jar water (60.5 ± 32.5 µg/L) and Day 15 jar water (60.5 ± 33.3 µg/L) (Table B.1, Figure 7). This is most likely attributed to high pH levels in Day 0 and 15 water (Figure 4a and 6a), as the concentration of the un-ionized ammonia form increases with increasing pH. Un-ionized ammonia concentrations from 2021 were low in 2.20% and 4.60% OSPW dilutions and their control (Figure 8a), almost meeting the EC's WQG, but gradually increased up to 115 ± 30.0 µg/L in 100% OSPW dilution. This is also correlated to increasing pH levels with decreasing OSPW dilutions. Only the pH-adjusted 100% OSPW decreased in un-ionized ammonia (61.8 ± 12.9 µg/L) compared to the unadjusted 100% OSPW (115 ± 30.0 µg/L), but it still exceeded the WQG. Un-ionized ammonia concentrations of adjusted and unadjusted 46.0% OSPW were similar, 26.2 ± 3.36 µg/L and 27.4 ± 11.9 µg/L, respectively.

Ammonia concentrations were lower in the 2022 experiment (Table B.2, Figure 7, Figure 8) than in 2021. Day 0 and 15 water from jars had on average un-ionized ammonia concentrations (47.4 ± 4.50 µg/L and 33.0 ± 3.13 µg/L, respectively) higher than the ammonia WQG, while Aged OSPW from buckets met the guideline (0.0162 ± 0.00177 µg/L). It is possible that un-ionized ammonia increased above WQG in Aged OSPW after fish exposure. Out of all the OSPW dilutions and their control, only 46.0% and 100% OSPW dilutions (4.80 ± 0.383 µg/L and 7.64 ± 0.512 µg/L, respectively) did not meet the water quality guideline.

Ammonia results were compared to other water quality guidelines to understand if high un-ionized ammonia concentrations in treated and untreated OPSW had possible harmful effects on RBT. CCME's environmental freshwater quality guidelines for ammonia (2010) indicates that 0.04 ± 0.021 mg/L of un-ionized ammonia at pH of 7.7 and 7.5-10 ºC is correlated to lesions in the gills and tissue degradation of the kidney of RBT. But these effects were observed after 4 months of a 5-year exposure experiment. A 30-day fish exposure with high un-ionized ammonia concentrations might not be

sufficient time to cause such impacts on RBT. A 90-day ammonia toxicity test at pH of 7.75 and 11.48 reports that the hatching success, survival, growth, biomass, and development of RBT alevins and swim-up fry were not affected by exposures ≤7.44 mg/L of total NH_3-N, with no visible gill lesions (Brinkman et al., 2009). None of the water samples, including pH-adjusted OSPW, have total NH_3-N concentrations above 7.44 mg/L (Table B.1 and A.2). Also, none of the samples are above USEPA's water quality guideline (2013) of 1.9 mg/L of total NH_3-N at pH 7 and 20°C. This indicates that ammonia in OSPW samples might not be the main cause of toxic effects on the RBT. However, endpoints in adjusted and unadjusted dilutions are further compared to determine if effects are due to pH-driven un-ionized ammonia toxicity.

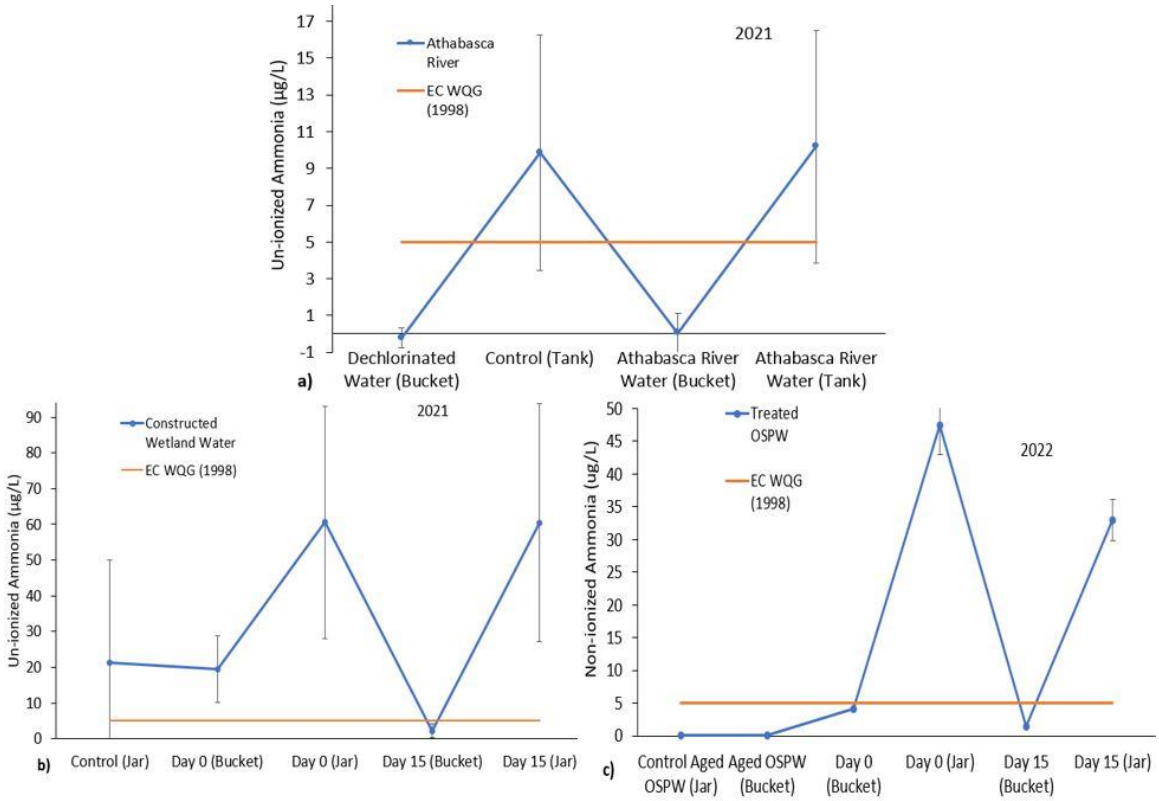

Figure 7. Un-ionized ammonia concentrations (µg/L) collected from buckets and tanks/jars of control dechlorinated water, Athabasca River water, Day 0 and Day 15 water, and aged OSPW in 2021 and 2022. Data points in blue represent the mean ± SD. Orange line represents the Environment Canada Water Quality Guideline (EC WQG) from 1998 for salmonid species, 5 µg/L.

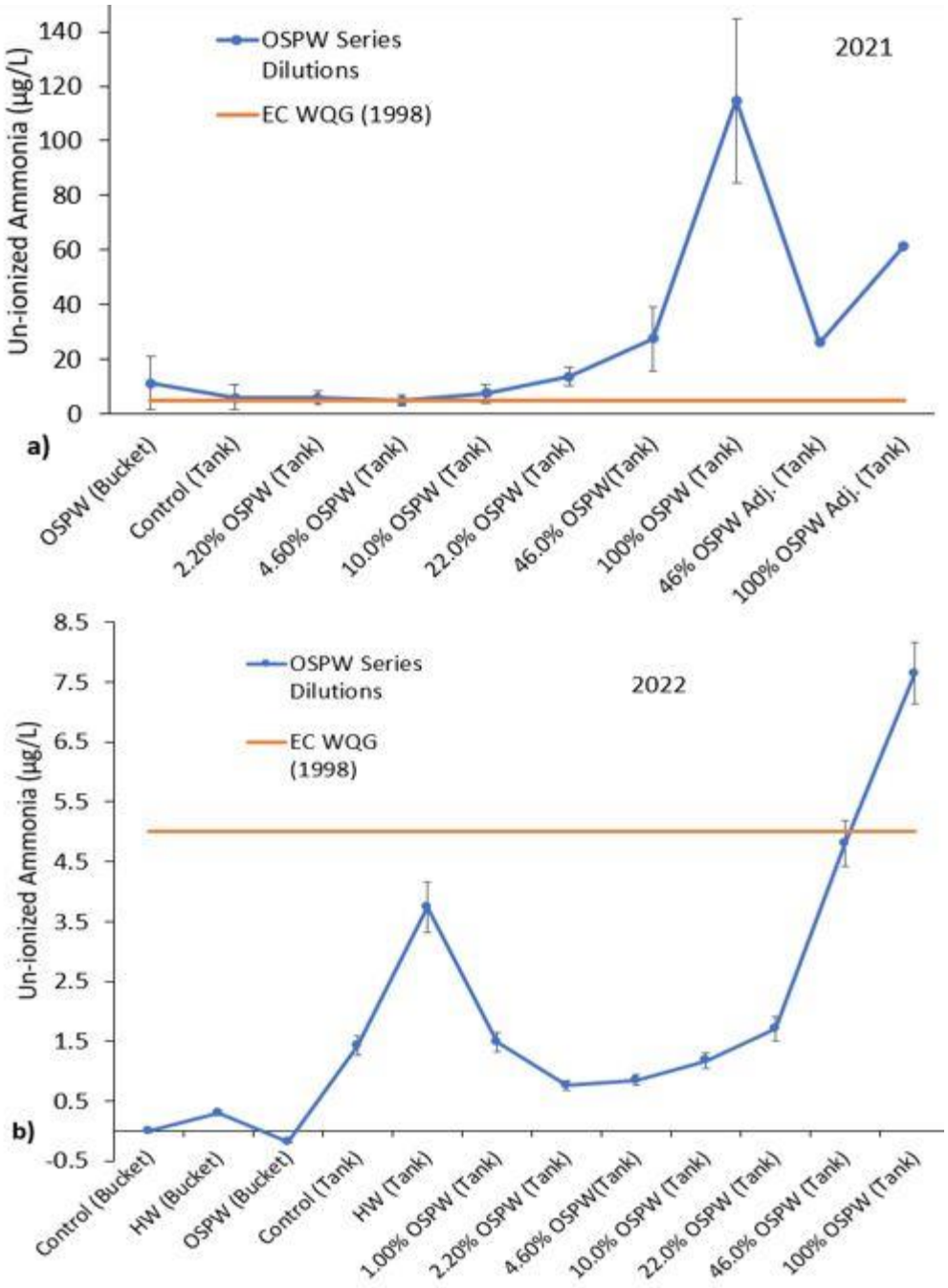

Figure 8. **Un-ionized ammonia concentrations (µg/L) collected from buckets and tanks of control dechlorinated water, hard water (HW), OSPW dilutions (%), and pH-adjusted OSPW dilutions (%) in 2021 and 2022. Data points in blue represent the mean ± SD. Orange line represents the Environment Canada Water Quality Guideline (EC WQG) from 1998 for salmonid species, 5 µg/L.**

3.1.2. Environmental Water Quality Parameters

Control dechlorinated water, Athabasca River water, and aged OSPW had water hardness (as CaCO3) between 15–150 mg $CaCO_3$/L, the recommended EC's water quality guideline (Table 3 and 4). Day 0 and 15 water had elevated water hardness ranging from 204-333 mg/L, with 2021 samples having hardness concentrations greater than 2022 samples. As expected, the hard water treatment also had elevated water hardness (162 mg/L) as it was prepared to approximately match the hardness of aged OSPW (126 mg/L). Before initiation of the experiments, pH levels of all water samples, except aged OSPW (pH = 8.61), met EC's water quality guidelines. The pH 8.27 and 8.31 of OSPW samples from 2021 and 2022, respectively, and the pH 8.28 of Athabasca River water are consistent with pH levels observed in raw OSPW (Mahaffey and Dubé, 2017) and the Athabasca River (Hebben, 2009). However, pH levels exceeded the guideline after exposure for some treatment waters (Table 1 and 2).

Total suspended solids (TSS) and total dissolved solids (TDS) contain particulate and dissolved, respectively, organic and inorganic compounds. In general, 2022 water samples had lower TSS (<3.0 - 9.1 mg/L) than 2021 samples (<3.0 - 16.1 mg/L). In both years, TSS were lower than the recommended TSS of 25 mg/L (EC, 1998). Athabasca River water had the highest TSS (16.1 mg/L) within both years. Certainly, a lot of soil and other material particles were observed in the initial Athabasca River water sample. Yet, it had the lowest TDS concentration (202 mg/L) between all the water samples. Similar TDS concentrations (107 – 310 mg/L) have been reported in the Athabasca River watershed (Hebben, 2009). In 2021, OSPW had the highest TDS (898 mg/L). TDS decreased when Day 0 water (848 mg/L) was treated for 15 days (810 mg/L) in the KT wetland. The opposite was observed in 2022. TDS increased when Day 0 water (954 mg/L) was treated for 15 days (1020 mg/L). Untreated OPSW had a TDS of 902 mg/L in 2022. Mesocosm treatment resulted in a TDS concentration of 736 mg/L, lower than the wetland treated OPSW and the untreated OSPW but higher than the Athabasca River water. Treated and untreated OSPW had TDS concentrations lower than reported values in the literature (Mahaffey and Dube, 2017; Hendrikse et al., 2018). As expected, the hard water treatment had 8.24 - 9.61 more TDS than control dechlorinated water samples due to the aggregated salts in the water.

Total organic carbon (TOC) and dissolved organic carbon (DOC) are great indicators for water contamination from natural and anthropogenic sources. Both indicators include the organic fraction of OSPW that contains naphthenic acids and other bitumen-derived organic compounds such as PAHs. In both years, TOC concentrations were close to DOC concentrations. Control dechlorinated water in 2022 had a negligible DOC concentration (<0.0015 mg/L). Control dechlorinated water in 2021 and hard water had DOC concentrations of 0.85 mg/L and 1.00 mg/L, respectively, which are below the recommended DOC concentration (5 mg/L) for source water (Moore, 1998). This indicates control dechlorinated water and hard water were clean. OSPW from 2021 had a higher DOC concentration (41.0 mg/L) than Day 0 water (35.4 mg/L) and Day 15 water (35.8 mg/L). OSPW from 2022 had a lower DOC concentration (33.1 mg/L) than treated OSPW. DOC content in Day 0 water (39.2 mg/L) increased after 15 days of wetland treatment (41.6 mg/L). Mesocosm treatment resulted in a DOC concentration of 40.9 mg/L, similar to Day 15 water. In both years, treatment of OSPW did not reduce TOC and DOC possibly because of the organic carbon released from the vegetation in the wetland and the mesocosm (Zhai et al., 2013; Davis et al., 2006; Alvarez and Becares, 2008) or from the decomposition of plants and microorganisms (Melnichuk, 2020) . Athabasca River water had a DOC concentration (21.7 mg/L) within the range of DOC concentrations (1.30 – 25.10 mg/L) reported in the Athabasca River watershed (Hebben, 2009), possibly due to soil and native vegetation and potential leakage from tailing ponds.

Besides naphthenic acids, PAHs were detected above detections limits in water samples from 2021 and were compared with available WQGs. 100% OSPW had the most types of PAHs, including acenaphthylene, anthracene, fluoranthene, and phenanthrene. Only anthracene was slightly above CCME's WQG (1999) of 0.012 µg/L. 46.0% OSPW had no PAH above the detection limit. 22.0% OSPW contained acenaphthylene but below WQGs. Benzo(a)pyrene and indeno(1,2,3-cd)pyrene were detected in 10.0% OSPW. Control dechlorinated water, 2.20% OSPW, and 4.60% OSPW also contained benzo(a)pyrene despite not being observed in treated OSPW and 46.0% and 100% OSPW. Benzo(a)pyrene was slightly above BC WQG (Nagpal, 1993) of 0.01 µg/L. Day 0 water had fluorene, phenanthrene, and anthracene and Day 15 water only phenanthrene, of which anthracene was the only PAH above WQGs. These detected PAHs probably contributed to the toxicity of OSPW and are included in the

organic fraction derived from biomimetic extraction in 2021. PAH measurements for 2022 samples were mostly non-detectable. Day 0 and 15 water collected from the KT wetland in 2017 and 2018 (Cancelli and Gobas, 2020) also had PAH concentrations below detection limits and WQGs.

Initial total nitrogen-ammonia (mg of NH_3-N/L) varied in water samples. Control dechlorinated water in 2021 and 2022 had NH_3-N concentrations below the lowest detection limit (0.005 mg/L) (Table 3 and 4) but increased up to 0.525 ± 0.395 mg/L in tanks/jars during fish exposure (Table B.1.). The initial NH_3-N concentration of the hard water treatment (0.0417 mg/L) was higher than in control dechlorinated water and later increased to 0.2089 mg/L in tanks during exposure. The initial NH_3-N concentration of Athabasca River water (0.0236 mg/L) also increased to 0.205 ± 0.073 mg/L in tanks after exposure. Both NH_3-N concentrations from Athabasca River water are within the range of NH_3-N concentrations (0.01 - 0.37 mg/L) observed in the Athabasca River watershed (Hebben, 2009).

NH_3-N concentration in the 2022 OSPW sample was below the lowest detection limit (Table 4), which was completely different to the 2021 OSPW sample (0.366 mg/L) (Table 3). This might explain why OSPW dilutions in 2022 had lower concentrations of un-ionized ammonia (µg/L) than in 2021 (Table B1 and A2). Diverse NH_3-N concentrations have been detected in OSPW from different tailing ponds, varying from <0.01 – 5.97 mg/L (Mahaffey and Dube, 2017; Hendrikse et al., 2018; McQueen et al., 2017). After exposure in tanks, NH_3-N concentrations in OSPW increased up to 0.929 ± 0.263 mg/L in the 100% OSPW dilution in 2021 and 0.2353 mg/L in the 4.60% OSPW dilution in 2022. However, none of the concentrations are above USEPA's water quality guideline (2013) for total ammonia of 1.9 mg/L.

Treatment of OSPW helped decrease the concentration of NH_3-N by approximately 20.60%, but fish exposure elevated NH_3-N to similar concentrations between Day 0 and Day 15 water. Day 0 water had an initial NH_3-N concentration of 0.336 mg/L in 2021 (Table 3) and 0.262 mg/L in 2022 (Table 4). After 15 days of wetland treatment, NH_3-N concentration decreased to 0.0687 mg/L in 2021 and 0.0542 mg/L in 2022. However, these NH_3-N concentrations increased up to 0.4131 mg/L - 0.5942 mg/L in both Day 0 and Day 15 water during fish exposure in both years. Aged OSPW had the lowest NH_3-N concentration (0.0102 mg/L) among the treated OSPW (Table 4), close to

the lowest concentration reported in the Athabasca River watershed (0.01 mg/L). Water sample for ammonia testing was not collected form jars containing aged OSPW (Figure 2.2), but it is probable that NH_3-N concentrations increased after fish exposure in a similar trend as the other water samples.

Table 3. Water quality parameters of control dechlorinated water, OSPW, Athabasca River water, and constructed wetland water (Day 0 and 15 water) from 2021 analyzed in ALS Environmental (Burnaby, BC). One sample per water treatment was collected upon arrival and before RBT exposures. It includes the lowest detection limit of each parameter.

Parameter	Lowest Detection Limit	Unit	Dechlorinated Water	OSPW	Athabasca River Water	Day 0 Water	Day 15 Water
Hardness (as CaCO3), from total Ca/Mg	0.60	mg/L	29.9	235	133	242	306
pH	0.10		7.67	8.27	8.28	8.30	8.30
Total dissolved solids (TDS)	10	mg/L	42.0	898	202	848	810
Total suspended solids (TSS)	3	mg/L	<3.0	6.70	16.1	7.20	7.80
Total ammonia (as N)	0.005	mg/L	<0.005	0.366	0.0236	0.336	0.0687
Dissolved organic carbon (DOC)	0.50	mg/L	0.850	41.0	21.7	35.4	35.8
Total organic carbon (TOC)	0.50	mg/L	0.700	43.6	21.3	35.6	36.1

Table 4. Water quality parameters of control dechlorinated water, hard water, OSPW, aged OSPW, and constructed wetland water (Day 0 and 15 water) from 2022 analyzed in ALS Environmental (Burnaby, BC). One sample per water treatment was collected upon arrival and before RBT exposures. It includes the lowest detection limit of each parameter.

Parameter	Lowest Detection Limit	Unit	Dechlorinated Water	Hard Water	OSPW	Aged OSPW	Day 0 Water	Day 15 Water
Hardness (as CaCO3), from total Ca/Mg	0.60	mg/L	19.9	162	204	126	227	333
pH	0.10		7.44	8.31	8.39	8.61	8.40	8.43
Total dissolved solids (TDS)	10	mg/L	36.0	346	902	736	954	1020
Total suspended solids (TSS)	3	mg/L	<3.0	<3.0	<3.0	<3.0	9.10	<3.0
Total ammonia (as N)	0.005	mg/L	<0.0050	0.0417	<0.0050	0.0102	0.262	0.0542
Dissolved organic carbon (DOC)	0.50	mg/L	<0.0015	1.00	33.1	40.9	39.2	41.6
Total organic carbon (TOC)	0.50	mg/L	<0.0016	0.670	33.0	40.6	41.4	40.3

Available water quality guidelines were used to analyze total and dissolved metals in each water sample. In both years, aluminum, manganese, and zinc were the metals that exceeded the most water quality guidelines (Table B.4 and A.5). All water samples in 2021 exceeded short- and long-term water quality guidelines for total aluminum. Total manganese concentrations also exceeded short- and long-term guidelines in all samples except for control dechlorinated water. In addition to manganese and aluminum, Athabasca River water had dissolved zinc and total iron concentrations above long-term guidelines. Control dechlorinated water also had total

and dissolved zinc concentrations above short and long-term guidelines. Total aluminum and manganese concentrations in Day 0 water were higher than concentrations in Day 15 water, above below water quality guidelines for both samples.

In 2022, control dechlorinated water, hard water, and aged OSPW did not exceed any available WQG (Table B.5). Total manganese concentrations were above short and long-term water quality guidelines for OSPW, Day 0 water, and Day 15 water. Total aluminum concentrations were observed only on OSPW and Day 0 water. Long-term water quality guidelines were exceeded by dissolved zinc in OSPW and total molybdenum in Day 0 water. Day 15 water only contained total manganese and it was above short and long-term water quality guidelines.

All metals above water quality guidelines observed in the water samples have been reported in the literature for OSPW, the Athabasca River, and Burnaby dechlorinated water. Aluminum, manganese, and zinc has been observed in raw OSPW used in other studies (Mahaffey and Dube, 2017; Hendrikse et al., 2018; McQueen et al., 2017), potentially coming from the bitumen extraction process. Aluminum, manganese, zinc, and iron has been reported in the Athabasca River watershed (Hebben, 2009), probably coming from natural sources such as rocks or soil erosion and anthropogenic sources. Aluminum and zinc concentrations have been reported in dechlorinated Burnaby municipal water (City of Burnaby, 2021).

3.1.3. BE-SPME and Total Naphthenic Acids

Some of the sampling amber containers broke on their way to InnoTech Alberta and EMBSI, and not all treatments were analyzed. Hence the cells in Table 5 with dashes. And for some treatments, only one sampling container was available for analysis. For treatments with more than one sampling container, results are presented as mean ± standard deviation (SD).

Overall, a non-linear increasing trend of BE-SPME concentration is observed as a function of decreasing OSPW dilution (Table 5 and Figure 9a), indicating some adsorption of the organic component of OSPW onto the surface of fish tanks and buckets. An interlaboratory study comparing BE-SPME concentrations measured at InnoTech Alberta, EMBSI, and SFU present a linear relationship with decreasing OSPW

dilutions (Letinski et al., 2022). However, these laboratories only included dilutions until 80% OSPW. A linear trend similar to the interlaboratory study is observed in BE-SPME concentrations, measured by InnoTech Alberta, from dilutions 0% OSPW (control dechlorinated water) to 46.0% OSPW of the present study (Figure 9), where the adsorption equilibrium constant (K_L) of each water treatment is close to 1 and the organic fraction of OSPW is more desorbed from the surface of fish tanks and buckets. Control dechlorinated water and dilutions of 2.20%, 4.60%, 10.0%, 22.0%, and 46.0% OSPW resulted in tank-derived BE-SPME concentrations (extracted at InnoTech Alberta) of 0.00 ± 0.00 μmol/mL, 4.36 ± 1.37 μmol/mL PDMS, 8.35 ± 1.76 μmol/mL PDMS, 19.4 ± 4.37 μmol/mL PDMS, 48.2 ± 10.1 μmol/mL PDMS, 79.5 ± 8.45 μmol/mL PDMS in 2021; and 0.00 ± 0.00 μmol/mL, 1.93 μmol/mL PDMS, 3.83 μmol/mL PDMS, 8.03 μmol/mL PDMS, 17.3 μmol/mL PDMS, and 34.9 μmol/mL PDMS in 2022, respectively (Table 5). In 100% OSPW, OSPW is the most concentrated (113 ± 6.43 μmol/mL PDMS in 2021 and 62.0 μmol/mL PDMS in 2022) and K_L is reduced lower than 1 as more organic fraction is adsorbed onto the surface of the fish tanks. Thus, less organic fraction of OSPW is available for biomimetic extraction in 100% OSPW, resulting in an overall non-linear curve (Figure 9) different from other laboratory measurements. BE-SPME concentrations extracted at EMBSI and SFU can be found in Table 6.

Letinski et al. (2022) also observed in their study inter-lab variability in BE-SPME concentrations extracted from the same samples of OSPW dilutions, EMBSI obtaining lower concentrations than InnoTech Alberta and SFU, and SFU obtaining the highest concentrations. Variability in BE-SPME values was also observed in the present study. BE-SPME concentrations from 2021 were higher than 2022 (Figure 9a; Table 5) when measured by InnoTech Alberta. This variability could be the result of OSPW samples from 2021 having more dissolved and total organic carbon (Table 3) than samples from 2022 (Table 4). OSPW samples from 2021 also had a darker appearance and a stronger odor than samples collected in 2022. BE-SPME concentrations extracted at SFU overlap with concentrations sampled in 2021 by InnoTech (Table B.6; Figure A.1.) but have a lower coefficient of determination due to high variability in concentrations. BE-SPME concentrations extracted at EMBSI were lower than SFU and InnoTech Alberta.

pH-adjusted 46.0% OSPW dilution had on average higher tank-derived BE-SPME concentration (89.7 ± 3.46 μmol/mL PDMS) than unadjusted 46.0% OSPW (79.5 ± 8.45 μmol/mL PDMS). But due to variability in results, BE-SPME values overlap. BE-

SPME concentration in pH-adjusted 100% OSPW dilution (117 ± 5.03 µmol/mL PDMS) is similar to unadjusted 100% OSPW (113 ± 6.43 µmol/mL PDMS). No samples from tanks and buckets of hard water (HW), Athabasca River water, and Day 15 water in 2021 were available for InnoTech to analyze. Samples from tanks were also not available for Day 0 water in 2021, but a sample from buckets resulted in a BE-SPME concentration of 113 µmol/mL PDMS, which is the same concentration obtained from 100% OSPW in 2021. In 2022, Day 0 water had a BE-SPME concentration of 50.0 µmol/mL PDMS and treated Day 15 water had a lower concentration of 44.5 µmol/mL PDMS. Both BE-SPME concentration fall between BE-SPME concentrations derived from 46.0% OSPW and 100% OSPW in 2022. Similar toxic effects are observed in Sections 3.2 – 3.4 between 100% OSPW and Day 0 water. Aged OSPW had a BE-SPME concentration (33.6 µmol/mL PDMS) lower than the wetland treated OSPW and closer to 46.0% OSPW from 2022 (34.9 µmol/mL PDMS). BE-SPME concentrations of treated OSPW measured at SFU (Table 6; Figure A.2.) were lower than concentrations measured by InnoTech Alberta in 2021 but higher than in 2022. And BE-SPME concentrations extracted at EMBSI were very similar to concentrations extracted at InnoTech Alberta in 2022 (Table 5 and 6).

A non-linear increasing curve of total naphthenic acid (NA) concentration is also observed as a function of decreasing OSPW dilution (Figure 9b). NA concentrations are also generally higher in 2021 than in 2022 (Table 5). Control dechlorinated water and dilutions at 2.20%, 4.60%, 10.0%, 22.0%, 46.0%, and 100% OSPW resulted in total NAs concentrations of 2.00 ± 0.00 µg/L, 369 µg/L, 807 µg/L, 1880 µg/L, 5280 µg/L, 10400 µg/L, and 15400 µg/L in 2021; and 4.05 µg/L, 468 µg/L, 976 µg/L, 1690 µg/L, 4170 µg/L, 7810 µg/L, 11100 µg/L in 2022, respectively. Some NAs in the most concentrated OSPW dilution (100% OSPW) could have been adsorbed onto the surface of the fish tanks and buckets in both years, leaving less NAs available in the samples used for NAs extraction. This could have contributed to the observed non-linear curve. But considering that NAs were obtained with a quantitative solvent-based extraction and not with a PDMS fiber, the adsorption capacity of the PDMS fiber cannot be used to explain the observed non-linearity. Alternatively, it could be explained by the chemical activity (a) of NAs, an approach that accounts for the chemical potential of non-ideal solutions in a system (Gobas et al., 2018). It describes how the NAs molecules interact between each other and with the solvent (water molecules). Gobas et al. (2018) describes that

chemical activity (a) is defined by the concentration of the solute (C) and the unitless activity coefficient (γ), a factor that accounts for the non-ideal conditions of the mixture:

$$a = \gamma * c \quad (2)$$

When OSPW is highly diluted, the concentration of NAs is low, and the molecules of NAs interact with each other less frequently. It requires strong intermolecular forces to contain the NAs molecules in the aqueous medium causing γ of NAs to increase. γ is also inversely proportional to the solubility or sorptive capacity (S) of the chemical in the medium (water). At high γ, NAs molecules are less soluble in water. This allows more NAs to get extracted with the methanol solvent used in SPE. It appears that γ remains high and constant from dilutions 2.20% OSPW to 46.0% OSPW, which could explain the linear trend observed between these dilutions. Meanwhile, 100% OSPW is highly concentrated with NAs molecules which interact with each other more frequently, requiring less activity to contain the NAs molecules in the aqueous medium and causing γ to decrease. If γ decreases at 100% OSPW, then NAs molecules are more soluble to water and methanol extracts less NAs than expected. This drop in γ and increase in S could explain the overall non-linear curve observed in both years when 100% OSPW is included (Figure 9b).

In 2021, pH-adjusted 46.0% OSPW (10900 µg/L) had a similar NA concentration as unadjusted 46.0% OSPW (10400 µg/L). Total NAs value in pH-adjusted 100% OSPW (14300 µg/L) was lower than unadjusted 100% OSPW (15400 µg/L). One bucket sample of Day 0 water in 2021 had a NAs concentration of 19600 µg/L, which was higher than the concentration from 100% OSPW in 2021. But wetland treatment in 2021 reduced total NAs concentration to 9100 µg/L in Day 15 water (a 2.15- fold decrease) and close to the concentration derived from 46.0% OSPW. Wetland treatment in 2022 reduced total NAs concentration from 12200 µg/L in Day 0 water to 9480 µg/L in Day 15 water, a 1.287-fold decrease. Both Day 0 and 15 water had concentrations that fall between dilutions 46% and 100% OSPW. Aged OSPW had a total NAs concentration (6870 µg/L) lower than wetland treatment but close to 46.0% OSPW from 2022.

Table 5. **BE-SPME (µmol/mL PDMS) and total naphthenic acids (NAs) (µg/L) from control dechlorinated water, hard water (HW), OSPW dilutions (%), pH-adjusted OSPW dilutions (%), Day 0 and 15 water, Athabasca River water, and aged OSPW from tanks/jars and buckets in 2021 and 2022. Samples were analyzed by InnoTech Alberta. Dashes represent samples that were not analyzed due to broken sampling containers. Values are presented as mean ± SD where more than one sample of the treatment was available.**

	2021				2022			
	BE-SPME (Tank)	BE-SPME (Bucket)	Total NAs (Tank)	Total NAs (Bucket)	BE-SPME (Tank)	BE-SPME (Bucket)	Total NAs (Tank)	Total NAs (Bucket)
Unit	**µmol/mL PDMS**	**µmol/mL PDMS**	**µg/L**	**µg/L**	**µmol/mL PDMS**	**µmol/mL PDMS**	**µg/L**	**µg/L**
Control	0.00 ± 0.00 (n=4)	0.00 ± 0.00 (n=4)	2 .00± 0.00 (n=2)	2.00 ± 0.00 (n=2)	0.00 (n=1)	-	4.05 (n=1)	-
HW	-	-	-	-	-	-	-	-
1.00% OSPW	-	-	-	-	-	0.590 (n=1)	-	249 (n=1)
2.20% OSPW	4.36 ± 1.37 (n=4)	4.95 ± 0.47 (n=4)	-	369 (n=1)	1.93 (n=1)	2.68 (n=1)	468 (n=1)	-
4.60% OSPW	8.35 ± 1.76 (n=4)	9.60 ± 2.32 (n=3)	807 (n=1)	-	3.83 (n=1)	-	976 (n=1)	-
10.0% OSPW	19.4 ± 4.37 (n=5)	22.7 ± 3.50 (n=5)	1880 (n=1)	1920 ± 70.71 (n=2)	8.03 (n=1)	7.99 ± 1.03 (n=2)	1690 (n=1)	1940 (n=1)
22.0% OSPW	48.2 ± 10.1 (n=4)	49.6 ± 6.93 (n=5)	5280 (n=1)	4995 ± 248 (n=2)	17.3 (n=1)	15.2 (n=1)	4170 (n=1)	4170 (n=1)
46.0% OSPW	79.5 ± 8.45 (n=4)	77.8 ± 11.7 (n=3)	-	10400 (n=1)	34.9 (n=1)	33.5 ± 5.16 (n=2)	7810 (n=1)	7210 (n=1)
100% OSPW	113.3 ± 6.43 (n=4)	111.4 ± 9.66 (n=5)	15400 (n=1)	14550 ± 2758 (n=2)	62.0 (n=1)	60.1 ± 15.8 (n=2)	11100 (n=1)	11750 ± 495 (n=2)
Adjusted 46.0% OSPW	89.8 ± 3.46 (n=3)	82.2 ± 9.18 (n=4)	-	10900 (n=1)	-	-	-	-
Adjusted 100% OSPW	116.7 ± 5.03 (n=3)	111 ± 4.79 (n=4)	-	14300 (n=1)	-	-	-	-
Day 0 water	-	113 (n=1)	-	19600 (n=1)	50.0 (n=1)	-	12200 (n=1)	-

	2021				2022			
	BE-SPME (Tank)	BE-SPME (Bucket)	Total NAs (Tank)	Total NAs (Bucket)	BE-SPME (Tank)	BE-SPME (Bucket)	Total NAs (Tank)	Total NAs (Bucket)
Unit	**µmol/mL PDMS**	**µmol/mL PDMS**	**µg/L**	**µg/L**	**µmol/mL PDMS**	**µmol/mL PDMS**	**µg/L**	**µg/L**
Day 15 water	-	-	-	9100 (n=1)	44.5 (n=1)	48.0 (n=1)	9480 (n=1)	-
Athabasca River	-	-	-	-	-	-	-	-
Aged OSPW	-	-	-	-	33.6 (n=1)	34.8 (n=1)	6870 (n=1)	-

Table 6. BE-SPME (µmol/mL PDMS) from control dechlorinated water, hard water (HW), OSPW dilutions (%), Day 0 and 15 water, and aged OSPW from tanks/jars and buckets in 2021 and 2022. Samples were analyzed at EBMSI in both years and at SFU only in 2022. Dashes represent samples that were not available. Values are presented as mean ± SD where more than one sample of the treatment was available.

	2021	2022		
Laboratory	**EBMSI**	**SFU**	**SFU**	**EBMSI**
Measurement	**BE-SPME (Bucket)**	**BE-SPME (Tank/Jar)**	**BE-SPME (Bucket)**	**BE-SPME (Bucket)**
Unit	**µmol/mL PDMS**	**µmol/mL PDMS**	**µmol/mL PDMS**	**µmol/mL PDMS**
Control	0.00 (n=1) †	40.6 (n=1)	9.48 (n=1)	0.00 (n=1) †
HW	--	33.8 (n=1)	17.0 (n=1)	--
1.00% OSPW	--	37.2 (n=1)	24.3 (n=1)	0.474 (n=1) †
2.20% OSPW	1.55 (n=1) †	35.1 (n=1)	24.4 (n=1)	1.55 (n=1) †
4.60% OSPW	2.97 (n=1) †	34.0 (n=1)	39.4 (n=1)	3.08 (n=1) †
10.0% OSPW	6.90 (n=1) †	44.8 (n=1)	27.1 (n=1)	6.45 (n=1) †
22.0% OSPW	17.13 (n=1) †	38.0 (n=1)	64.5 (n=1)	13.9 (n=1) †
46.0% OSPW	28.26 (n=1) †	78.1 (n=1)	106 (n=1)	28.0 (n=1) †
100% OSPW	47.4 ± 0.436 (n=3)	103 (n=1)	133 (n=1)	49.8 (n=1)
Day 0 water	47.4 ± 0.436 (n=3)	70.9 (n=1)	70.3 (n=1)	48.6 ± 4.90 (n=3)
Day 15 water	42.8 ± 3.04 (n=3)	70.1 (n=1)	76.2 (n=1)	48.0 ± 6.52 (n=3)
Aged OSPW	--	63.0 (n=1)	65.0 (n=1)	36.0 (n=1)

† Estimated BE-SPME concentrations with the observed % of Cmax

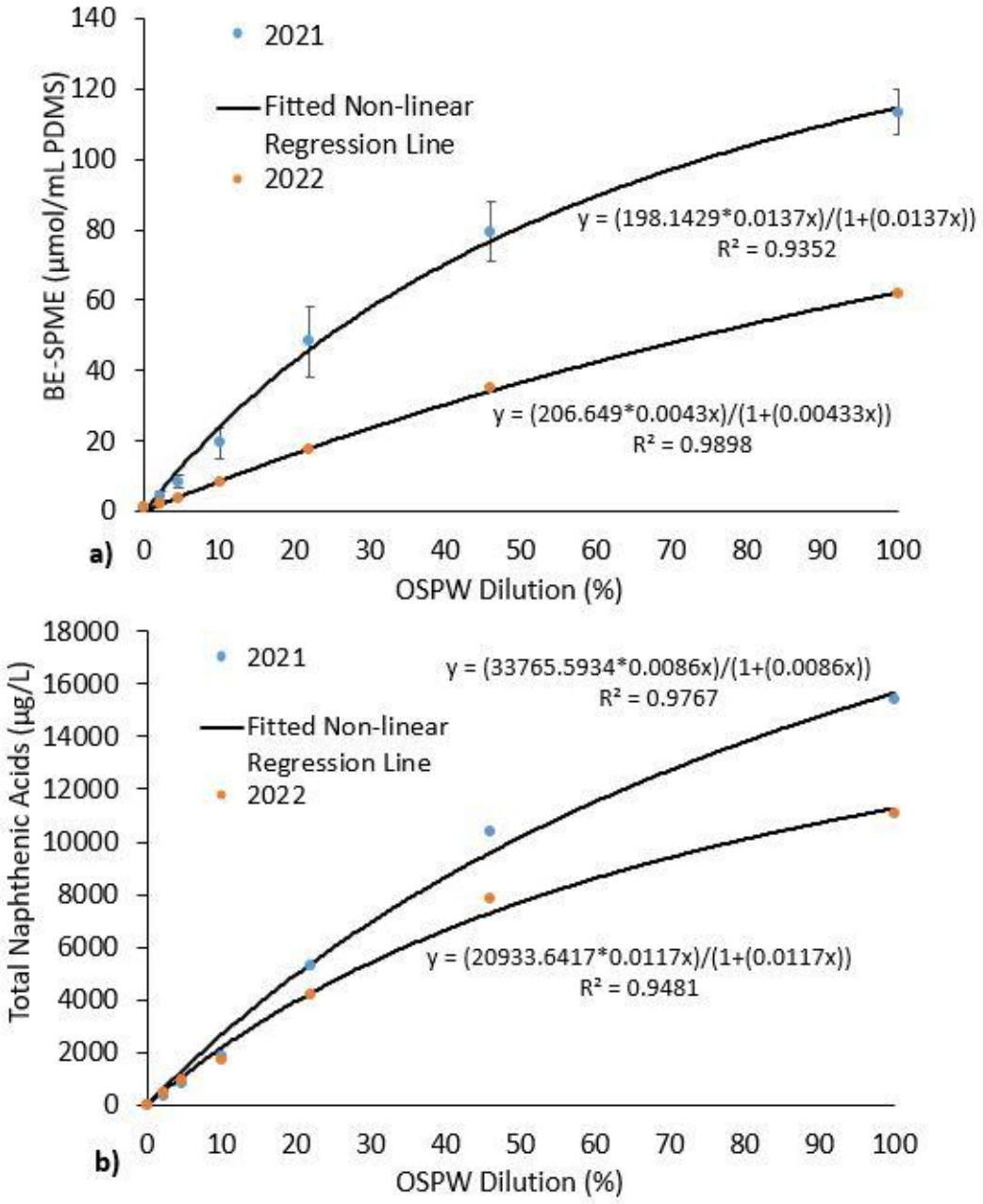

Figure 9. **Non-linear Langmuir adsorption curves for a) BE-SPME measured concentrations (µmol/mL PDMS) as a function of OSPW dilutions (%) in 2021 and 2022, and b) total naphthenic acid concentrations (µg/L) as a function of OSPW dilutions (%) in 2021 and 2022. Concentrations were obtained by InnoTech Alberta. Dilution 1.00% OSPW was ommited in 2022. Black trendline represent the fitted non-linear reggression line calculated in JMP. Non-linear Langmuir adsorption equations and their corresponding correlation coefficient (R^2) calculated in JMP are included for each curve. Values are presented as mean ± standard deviation where more than one sample of the treatment was available.**

3.2. Hatching Success and Mortality

Hatching success was only observed in 2021. Embryos started to hatch first in dilution 4.60% OSPW in day 3. By day 4, embryos in all dilutions and the control dechlorinated water started to hatch, with no significant differences (Kruskal-Wallis, pairwise comparison, $p>0.05$) compared to the control (Figure 10a, Table B.6). Between 95% and 100% embryos in control dechlorinated water, 2.20%, 4.60%, 10.0%, 22.0%, and 46.0% OSPW successfully hatched by day 7 with no significant differences compared to the control. Dead embryos had a white or cloudy appearance, and some unsuccessful hatchings had yolk sac edema. The most concentrated OSPW dilution (100% OSPW, 113 µmol/mL PDMS, or 15400 µg/L) delayed maximum hatching success by 3 days (Figure 10a-c). That is, 70.46% ± 2.041% of embryos in 100% OSPW successfully hatched in day 10, with a significant difference (Kruskal-Wallis, pairwise comparison, $p<0.05$) compared to the control. Piggott (2022) observed similar hatching periods and a significant decrease in hatching rate of RBT exposed to 100% OSPW. The hatching success and hatch viability of fathead minnow and zebrafish embryos decreased with exposure to increasing concentrations of naphthenic acid fraction compounds extracted from OSPW (Reynolds et al., 2022; Wang et al., 2015). Hatching success of exposure to pH-adjusted and unadjusted 46.0% OSPW were not significantly different (Kruskal-Wallis, pairwise comparison, $p>0.05$) to each other. pH adjustment in 100% OSPW resulted in 100.00% ± 0.000% hatching success compared to unadjusted 100% OSPW that had a significantly lower hatching success of 70.5% ± 2.04% (Kruskal-Wallis, pairwise comparison, $p<0.05$). This difference in hatching success could have happened because exposure to pH-adjusted 46.0% and 100% OSPW started 7 days after exposure to unadjusted OSPW dilutions initiated. Embryos in the incubation tank were probably more developed by the time parallel testing started and were already close to hatching regardless of exposure to OSPW, resulting in high hatching success.

Wetland treatment successfully decreased impacts on hatching success. In day 3, 100.0% ± 0.000% of embryos hatched in Day 15 water with no significant difference (Kruskal-Wallis, pairwise comparison, $p>0.05$) compared to control dechlorinated water (Figure 10d, Table B.6). Initially, embryos in Day 0 water had a significantly (Kruskal-Wallis, pairwise comparison, $p<0.05$) lower hatching success of 5.0% ± 5.0% in day 2 compared to the control that had a hatching success of 70% ± 17%. But 95% ± 5.0% of

embryos in Day 0 water hatched by the next day, with no significant difference compared to the control (Kruskal-Wallis, pairwise comparison, p>0.05). Although Day 0 water had a BE-SPME concentration (113 µmol/mL PDMS) similar to 100% OSPW (113 ± 6.43 µmol/mL PDMS), Day 0 water had a hatching success comparable to 10.0% OSPW with a BE-SPME concentration of 19.4 ± 4.37 µmol/mL PDMS. All embryos exposed to Athabasca River water successfully hatched by day 4.

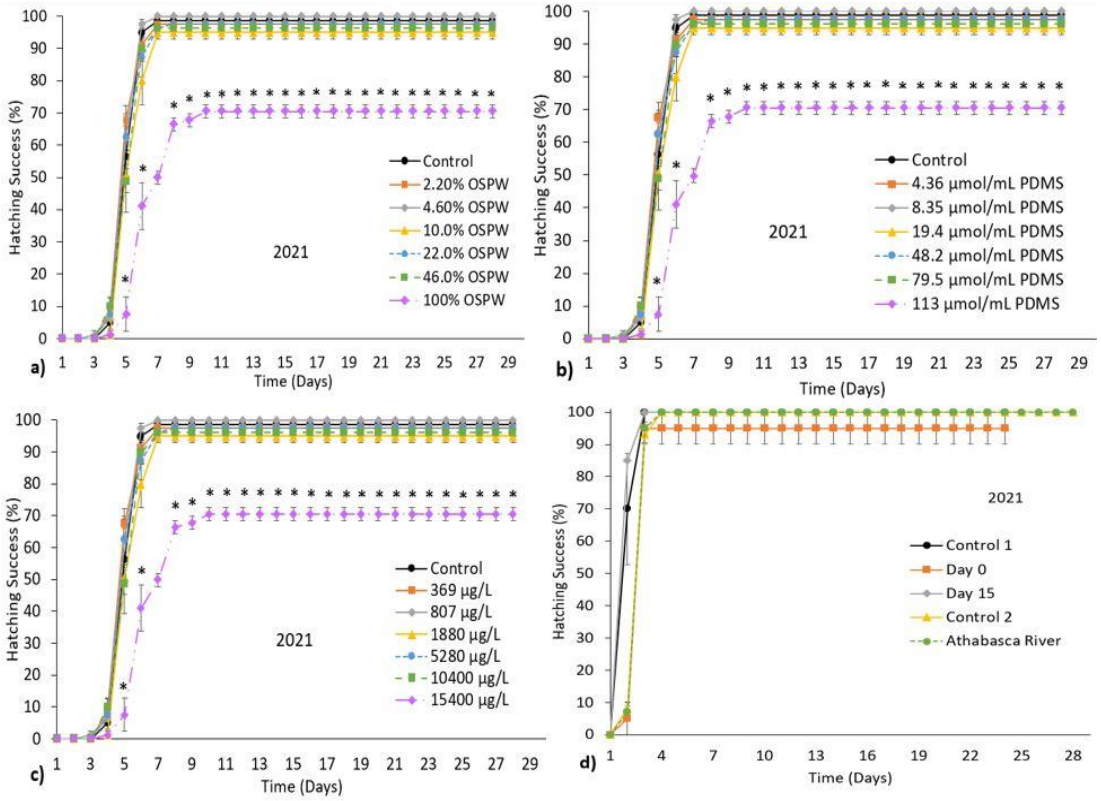

Figure 10. **Hatching success (%) of rainbow trout eyed-stage embryos in 2021. a) Exposure to OSPW series dilutions (%) in 2021 (n=20 per treatment, 4 replicates). b) Exposure to OSPW-derived BE-SPME (µmol/mL PDMS) in 2021 (n=20 per treatment, 4 replicates). c) Exposure to OSPW-derived total naphthenic acids (µg/L) in 2021 (n=20 per treatment, 4 replicates). d) Exposure to Day 0 and Day 15 water and Athabasca River water in 2021 (n=5 per treatment, replicates). BE-SPME and total NAs concentrations were obtained from InnoTech Alberta. Data points represent the mean ± SE. Astherics (*) represent significant differences compared to the control, where a), b), and c) are determined by Kruskal Wallis and pairwise comparisons (p<0.05). Exposure to 100% OSPW, 113 µmol BE-SPME/mL PDMS, and 15400 µg NAs/L significantly reduced hatching success from day 5 to termination. Day 0 water significantly (Kruskal Wallis, pairwise comparisons, p<0.05) reduced hatching success only in day 2. Athabasca River water had no effect in hatching success (Mann-Whitney U test, p>0.05).**

Table 7. Summary of mortality (%) and deformities (%) for exposure to OSPW series dilutions (n=20 per treatment, 4 replicates), pH-adjusted OSPW dilutions (n=18), constructed wetland treatment (Day 0 and Day 15) (n=5 each, 4 replicates), and Athabasca River water (n=20 per treatment, 4 replicates) in 2021. Values are mean ± standard error (S.E.). Aesthetics (*) represent significant difference compared to the control and letters represent the hypothesis testing method (see note). pH-adjusted 46.0% and 100% OSPW were statistically compared to unadjusted 46.0% and 100% OSPW. Total deformities represent the percentage of individuals with at least one type of deformity.

	Mortality (%)	Skeletal deformities (%)	Craniofacial deformities (%)	Finfold deformities (%)	Edemas (%)	Total deformities (%)
Treatment	Mean ± S. E	Mean ± S. E	Mean ± S. E	Mean ± S. E	Mean ± S. E	Mean ± S. E
Control	5.0 ± 2.0	1.25 ± 1.25	8.75 ± 3.75	2.5 ± 2.5	0.0 ± 0.0	12.5 ± 3.23
2.20% OSPW	6.25 ± 6.25	1.25 ± 1.25	2.5 ± 1.4	1.25 ± 1.25	0.0 ± 0.0	5.0 ± 2.0
4.60% OSPW	1.25 ± 1.25	1.25 ± 1.25	12.5 ± 9.47	0.0 ± 0.0	1.25 ± 1.25	12.5 ± 9.47
10.0% OSPW	43 ± 10	20.5 ± 7.29	78 ± 3.9 * [a]	6.3 ± 1.2	44 ± 5.1 * [a]	91.3 ± 3.15 * [a]
22.0% OSPW	96 ± 2.4 * [a]	27.5 ± 7.77 * [a]	45 ± 5.4	0.0 ± 0.0	66.3 ± 3.75 * [a]	76.3 ± 4.27 * [a]
46.0% OSPW	100 ± 0.0 * [a]	47.5 ±13.0* [a]	45 ± 11	5.0 ± 2.0	46 ± 8.3 * [a]	65 ± 12
100% OSPW	100 ± 0.0 * [a]	45 ± 6.1 * [a]	27 ± 7.1	1.25 ± 1.25	23 ± 10 * [a]	58 ± 9.5
pH-adjusted 46.0% OSPW	97 ± 2.8	23.6 ± 13.3	29 ± 15	5.56 ± 3.93	36 ± 14	48.6 ± 17.8
pH-adjusted 100% OSPW	100 ± 0.0	48.6 ± 9.98	34.7 ± 5.73	2.78 ± 1.60	26 ± 7.7	69.3 ± 3.99
Control	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	10 ± 5.8	5.0 ± 5.0	10 ± 5.8
Day 0 water	100 ± 0.0 * [a]	50 ± 13 * [a]	45 ± 17 * [a]	5.0 ± 5.0	50 ± 17	85 ± 5.0*
Day 15 water	10 ± 5.7	0.0 ± 0.0	50 ± 10 * [a]	0.0 ± 0.0	5.0 ± 5.0	50 ± 10 * [b]
Control	0.0 ± 0.0	0.0 ± 0.0	2.6 ± 2.6	0.0 ± 0.0	2.6 ± 2.6	5.3 ± 3.0
Athabasca River water	0.0 ± 0.0	0.0 ± 0.0	8.8 ± 2.4	0.0 ± 0.0	1.25 ± 1.25	10.1 ± 1.99

[a] Kruskal Wallis and pairwise comparison, $p<0.05$

[b] One-Way ANOVA and Dunnett's test, $p<0.05$

Table 8. Summary of mortality (%) and deformities (%) for exposure to OSPW series dilutions (n=20 per treatment, 4 replicates), hard water (n=20, 4 replicates), constructed wetland treatment (Day 0 and Day 15) (n=5 each, 4 replicates), and Aged OSPW (n=5, 4 replicates) in 2022. Values are mean ± standard error (S.E.). Aesthetics (*) represent significant difference compared to the control and letters represent the hypothesis testing method (see note). Total deformities represent the percentage of individuals with at least one type of deformity.

	Mortality (%)	Skeletal deformities (%)	Craniofacial deformities (%)	Finfold deformities (%)	Edemas (%)	Total deformities (%)
Treatment	Mean ± S.E	Mean ± S.E	Mean ± S.E	Mean ± S.E	Mean ± S.E	Mean ± S.E
Control	3.75 ± 1.25	20 ± 8.4	5.0 ± 2.0	1.25 ± 1.25	2.5 ± 1.4	22.5 ± 7.22
Hard Water	1.25 ± 1.25	26 ± 14	3.75 ± 2.39	1.25 ± 1.25	1.25 ± 1.25	27.5 ± 14.8
1.000%	3.75 ± 2.39	25.0 ± 11.7	3.75 ± 1.25	0.0 ± 0.0	2.5 ± 1.4	27.5 ± 11.6
2.20%	3.8 ± 1.3	39.5 ± 22.1	7.6 ± 1.5	3.9 ± 2.5	5.1 ± 2.2	44.7 ± 21.7
4.60%	3.75 ± 2.39	36 ± 17	8.75 ± 2.39	5.0 ± 2.9	3.75 ± 2.39	42.5 ± 14.5
10.0%	22.5 ± 4.79	40 ± 11	21 ± 4.7	6.25 ± 2.39	16 ± 3.2	41 ± 11
22.0%	95 ± 3.5 * [a]	87.5 ± 5.20 * [a]	95 ± 3.5 * [a]	28.8 ± 7.47 * [a]	100 ± 0.0 * [a]	98.8 ± 1.25 * [a]
46.0%	97.5 ± 1.44 * [a]	96 ± 2.4 * [a]	98.8 ± 1.25 * [a]	28.8 ± 4.27 * [a]	97.5 ± 1.44 * [a]	100 ± 0.0 * [a]
100%	100 ± 0.0 * [a]	92.5 ± 4.33 * [a]	95 ± 2.9 * [a]	22.5 ± 1.44 * [a]	93.8 ± 2.39 * [a]	97.5 ± 1.44 * [a]
Control	100 ± 0.0	0.0 ± 0.0	5.0 ± 5.0	0.0 ± 0.0	0.0 ± 0.0	5.0 ± 5.0
Day 0 water	100 ± 0.0 * [a]	90 ± 10 * [a]	100 ± 0.0 * [a]	50 ± 10 * [a]	95 ± 5.0 * [a]	100 ± 0.0 * [a]
Day 15 water	5.0 ± 5.0	5.0 ± 5.0	10 ± 5.8	0.0 ± 0.0	5.0 ± 5.0	10 ± 5.8
Control	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Aged OSPW	20 ± 14	20 ± 14	25 ± 15	0.0 ± 0.0	15 ± 9.6	25 ± 15

[a] Kruskal Wallis and pairwise comparison, $p<0.05$

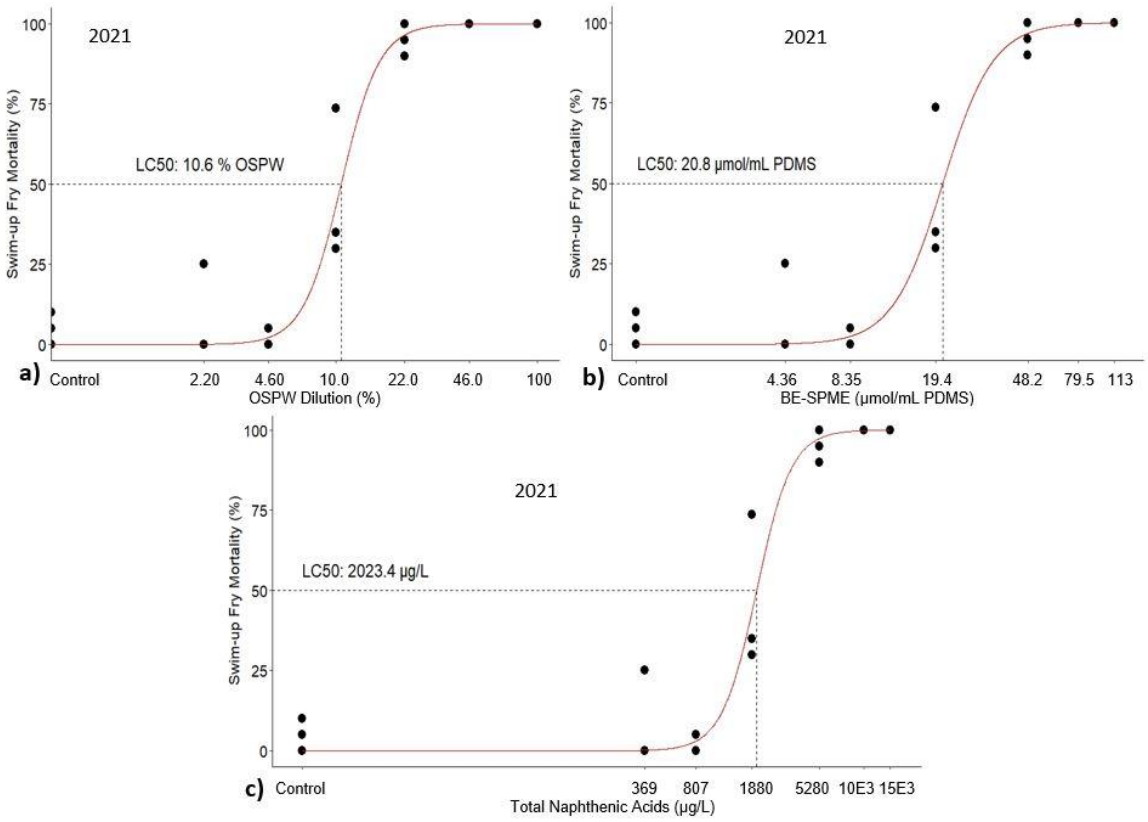

Figure 11. **OSPW dose-response curves for mortality (%) of rainbow trout until the swim-up fry stage in 2021. a) OSPW series dilutions (%) curve with LC50 value (n=20 per treatment, 4 replicates). b) OSPW-derived BE-SPME (µmol/mL PDMS) curve with LC50 value (n=20 per treatment, 4 replicates). c) OSPW-derived total naphthenic acids (µg/L) curve with LC50 value (n=20 per treatment, 4 replicates). BE-SPME and total NAs concentrations were obtained from InnoTech Alberta. Data points (●) represent the average percentage of dead swim-up fry per replicate of each series dilution treatment. Red line (─) is the estimated dose-response trendline. Graphed in R.**

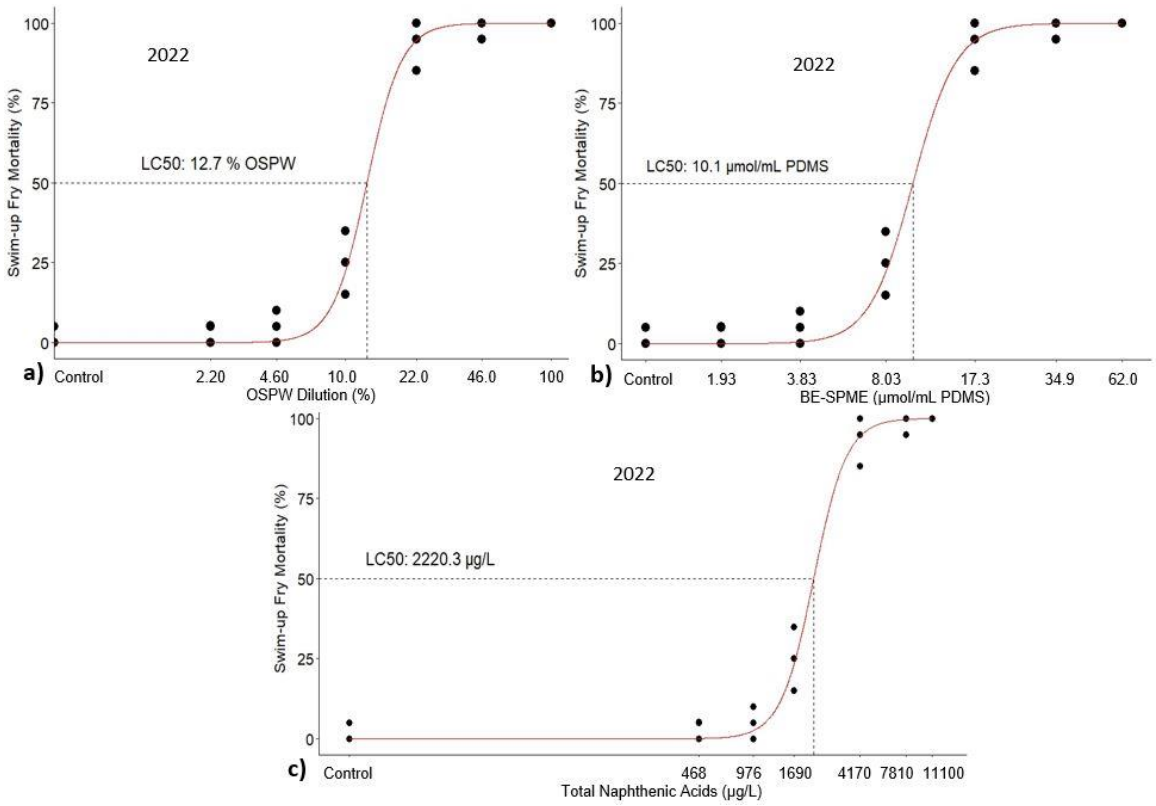

Figure 12. OSPW dose-response curves for mortality (%) of rainbow trout until the swim-up fry stage in 2022. a) OSPW series dilutions (%) curve with LC50 value (n=20 per treatment, 4 replicates). b) OSPW-derived BE-SPME (µmol/mL PDMS) curve with LC50 value (n=20 per treatment, 4 replicates). c) OSPW-derived total naphthenic acids (µg/L) curve with LC50 value (n=20 per treatment, 4 replicates). BE-SPME and total NAs concentrations were obtained from InnoTech Alberta. Data points (●) represent the average percentage of dead swim-up fry per replicate of each series dilution treatment. Red line (─) is the estimated fitted dose-response trendline. Graphed in R.

Mortality was observed from onset to termination of exposure experiments. Embryonic and larval mortality are included. The average mortality percentage until termination in control dechlorinated water was 5.0% ± 2.0% in 2021 and 3.75% ± 1.25% in 2022 (Table 7 and 8), meeting the test validity requirement of less than 35% mortality in the control. RBT exposure to whole OSPW exhibited an increasing sigmoidal concentration-mortality relationship (Figure 11 and 12) similar to exposure of fathead minnow and zebrafish to OSPW acid extractable organics and commercial mixtures of NAs (Redman et al., 2018b). Steep concentration-response curves observed in these species are representative of a narcotic toxicity mode of action for non-polar organics (Redman et al., 2018b; Morandi et al., 2016).

Significant mortalities were observed in the lowest three OSPW dilutions (22.0% OSPW, 46.0% OSPW, and 100% OSPW) in 2021 and 2022 (Kruskal-Wallis, pairwise comparison, $p<0.05$), with 95% - 100% RBT mortalities (Table 7 and 8). All RBT exposed to 100% OSPW died the fastest in 13 days in 2021 and 15 days in 2022, which are consistent to survival times observed by Piggott (2022). An acute embryonic mortality of ~29.5% contributed to the overall mortality in 100% OSPW. It required a high BE-SPME (113 ± 6.43 µmol/mL PDMS) and total NAs concentration (15400 µg/L) to cause early embryonic mortality in 2021. Reynolds et al. (2022) and Wang et al. (2015) also observed significantly high embryonic mortalities with exposure to increasing OSPW-extracted NAs concentrations. Considering that more than 95% of embryos hatched in 22.0% OSPW and 46.0% OSPW (Figure 10a), mortality in these two dilutions is mostly due to chronic effects that impact the larval stage. BE-SPME and total NAs derived concentrations of OSPW dilutions are reported in Table 5. pH adjustment had no significant impact (Kruskal-Wallis, pairwise comparison, $p<0.05$) on RBT mortality (97% ± 2.8% in adjusted 46.0% OSPW and 100% ± 0.0% in adjusted 100% OSPW) compared to dilutions with no pH adjustment (Table 7). Difference in water hardness had no significant (Kruskal-Wallis, pairwise comparison, $p<0.05$) impact on RBT mortality (Table 7). No mortality was observed in Athabasca River water (Figure 13a).

OSPW-induced mortality of RBT could be explained by the non-specific narcosis mode of action of NAs. The toxic potency of NAs is related to its hydrophobicity, polarity, and octanol−water partition coefficient (K_{ow}). Neutral hydrophobic NAs, dissolved in OSPW, with a high K_{ow} accumulate in phospholipid bilayer of cell membranes disrupting membrane fluidity, cell tight junctions, membrane-bound enzymes, and cell-to-cell communication, ultimately causing narcosis (Redman et al., 2018b; Frank et al., 2010). BE-SPME values are expected to predict the accumulation potential of the organic fraction of OSPW into biological lipid tissues and thus estimate the narcotic toxicity of OSPW.

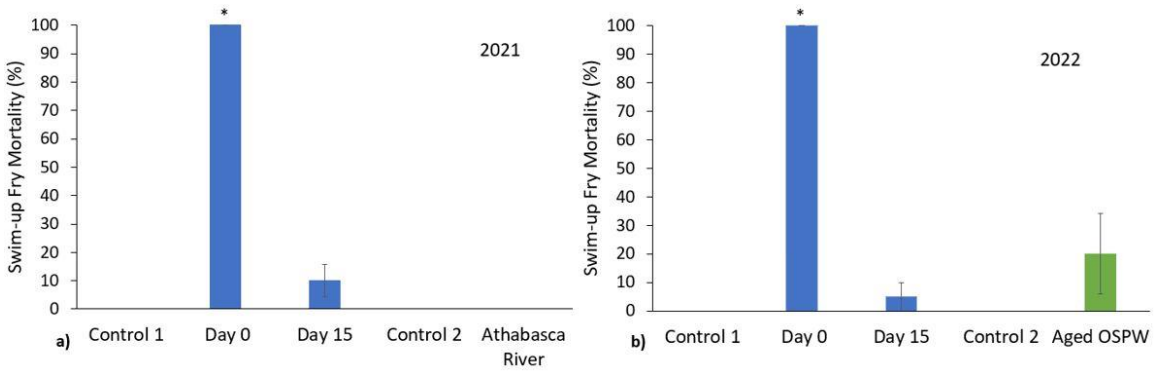

Figure 13. **Mortality (%) of rainbow trout until the swim-up fry stage. a) Exposure to constructed wetland water (Day 0 and Day 15) (n=5 per treatment, 4 replicates) and Athabasca River water (n=20 per treatment, 4 replicates) in 2021 with corresponding controls. b) Exposure to constructed wetland (Day 0 and Day 15) and Aged OSPW (n=5 per treatment, 4 replicates) in 2022 with corresponding controls. Values represent mean ± SE. Asterisks (*) represents significant difference compared to controls ($p<0.05$). Significant differences were observed only for Day 0 water in 2021 and 2022 (Kruskal Wallis, pairwise comparisons, $p<0.05$).**

In 2021 and 2022, Day 0 water had 100 ± 0.0% mortality (Figure 13a and 13b, Table 7 and 8), which is the same mortality percentage observed in 100% OSPW. Day 0 water had BE-SPME derived concentrations of 113 µmol/mL PDMS and 50.0 µmol/mL PDMS in 2021 and 2022, respectively (Table 5). These values are similar to the BE-SPME concentrations extracted from 100% OSPW (113 ± 6.43 µmol/mL PDMS in 2021 and 62.0 µmol/mL PDMS in 2022). Wetland treatment in Day 15 water significantly (Kruskal-Wallis, pairwise comparison, $p<0.05$) decreased mortality to 10.0 ± 5.77% in 2021 and 5.0 ± 5.0% in 2022. Mortality from Day 15 water is similar to mortality percentages observed in control dechlorinated water, 1.00% OSPW, 2.20% OSPW, and 4.60% OSPW. However, BE-SPME (44.5 µmol/mL PDMS) and total NAs (9480 µg/L) concentrations from Day 15 water correlate to concentrations derived from 46.0% OSPW and 100% OSPW. Although Aged mesocosm OSPW had less BE-SPME (33.6 µmol/mL PDMS) and total NAs (6870 µg/L) concentrations than Day 15 water, mesocosm treatment was less effective at reducing mortality than wetland treatment, with a 20 ± 14% mortality not significantly different (Mann-Whitney U Test, $p<0.05$) to the control (Figure 13b). Mortality in aged OSPW compares to mortality observed in 10.0% OSPW (22.5 ± 4.79% mortality). However, biomimetic extraction would indicate that Day

15 water and aged OSPW should have a lethal effect similar to 46.0% OSPW and 100% OSPW.

Table 9. LC_{50}, lowest observed effect concentration (LOEC), and no observed effect concentration (NOEC) values for mortality from exposure to OSPW dilutions (%), BE-SPME (µmol/ml PDMS), and total naphthenic acids (µg/L) in 2021 and 2022. BE-SPME and total NAs concentrations were obtained from InnoTech Alberta. Values represent the estimated value, standard deviation (S.D.), and 95% confidence interval for mortality calculated in R.

	2021			2022		
OSPW Dilution (%)	**Estimate**	**S.D.**	**95% Confidence Interval**	**Estimate**	**S.D.**	**95% Confidence Interval**
LC_{50}	10.6	0.457	9.67 - 11.6	12.7	0.456	11.7 - 13.6
LOEC Mortality	22. 0	5.69	10.3 - 33.7	22.0	2.22	17.4 - 26.6
NOEC Mortality	4.96	1.16	2.56 - 7.35	4.62	0.539	3.51 - 5.73
BE-SPME (µmol/mL PDMS)	**Estimate**	**S.D**	**95% Confidence Interval**	**Estimate**	**S.D**	**95% Confidence Interval**
LC_{50}	20.8	1.02	18.7 - 22.9	10.1	0.353	9.39 - 10.8
LOEC Mortality	48.2	14.1	19.2 - 77.2	17.3	1.69	13.8 - 20.8
NOEC Mortality	8.35	2.29	3.63 - 13.1	3.82	0.428	2.94 - 4.70
Total Naphthenic Acids (µg/L)	**Estimate**	**S.D**	**95% Confidence Interval**	**Estimate**	**S.D**	**95% Confidence Interval**
LC_{50}	2023	114	1789 - 2258	2220	86.9	2042 - 2399
LOEC Mortality	5280	1949	1274 - 9287	4169	437	3271 - 5067
NOEC Mortality	807	204	313 - 1301	976	80.0	812 - 1140

The LC_{50} values for OSPW dilutions are 10.6% ± 0.457% OSPW in 2021 and 12.7% ± 0.456% in 2022 (Table 9; Figure 11a and 12a). The LC_{50} value for BE-SPME analyzed by InnoTech Alberta was lower in 2022 (10.1 ± 0.353 µmol/mL PDMS) than in 2021 (20.8 ± 1.02 µmol/mL PDMS) (Figure 11b and 12b) due to higher BE-SPME concentrations measured in 2021 (Figure 9a). When considering LC_{50} values estimated from EMBSI's BE-SPME extractions (Table 6), the LC_{50} concentrations from the present

study in both years (7.38 ± 0.37 µmol/mL PDMS in 2021 and 8.12 ± 0.28 µmol/mL PDMS in 2022 from EMBSI) were lower than 4-day acute BE-SPME LC_{50} values for fathead minnow (19.5 ± 1.6 µmol/mL PDMS), rainbow trout (38.9 ± 1.2 µmol/mL PDMS), and zebrafish (45.7 ± 1.7 µmol/mL PDMS) derived by Redman et al. (2018a,b) (Figure 14). This indicates that rainbow trout used in the present study was highly sensitive to whole OSPW, more than acute exposures of other fish species exposed to acid extractable organics from OSPW and commercial mixtures of NAs.

Acute to chronic ratios (ACRs) can be used to extrapolate the mode of action of a compound by dividing the acute LC_{50} by the chronic toxicity of a substance. An ACR less than 10.4 is indicative of non-specific narcosis or baseline toxicity (Hoff et al., 2010), with low ACRs associated to non-polar narcosis (May et al., 2016). The ACR values for the present study were calculated using the 4-day acute LC_{50} of rainbow trout reported by Redman et al. (2018b) and the chronic LC_{50} from the present study derived from EMBSI concentrations: 5.27 in 2021 and 4.79 in 2022. Both ACRs, in addition to the steep concentration-response curves observed in Figures 11 and 12, denote that the observed toxicity is due to non-specific narcosis. Redman et al. (2018b) calculated ACR values of 2.0 for both zebrafish and fathead minnow, demonstrating that the mode of action for these two species is more related to non-polar narcosis compared to rainbow trout of the present study that had higher ACR values.

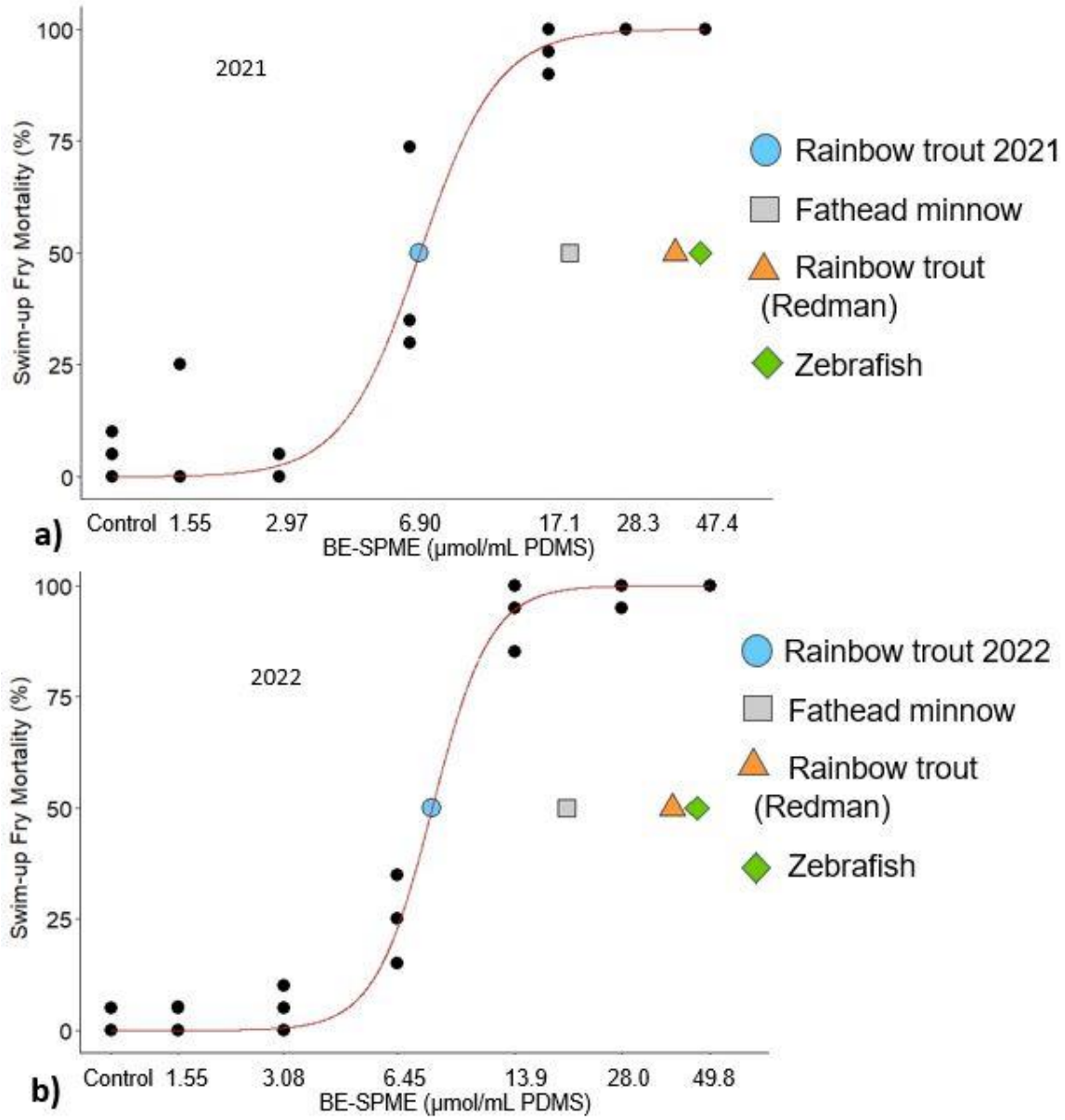

Figure 14. **Comparison of BE-SPME-derived LC_{50} concentrations estimated from EMBSI extraction values . a) 30-day exposure to rainbow trout (●) (7.38 ± 0.37 µmol/mL PDMS; present study); and 4-day acute exposures to fathead minnow (■) (19.5 ± 1.6 µmol/mL PDMS), rainbow trout (▲) (38.9 ± 1.2 µmol/mL PDMS), and zebrafish (♦) (45.7 ± 1.7 µmol/mL PDMS) in 2021. b) 30-day exposure to rainbow trout (●) (8.12 ± 0.28 µmol/mL PDMS; present study); and 4-day acute exposures to fathead minnow, rainbow trout, and zebrafish in 2022. 4-day acute exposures were obtained from Redman et al. (2018a,b). Data points (●) represent the average percentage of dead swim-up fry per replicate of each OSPW series dilution. Red line (—) is the estimated fitted dose-response trendline. Graphed in R.**

The LC_{50} value for total naphthenic acids concentration in 2021 was slightly lower (2023 ± 114 µg/L) than in 2022 (2220 ± 8 µg/L) (Table 9; Figure 11c and 12c), with high variability and overlap in NAs values. This could be explained to similar NAs concentrations observed in dilutions 2.20% OSPW to 10.0% OSPW in both years (Figure 9). LC_{50} values are below the lowest observed effect concentrations (LOECs) for mortality (22% OSPW) in both years (Table 9). It required a high OPSW dilution of 4.60% to obtain no observed effect concentrations (NOECs) for mortality. This is equivalent to BE-SPME concentrations of 8.35 ± 2.29 µmol/mL PDMS in 2021 and 3.82 ± 0.428 µmol/mL PDMS in 2022, and total NAs concentrations of 807 ± 240 µg/L in 2021 and 976 ± 80.0 µg/L in 2022.

In addition to narcosis, cardiovascular effects and alterations of gene expression could further explain mortality and delay in hatching of RBT exposed to OSPW. When exposed to OSPW, NAs penetrate the chorion, a semipermeable membrane that surrounds the embryo for protection (Mylroie et al., 2021). Zebrafish embryos exposed to dimethyl sulfoxide (DMSO), an amphipathic organic solvent used in embryonic tests, demonstrated accumulation of DMSO in the heart and common cardiac veins (ducts of Cuvier) that control blood circulation during embryonic development (Kais et al., 2013). NAs accumulation in the heart area and veins (Young et al., 2011) could cause blood flow restriction that leads to cardiovascular problems that could affect embryonic development and persist during the larval or alevin stage (Reynolds et al., 2022). Many alevins in the current study had pericardial and spinal hemorrhages.

NAs also affect gene networks involved with calcium ion export and mobilization, which are important roles for the function of cardiac muscle cells (Loughery et al., 2019). NAs activates the aryl-hydrocarbon receptor (AhR) that regulates the gene expression of enzymes (cytochrome P450) involved in the metabolism of aromatic hydrocarbons (Marentette et al., 2017; Wang et al., 2016; Granados et al., 2022). However, metabolism of hydrocarbons could produce free radicals in excess that damage lipids, proteins, and the DNA of cells, causing oxidative stress and apoptosis (Marentette et al., 2017; Granados et al., 2022; Reynolds et al., 2022). Yolk sac edemas observed in embryos could have prevented movement inside the chorion which is necessary for exercising, muscle contraction and development, and distribution of hatching enzymes (Vines et al., 2000; Peters et al., 2007). This would result in weak embryos unable to rupture the chorion for hatching. Impacts from weakened cardiac muscles and oxidative

stress could have contributed to post-hatch mortality in OSPW treatments. Reduced mortality in Day 15 water and Aged OSPW might be the result of wetland and mesocosm treatment biodegrading toxic NAs that induce cardiovascular and developmental problems in the early life stages of RBT.

3.3. Deformities

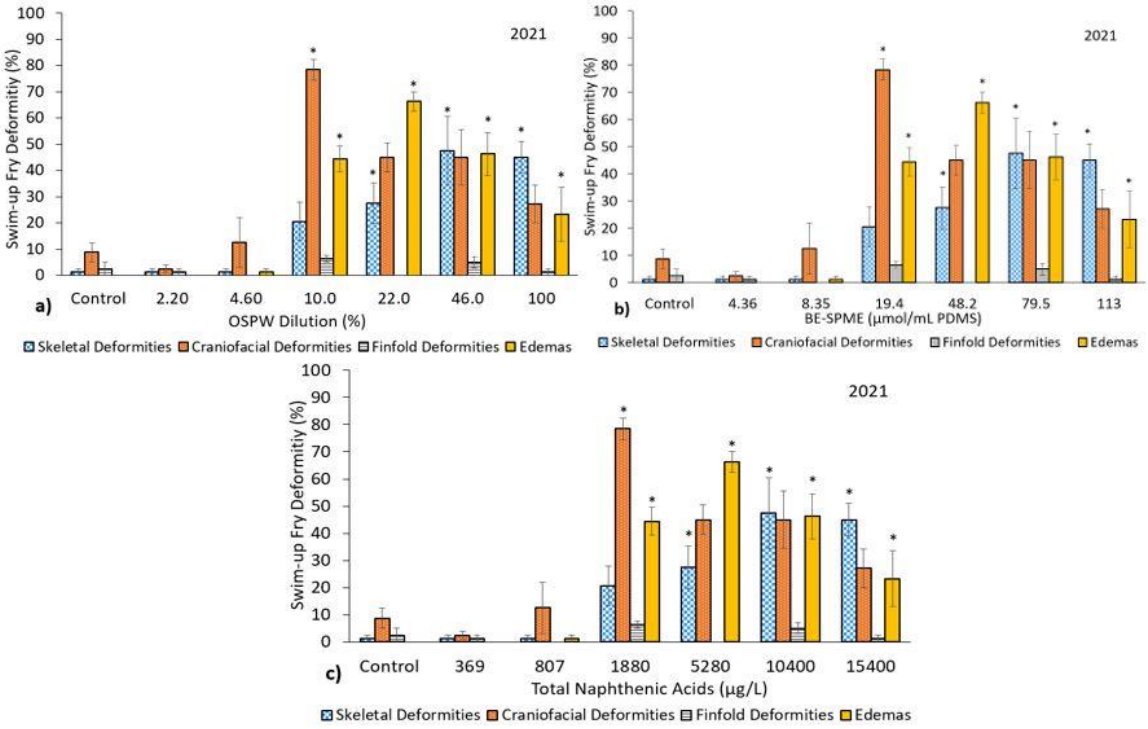

Figure 15. **Deformity (%) of rainbow trout until the swim-up fry stage in 2021. a) Exposure to OSPW series dilutions (%). b) Exposure to BE-SPME (µmol/mL PDMS). c) Exposure to total naphthenic acids (µg/L). Deformities include skeletal, craniofacial, and finfold deformities and edemas. BE-SPME and total NAs concentrations were obtained from InnoTech Alberta. Values are presented as the mean ± SE (n=20 per treatment, 4 replicates). Asterisks (*) represent significant difference compared to the control ($p<0.05$). Significant differences were observed in dilutions from 10.0% OSPW to 100% OSPW (Kruskal-Wallis test and pairwise comparison, $p<0.05$).**

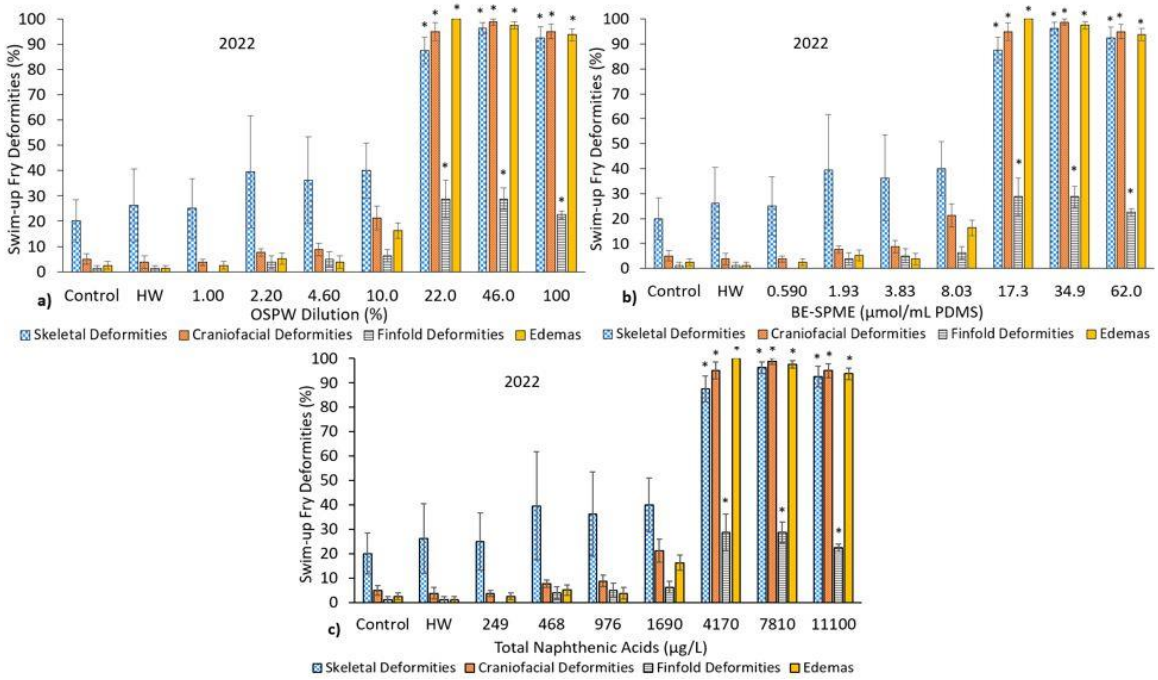

Figure 16. **Deformity (%) of rainbow trout until the swim-up fry stage in 2022. a) Exposure to OSPW series dilutions (%). b) Exposure to BE-SPME (µmol/mL PDMS). c) Exposure to total naphthenic acids (µg/L). HW represents exposures to hard water. Deformities include skeletal, craniofacial, and finfold deformities and edemas. BE-SPME and total NAs concentrations were obtained from InnoTech Alberta. Values are presented as the mean ± SE (n=20 per treatment, 4 replicates). Asterisks (*) represent significant difference compared to the control ($p<0.05$). Significant differences were observed in dilutions from 22.0% OSPW to 100% OSPW (Kruskal-Wallis test and pairwise comparison, $p<0.05$).**

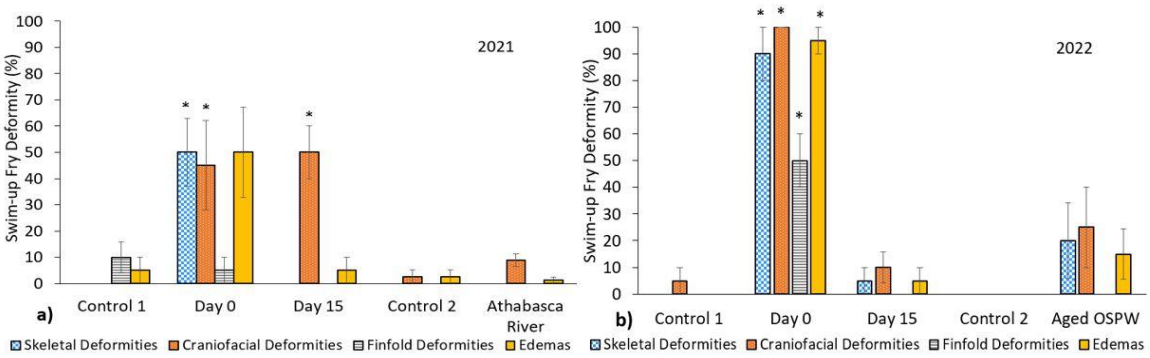

Figure 17. **Deformity (%) of rainbow trout until the swim-up fry stage. a) Exposure to constructed wetland water (Day 0 and Day 15) (n=5 per treatment, 4 replicates) and Athabasca River water (n=20 per treatment, 4 replicates) in 2021 with corresponding controls. b) Constructed wetland (Day 0 and Day 15) and Aged OSPW (n=5 per treatment, 4 replicates) in 2022 with corresponding controls. Deformities include skeletal, craniofacial, and finfold deformities and edemas. Values are presented as the mean ± SE. Asterisks (*) represent significant difference compared to corresponding controls (P<0.05). Significant differences were mainly observed in Day 0 water (Kruskal-Wallis test and pairwise comparison, p<0.05). Exposure to Day 15 water in 2021 had significantly high craniofacial deformities (Kruskal-Wallis test and pairwise comparison, p<0.05).**

Table 10. **Summary of yolk sac development (%), total length (mm), and wet weight (g) for exposure to OSPW series dilutions (n=20), pH-adjusted OSPW dilutions (n=18), Day 0 and Day 15 water (n=5 each), and Athabasca River water (n=20) in 2021. Yolk sac score 1 represents complete absorption of the yolk sac, 2 represents some visible yolk sac with a separated epidermis, and 3 represents a protruding yolk sac indicating the least development. Values are mean ± standard error (S.E.). Aesthetics (*) represent statistical difference compared to the control and letters represent the hypothesis testing method (see notes). pH-adjusted 46.0% and 100% OSPW dilutions were statistically compared to unadjusted 46.0% and 100% OSPW dilutions.**

	Yolk Sac Score 1 (%)	Yolk Sac Score 2 (%)	Yolk Sac Score 3 (%)	Total Length (mm)	Wet Weight (g)
Treatment	**Mean ± S.E**	**Mean ± S.E**	**Mean ± S.E**	**Mean ± S.E**	**Mean ± S.E**
Control	95 ± 2.0	1.25 ± 1.25	3.75 ± 2.39	24.4 ± 0271	0.099 ± 0.0023
2.20% OSPW	92.5 ± 7.50	1.25 ± 1.25	6.25 ± 6.25	23.8 ± 0.725	0.092 ± 0.0015
4.60% OSPW	74.9 ± 10.5	23.9 ± 9.59 * [a]	1.25 ± 1.25	24.5 ± 0.118	0.092 ± 0.0018
10.0% OSPW	3.75 ± 2.39 * [a]	59 ± 4.2 * [a]	36 ± 5.5	20.9 ± 0.328	0.12 ± 0.012
22.0% OSPW	0.0 ± 0.0 * [a]	13.8 ± 6.25	86 ± 6.3 * [a]	16.9 ±	0.11 ±

	Yolk Sac Score 1 (%)	Yolk Sac Score 2 (%)	Yolk Sac Score 3 (%)	Total Length (mm)	Wet Weight (g)
Treatment	**Mean ± S.E**	**Mean ± S.E**	**Mean ± S.E**	**Mean ± S.E**	**Mean ± S.E**
				0.0902 * [a]	0.0060
46.0% OSPW	0.0 ± 0.0 * [a]	0.0 ± 000	100 ± 0.0 * [a]	15.2 ± 0.767 * [a]	0.087 ± 0.0045 * [a]
100% OSPW	0.0 ± 0.0 * [a]	0.0 ± 0.0	100 ± 0.0 * [a]	10.2 ± 0.949 * [a]	0.079 ± 0.0028 * [a]
Adjusted 46.0% OSPW	0.0 ± 000	0.0 ± 000	100 ± 0.0	16.5 ± 0.299	0.087 ± 0.0058
Adjusted 100% OSPW	0.0 ± 0.0	0.0 ± 0.0	100 ± 0.0	15.4 ± 0.766 * [a]	0.088 ± 0.0059
Control	100 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	24.7 ± 0.103	0.093 ± 0.00094
Day 0 water	0.0 ± 0.0 * [a]	0.0 ± 0.0	100 ± 0.0 * [a]	15.1 ± 0.832 * [a]	0.093 ± 0.0054
Day 15 water	65 ± 5.0	25 ± 9.6 * [a]	10 ± 5.8	22.7 ± 0.577	0.093 ± 0.0051
Control	100 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	23.7 ± 0.255	0.092 ± 0.0031
Athabasca River water	100 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	23.6 ± 0.0399	0.082 ± 0.0017 * [b]

[a] Kruskal-Wallis test and pairwise comparison, $p<0.05$
[b] Independent samples t-test, $p<0.05$

Table 11. Summary of yolk sac development (%), total length (mm), and wet weight (g) for exposure to OSPW series dilutions (n=20), hard water (n=20), constructed wetland treatment (Day 0 and Day 15) (n=5 each), and aged OSPW (n=5) in 2022. Yolk sac score 1 represents complete absorption of the yolk sac, 2 represents some visible yolk sac with a separated epidermis, and 3 represents a protruding yolk sac indicating the least development. Values are mean ± standard error (S.E.). Aesthetics (*) represent statistical difference compared to the control and letters represent the hypothesis testing method (see notes).

	Yolk Sac Score 1 (%)	Yolk Sac Score 2 (%)	Yolk Sac Score 3 (%)	Total Length (mm)	Wet Weight (g)
Treatment	**Mean ± S.E**	**Mean ± S.E**	**Mean ± S.E**	**Mean ± S.E**	**Mean ± S.E**
Control	96.3 ± 1.25	2.5 ± 1.4	1.25 ± 1.25	23.8 ± 0.076	0.0849 ± 0.00109
Hard Water	97.5 ± 1.44	1.25 ± 1.25	1.25 ± 1.25	24.1 ± 0.180	0.0886 ± 0.00083
1.00%	97.5 ± 1.44	0.0 ± 0.0	2.5 ± 1.4	24.1 ± 0.0842	0.0876 ± 0.00185
2.20%	91 ± 5.5	5.0 ± 5.0	3.8 ± 1.3	24.2 ± 0.121	0.0913 ± 0.00157

	Yolk Sac Score 1 (%)	Yolk Sac Score 2 (%)	Yolk Sac Score 3 (%)	Total Length (mm)	Wet Weight (g)
Treatment	**Mean ± S.E**	**Mean ± S.E**	**Mean ± S.E**	**Mean ± S.E**	**Mean ± S.E**
4.60%	96 ± 2.4	1.25 ± 1.25	2.0 ± 2.5	24.0 ± 0.405	0.0917 ± 0.00216
10.0%	62.5 ± 9.68	20 ± 7.4	17.5 ± 3.23	22.6 ± 0.359	0.0919 ± 0.00264
22.0%	0.0 ± 0.0 *[a]	2.5 ± 1.4	97.5 ± 1.44 *[a]	18.2 ± 0.723	0.113 ± 0.00461 *[a]
46.0%	0.0 ± 0.0 *[a]	2.5 ± 2.5	97.5 ± 2.50 *[a]	17.4 ± 0.803 *[a]	0.120 ± 0.0124 *[a]
100%	0.0 ± 0.0 *[a]	0.0 ± 0.0	100 ± 0.0 *[a]	15.7 ± 0.773 *[a]	0.0941 ± 0.00800
Control	100 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	24.4 ± 0.258	0.0889 ± 0.00337
Day 0 water	0.0 ± 000 *[a]	5.0 ± 5.0	95 ± 5.0 *[a]	20.7 ± 0.234 *[b]	0.0989 ± 0.00302
Day 15 water	75.0 ± 18.9	20 ± 20	5.0 ± 5.0	23.2 ± 0.249 *[b]	0.0948 ± 0.00193
Control	100 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	24.4 ± 0.105	0.0897 ± 0.00203
Aged OSPW	80 ± 14	5.0 ± 5.0	15. ± 9.6	22.5 ± 0.463 *[C]	0.0876 ± 0.00503

[a] Kruskal-Wallis test and pairwise comparison, $p<0.05$
[b] One-Way ANOVA and Dunnett's test, $p<0.05$
[c] Independent samples t-test, $p<0.05$

3.3.1. Skeletal Deformities

Lordosis was the most common skeletal deformity in 2021 and 2022, followed by scoliosis and kyphosis. Hemorrhage was observed along the spine of some alevin and swim-up fry, mostly in 22.0%, 46.0% and 100% OSPW. Overall, the frequency of skeletal deformities increased with decreasing OSPW dilutions or increasing BE-SPME and total NAs concentrations in both years (Figure 15 and 16). A similar increasing trend in skeletal deformity incidences has been observed in zebrafish and fathead minnow exposed to increasing concentrations of oil sands extracted fraction of NAs (Wang et al., 2015; Marentette et al., 2015). But it required lower BE-SPME concentrations in 2022 to cause more skeletal deformity frequencies than in 2021. OSPW dilutions 22.0%, 46.0%, and 100% had significant (Kruskal-Wallis, pairwise comparison, $p<0.05$) skeletal deformities of 27.5% ± 7.77%, 47.5% ± 13.0%, and 45% ± 6.1% in 2021 (Table 7); and 87.5% ± 5.20%, 96% ± 2.4%, and 92.5% ± 4.33% in 2022 (Table 8), respectively.

Skeletal effects in 2021 were almost negligible in control dechlorinated water and dilutions 2.20% and 4.60% OSPW (1.25% ± 1.25% each). Despite no significant difference (Kruskal-Wallis test, pairwise comparison, $p<0.05$), skeletal deformity frequencies in 2022 where high in control dechlorinated water (20% ± 8.4%), 1.00% OSPW (25% ± 12%), 2.20% OSPW (39.5% ± 22.1%), and 4.60% OSPW (36% ± 17%). But skeletal deformities in less concentrated OSPW dilutions mostly had a score index of 1 or slight severity compared to more concentrated OSPW dilutions that had scores of 2 and 3. Difference in water hardness and pH adjustment to 46.0% and 100% OSPW had no significant (Kruskal-Wallis test, pairwise comparison, $p>0.05$) impact on skeletal deformities. Although not significantly different, pH-adjustment in 46.0% OSPW deceased skeletal deformity frequency by 49.71% on average.

Wetland treatment effectively decreased skeletal deformities in 2021 and 2022 (Figure 17). Day 0 water in 2021 with a BE-SPME concentration of 133 µmol/mL PDMS had a skeletal deformity percentage (50% ± 13%) similar to 46.0% OSPW (47.5% ± 13.0%) and 100% OSPW (45% ± 6.1%). No skeletal deformities were observed in Day 15 water in 2021. Day 0 water in 2022 (50.0 µmol/mL PDMS) had a skeletal deformity percentage (90% ± 10%) similar to 22.0%, 46.0% and 100% OSPW but was significantly reduced (Kruskal-Wallis test, pairwise comparison, $p<0.05$) to 5.0% ± 5.0% after 15 days (44.5 µmol/mL PDMS) of wetland treatment. OSPW treated by wetland treatment in both years resulted in less skeletal deformities than any OSPW dilution despite having a BE-SPME comparable to 46.0% OSPW and 100% OSPW. Similar to RBT mortality, mesocosm pond treatment (aged OSPW) decreased skeletal deformities but was less effective than wetland treatment. Aged OSPW resulted in 20% ± 14% skeletal deformities with no significant difference (Mann-Whitney U Test, $p>0.05$) compared to the control. Athabasca River water did not cause skeletal deformities.

3.3.2. Craniofacial Deformities

The most common craniofacial deformities observed in 2021 and 2022 were bumps on the head, reduced upper and/or lower jar, and enlarged eye(s). Fathead minnow exposed to NAs also resulted in underdeveloped jaw and head (Reynolds et al., 2022). Hemorrhaging was prevalent in the head, eyes, and heart. Some alevins and swim-up fries had edema around their eyes. RBT exposed to 10.0%, 22.0%, 46.0%, and 100% OSPW had the most craniofacial deformities in 2021 and 2022 (Figure 15 and 16).

But the OPSW sample from 2022 with lower BE-SPME and total NAs concentrations caused more craniofacial deformities than the sample from 2021. Significant difference (Kruskal-Wallis test, pairwise comparison, $p<0.05$) was only observed in 10.0% OSPW from 2021 with 78% ± 3.9% craniofacial deformities. OSPW dilutions 22.0%, 46.0%, and 100% from 2022 had on average more than 95% craniofacial deformities with significant differences (Kruskal-Wallis test, pairwise comparison, $p<0.05$) compared to the control. pH adjustment and water hardness had no significant (Kruskal-Wallis test, pairwise comparison, $p<0.05$) impact on the percentage of craniofacial deformities.

Wetland treatment was only effective in 2022 at significantly (Kruskal-Wallis test, pairwise comparison, $p<0.05$) reducing the percentage of craniofacial deformities from 100% ± 0.0% in Day 0 water to 10% ± 5.8% in Day 15 water (Figure 17). Day 15 water with a BE-SPME concentration of 44.5 µmol/mL PDMS had a similar craniofacial deformity frequency as 2.20% OSPW (7.6% ± 1.5%) and 4.60% OSPW (8.75% ± 2.39%). The percentage of craniofacial deformities remained high in Day 0 and 15 water in 2021, 45% ± 17% and 50% ± 10%, respectively. Craniofacial deformities in aged OSPW (20% ± 14%) and Athabasca River water (0.0% ± 0.0%) were not significantly (Mann-Whitney U Test, $p>0.05$) different than control dechlorinated water.

3.3.3. Finfold Deformities

Finfold deformities were the least observed deformities in 2021 and 2022 (Figure 15 and 16). Results for finfold deformities are conflicting in other studies. Marentette et al. (2015) did not observe significant finfold malformations in fathead minnow exposed to NAFC extracted from OSPW, but a similar study conducted with walleye resulted in caudal finfold deformities (Marentette et al., 2017). Some alevins and swim-up fries from the present study had small tail fin (caudal fin) with hemorrhage. Tail fin was not fully formed in the least developed alevins. On average, the percentage of finfold deformities in 2021 was less than 6.3% ± 1.2%, and no OSPW dilution was significantly (Kruskal-Wallis test, pairwise comparison, $p>0.05$) different than the control. In 2022, RBT exposed to control dechlorinated water, 1.00% OSPW, 2.20% OSPW, 4.60% OSPW, and 10.0% OSPW had less than 6.25% ± 2.39% of finfold deformities. The percentage of finfold deformities in 2022 significantly (Kruskal-Wallis test, pairwise comparison, $p<0.05$) increased to 28.8% ± 7.47% in 22.0% OSPW, 28.8% ± 4.27% in 46.0% OSPW, and 22.5% ± 1.44% in 100% OSPW. pH adjustment and water hardness did not have a

significant (Kruskal-Wallis test, pairwise comparison, $p>0.05$) effect on the percentage of finfold deformities.

Day 0 water had finfold deformity percentages of 5.0% ± 5.0% in 2021 and 50% ± 10% in 2022 (Figure 17). Day 0 water from 2022 has a BE-SPME concentration (50.0 µmol/mL PDMS) similar to 100% OSPW (62.0 µmol/mL PDMS), yet Day 0 water had a higher finfold deformity percentage than any OSPW dilution. No finfold deformities were observed in Day 15 water of both years and in aged OSPW, indicating successful wetland and mesocosm treatment of OSPW despite having high BE-SPME and total NAs concentrations. No finfold deformities were observed in Athabasca River water.

3.3.4. Edemas

The most observed edemas were yolk sac and pericardial edemas with hemorrhaging from vitelline vessels in alevins and swim-up fries involved with blood circulation from and to the yolk sac. Hemorrhages and edemas were also observed in eyes and the head of some alevins and fries. Yolk sac and pericardial edemas have also been observed in zebrafish and fathead minnow exposed to oil sands extracted NAs, most notably when exposed to high concentrations of NAs (Wang et al., 2015; Reynolds et al., 2022). RBT mostly had edemas with a severity score index of 1 (slight severity) when exposed to less concentrated OSPW dilutions and moderate to extreme edemas when exposed to highly concentrated OSPW dilutions. RBT exposed to control dechlorinated water, 2.20% OSPW, and 4.60% OSPW in 2021 had less than 1.25% ± 1.25% of edemas (Table 7; Figure 15). The percentage of edemas significantly (Kruskal-Wallis test, pairwise comparison, $p<0.05$) increased to 44% ± 5.1% in 10.0% OSPW, 66% ± 3.8% in 22.0% OSPW, 46% ± 8.3% in 46.0% OSPW, and 23% ± 10% in 100% OSPW. In 2022, RBT exposed to control dechlorinated water, 1.00% OSPW, 2.20% OSPW, 4.60% OSPW, and 10.0% OSPW had less than 16% ± 3.2% of edemas (Table 8; Figure 16). The percentage of edemas significantly (Kruskal-Wallis test, pairwise comparison, $p<0.05$) increased to 100% ± 0.0% in 22.0% OSPW, 97.5% ± 1.44% in 46.0% OSPW, and 93.8% ± 2.39% in 100% OSPW. pH adjustment and water hardness had no significant (Kruskal-Wallis test, pairwise comparison, $p>0.05$) impact on the percentage of edemas.

Wetland treatment significantly (Kruskal-Wallis test, pairwise comparison, $p<0.05$) decreased the percentage of edemas in Day 0 water from 50% ± 17% in 2021 (Table 7; Figure 17) and 95% ± 5.0% in 2022 (Table 8) to 5.0% ± 5.0% in Day 15 water each year. Mesocosm pond treatment (aged OSPW) was less effective at reducing edemas to 15% ± 9.6% but it was not significantly (Mann-Whitney U Test, $p>0.05$) different than the control. Athabasca River water had no significant impact (Mann-Whitney U Test, $p>0.05$) on RBT.

3.3.5. Total Deformities

Craniofacial malformations and edemas were the most common deformities observed in both years. And finfold deformities were the least observed deformities. In general, high percentage of total deformities accumulated among the lowest OSPW dilutions (10.0% to 100% OSPW) or highest BE-SPME and total NAs concentrations in both years (Figure 18 and 19). Results are consistent with other studies. Fathead minnow exposed to increasing concentrations of Nas resulted in significantly increasing frequencies of total malformations (Reynolds et al., 2022; Marentette et al., 2015). In the present study, significant (Kruskal-Wallis test, pairwise comparison, $p<0.05$) effects started to occur in both years at similar and overlapping BE-SPME concentrations (19.4 ± 4.37 µmol/mL PDMS in 2021 and 17.3 µmol/mL PDMS in 2022) but at different total NAs concentrations (1880 µg/L in 2021 and 4170 µg/L in 2022). Despite requiring a higher NAs concentration to start causing significant malformations, 2022 OPSW dilutions induced higher percentages of total deformities (97.5% ± 1.44% to 100% ± 0.0%) than 2021 OPSW dilutions (58% ± 9.5% to 91% ± 3.2%). Possibly the 2022 OSPW sample had more toxic NA congeners or the BE-SPME extracted concentration had more organic and inorganic compounds that contributed to chronic toxicity.

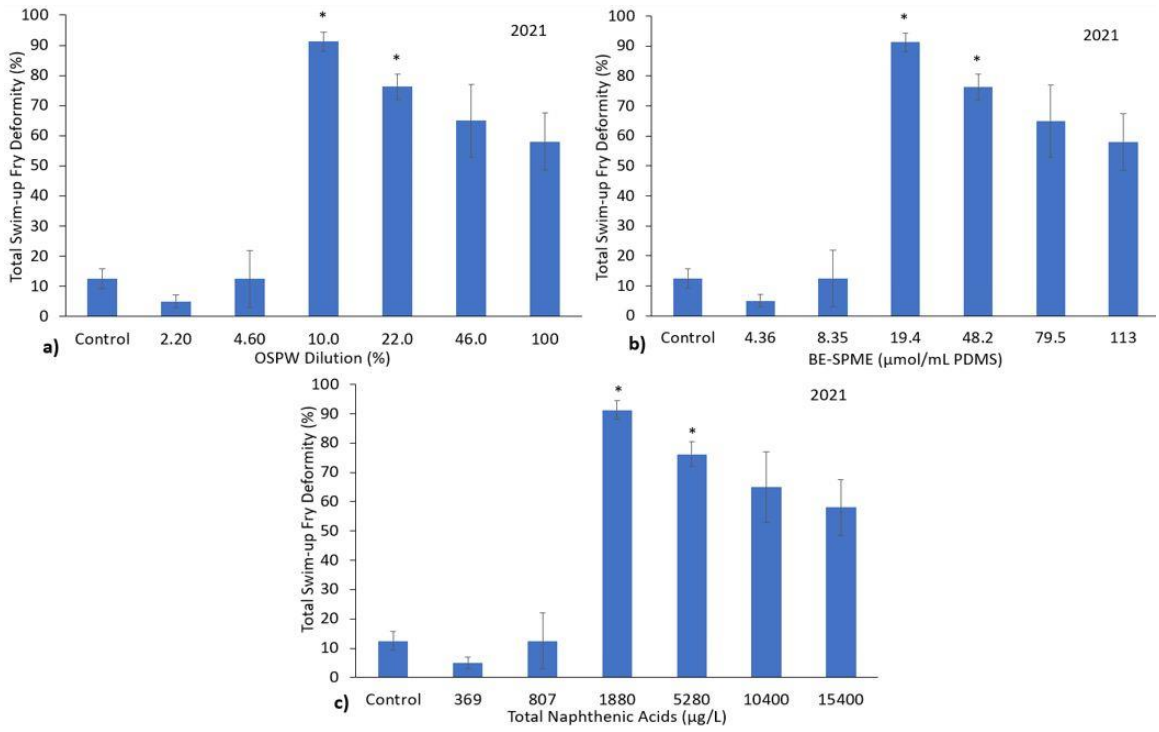

Figure 18. **Total deformity (%) of rainbow trout until the swim-up fry stage in 2021. a) Exposure to OSPW series dilutions (%). b) Exposure to BE-SPME (µmol/mL PDMS). c) Exposure to total naphthenic acids (µg/L). Total deformity refers to the percentage individuals with at least one type of deformity (skeletal, craniofacial, and finfold deformities and edemas). BE-SPME and total NAs concentrations were obtained from InnoTech Alberta. Values are presented as the mean ± SE (n=20 per treatment, 4 replicates). Asterisks (*) represent significant difference compared to the control ($p<0.05$). Significant differences were observed in dilutions 10.0% OSPW and 22.0% OSPW (Kruskal-Wallis test and pairwise comparison, $p<0.05$).**

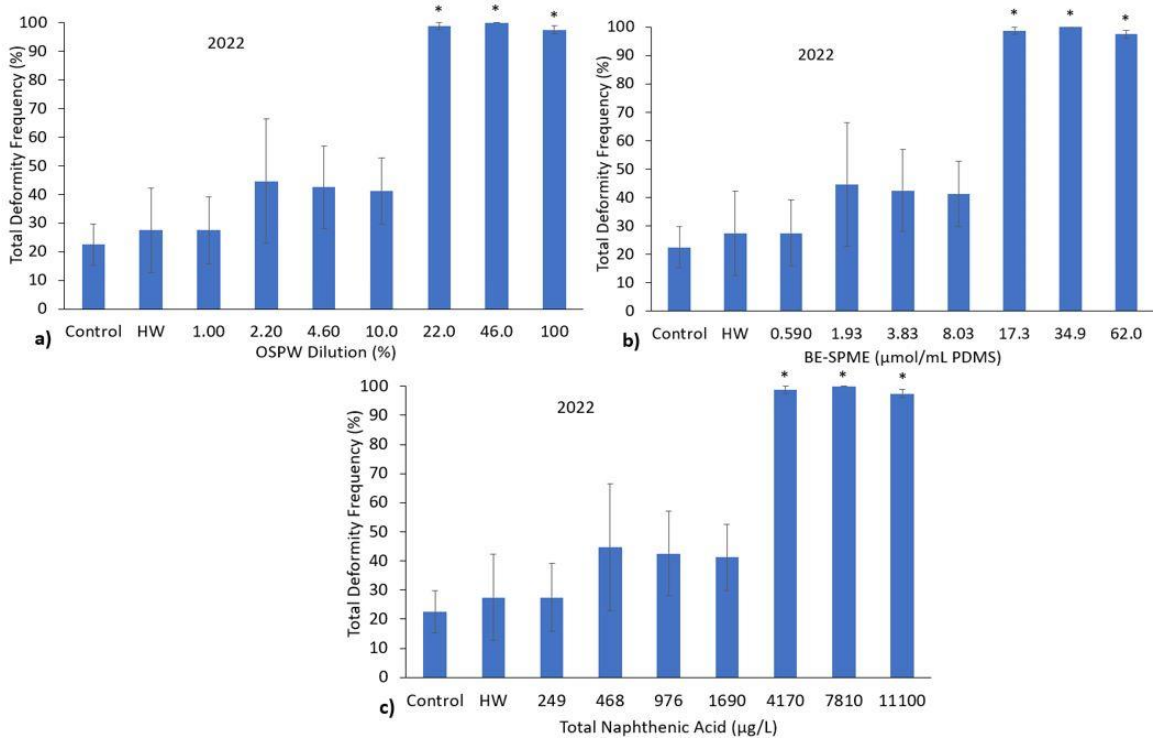

Figure 19. **Total deformity (%) of rainbow trout until the swim-up fry stage in 2022. a) Exposure to OSPW series dilutions (%). b) Exposure to BE-SPME (µmol/mL PDMS). c) Exposure to total naphthenic acids (µg/L). HW represents exposures to hard water. Total deformity refers to the percentage individuals with at least one type of deformity (skeletal, craniofacial, and finfold deformities and edemas). BE-SPME and total NAs concentrations were obtained from InnoTech Alberta. Values are presented as the mean ± SE (n=20 per treatment, 4 replicates). Asterisks (*) represent significant difference compared to the control ($p<0.05$). Significant differences were observed in dilutions 22.0% OSPW to 100% OSPW (Kruskal-Wallis test and pairwise comparison, $p<0.05$).**

Wetland treatment in 2022 was more effective at decreasing the percentage of total deformities than in 2021 (Figure 20). All individuals exposed to Day 0 water from 2022 resulted with at least one type of deformity and had a 10-fold decrease in total deformities after wetland treatment (Day 15 water). No significant difference was observed between Day 15 water and the control (Kruskal-Wallis test, pairwise comparison, $p>0.05$). Wetland treatment in 2022 had a lower malformation percentage than any OSPW dilution and more comparable to Athabasca River water, which had a total deformity percentage (10.1% ± 1.99%) not significantly (Mann-Whitney U Test, $p>0.05$) different than the control. Mesocosm pond treatment was slightly less effective than wetland treatment in 2022 but it was able to decrease the percentage of total

deformities to 25% ± 15% with no significant (Mann-Whitney U Test, $p<0.05$) difference compared to the control. In 2021, 85% ± 5.0% of individuals exposed to Day 0 water had at least one type of deformity, mostly of moderate severity, and had a 1.7-fold decrease in total deformities after 15 days of wetland treatment. Total deformities in Day 15 water from 2021 (50% ± 10%) was high mainly due to a high percentage of slightly severe craniofacial deformities and it was significantly (Kruskal-Wallis test, pairwise comparison, $p<0.05$) different than the control. pH adjustment and water hardness had no significant (Kruskal-Wallis test, pairwise comparison, $p>0.05$) impact on the percentage of total deformities.

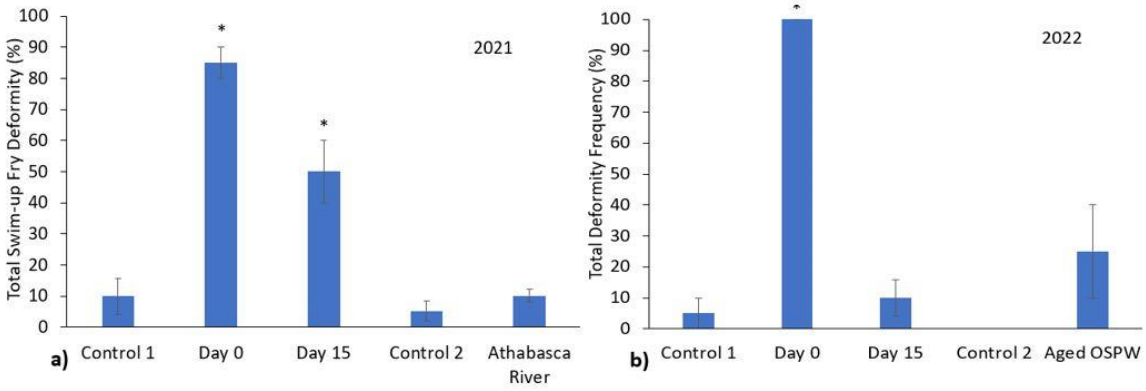

Figure 20. **Total deformity (%) of rainbow trout until the swim-up fry stage. a) Exposure to constructed wetland water (Day 0 and Day 15) (n=5 per treatment, 4 replicates) and Athabasca River water (n=20, 4 replicates) in 2021 with corresponding controls. b) Exposure to constructed wetland water (Day 0 and Day 15) and Aged OSPW (n=5 per treatment, 4 replicates) in 2022 with corresponding controls. Total deformity refers to the percentage individuals with at least one type of deformity (skeletal, craniofacial, and finfold deformities and edemas). Values are presented as the mean ± SE. Asterisks (*) represent significant difference compared to the control ($P<0.05$). Significant differences were observed in Day 0 and Day 15 water in 2021 (One-way ANOVA and Dunnett's test, $p<0.05$) and in Day 15 water in 2022 (Kruskal-Wallis test and pairwise comparison, $p<0.05$).**

Impacts on cardiovascular and skeletal development during the embryonic stage could have induced chronic morphological problems that worsen during the larval stage. NAs alter the expression of cardiac developmental genes that affect calcium ion homeostasis, muscle cell function, blood flow, oxidative stress, and overall cardiovascular function. (Reynolds et al., 2022; Loughery et al., 2019; Marentette et al., 2017; Wang et al., 2016). This could lead to the development of cardiovascular problems such as yolk sac and pericardial edemas and hemorrhaging. In addition to cardiotoxicity,

craniofacial malformations such as reduced jaw length or short snout and skeletal malformations observed in this study could be related to alterations of skeletal gene networks involved with chondrocyte development and endochondral ossification, when cartilage cells differentiate into osseous tissue during embryonic development (Loughery et al., 2019).

The EC_{50} values for OSPW dilutions are 10.6% ± 3.94% OSPW in 2021 and 11.9% ± 3.83% in 2022 (Table 12), with overlap in values. The EC_{50} value for BE-SPME was lower in 2022 (10.3 ± 2.98 µmol/mL PDMS) than in 2021 (20.2 ± 6.35 µmol/mL PDMS) due to higher BE-SPME concentrations measured in 2021 (Figure 9a). The EC_{50} value from 2022 compares to another 30-day BE-SPME derived EC_{25} value for RBT exposed to OSPW (9.53 µmol/mL PDMS) (Piggott, 2022). The EC_{50} value for total NAs concentration in 2021 was lower (2106 ± 792 µg/L) than in 2022 (3040 ± 1257 µg/L), with high variability and overlap in NAs values. EC_{50} values from 2021 overlap with LOEC values for total deformities, while EC_{50} values from 2022 are lower than the LOEC values. It required dilutions of 4.63% OSPW in 2021 and 10.0% OSPW in 2022 to obtain NOEC values for total deformities.

Table 12. EC_{50}, lowest observed effect concentration (LOEC), and no observed effect concentration (NOEC) values for total deformities from exposure to OSPW series dilutions (%), BE-SPME (µmol/mL PDMS), and total naphthenic acids (µg/L) in 2021 and 2022. BE-SPME and total NAs concentrations were obtained from InnoTech Alberta. Values represent the estimated value, standard deviation (S.D.), and 95% confidence interval for total deformities calculated in R.

	2021			2022		
OSPW Dilution (%)	**Estimate**	**S.D.**	**95% Confidence Interval**	**Estimate**	**S.D.**	**95% Confidence Interval**
EC_{50} Total Deformities	10.6	3.94	2.52 – 18.7	11.9	3.83	4.06 – 19.8
LOEC Total Deformities	10. 0	3.66	2.51 – 17.6	22.0	9.32	2.85 – 41.2
NOEC Total Deformities	4.63	1.92	0.672 – 8.58	10.0	3.07	3.70 - 16.3
BE-SPME (µmol/mL PDMS)	**Estimate**	**S.D**	**95% Confidence Interval**	**Estimate**	**S.D**	**95% Confidence Interval**
EC_{50} Total Deformities	20.2	6.35	7.13 – 33.2	10.3	2.98	4.16 – 16.4
LOEC Total	19.4	6.07	6.93 - 31.9	17.3	1.69	13.8 - 20.8

Deformities						
NOEC Total Deformities	8.35	3.12	1.95 - 14.8	17.3	6.32	4.29 - 30.3
Total Naphthenic Acids (µg/L)	**Estimate**	**S.D**	**95% Confidence Interval**	**Estimate**	**S.D**	**95% Confidence Interval**
EC_{50} Total Deformities	2106	792	478 - 3734	3040	1257	456 - 5625
LOEC Total Deformities	1882	698	446 - 3317	4170	1951	159 - 8181
NOEC Total Deformities	369	244	-132 - 871	1690	638	378 - 3002

3.4. Development

3.4.1. Yolk Sac Absorption

Embryos and alevins absorb their yolk sac for nutrition as they grow. Their yolk sac should be fully absorbed when they reach the swim-up fry stage, demonstrating larval full development. A delay of yolk sac absorption was observed in alevins exposed to the lowest OSPW dilutions or highest BE-SPME and total NAs concentrations in both years (Figure 21 and 22; Table 10 and 11). Most alevins exposed to 22.0% OSPW, 46.0% OSPW, and 100% OSPW in 2021 and 2022 stayed at yolk sac score 3, indicating a delay in alevin development, with significant differences (Kruskal-Wallis test, pairwise comparison, $p<0.05$) compared to the control. Visible protruding yolk sacs with edema and hemorrhage were observed in these three dilution treatments. It required a similar BE-SPME concentration in 2021 (8.35 ± 1.76 µmol/mL PDMS in 4.60% OSPW) and 2022 (8.03 µmol/mL PDMS in 10.0% OSPW) to start observing an effect on yolk sac absorption, in which, on average, 20.0% to 23.9% alevins had a yolk sac score of 2. In 2021, almost all alevins exposed to control dechlorinated water (95% ± 2.0%) and 2.20% OSPW (92.5% ± 7.50%) had a score 1 of yolk sac absorption, indicating full alevin development. Similarly in 2022, 91.2% to 97.5% of alevins exposed to control, 1.00% OSPW, 2.20% OSPW, and 4.60% OSPW fully absorbed their yolk sac and had a sealed abdominal epidermis (Score 1). pH adjustment and water hardness had no significant impact on yolk sac absorption.

LOEC values for yolk sac score of 1 were similar both years. The LOEC value for BE-SPME was slightly lower in 2022 (17.3 ± 12.3 µmol/mL PDMS) than in 2021 (19.3 ± 3.71 µmol/mL PDMS) but with overlap due to high variability in values (Table 13). Yet LOEC values for total NAs was lower in 2021 (1880 µg/L) than in 2022 (4170 ± 1470 µg/L). LOEC values for yolk sac score 1 compared to LOEC values for total deformities. NOEC values for yolk sac score 1 were 4.36 ± 0.797 µmol/mL PDMS in 2021 equivalent to a OSPW dilution of 2.19% and 7.78 ± 5.74 µmol/mL PDMS in 2022 equivalent to a OSPW dilution of 4.60%.

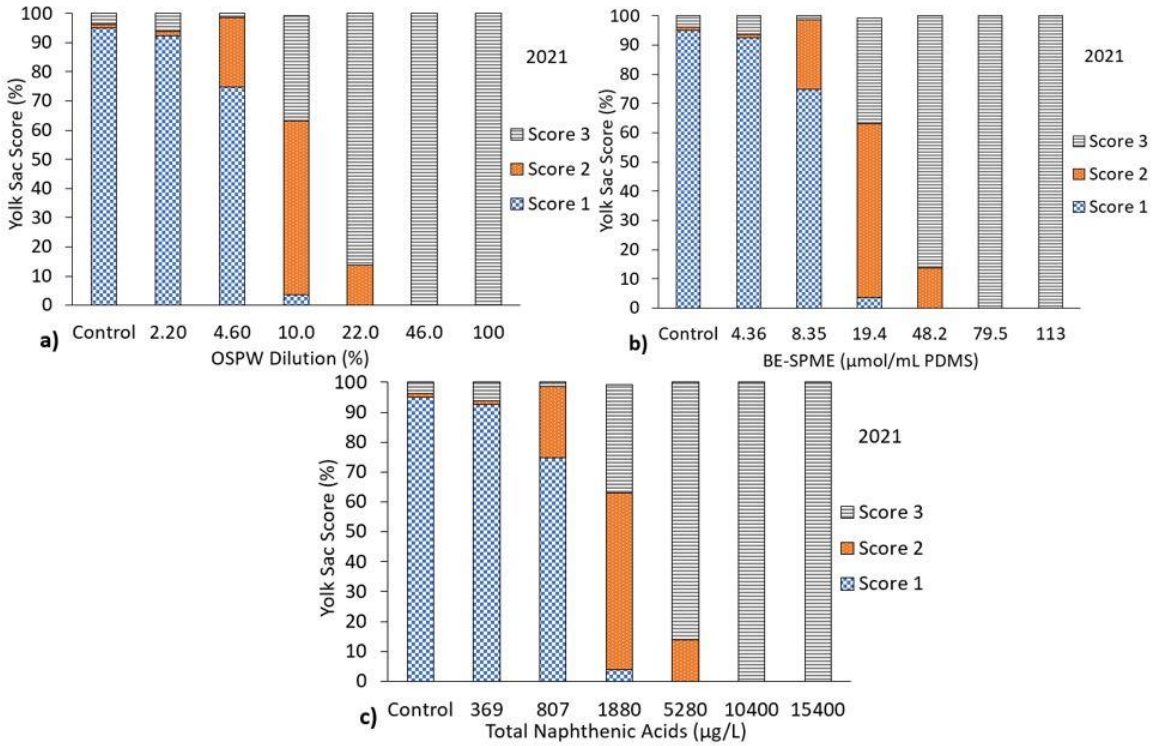

Figure 21. **Yolk sac absorption (%) of rainbow trout until the swim-up fry stage in 2021. a) Exposure to OSPW series dilutions (%). b) Exposure to BE-SPME (µmol/mL PDMS). c) Exposure to total naphthenic acids (µg/L). Score 1 = completely absorbed yolk sac with sealed abdominal epidermis indicating full development. Score 2 = slightly absorbed yolk sac. Score 3 = no absorption of yolk sac indicating delay in development. BE-SPME and total NAs concentrations were obtained from InnoTech Alberta. Values are presented as the mean (n=20 per treatment, 4 replicates). SE is not included for simplicity (see Table 10). Significant differences for yolk sac score 1 were observed in dilutions 10.0% OSPW to 100% OSPW; for score 2 in dilutions 4.60% OSPW and 10.0% OSPW; and for score 3 in dilutions 22.0% OSPW to 100% OSPW (Kruskal-Wallis test and pairwise comparison, $p<0.05$).**

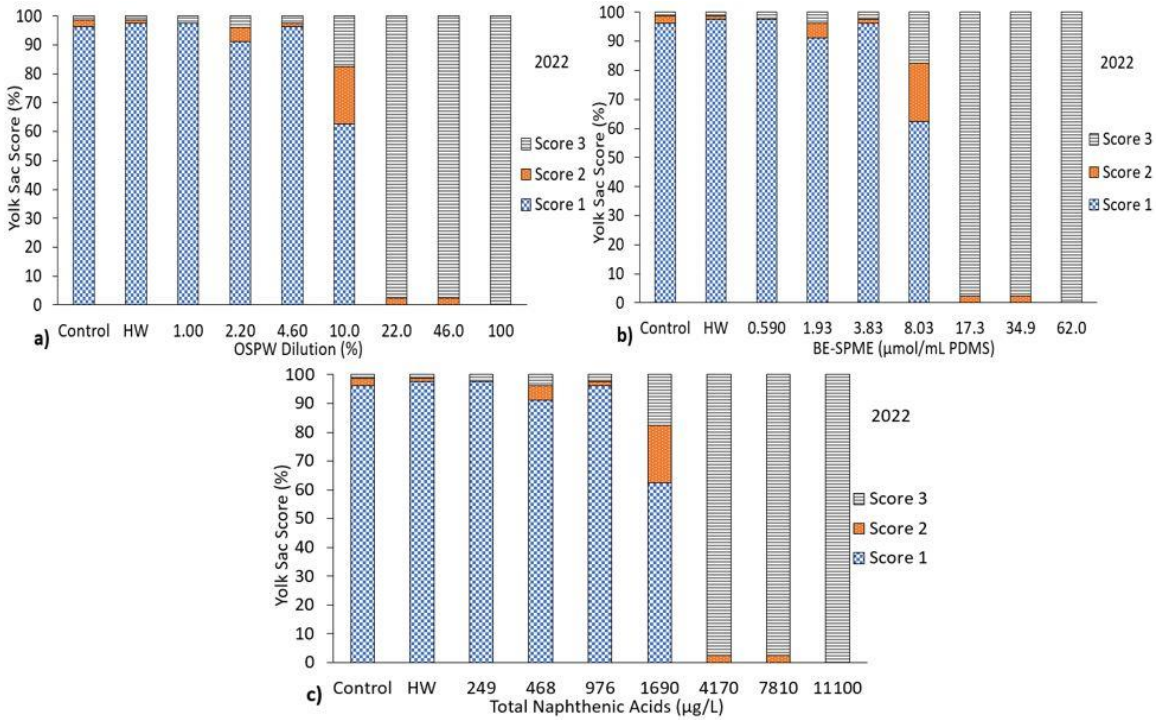

Figure 22. **Yolk sac absorption (%) of rainbow trout until the swim-up fry stage in 2022. a) Exposure to OSPW series dilutions (%). b) Exposure to BE-SPME (µmol/mL PDMS). c) Exposure to total naphthenic acids (µg/L). HW represents exposures to hard water. Score 1 = completely absorbed yolk sac with sealed abdominal epidermis indicating full development. Score 2 = slightly absorbed yolk sac. Score 3 = no absorption of yolk sac indicating delay in development. BE-SPME and total NAs concentrations were obtained from InnoTech Alberta. Values are presented as the mean (n=20 per treatment, 4 replicates). SE is not included for simplicity (see Table 11). Significant differences for yolk sac score 1 and 3 were observed in dilutions 22.0% OSPW to 100% OSPW (Kruskal-Wallis test and pairwise comparison, $p<0.05$).**

Table 13. **Lowest observed effect concentration (LOEC) and no observed effect concentration (NOEC) values for yolk sac score 1 from exposure to OSPW series dilutions (%), BE-SPME (µmol/ml PDMS), and total naphthenic acids (µg/L) in 2021 and 2022. BE-SPME and total NAs concentrations were obtained from InnoTech Alberta. Values represent the estimated value, standard deviation (S.D.), and 95% confidence interval calculated in R. Dashes represent values that could not be calculated in R.**

	2021			2022		
OSPW Dilution (%)	**Estimate**	**S.D.**	**95% Confidence Interval**	**Estimate**	**S.D.**	**95% Confidence Interval**
LOEC Yolk	10.0	1.98	5.93 – 14.1	21.6	8.65	3.79 – 39.3

Sac Score 1						
NOEC Yolk Sac Score 1	2.19	0.48	1.20 – 3.17	4.60	0.222	4.14 - 5.06
BE-SPME (µmol/mL PDMS)	**Estimate**	**S.D**	**95% Confidence Interval**	**Estimate**	**S.D**	**95% Confidence Interval**
LOEC Yolk Sac Score 1	19.3	3.71	11.7 – 27.0	17.3	12.7	-8.79 – 43.4
NOEC Yolk Sac Score 1	4.36	0.797	2.72 – 5.99	7.78	5.74	-4.02 - 19.6
Total Naphthenic Acids (µg/L)	**Estimate**	**S.D**	**95% Confidence Interval**	**Estimate**	**S.D**	**95% Confidence Interval**
LOEC Yolk Sac Score 1	1880	-	-	4170	1470	1148 - 7191
NOEC Yolk Sac Score 1	369	-	-	976	211	543 - 1408

Wetland treatment improved larval development. Almost all alevins in 2021 and 2022 exposed to Day 0 water had a significant score of 3 and none had a score of 1 (Figure 23) (Kruskal-Wallis test, pairwise comparison, $p<0.05$). This is comparable to results from exposure to 22.0% OSPW, 46.0% OSPW, and 100% OSPW dilutions. After 15 days of treatment, 65% ± 5.0% of alevins in 2021 and 75% ± 19% in 2022 had fully utilized their yolk sac (score 1) not significantly different than the control (Kruskal-Wallis test, pairwise comparison, $p>0.05$). Results from Day 15 water are similar to results from exposure to 4.60% OSPW (74.9% ± 10.5% for score 1) in 2021 and to 10.0% OSPW (62.5% ± 9.68% for score 1) in 2022. However, biomimetic extraction indicates that exposure to Day 15 water (44.5 µmol/mL PDSM) in 2022 should have comparable results to 46.0% OSPW (34.9 µmol/mL PDSM) where no alevin absorbed their yolk sac. Exposure to aged OSPW resulted in 80% ± 14% of alevins with a score of 1 with no significant difference compared to the control (Mann-Whitney U Test, $p>0.05$), demonstrating that mesocosm pond treatment is also helpful at improving alevin development. Aged OSPW (33.6 µmol/mL PDSM) had an impact on yolk sac absorption similar to 2.20% OSPW (1.93 µmol/mL PDSM) and 4.60% OSPW (3.83 µmol/mL PDSM), yet biomimetic extraction indicates that exposure to aged OSPW should have results similar to 46.0% OSPW (or 34.9 µmol/mL PDSM). All embryos and alevins exposed to Athabasca River water utilized their yolk sac.

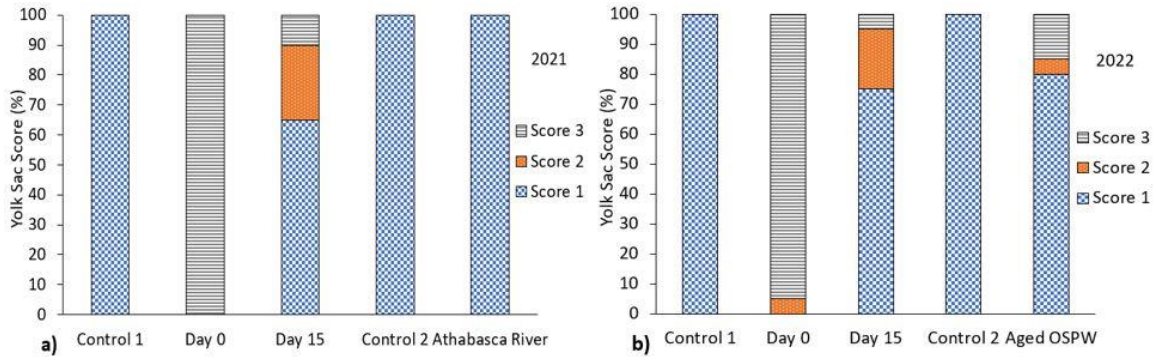

Figure 23. Yolk sac absorption (%) of rainbow trout until the swim-up fry stage. a) Exposure to constructed wetland water (Day 0 and Day 15) (n=5 per treatment, 4 replicates) and Athabasca River water (n=20, 4 replicates) in 2021 with corresponding controls. b) Exposure to constructed wetland water (Day 0 and Day 15) and Aged OSPW (n=5 per treatment, 4 replicates) in 2022 with corresponding controls. Score 1 = completely absorbed yolk sac with sealed abdominal epidermis indicating full development. Score 2 = slightly absorbed yolk sac. Score 3 = no absorption of yolk sac indicating delay in development. Values are presented as the mean. SE is not included for simplicity (see Table 10 and 11). Significant differences for yolk sac score 1 and 2 in Day 0 water of both years; for score 2 in Day 15 water in 2021 (Kruskal-Wallis test and pairwise comparison, $p<0.05$).

Decreased yolk sac absorption was also observed in RBT by Nina (2022) and in Japanese medaka (Peters et al., 2007). Peters et al. (2007) suggests that this might be the result of NAs-induced metabolic complications. Zebrafish exposed to commercial NAs caused changes of energy metabolic pathways (Zhang et al., 2023) that could also be affecting RBT metabolism. In addition, problems in cardiovascular development and the circulatory system and the presence of severe yolk sac and pericardial edemas could have impacted the absorption rate of yolk sac nutrients, especially in embryos and alevins exposed to highly concentrated OSPW. Alevins exposed to Day 0 water, 22.0% OSPW, 46.0% OSPW, and 100% OSPW treatments remained at the bottom of the tanks/jars and barely moved when inspected. While alevins and swim-up fries in the controls, less concentrated OSPW treatments, Day 15 water, aged OSPW, and Athabasca River water moved freely within the tanks/jars and were receptive to any disturbance. This suggests that alevins in highly concentrated OSPW treatments lacked energy due to inadequate utilization of their yolk, affecting their normal swimming behaviour and ability to avoid environmental disturbances.

3.4.2. Total Length

Total length decreased with decreasing OSPW dilutions or increasing BE-SPME and total NAs concentrations, indicating a delay in growth (Figure 24 and 25). Yellow perch and Japanese medaka also decreased in length with exposure to increasing concentrations of NAs (Peters et al., 2007). In 2021 and 2022, RBT exposed to control dechlorinated water, 1.00% OSPW, 2.20% OSPW, and 4.60% OSPW had on average a total length of 23.8mm - 24.5 mm (Table 10 and 111). Total length starts to decrease to 18.2 mm - 20.9 mm at similar BE-SPME concentrations, 19.4 µmol/mL PDMS (for 10.0% OSPW) in 2021 and 17.3 µmol/mL PDMS (for 22.0% OSPW) in 2022. In 2021, total length significantly (Kruskal-Wallis test, pairwise comparison, $p<0.05$) reduced to 16.9 ± 0.09 mm in 22.0% OSPW, 15.2 ± 0.77 mm in 46.0% OSPW, and 10.2 ± 0.95 mm in 100% OSPW (Figure 24). Overall, exposure to OSPW dilutions in 2021 resulted in a bi-linear trendline that is first stable and then starts to decrease at dilution 10.0% OSPW (Figure 24a). In 2022, total length also significantly (Kruskal-Wallis test, pairwise comparison, $p<0.05$) decreased to 17.4 ± 0.80 mm in 46.0% OSPW and 15.7 ± 0.77 mm in 100% OSPW (Figure 25). Overall, exposure to OSPW dilutions in 2022 also resulted in a bi-linear trendline that starts to decrease at dilution 4.60% OSPW but was first stable at less concentrated OSPW dilutions. RBT from Nina's (2022) study had an average length of 23.2 ± 0.39 mm when exposed to control dechlorinated water and also became significantly smaller when exposed to 10.0% OSPW dilution (21.4 ± 0.39 mm).

RBT exposed to pH-adjusted 100% OSPW retained a similar total length (15.4 ± 0.766 mm) as RBT exposed to pH-adjusted 46.0% OSPW (16.5 ± 0.299 mm). But total length from exposure to unadjusted 100% OSPW (10.2 ± 0.949 mm) was significantly (Kruskal-Wallis test, pairwise comparison, $p<0.05$) smaller than pH-adjusted 100% OSPW (Table 10). A reduction of un-ionized ammonia concentration in pH-adjusted 100% OSPW could have helped increase the total length of RBT relative to unadjusted 100% OSPW. However, exposure to pH-adjusted 100% OSPW is still considered toxic as its total length is smaller than the control by a factor of 1.61 and significantly (Kruskal-Wallis test, pairwise comparison, $p<0.05$) comparable to 46.0% OSPW. Water hardness had no impact on total length.

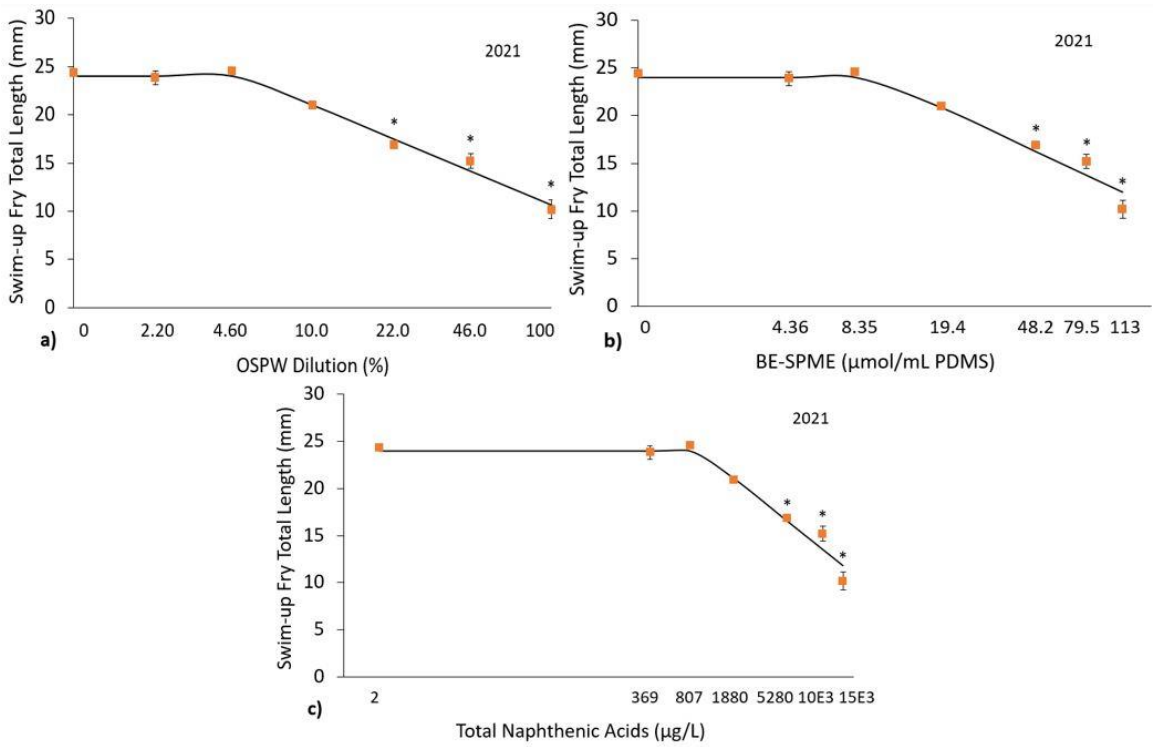

Figure 24. **Total length (mm) of rainbow trout until the swim-up fry stage in 2021. a) Exposure to OSPW series dilutions (%). b) Exposure to BE-SPME (µmol/mL PDMS). c) Exposure to total naphthenic acids (µg/L). BE-SPME and total NAs concentrations were obtained from InnoTech Alberta. Values are presented as the mean ± SE (n=20 per treatment, 4 replicates). Decreasing bi-linear trendline was included in each graph. Asterisks (*) represent significant difference compared to the control ($p<0.05$). Significant differences were observed in dilutions 22.0% OSPW to 100% OSPW (Kruskal-Wallis test and pairwise comparison, $p<0.05$).**

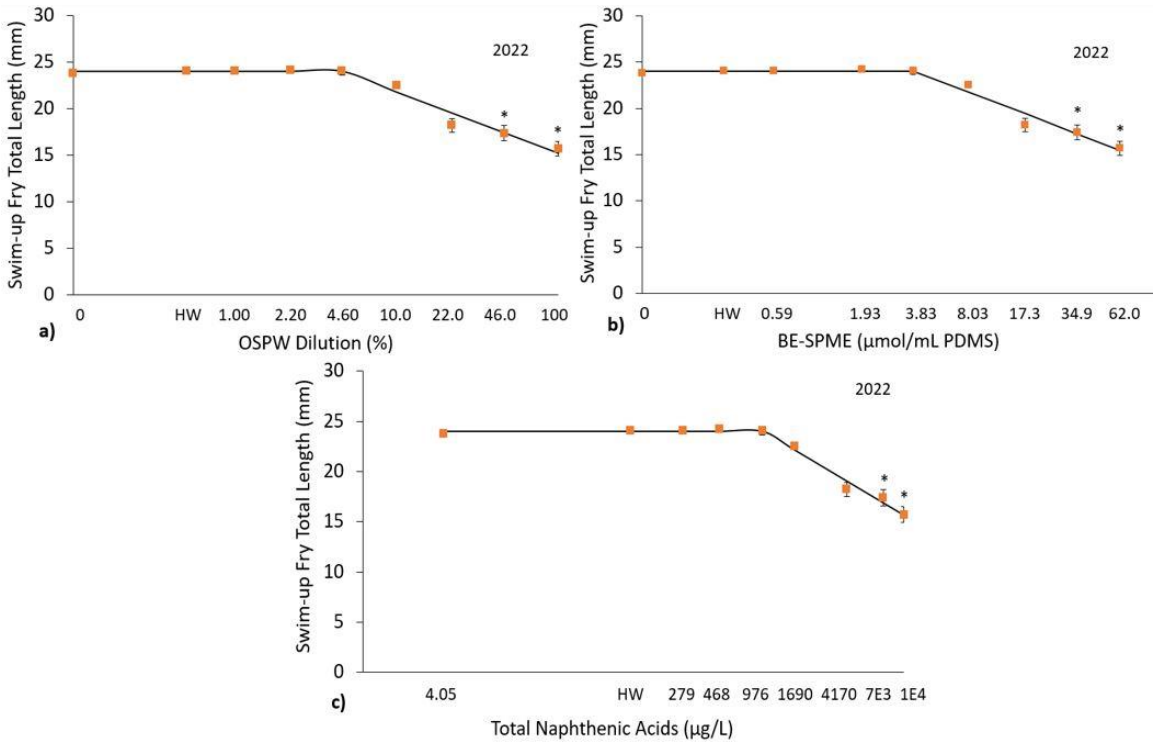

Figure 25. Total length (mm) of rainbow trout until the swim-up fry stage in 2022. a) Exposure to OSPW series dilutions (%) and hard water (HW). b) Exposure to BE-SPME (µmol/mL PDMS). c) Exposure to total naphthenic acids (µg/L). BE-SPME and total NAs concentrations were obtained from InnoTech Alberta. Values are presented as the mean ± SE (n=20 per treatment, 4 replicates). Decreasing bi-linear trendline was included in each graph. Asterisks (*) represent significant difference compared to the control (p<0.05). Significant differences were observed in dilutions 46.0% OSPW and 100% OSPW (Kruskal-Wallis test and pairwise comparison, p<0.05).

The total length of RBT exposed to Day 0 water was significantly (Kruskal-Wallis test, pairwise comparison, p<0.05) smaller (15.1 ± 0.832 mm in 2021 and 20.7 ± 0.234 mm in 2022) compared to the controls (24.7 ± 0.103 mm in 2021 and 24.4 ± 0.258 mm in 2022) (Figure 26; Table 10 and 11). Results from Day 0 water exposure are comparable to results from exposure to 22.0% OSPW and 46.0% OPW despite having higher BE-SPME and total NAs concentrations than these two dilutions. 15 days of wetland treatment effectively maintained a total length of 22.7 ± 0.577 mm in 2021 (Kruskal-Wallis test, pairwise comparison, p>0.05) and 23.2 ± 0.249 mm in 2022 (One way ANOVA, Dunnett's test, p>0.05), with no significant differences compared to the controls. Mesocosm treatment also successfully retained a total length (22.5 ± 0.463 mm) similar to the control (24.4 mm ± 0.105 mm) (Independent samples t-test, p>0.05).

Wetland and mesocosm treatment of OSPW had total length results similar to 4.60% OSPW and10.0% OSPW despite treated OSPW having higher BE-SPME and total NAs concentrations than these dilution treatments. Athabasca River water had no significant impact on RBT total length (Independent samples t-test, p>0.05).

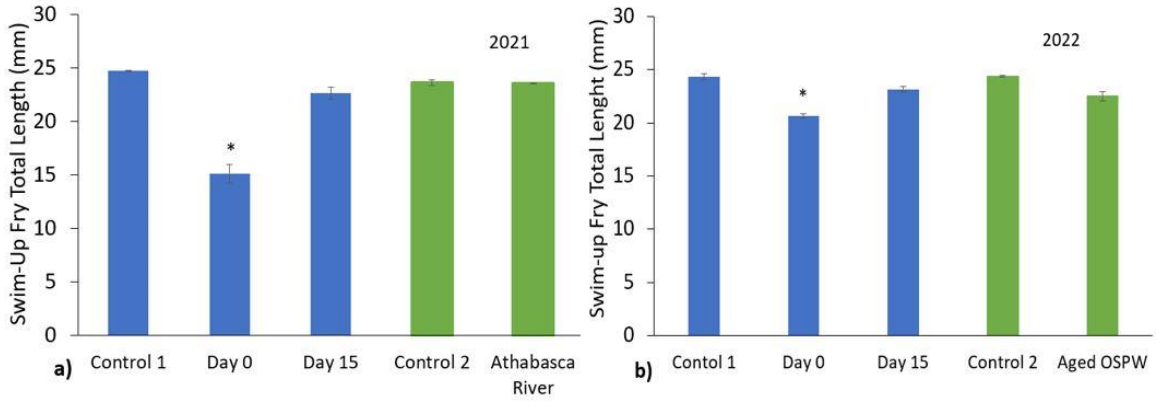

Figure 26. Total length (mm) of rainbow trout until the swim-up fry stage. a) Exposure to constructed wetland water (Day 0 and Day 15) (n=5 per treatment, 4 replicates) and Athabasca River water (n=20, 4 replicates) in 2021 with corresponding controls. b) Exposure to constructed wetland water (Day 0 and Day 15) and Aged OSPW (n=5 per treatment, 4 replicates) in 2022 with corresponding controls. Values are presented as the mean ± SE. Asterisks (*) represent significant difference compared to the control (P<0.05). Significant differences were observed in Day 0 water in 2021 (Kruskal-Wallis test and pairwise comparison, p<0.05) and in 2022 (One-way ANOVA and Dunnett's test, p<0.05).

Embryos and alevins that did not absorb their yolk sac in Day 0 water (Figure 23) and dilutions treatments 22.0% OSPW, 46.0% OSPW, and 100% OSPW (Figure 21 and 22) most likely did not utilize the lipid, protein, vitamin, and amino acid contents of their yolk necessary for energy and growth. This could have resulted in smaller individuals (Figure 24, 25, 26) compared to the control in which most embryos and alevins completely absorbed their yolk sac.

In addition to cardiovascular problems and oxidative stress, NAs exposure at the embryonic stage also has other endocrine-disrupting effects that could contribute to development and growth problems (Wang et al., 2015). Zebrafish exposed to oil sands extracted NAFC resulted in increased gene expression of enzymes involved in steroidogenesis: CYP19b (cytochrome P450 aromatase), Er_{α}, and $Er_{\beta 2}$ (estrogen receptors) (Wang et al., 2015). NAs bind to estrogen receptors (Er_{α} and $Er_{\beta 2}$) which bind

to the estrogen responsive element (ERE), a promoter region of the CYP19b gene located in the brain that encodes cytochrome P450 (Dalla Valle et al., 2005; Trant et al., 2001). Cytochrome P450 aromatase enzyme catalyzes the conversion of androgens to estrogens. This disrupts the appropriate androgen to estrogen ratio important for fish development, growth, and reproduction. Excessive increased production of estrogen could disrupt pathways for lipid metabolism in the liver (Wojnarowski et al., 2022) resulting in high fat deposition mainly in female fish (Sun et al., 2020) and could activate the G-protein–coupled estrogen receptor 1 (GPER1) involved in the proliferation of hepatocytes (liver cells) resulting in liver growth and cancer (Chaturantabut et al., 2019). If male fish are exposed to high NAs concentrations in the wild, increased estrogen levels could induce metabolic and reproductive feminization (Sun et al., 2020) which would eventually affect reproduction of fish populations in the wild.

3.4.3. Wet Weight

A slight increase followed by a decrease in wet weight was observed in RBT exposed to decreasing OSPW dilutions or increasing BE-SPME/total naphthenic acid concentrations (Figure 27 and 28). This fluctuating trend occurred at similar BE-SPME concentrations in both years. Wet weight increased from 0.0991 g ± 0.00226 g in control dechlorinated water to 0.118 ± 0.0119g in 10.0% OSPW (19.4 µmol/mL PDMS) in 2021 (Table 10), and from 0.0849 ± 0.00109 g in the control to 0.113 ± 0.00461 g in 22.0% OSPW (17.3 µmol/mL PDMS) in 2022 (Table 11). However, only the increase in wet weight in 2022 was significantly (Kruskal-Wallis test, pairwise comparison, $p<0.05$) different than the control, which continued to be significantly high (0.120 ± 0.0124 g) in 46.0% OSPW (34.9 µmol/mL PDMS). Then in 2022, it decreased back to 0.0941 ± 0.00800 g in 100% OSPW (62 µmol/mL PDMS), with no significant (Kruskal-Wallis test, pairwise comparison, $p>0.05$) difference compared to the control.. Wet weight in 2021 significantly decreased to 0.0865 ± 0.00451 g in 46.0% OSPW (79.48 µmol/mL PDMS) and 0.0794 ± 0.00279 g in 100% OSPW (113.3 µmol/mL PDMS). Overall, wet weight in 2021 and 2022 had a polynomial trendline with an order of 3 and 5, respectively. pH adjustment (One way ANOVA, Dunnett's test, $p>0.05$) and water hardness (Kruskal-Wallis test, pairwise comparison, $p>0.05$) had no significant effect on RBT wet weight.

Alevins exposed to 22.0% OSPW, 46.0% OSPW, and 100% OSPW did not absorb their yolk sac and had high percentages of yolk sac and pericardial edemas,

contributing additional weight. Thus, wet weight results for the last three OSPW dilutions might be overestimated than expected. If alevins did not have that extra fluid, wet weight in those three dilutions might be slightly lower. Dry weight might have been a better alternative to not account for extra fluids.

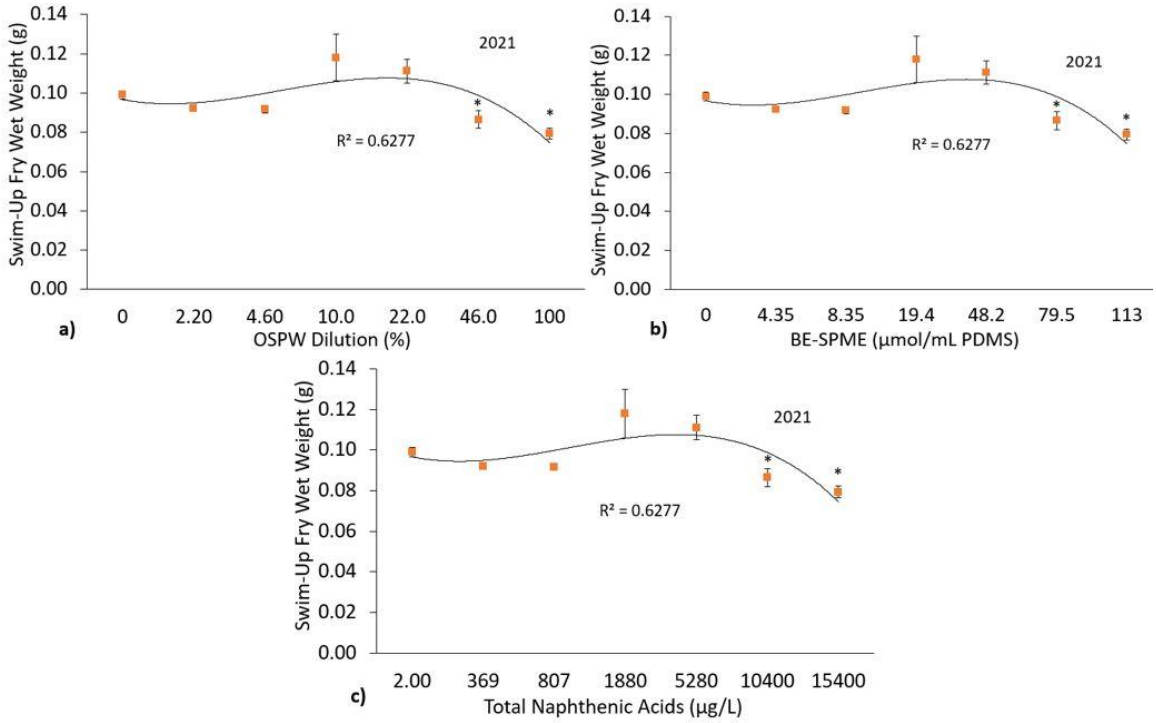

Figure 27. **Wet weight (g) of rainbow trout until the swim-up fry stage in 2021. a) Exposure to OSPW series dilutions (%). b) Exposure to BE-SPME (µmol/mL PDMS). c) Exposure to total naphthenic acids (µg/L). BE-SPME and total NAs concentrations were obtained from InnoTech Alberta. Values are presented as the mean ± SE (n=20 per treatment, 4 replicates). Asterisks (*) represent significant difference compared to the control ($p<0.05$). Significant differences were observed in dilutions 46.0% OSPW and 100% OSPW (Kruskal-Wallis test and pairwise comparison, $p<0.05$).**

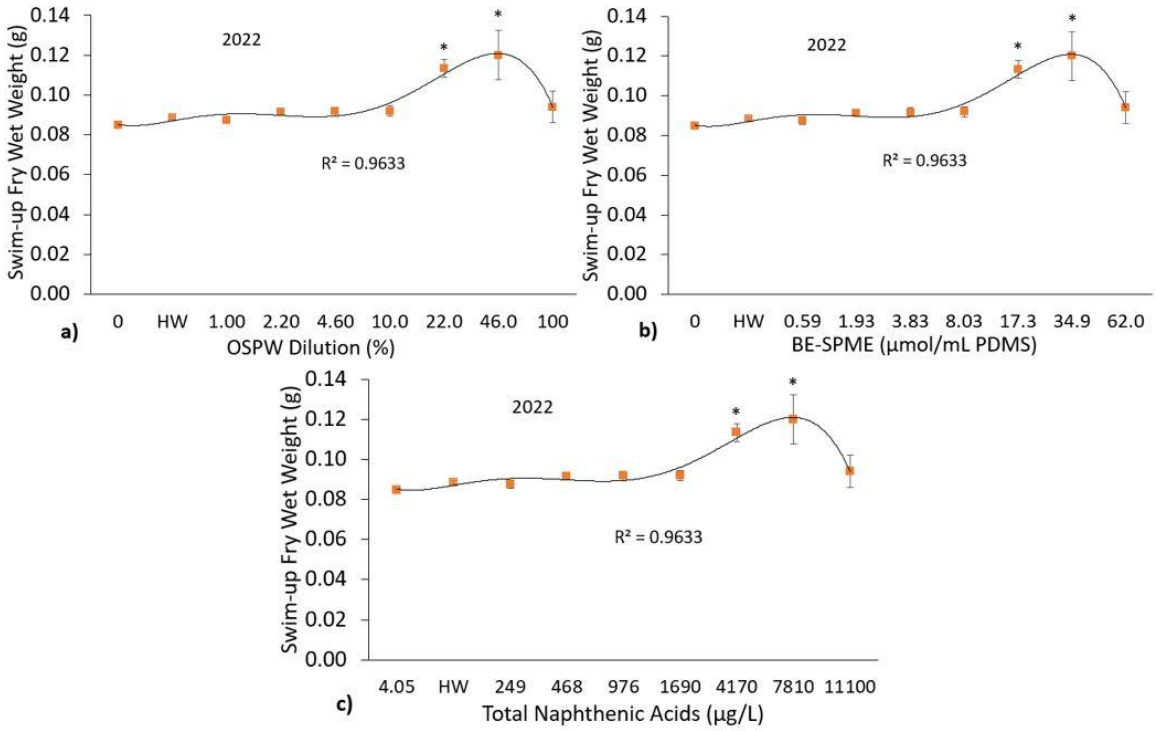

Figure 28. Wet weight (g) of rainbow trout until the swim-up fry stage in 2022. a) Exposure to OSPW series dilutions (%). b) Exposure to BE-SPME (µmol/mL PDMS). c) Exposure to total naphthenic acids (µg/L). BE-SPME and total NAs concentrations were obtained from InnoTech Alberta. Values are presented as the mean ± SE (n=20 per treatment, 4 replicates). Asterisks (*) represent significant difference compared to the control (p<0.05). Significant differences were observed in dilutions 22.0% OSPW and 46.0% OSPW (Kruskal-Wallis test and pairwise comparison, p<0.05).

The trend observed in RBT wet weight could be considered a hormetic biphasic response to exposure to increasing BE-SPME and total NAs concentrations in OSPW. A hormetic biphasic response occurs when low concentrations of a substance elicits a favorable stimulatory response followed by harmful inhibitory response at higher concentrations (Kendig et al., 2010; Calabrese and Baldwin, 2002). Calabrese and Baldwin (2002) suggest that the stimulatory response is an adaptive and genetically conserved compensatory process as the result of homeostasis disruption during low levels of stress. In the current study, the stimulatory response would start to occur at similar concentrations in both years, 19.4 ± 4.37 µmol BE-SPME/mL PDMS (or 10.0% OSPW) in 2021 and 17.3 µmol BE-SPME/mL PDMS (or 22.0% OSPW) in 2022. Mortality, total deformities, yolk sac absorption, and total length worsen when RBT were exposed to the former two BE-SPME concentrations, triggering the compensatory

response. This suggests that increased wet weight could be a physiological response to overcome toxic effects at low concentrations in an attempt to restore homeostasis. The inhibitory response in 2021 would occur at a BE-SPME concentration slightly lower than 79.48 ± 8.45 µmol/mL PDMS (or 46.0% OSPW) when wet weight is lower than the weight in the control. At this point, BE-SPME and total NAs concentrations are too high and toxic effects are too severe to overcompensate, and the organism succumbs to high levels of stress. In 2022, exposure to 62 µmol BE-SPME/mL PDMS decreases wet weight back to a level similar to the control. The inhibitory response would likely be observed if RBT in 2022 were exposed to higher BE-SPME concentrations. However, it is probable that the stimulatory response might be the result of alevins and fries with edemas and unabsorbed yolk sac and the inhibitory response the result of reduced total length relative to the control. Measuring dry weight might have eliminated the stimulatory response in both years and enhanced the harmful response phase.

Wetland and mesocosm treatment had no significant impact on wet weight (Figure 29). RBT exposed to Day 0 and 15 water in 2021 had a similar average wet weight as in the control (0.0933 ± 0.000940 g) despite having higher BE-SPME and total NAs concentrations than the control (Table 10 and 11). The wet weight of RBT exposed to Day 0 water in 2022 slightly increased to 0.0989 ± 0.00302 g but was not significantly (One Way ANOVA, Dunnett's test, $p>0.05$) different compared to the control (0.0889 ± 0.00337g). Exposure to Day 15 water increased wet weight to 0.0948 ± 0.00193 g but not significantly (One Way ANOVA, Dunnett's test, $p>0.05$) different compared to the control. The wet weight in aged OSPW (0.0876 ± 0.00503 g) remained similar to the control (0.0897 ± 0.00203 g), with no significant differences (independent t-test, $p>0.05$). Surprisingly, RBT exposed to Athabasca River water had significantly lower wet weight (0.0822 ± 0.00166g) than the control (0.0921 ± 0.00315 g) (Figure 29a). However, this slight drop in weight did not affect their overall survival and development (yolk sac utilization and total length).

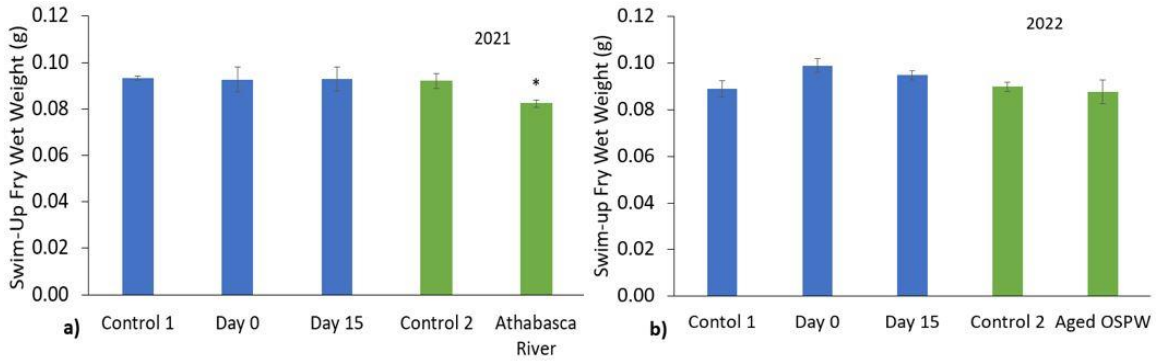

Figure 29. **Wet weight (g) of rainbow trout until the swim-up fry stage. a) Exposure to constructed wetland water (Day 0 and Day 15) (n=5 per treatment, 4 replicates) and Athabasca River water (n=20, 4 replicates) in 2021 with corresponding controls. b) Exposure to constructed wetland water (Day 0 and Day 15) and Aged OSPW (n=5 per treatment, 4 replicates) in 2022 with corresponding controls. Values are presented as the mean ± SE. Asterisks (*) represent significant difference conpared to the control (P<0.05). Significant differences were observed only in Athabasca River water in 2021 (Independent samples t-test, p<0.05).**

3.5. Application of BE-SPME Passive Samplers and Total Naphthenic Acids

BE-SPME passive sampling is a novel method that has the potential to measure contamination of water samples without resorting to animal-based testing. Passive samplers are applied to OSPW to extract and determine its dissolved organic fraction concentration and thus estimate acute or chronic toxicity. In the present study, BE-SPME concentrations of Day 0 water, Day 15 water, and Aged OSPW were compared to BE-SPME concentrations of the OSPW series dilutions, in 2021 and 2022, to verify if biomimetic extraction can predict toxicity results. Comparisons were also conducted with total NAs. All concentrations used for comparisons were obtained by InnoTech Alberta.

Untreated Day 0 water in 2021 had a BE-SPME concentration of 113 µmol/mL PDMS similar to 100% OSPW (113 ± 6.43 µmol/mL PDMS). Total NAs concentration in Day 0 water (19600 µg/L) was higher than the concentration in 100% OSPW (15400 µg/L). In 2022, Day 0 water had a BE-SPME concentration (50 µmol/mL PDMS) close to the concentration derived from 100% OSPW (62 µmol/mL PDMS) and a total NAs concentration (12200 µg/L) slightly higher than 100% OSPW (11100 µg/L). Thus, it is expected that Day 0 water has a toxic effect similar to 100% OSPW. Exposure to Day 0 water and 100% OSPW had the same results for mortality (100% ± 0.00% each; Figure

30a,b) and yolk sac score 1 (0.00% ± 0.00% each; Figure 32a,b) in both years. Day 0 water in 2021 had total deformity percentages similar to 10.0% OSPW (19.4 ± 4.37 µmol/mL PDMS) (Figure 31a) but with overlaps with 22.0% OSPW (48.2 ± 10.1 µmol/mL PDMS), 46.0% OSPW (79.5 ± 8.45 µmol/mL PDMS), and 100% OSPW (113 ± 6.43 µmol/mL PDMS) due to high variability in results. In 2022, Day 0 water resulted in the same total deformity percentage as 100% OSPW. These results demonstrate that BE-SPME and total NAs concentrations from Day 0 water predicts mortality, yolk sac score 1 (indicating full development), and total deformities on RBT. But this is not always the case. BE-SPME concentrations estimate that exposure to Day 0 water should have resulted in approximately 70.46% ± 2.041% successful hatchings as observed in 100% OSPW (Figure 10), yet Day 0 water had a hatching success (95.00% ± 5.000%) similar to the control and 10.0% OSPW (Figure 10d).

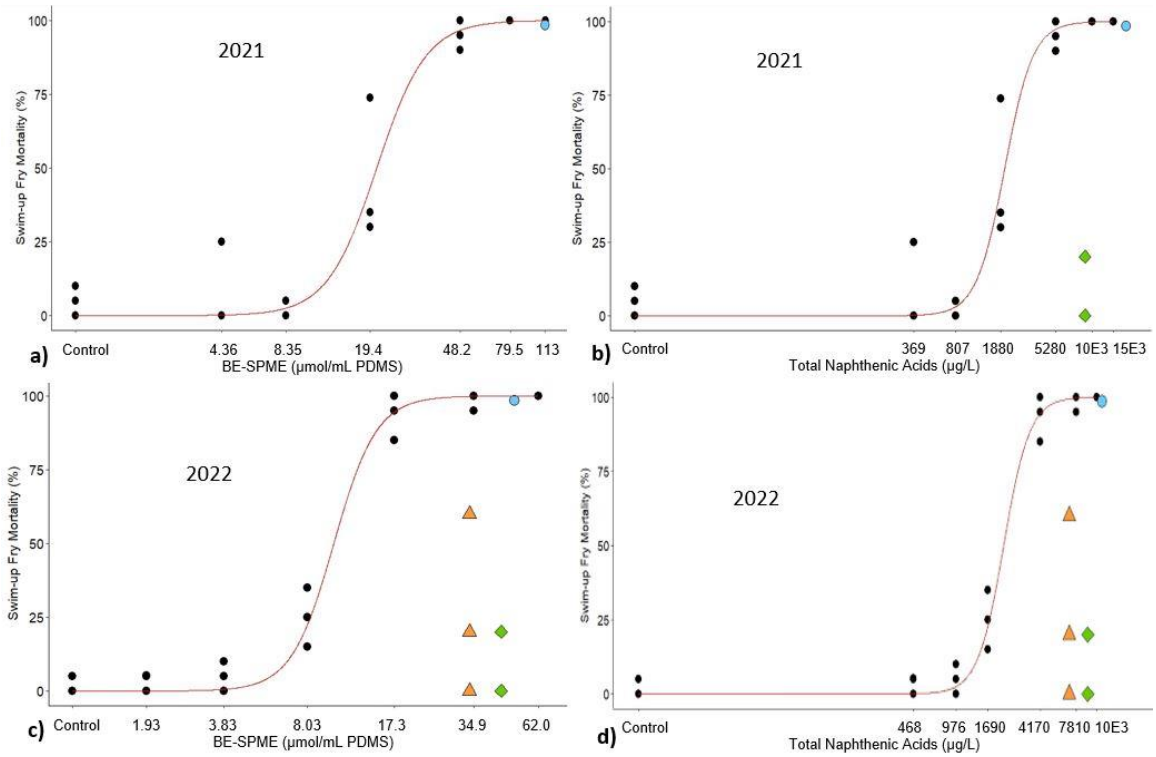

Figure 30. **Comparisons of mortality (%) on rainbow trout until the swim-up fry stage from exposure to BE-SPME and total naphthenic acids against OSPW series dilutions (●; n=20, 4 replicates). a) BE-SPME (µmol/mL PDMS) from Day 0 water (●) in 2021. b) Total naphthenic acids (µg/L) from Day 0 water (●) and Day 15 water (♦) in 2021. c) BE-SPME (µmol/mL PDMS) from Day 0 water (●), Day 15 water (♦), and aged OSPW (▲) in 2022. d) Total naphthenic acids (µg/L) from Day 0 water (●), Day 15 water (♦), and aged OSPW (▲) in 2022. BE-SPME and total naphthenic acids concentrations were obtained from InnoTech Alberta. Data points represent the percentage of dead individuals of each replicate per treatment. Day 0 and Day 15 water and aged OSPW each had n=5, 4 replicates. Red line (—) is the estimated dose-response trendline. Graphed in R.**

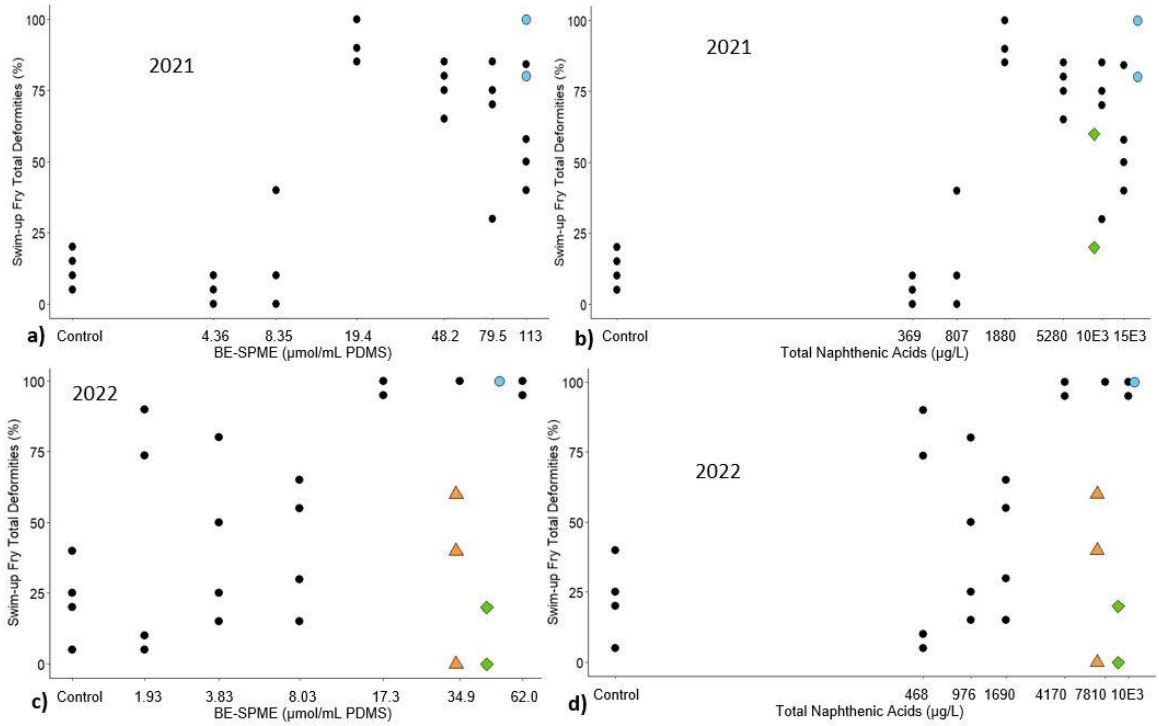

Figure 31. **Comparisons of total deformities (%) on rainbow trout until the swim-up fry stage from exposure to BE-SPME and total naphthenic acids against OSPW series dilutions (●; n=20, 4 replicates). a) BE-SPME (µmol/mL PDMS) from Day 0 water (●) in 2021. b) total naphthenic acids (µg/L) from Day 0 water (●) and Day 15 water (♦) in 2021. c) BE-SPME (µmol/mL PDMS) from Day 0 water (●), Day 15 water (♦), and aged OSPW (▲) in 2022. d) total naphthenic acids (µg/L) from Day 0 water (●), Day 15 water (♦), and aged OSPW (▲) in 2022. BE-SPME and total naphthenic acids concentrations were obtained from InnoTech Alberta. Data points represent the percentage of total deformities of each replicate per treatment. Deformities include skeletal, craniofacial, finfold, and edemas deformities. Day 0 and Day 15 water and aged OSPW each had n=5, 4 replicates. Graphed in R.**

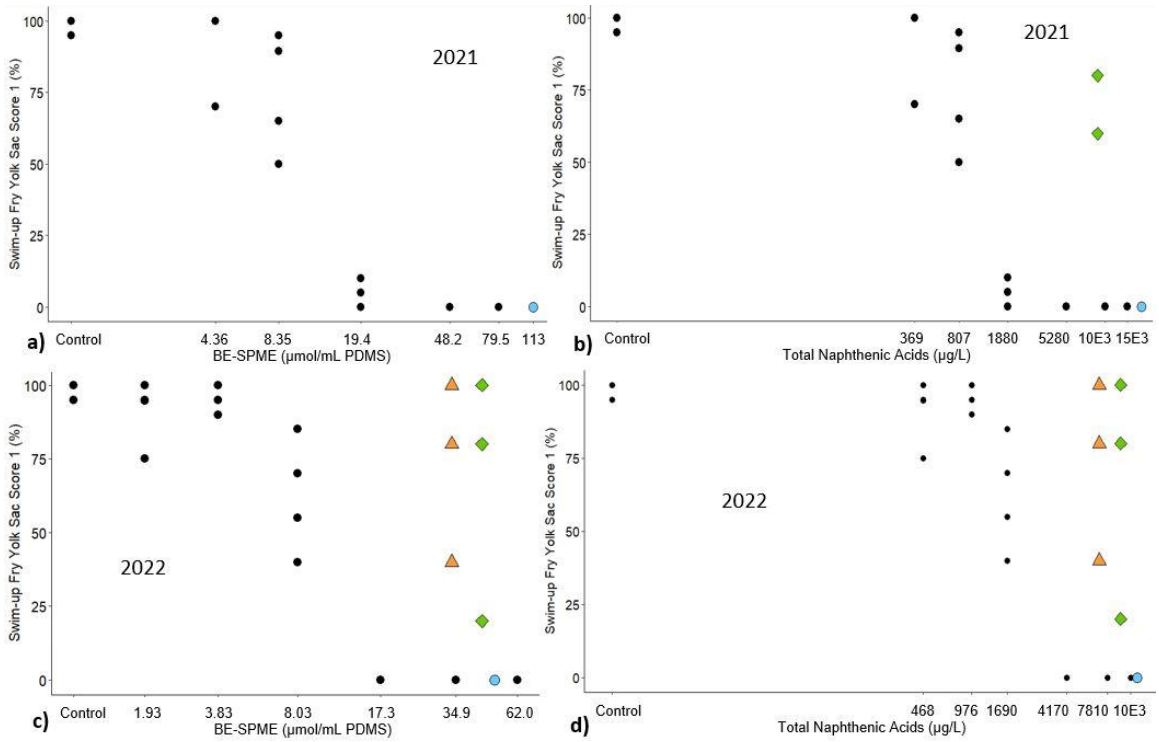

Figure 32. **Comparisons of yolk sac score 1 (%) on rainbow trout until the swim-up fry stage from exposure to BE-SPME and total naphthenic acids against OSPW series dilutions (●; n=20, 4 replicates). a) BE-SPME (µmol/mL PDMS) from Day 0 water (●) in 2021. b) total naphthenic acids (µg/L) from Day 0 water (●) and Day 15 water (♦) in 2021. c) BE-SPME (µmol/mL PDMS) from Day 0 water (●), Day 15 water (♦), and aged OSPW (▲) in 2022. d) total naphthenic acids (µg/L) from Day 0 water (●), Day 15 water (♦), and aged OSPW (▲) in 2022. BE-SPME and total naphthenic acids concentrations were obtained from InnoTech Alberta. Data points represent the percentage of yolk sac score 1 of each replicate per treatment. Score 1 represets full absorption of yolk sac and full development. Day 0 and Day 15 water and aged OSPW each had n=5, 4 replicates. Graphed in R.**

BE-SPME and total NAs concentrations are not very successful at predicting toxic effects of treated OSPW (Day 15 water and Aged OSPW). Day 15 water from 2021 (9100 µg/L of total NAs) should have toxic effects comparable to 46.0% OSPW (10400 µg/L of total NAs). Day 15 water from 2022 (44.5 µmol BE-SPME/mL PDMS and 9480 µg total NAs/L) and aged OSPW (33.6 µmol BE-SPME/mL PDMS and 6870 µg total NAs/L) should have toxic effects similar to 46.0% OSPW (34.9 µmol BE-SPME/mL PDMS and 7810 µg/L of total NAs) and 100% OSPW (62 µmol BE-SPME/mL PDMS and 11100 µg/L of total NAs). However, Day 15 water and aged OSPW in 2021 and 2022

had in general mortality, yolk sac score 1, and total deformity results similar to control dechlorinated water and dilutions 2.20% OSPW, 4.60% OSW, and 10.0% OSPW (Figure 30, 31, 32). Only total NAs concentrations from Day 15 water in 2021 correctly predicts total deformity percentages, which overlaps with results from 46.0% OSPW and 100% OSPW (Figure 31b).

The composition of naphthenic acids in the organic fraction of treated OSPW could have impacted predictions of toxic results. In the present study, treatment of OSPW (Day 15 water and aged OSPW) slightly decreased BE-SPME and total NAs concentrations relative to untreated OSPW (Day 0 water and 100% OSPW) (Table 5) and successfully reduced acute and chronic effects on RBT. Other small-scale constructed wetland treatment systems decreased total NAs and metals concentrations and reduced acute toxicity on *C. dubia* and rainbow trout (McQueen et al., 2017; Hendrikse et al., 2018; Toor et al., 2013). The removal efficiency of the KT wetland increased with increasing carbon content (or molecular weight) and decreasing double bond equivalent (DBE or the negative z integer in the classical NAs formula) (Cancelli and Gobas, 2022). High molecular weight NAs with less cyclicity and alkyl side chain branching are more hydrophobic and biodegradable than NAs with low molecular weight and high cyclicity and alkyl side branching (Frank et al., 2009). Day 15 water in figures A.3 and A.4 reveal that wetland treatment mostly removed NAs with high carbon numbers (C16-C23) and BDE of 3-8 while Day 0 water, 46% OSPW and 100% OSPW had relatively unchanged distributions of NA congeners. This indicates that dilution alone does not impact NAs composition. Dilution only decreases the overall concentration of dissolved NAs regardless of their chemical structure. While microorganisms in the wetland treatment most likely biodegraded the highly hydrophobic and toxic NAs from Day 0 water resulting in decreased toxic impacts on RBT similar to highly diluted OSPW treatments such as 2.20% OSPW, 4.60% OSPW and 10.0% OSPW. The remaining low molecular weight NAs could have caused the chronic malformations and delay in development (yolk sac scores 2 and 3) observed in Day 15 water.

Cancelli and Gobas (2020) also found that the KT wetland pilot removes certain PAHs. Only water samples in 2021 contained PAHs above available water quality guidelines. Day 15 water had less PAHs compounds compared to Day 0 water and 100% OSPW. These PAHs compounds could have also contributed to the chronic effects observed in exposure to Day 15 water.

The removal efficiency of NAs is yet to be studied for the aged mesocosm pond treatment. Figures A.3 and A.4 indicate that mesocosm treatment mostly removed NAs with high carbon number (C16-C21) and BDE of 3-8. This suggests that mesocosm treatment works similarly to the KT treatment at removing high molecular NAs, resulting in toxic results comparable to Day 15 water. However, mesocosm pond treatment could take years to biodegrade NAs to the same level as the wetland treatment. Wetland treatment is more efficient by only requiring 14 days of operation to reduce toxic impacts. It is possible that mesocosm treatment could have biodegraded more toxic NAs than wetland treatment if OSPW was left aging more than 2 years. It is important to consider that PAHs, metals, un-ionized ammonia, and other constituents not captured by biomimetic extraction could also have contributed to the overall toxicity of treated and untreated OSPW.

The following Excel spreadsheets were submitted as supplementary materials:

- Supplementary–Figures_and_Table.xlsx: a compilation of Figures 3-33, Tables 1-13, Figures A.1-A.4, and Tables B.1-B.6.
- Supplementary–Figure 24_and_25.xlsx: figures for bi-linear fit of total length for OSPW series dilutions in 2021 and 2022.
- Supplementary–Original_Exposure_Data_2021.xlsx: original data for laboratory water quality parameters and embryo exposure counts in 2021.
- Supplementary-Original_Exposure_Data_2022.xlsx: original data for laboratory water quality parameters and embryo exposure counts in 2022.

Chapter 4. Conclusions

Exposure to whole OSPW impacted the early life stages of rainbow trout. In general, highly concentrated OSPW dilutions (22% OSPW, 46% OSPW, and 100% OSPW) induced significantly high percentages of mortality, deformities, and delay in development in 2021 and 2022. It required similar BE-SPME concentrations in both years to start observing significant deformities (19.4 µmol/mL PDMS in 2021 and 17.3 µmol/mL PDMS in 2022), delay in yolk sac absorption (8.35 µmol/mL PDMS in 2021 and 8.03 µmol/mL PDMS in 2022), and decrease in total length (48.2 µmol/mL PDMS in 2021 and 34.9 µmol/mL PDMS in 2022). Maximum hatching success was delayed by 3 days in the most concentrated OSPW dilution (100% OSPW; 113 µmol BE-SPME/mL PDMS in 2021) with a significantly lowered hatching success compared to the control. Wetland and mesocosm pond treatments significantly reduced mortality, hatching delay, and deformities; and maintained yolk sac adsorption and total length similar to the control. Un-ionized ammonia, metals, and other organic compounds such as PAHs could have contributed to the toxicity of OSPW. Athabasca River water had no significant impact on RBT except for a slight decrease in wet weight that is not drastically different from the control.

OSPW samples from 2021 had higher BE-SPME and total NAs concentrations than in 2022. The BE-derived LC_{50} concentrations (20.8 ± 1.02 µmol/mL PDMS in 2021 and 10.1 ± 0.353 µmol/mL PDMS in 2022) were lower than the lowest observed effect concentrations (LOECs) for mortality (48.2 ± 14.1 µmol/mL PDMS in 2021 and 17.3 ± 1.69 µmol/mL PDMS in 2022) (Figure 33a,c). BE-derived EC_{50} values for deformities (20.2 ± 6.35 µmol/mL PDMS in 2021 and 10.3 ± 2.98 µmol/mL PDMS in 2022), which mostly include craniofacial, skeletal, and edema deformities as well as hemorrhaging demonstrating cardiovascular problems, were close to LC_{50} values indicating that it requires similar BE-SPME concentrations to cause lethal and sublethal effects. LOEC values for total deformities and yolk sac score 1 (as an indicator for full development) were 19.4 µmol/mL PDMS each in 2021 and 17.3 µmol/mL PDMS each in 2022. These lethal and sublethal concentrations could be useful tools for adaptive management of OSPW. No observed effect concentrations (NOECs) for mortality, deformities, and full development vary between 3.83 and 8.35 µmol/mL PDMS in both years. NOECs could be considered for developing water quality guidelines for OSPW or NAs.

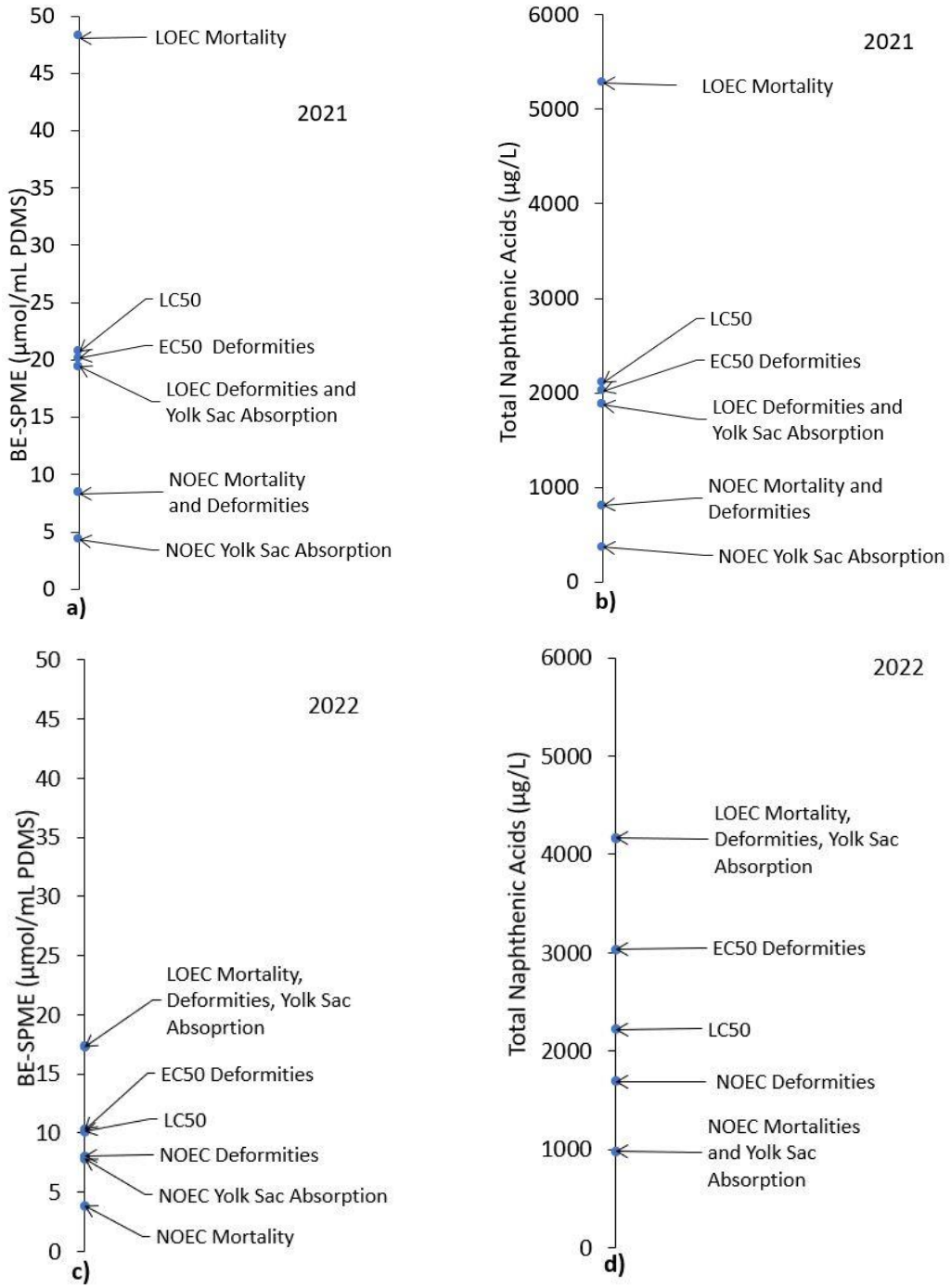

Figure 33. Summary of critical endpoints of mortality (LC_{50}, LOEC, and NOEC), total deformities (EC_{50}, LOEC, and NOEC), and yolk sac absorption score 1 representing full development (LOEC, and NOEC). Exposure to a) BE-SPME (µmol/mL PDMS) in 2021, b) total naphthenic acids (µg/L) in 2021, c) BE-SPME (µmol/mL PDMS) in 2022, and d) total naphthenic acids (µg/L) in 2022. BE-SPME and total naphthenic acids concentrations were obtained from InnoTech Alberta.

Results from this study demonstrates the complexity of working with whole OSPW because its dissolved organic fraction can vary spatially and temporally in composition, which can complicate the application of BE samplers as rapid assessment tools. BE-SPME and total NAs values derived from treated OSPW (Day 15 water and aged OSPW) did not always predict toxicity due to a compositional change of NAs congeners that occurs during treatment. This results in an overestimation of the toxicity of treated OSPW. BE-SPME and total NAs concentrations from untreated OSPW (Day 0 water) correspond to values derived from 100% OSPW, generally predicting toxic impacts. More research is needed to understand how BE can be used to successfully gauge toxicity of treated OSPW. Nevertheless, this provides to the oil industry a promising approach that could contribute to the remediation of oil sands tailing ponds and potentially other water effluents contaminated with organic compounds.

Chapter 5. Future Work

The embryonic stage is sensitive to external disturbances and contaminants. The period between egg fertilization and hatching (incubation period) is crucial because cell division occurs 2 to 72 hrs post-fertilization and cell differentiation occurs at day 4 to 14 post-fertilization (EC, 1998). For this reason, most acute toxicity tests are conducted a few hours post-fertilization. The current study exposed embryos to treatment water after ~25 days post-fertilization (dpf) due to logistics on water samples shipment and lack of laboratory equipment for egg fertilization. It is possible that embryos absorbed less treatment water than if exposure happened earlier in their developmental stage, which could have alleviated acute toxic effects. Future work with RBT should consider exposing embryos a few hours after fertilization to maximize the potential acute toxic effects of whole OSPW and minimize accidental disturbances and exposure to other toxic solutions.

Another limitation for this experiment is that physiological effects on a cellular level were not observed, only external effects (skeletal, craniofacial, finfold, and edemas deformities). Observing external effects is not enough to certainly explain in detail underlying causes and mechanisms for the presented results. Future work should include a close inspection of immunological and endocrine-disrupting responses on a cellular and molecular level after exposure to whole OSPW.

Acute effects during the developmental stage could have caused chronic effects post hatching. The present study was conducted for approximately 30 days (~55 pdf), when 50% of alevins in the control transitioned to the swim-up fry stage. An exposure study longer than 30 days could help observe if RBT can survive on the long run to treated OSPW and highly diluted OSPW (i.e., 1.00% OSPW, 2.20% OSPW, or 4.60% OSPW), as it is possible that the chronic effects observed in surviving alevins and fries could impact later growth stages, fitness, and fecundity. Reynolds et al. (2022) exposed fathead minnow to naphthenic acid fraction compounds extracted from OSPW for 7 days and surviving individuals were later exposed to uncontaminated lake water for 1 month to observe if acute effects from naphthenic acids impacted the larval stage. Similarly, RBT could be exposed to Athabasca River water or control dechlorinated water for more

than 30 days after exposure to treated OSPW and diluted OSPW to observe if RBT can later adapt and survive after being removed from OSPW.

OSPW is a complex mixture comprised of naphthenic acids and other organic compounds of diverse compositions, each with different mechanisms of toxicity. The composition of OSPW also varies depending on the location of the oil sands pond, the method of bitumen extraction, and the time of OSPW sampling. Therefore, not all OSPW-derived BE-SPME samples will have the same magnitude of lethal and sublethal effects. This was observed when the 2022 OSPW sample resulted in higher percentages of deformities than the 2021 OSPW sample and when different laboratories resulted in different BE-SPME concentrations despite using the same extraction method. For this reason, biomimetic extraction might not always correctly predict toxic effects, specially for treated OSPW. This makes it particularly difficult to compare critical endpoints or species sensitivities using results from different laboratories and to develop water quality guidelines for the protection of aquatic life. The present study mainly focused on NAs as a whole, and available individual NAs congeners were not observed in much detail. A closer examination on the compositional change of individual NAs (e.g., carbon number, cyclicity, alkyl side chain) after wetland and mesocosm treatment and how this affects biomimetic extraction predictions in addition to further inspection on the biomimetic extraction process used in different laboratories would provide insightful information to improve this screening method.

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Appendix A. Supplementary Figures

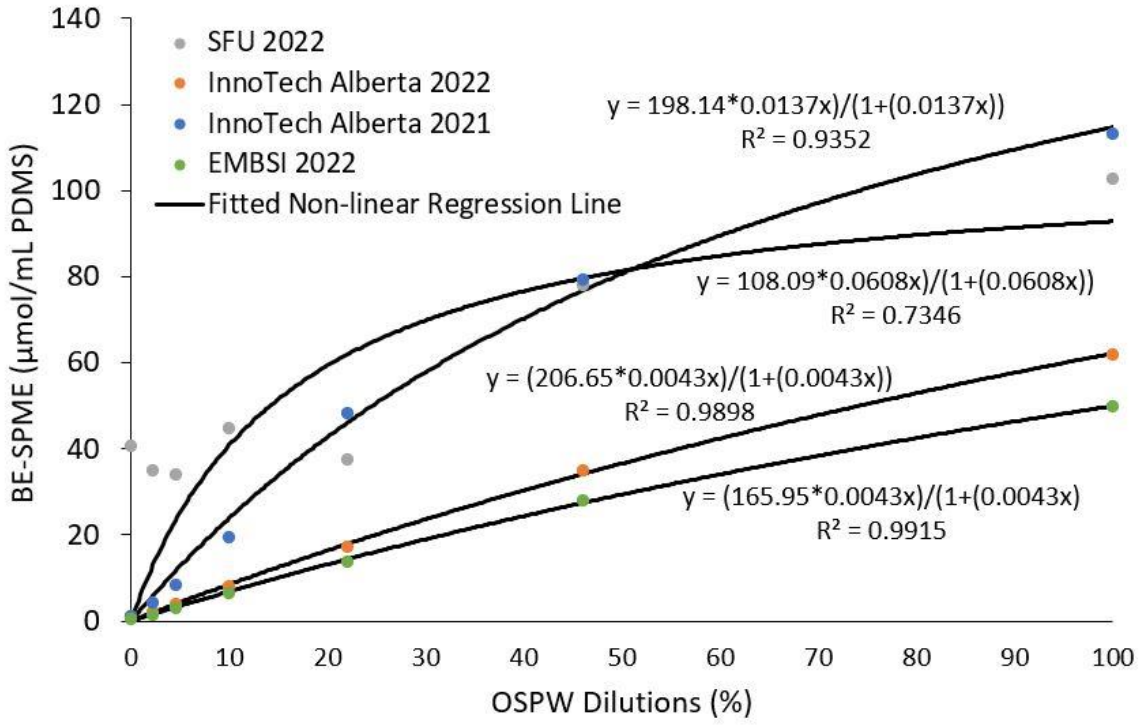

Figure A.1. **Non-linear Langmuir adsorption curves for BE-SPME concentrations (µmol/mL PDMS) measured at InnoTech Alberta in 2021 (●) and in 2022 (●), SFU in 2022 (●), and EMBSI in 2022 (●). Black trendline represent the fitted non-linear reggression line. Non-linear Langmuir adsorption equations and their corresponding correlation coefficient (R^2) in each curve were calculated in JMP. Data points are presented as mean ± standard deviation where more than one sample of the treatment was available.**

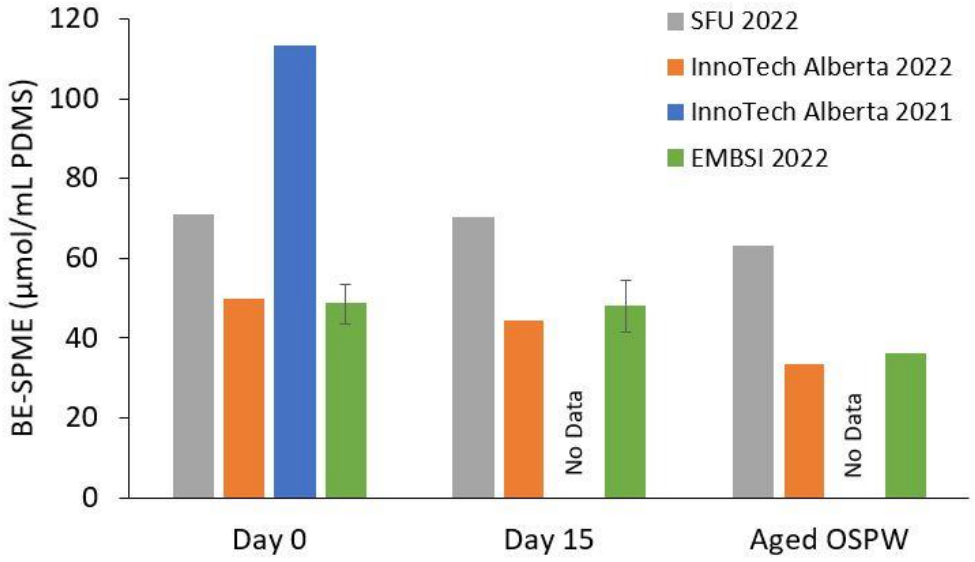

Figure A.2. **BE-SPME concentrations (µmol/mL PDMS) measured at InnoTech Alberta in 2021 (■) and in 2022 (■), SFU in 2022 (■), and EMBSI in 2022 (■). One sample container was available per treatment water for each facility. Only sampler container of Day 15 water in 2021 broke down on the way to InnoTech Alberta, and aged OSPW was not used for exposure tests in 2021.**

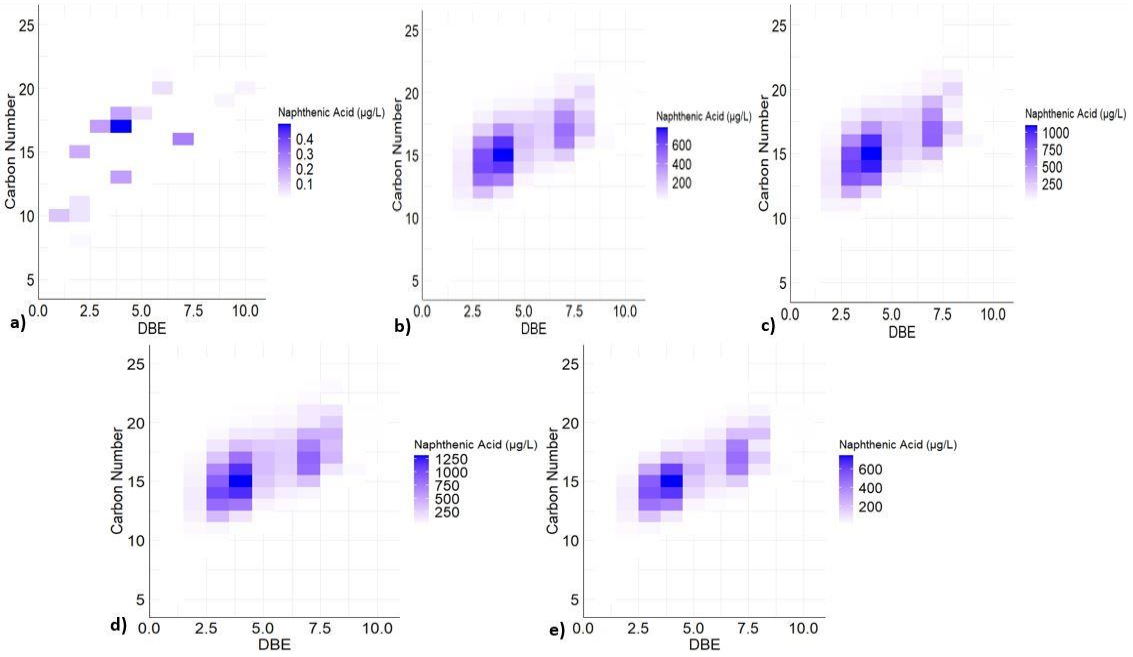

Figure A.3. **Distribution of naphthenic acid congeners (µg/L) in a) control dechlorinated water, b) 46.0% OSPW, c) 100% OSPW, d) Day 0 water, and e) Day 15 water from 2021 relative to their carbon number and double bond equivalent (DBE). Dark blue represents high concentrations and light blue represents low concentrations. Graphed in R.**

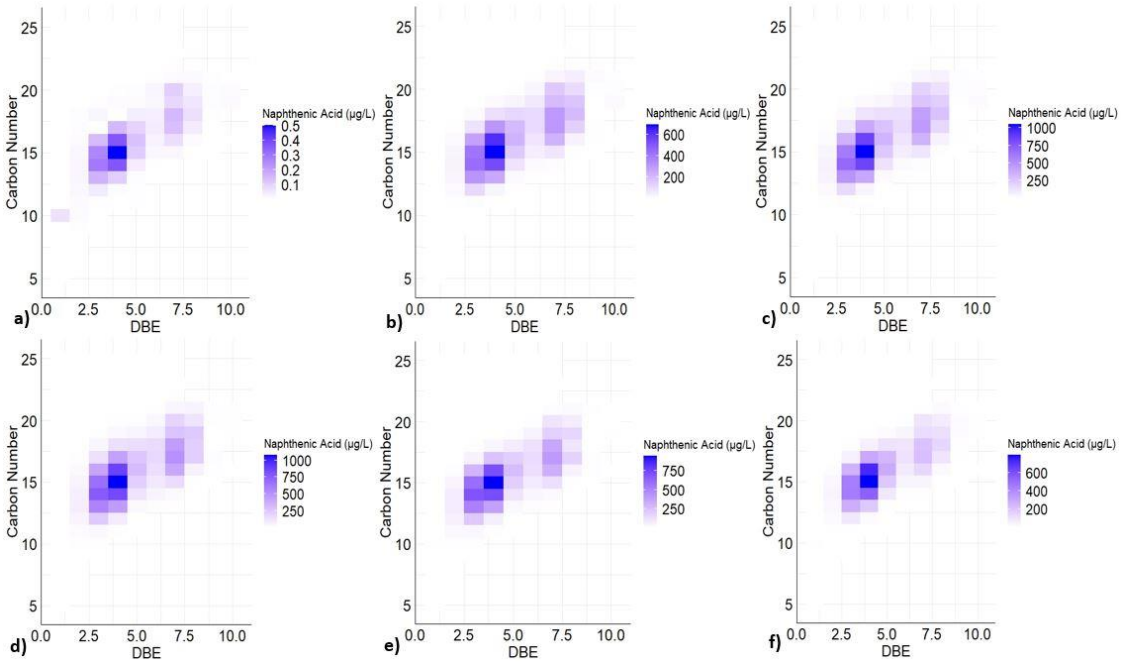

Figure A.4. Distribution of naphthenic acid congeners (µg/L) in a) control dechlorinated water, b) 46.0% OSPW, c) 100% OSPW, d) Day 0 water, e) Day 15 water, and f) aged OSPW from 2022 relative to their carbon number and double bond equivalent (DBE). Dark blue represents high concentrations and light blue represents low concentrations. Graphed in R.

Appendix B. Supplementary Tables

Table B.1. Nitrogen-ammonia (mg/L) and un-ionized ammonia (mg/L and µg/L) results of treatment water and control water from buckets and tanks/jars in 2021 analyzed at SFU. Temperature and pH of each exposure water were collected on the day of sampling. Results are expressed as mean ± SD.

Treatment	Nitrogen-Ammonia (NH_3-N)	Un-ionized Ammonia	Un-ionized Ammonia	Temperature	pH
Unit	**mg/L**	**mg/L**	**µg/L**	**ºC**	
Dechlorinated water (bucket)	< 0.0005 (n=8)	< 0.0005 (n=8)	< 0.0005 (n=8)	13.8 ± 0.707 (n=8)	7.99 ± 0.0389 (n=8)
Control (tank)	0.236 ± 0.0718 (n=8)	0.00986 ± 0.00639 (n=8)	9.86 ± 6.39 (n=8)	14.4 ± 0.450 (n=8)	8.17 ± 0.175 (n=8)
Athabasca River water (bucket)	0.000642 ± 0.0221 (n=8)	< 0.0005 (n=8)	0.0467 ± 1.08 (n=8)	13.7 ± 0.822 (n=8)	8.02 ± 0.206 (n=8)
Athabasca River water (tank)	0.205 ± 0.0727 (n=8)	0.0102 ± 0.00633 (n=8)	10.2 ± 6.33 (n=8)	14.0 ± 0.276 (n=8)	8.25 ± 0.171 (n=8)
Control (jar)	0.525 ± 0.395 (n=3)	0.0212 ± 0.0289	21.2 ± 28.9 (n=3)	13.6 ± 1.21 (n=3)	7.85 ± 0.625 (n=3)
Day 0 (bucket)	0.362 ± 0.149 (n=3)	0.0196 ± 0.00931 (n=3)	19.6 ± 9.31 (n=3)	14.2 ± 0.737 (n=3)	8.32 ± 0.320 (n=3)
Day 0 (jar)	0.438 ± 0.0993 (n=3)	0.0605 ± 0.0325 (n=3)	60.5 ± 32.5 (n=3)	13.9 ± 0.0764 (n=3)	8.77 ± 0.161 (n=3)
Day 15 (bucket)	0.0314 ± 0.0251 (n=3)	0.00219 ± 0.00189 (n=3)	2.19 ± 1.89 (n=3)	14.0 ± 0.702 (n=3)	8.35 ± 0.375 (n=3)
Day 15 (jar)	0.468 ± 0.348 (n=3)	0.0605 ± 0.0333 (n=3)	60.5 ± 33.3 (n=3)	14.1 ± 0.0289 (n=3)	8.82 ± 0.151 (n=3)
Control (tank)	0.185 ± 0.158 (n=4)	0.00615 ± 0.00461 (n=4)	6.15 ± 4.61 (n=4)	14.2 ± 0.141 (n=4)	8.15 ± 0.107 (n=4)
2.2% OSPW (tank)	0.236 ± 0.130 (n=4)	0.00599 ± 0.00234 (n=4)	5.99 ± 2.34 (n=4)	14.0 ± 0.103 (n=4)	8.01 ± 0.123 (n=4)
4.6% OSPW (tank)	0.231 ± 0.141 (n=4)	0.00504 ± 0.00211 (n=4)	5.04 ± 2.11 (n=4)	13.7 ± 0.287 (n=4)	8.00 ± 0.105 (n=4)
10% OSPW (tank)	0.317 ± 0.158 (n=4)	0.00757 ± 0.00335 (n=4)	7.57 ± 3.35 (n=4)	13.9 ± 0.222 (n=4)	8.02 ± 0.175 (n=4)
22% OSPW (tank)	0.344 ± 0.0779 (n=4)	0.0137 ± 0.00362 (n=4)	13.7 ± 3.62 (n=4)	13.7 ± 0.214 (n=4)	8.24 ± 0.0707 (n=4)
46% OSPW (tank)	0.411 ± 0.0602 (n=4)	0.0274 ± 0.0119 (n=4)	27.4 ± 11.9 (n=4)	13.8 ± 0.239 (n=4)	8.43 ± 0.198 (n=4)
100% OSPW (tank)	0.929 ± 0.263 (n=4)	0.115 ± 0.0300 (n=4)	115 ± 30.0 (n=4)	13.6 ± 0.176 (n=4)	8.76 ± 0.0435 (n=4)
100% OSPW (bucket)	0.581 ± 0.122 (n=4)	0.0112 ± 0.00964 (n=4)	11.2 ± 9.64 (n=4)	13.43 ± 0.831 (n=4)	7.64 ± 0.839 (n=4)

Treatment	Nitrogen-Ammonia (NH_3-N)	Un-ionized Ammonia	Un-ionized Ammonia	Temperature	pH
Unit	**mg/L**	**mg/L**	**µg/L**	**ºC**	
Adjusted 46% OSPW (tank)	0.353 ± 0.0839 (n=2)	0.0262 ± 0.00336 (n=2)	26.2 ± 3.36 (n=2)	13.7 ± 0.247 (n=2)	8.53 ± 0.0424 (n=2)
Adjusted 100% OSPW (tank)	0.566 ± 0.0786 (n=2)	0.0618 ± 0.0129 (n=2)	61.8 ± 12.9 (n=2)	13.9 ± 0.00 (n=2)	8.69 ± 0.0354 (n=2)

Table B.2. Nitrogen-ammonia (mg/L) and un-ionized ammonia (mg/L and µg/L) results of treatment water and control water from buckets and tanks/jars in 2022 analyzed at SFU. Temperature and pH of each exposure water were collected on the day of sampling. Results are expressed as mean ± SD.

Treatment	Nitrogen-Ammonia (NH3-N)	Un-ionized Ammonia	Un-ionized Ammonia	Temperature	pH
Unit	**mg/L**	**mg/L**	**µg/L**	**ºC**	
Aged OSPW (bucket)	< 0.0005 (n=1)	< 0.0005 (n=2)	0.0162 ± 0.00177 (n=2)	13.8 (n=1)	8.39 (n=1)
Control aged OSPW (jar)	0.00392 (n=1)	< 0.0005 (n=2)	0.0282 ± 0.00443 (n=2)	13.9 (n=1)	7.45 (n=1)
Day 0 (bucket)	0.306 (n=1)	0.00415 ± 0.000411 (n=2)	4.15 ± 0.411 (n=2)	15.2 (n=1)	7.75 (n=1)
Day 0 (jar)	0.594 (n=1)	0.0474 ± 0.00450 (n=2)	47.4 ± 4.50 (n=2)	14.6 (n=1)	8.71 (n=1)
Day 15 (bucket)	0.0453 (n=1)	0.00146 ± 0.000231 (n=2)	1.46 ± 0.231 (n=2)	16.3 (n=1)	8.05 (n=1)
Day 15 (jar)	0.413 (n=1)	0.0330 ± 0.00313 (n=2)	33.0 ± 3.13 (n=2)	14.6 (n=1)	8.70 (n=1)
Control (bucket)	< 0.0005 (n=1)	< 0.0005 (n=2)	< 0.0005 (n=2)	11.2 (n=1)	7.25 (n=1)
Hard water (bucket)	0.0197 (n=1)	< 0.0005 (n=2)	0.300 ± 0.0336 (n=2)	11.2 (n=1)	7.89 (n=1)
Control (tank)	0.197 (n=1)	0.00142 ± 0.000223 (n=2)	1.42 ± 0.158 (n=2)	14.2 (n=1)	7.42 (n=1)
Hard water (tank)	0.210 (n=1)	0.00373 ± 0.000593 (n=2)	3.73 ± 0.420 (n=2)	14.4 (n=1)	7.88 (n=1)
1.1% OSPW (tank)	0.207 (n=1)	0.00149 ± 0.000234 (n=2)	1.49 ± 0.165 (n=2)	14.3 (n=1)	7.44 (n=1)
2.2% OSPW (tank)	0.211 (n=1)	0.000760 ± 0.000119 (n=2)	0.760 ± 0.0844 (n=2)	14.3 (n=1)	7.18 (n=1)

Treatment	Nitrogen-Ammonia (NH3-N)	Un-ionized Ammonia	Un-ionized Ammonia	Temperature	pH
Unit	mg/L	mg/L	µg/L	ºC	
4.6% OSPW (tank)	0.235 (n=1)	0.000847 ± 0.000133 (n=2)	0.847 ± 0.094 (n=2)	13.4 (n=1)	7.17 (n=1)
10% OSPW (tank)	0.204 (n=1)	0.00117 ± 0.000187 (n=2)	1.17 ± 0.133 (n=2)	14.3 (n=1)	7.34 (n=1)
22% OSPW (tank)	0.151 (n=1)	0.00171 ± 0.000278 (n=2)	1.71 ± 0.197 (n=2)	14.1 (n=1)	7.64 (n=1)
46% OSPW (tank)	0.167 (n=1)	0.00480 ± 0.000542 (n=2)	4.80 ± 0.383 (n=2)	13.6 (n=1)	8.10 (n=1)
100% OSPW (tank)	0.0957 (n=1)	0.00764 ± 0.000724 (n=2)	7.64 ± 0.512 (n=2)	14.2 (n=1)	8.51 (n=1)
100% OSPW (bucket)	< 0.0005 (n=1)	< 0.0005 (n=2)	< 0.0005 (n=2)	11.2 (n=1)	7.79 (n=1)

Table B.3. Polycyclic aromatic hydrocarbons (PAHs) (µg/L) of control dechlorinated water, OSPW series dilutions, pH-adjusted OSPW, and constructed wetland water from buckets and tanks/jars in 2021 analyzed in InnoTech Alberta (Vegreville, Alberta). Available water quality guidelines (WQG) and lowest detection limits are included. Results are expressed as mean ± SD where more than one sample was available. Dashes represent no samples were available.

Treatment	Acenaphthylene	Anthracene	Benzo(a)pyrene	Fluoranthene	Fluorene	Indeno(1,2,3-cd)pyrene	Phenanthrene
Unit	µg/L	µg/L	µg/L	µg/L	µg/L	µg/L	µg/L
BCWQG (1993) †	-	4.00	0.01	4.00	12	-	0.3
CCME (1998) [a]	-	0.012	0.015	0.04	3	-	0.4
Lowest Detection Limit	0.01	0.007	0.005	0.008	0.006	0.008	0.007
Control dechlorinated water (bucket)	<0.01 (n=2)	<0.007 (n=2)	<0.005 (n=2)	<0.008 (n=2)	<0.006 (n=2)	<0.008 (n=2)	<0.007 (n=2)

Treatment	Acenaphthylene	Anthracene	Benzo(a)pyrene	Fluoranthene	Fluorene	Indeno(1,2,3-cd)pyrene	Phenanthrene
Unit	**µg/L**	**µg/L**	**µg/L**	**µg/L**	**µg/L**	**µg/L**	**µg/L**
Control dechlorinated water (tank)	<0.01 (n=2)	<0.007 (n=2)	0.012 (n=1)	<0.008 (n=2)	<0.006 (n=2)	<0.008 (n=2)	<0.007 (n=2)
2.20% OSPW (bucket)	<0.01 (n=2)	<0.007 (n=1)	0.012 (n=1)	<0.008 (n=1)	<0.006 (n=1)	<0.008 (n=1)	<0.007 (n=1)
2.20% OSPW (tank)	<0.01 (n=2)	-	-	-	-	-	-
4.60% OSPW (bucket)	-	-	-	-	-	-	-
4.60% OSPW (tank)	<0.01 (n=1)	<0.007 (n=1)	0.012 (n=1)	<0.008 (n=1)	<0.006 (n=1)	<0.008 (n=1)	<0.007 (n=1)
10.0% OSPW (bucket)	<0.01 (n=2)	<0.007 (n=2)	0.011 (n=1)	<0.008 (n=2)	<0.006 (n=2)	<0.008 (n=2)	<0.007 (n=2)
10.0% OSPW (tank)	<0.01 (n=1)	<0.007 (n=1)	<0.005 (n=1)	<0.008 (n=1)	<0.006 (n=1)	0.009 (n=1)	<0.007 (n=1)
22.0% OSPW (bucket)	<0.01 (n=2)	<0.007 (n=2)	<0.005 (n=2)	<0.008 (n=2)	<0.006 (n=2)	<0.008 (n=2)	<0.007 (n=2)
22.0% OSPW (tank)	0.01 (n=1)	<0.007 (n=2)	<0.005 (n=2)	<0.008 (n=2)	<0.006 (n=2)	<0.008 (n=2)	<0.007 (n=2)
46.0% OSPW (bucket)	<0.01 (n=1)	<0.007 (n=1)	<0.005 (n=1)	<0.008 (n=1)	<0.006 (n=1)	<0.008 (n=1)	<0.007 (n=1)
46.0% OSPW (tank)	-	-	-	-	-	-	-
100% OSPW (bucket)	0.01 ± 0.00 (n=2)	0.018 ± 0.0078 (n=2)	<0.005 (n=2)	0.008 (n=1)	<0.006 (n=2)	<0.008 (n=2)	<0.007 (n=2)
100% OSPW (tank)	0.01 (n=1)	0.021 (n=1)	<0.005 (n=2)	<0.008 (n=2)	<0.006 (n=2)	<0.008 (n=2)	0.021 (n=1)
Adjusted	<0.01 (n=1)	<0.007 (n=1)	<0.005 (n=1)	<0.008 (n=1)	<0.006 (n=1)	<0.008 (n=1)	0.007 (n=1)

Treatment	Acenaphthylene	Anthracene	Benzo(a)pyrene	Fluoranthene	Fluorene	Indeno(1,2,3-cd)pyrene	Phenanthrene
Unit	**µg/L**	**µg/L**	**µg/L**	**µg/L**	**µg/L**	**µg/L**	**µg/L**
46.0% OSPW (bucket)							
Adjusted 46.0% OSPW (tank)	<0.01 (n=1)	<0.007 (n=1)	<0.005 (n=1)	<0.008 (n=1)	<0.006 (n=1)	<0.008 (n=1)	<0.007 (n=1)
Adjusted 100% OSPW (bucket)	<0.01 (n=1)	0.008 (n=1)	<0.005 (n=1)	<0.008 (n=1)	<0.006 (n=1)	<0.008 (n=1)	0.011 (n=1)
Adjusted 100% OSPW (tank)	<0.01 (n=1)	0.011 (n=1)	<0.005 (n=1)	<0.008 (n=1)	<0.006 (n=1)	<0.008 (n=1)	0.013 (n=1)
Day 0 water (jar)	<0.01 (n=1)	0.014 (n=1)	<0.005 (n=1)	<0.008 (n=1)	0.008 (n=1)	<0.008 (n=1)	0.01 (n=1)
Day 15 water (jar)	<0.01 (n=1)	<0.007 (n=1)	<0.005 (n=1)	<0.008 (n=1)	<0.006 (n=1)	<0.008 (n=1)	0.01 (n=1)

† British Columbia water quality guidelines (BCWQGs) for polycyclic aromatic hydrocarbons (PAHs) (Nagpal, 1993)

[a] Canadian Council of Ministers of the Environment's water quality guidelines for polycyclic aromatic hydrocarbons (PAHs) (CCME, 1999)

Table B.4. Total and dissolved metals (mg/L) of control dechlorinated water, Athabasca River water, OSPW, and Day 0 and 15 water from 2021 analyzed in ALS Environmental (Burnaby, BC). Samples were collected upon water arrival and before RBT exposures. Hardness, dissolved organic carbon (DOC), and pH are included to compare results with available water quality guidelines for the protection of aquatic life.

Water Sample	Lowest Detection Limit	Dechlorinated Water	Athabasca River	OSPW	Day 0	Day 15
Alkalinity (as CaCO3) (mg/L)	1.0	31.1	117	222	226	264
Hardness	0.60	29.9	133	227	240	302
Dissolved Organic	0.50	0.72	21.7	41	35.4	35.8

Water Sample	Lowest Detection Limit	Dechlorinated Water	Athabasca River	OSPW	Day 0	Day 15
Carbon (DOC) (mg/L)						
pH	0.10	7.74	8.28	8.27	8.3	8.3
Turbidity	0.10	<0.10	11.2	7.19	12.8	5.53
Anions and Nutrients (Matrix: Water)	**mg/L**	**mg/L**	**mg/L**	**mg/L**	**mg/L**	**mg/L**
Bromide	0.005	<0.050	<0.050	<0.250	<0.250	<0.250
Chloride	0.005	2.62	4.66	25.8	24.7	20.1
Fluoride	0.020	<0.020	0.082	3.61	3.33	2.33
Nitrate (as N)	0.005	0.0316	0.0683	0.0964	0.0988	<0.0250
Nitrate + nitrite (as N)	0.005	0.0316	0.0745	0.126	0.0312	<0.0050
nitrite (as N)	0.001	<0.0010	0.0062	0.0296	0.814	0.585
sulfate (as SO_4)	0.30	1.82	24.9	384	373	342
Sulfide, total (as S)	0.010	<0.010	<0.010	<0.010	<0.010	<0.010
Sulfide, total (as H2S)	0.011	<0.011	<0.011	<0.011	<0.011	<0.011
Total Metals (Matrix: Water)	**mg/L**	**mg/L**	**mg/L**	**mg/L**	**mg/L**	**mg/L**
Aluminum, total	0.0030	0.0606 †	0.333 †[a]	0.528 †[a]	0.205 †[a]	0.0805 †
Antimony, total	0.00010	<0.00010	<0.00010	0.00143	0.00131	0.00059
Arsenic, total	0.00010	0.00022	0.00037	0.00222	0.00184	0.00084
Barium, total	0.00010	0.00137	0.0284	0.182	0.170	0.0984
Beryllium, total	0.000100	<0.000100	<0.000100	<0.000100	<0.000100	<0.000100
Bismuth, total	0.000050	<0.000050	<0.000050	<0.000050	<0.000050	<0.000050
Boron, total	0.010	<0.010	0.046	1.19	1.08	0.818
Cadmium, total	0.0000050	<0.0000050	<0.0000050	<0.0000150	<0.0000150	<0.0000100
Calcium, total	0.050	11.2	38.6	51.3	53.2	71.9
Cesium, total	0.000010	<0.000010	0.000042	0.000133	0.000104	0.000022

Water Sample	Lowest Detection Limit	Dechlorinated Water	Athabasca River	OSPW	Day 0	Day 15
Chromium, total	0.00050	<0.00050	<0.00050	0.00061	<0.00050	<0.00050
Cobalt, total	0.00010	<0.00010	0.00025	0.00140	0.00145	0.00038
Copper, total	0.00050	<0.00050	<0.00050	<0.00050	<0.00050	<0.00050
Iron, total	0.010	<0.010	0.885 [a]	0.094	0.139	0.155
Lead, total	0.000050	<0.000050	0.000152	0.000106	0.000074	<0.000050
Lithium, total	0.0010	<0.0010	0.0083	0.0952	0.103	0.0796
Magnesium, total	0.0050	0.480	8.95	24.1	26.1	29.7
Manganese, total	0.00010	0.00028	0.0596 †	0.0861 †	0.0861 †	0.0141 †
Mercury, total	0.0000050	<0.0000050	<0.0000050	<0.0000050	<0.0000250	<0.0000050
Molybdenum, total	0.000050	0.000232	0.000126	0.0667	0.0558	0.0326
Nickel, total	0.00050	<0.00050	0.00088	0.00778	0.00641	0.00380
Phosphorus, total	0.050	<0.050	<0.050	<0.050	<0.050	<0.050
Potassium, total	0.050	0.344	1.38	13.0	13.8	12.1
Rubidium, total	0.00020	0.00040	0.00180	0.0176	0.0174	0.0100
Selenium, total	0.000050	<0.000050	<0.000050	0.000158	0.000124	0.000068
Silicon, total	0.10	1.37	2.38	5.98	5.39	5.95
Silver, total	0.000010	<0.000010	<0.000010	<0.000010	<0.000010	<0.000010
Sodium, total	0.050	4.43	10.1	176	184	149
Strontium, total	0.00020	0.0424	0.0975	1.05	1.03	0.751
Sulfur, total	0.50	0.57	8.64	142	136	126
Tellurium, total	0.00020	<0.00020	<0.00020	<0.00020	<0.00020	<0.00020
Thallium, total	0.000010	<0.000010	<0.000010	<0.000010	<0.000010	<0.000010
Thorium, total	0.00010	<0.00010	<0.00010	<0.00010	<0.00010	<0.00010
Tin, total	0.00010	<0.00010	<0.00010	<0.00010	<0.00010	<0.00010
Titanium, total	0.00030	<0.00030	<0.0120	0.0113	0.00334	<0.00270
Tungsten, total	0.00010	<0.00010	<0.00010	0.00106	0.00081	0.00022

Water Sample	Lowest Detection Limit	Dechlorinated Water	Athabasca River	OSPW	Day 0	Day 15
Uranium, total	0.000010	0.000547	0.000124	0.00238	0.00232	0.00161
Vanadium, total	0.00050	0.00064	0.00087	0.00779	0.00587	0.00090
Zinc, total	0.0030	0.259 †	<0.0030	0.0062	<0.0030	<0.0030
Zirconium, total	0.00020	0.00092	0.00037	0.00076	0.00094	0.00024
Dissolved Metals (Matrix: Water)	**mg/L**	**mg/L**	**mg/L**	**mg/L**	**mg/L**	**mg/L**
Aluminum, dissolved	0.0010	0.0559	0.0067	0.0044	0.0011	0.0051
Antimony, dissolved	0.00010	<0.00010	<0.00010	0.00145	0.00126	0.00062
Arsenic, dissolved	0.00010	0.00019	0.00028	0.00214	0.00156	0.00072
Barium, dissolved	0.00010	0.00135	0.0245	0.173	0.178	0.104
Beryllium, dissolved	0.000100	<0.000100	<0.000100	<0.000100	<0.000100	<0.000100
Bismuth, dissolved	0.000050	<0.000050	<0.000050	<0.000050	<0.000050	<0.000050
Boron, dissolved	0.010	<0.010	0.040	1.18	1.09	0.850
Cadmium, dissolved	0.0000050	<0.0000050	<0.0000050	<0.0000200	<0.0000150	<0.0000050
Calcium, dissolved	0.050	11.7	38.0	53.1	54.6	74.5
Cesium, dissolved	0.000010	<0.000010	<0.000010	0.000095	0.000076	0.000019
Chromium, dissolved	0.00050	<0.00050	<0.00050	<0.00050	<0.00050	<0.00050
Cobalt, dissolved	0.00010	<0.00010	<0.00010	0.00053	0.00043	0.00024
Copper, dissolved	0.00020	<0.00020	<0.00020	<0.00020	<0.00020	0.00024
Iron, dissolved	0.010	<0.010	0.206	<0.010	<0.010	0.039
Lead, dissolved	0.000050	<0.000050	<0.000050	<0.000050	<0.000050	<0.000050
Lithium, dissolved	0.0010	<0.0010	0.0072	0.0951	0.0937	0.0752
Magnesium, dissolved	0.0050	0.476	9.54	24.8	25.8	29.1
Manganese, dissolved	0.00010	0.00020	0.00142	0.00794	0.00028	0.00079
Mercury, dissolved	0.0000050	<0.0000050	<0.0000050	<0.0000050	<0.0000050	<0.0000050

Water Sample	Lowest Detection Limit	Dechlorinated Water	Athabasca River	OSPW	Day 0	Day 15
Molybdenum, dissolved	0.000050	0.000195	0.000122	0.0653	0.0566	0.0332
Nickel, dissolved	0.00050	<0.00050	0.00062	0.00676	0.00527	0.00348
Phosphorus, dissolved	0.050	<0.050	<0.050	<0.050	<0.050	<0.050
Potassium, dissolved	0.050	0.295	1.23	13.6	13.6	12.2
Rubidium, dissolved	0.00020	0.00045	0.00122	0.0188	0.0162	0.00944
Selenium, dissolved	0.000050	<0.000050	<0.000050	0.000112	0.000110	0.000066
Silicon, dissolved	0.050	1.35	1.58	4.72	4.99	5.67
Silver, dissolved	0.000010	<0.000010	<0.000010	<0.000010	<0.000010	<0.000010
Sodium, dissolved	0.050	4.04	11.6	178	181	146
Strontium, dissolved	0.00020	0.0399	0.0990	1.05	1.01	0.781
Sulfur, dissolved	0.50	0.73	8.30	124	143	125
Tellurium, dissolved	0.00020	<0.00020	<0.00020	<0.00020	<0.00020	<0.00020
Thallium, dissolved	0.000010	<0.000010	<0.000010	<0.000010	<0.000010	<0.000010
Thorium, dissolved	0.00010	<0.00010	<0.00010	<0.00010	<0.00010	<0.00010
Tin, dissolved	0.00010	<0.00010	<0.00010	<0.00010	<0.00010	<0.00010
Titanium, dissolved	0.00030	<0.00030	0.00031	<0.00030	<0.00030	<0.00030
Tungsten, dissolved	0.00010	<0.00010	<0.00010	0.00103	0.00081	0.00023
Uranium, dissolved	0.000010	0.000530	0.000101	0.00241	0.00239	0.00172
Vanadium, dissolved	0.00050	0.00073	<0.00050	0.00667	0.00465	0.00058
Zinc, dissolved	0.0010	0.215 [a]	0.104 [a]	0.0035	<0.0010	<0.0010
Zirconium, dissolved	0.00020	<0.00020	<0.00020	<0.00020	<0.00020	<0.00020

† Exceeds acute and/or chronic ambient water quality guidelines for the protection of aquatic life from BC Ministry of Environment and Climate Change Strategy.

[a] Exceeds acute and/or chronic environmental quality guidelines for the protection of aquatic life from Canadian Council of Ministers of the Environment.

Table B.5. Total and dissolved metals (mg/L) of control dechlorinated water, hard water, aged mesocosm OSPW, OSPW, and Day 0 and Day 15 water from 2022 analyzed in ALS Environmental (Burnaby, BC). Samples were collected upon water arrival and before RBT exposures. Hardness, dissolved organic carbon (DOC), and pH are included to compare results with available water quality guidelines for the protection of aquatic life.

Water Sample	Lowest Detection Limit	Dechlorinated Water	Hard Water	Aged OSPW	OSPW	Day 0	Day 15
Alkalinity (as CaCO3) (mg/L)	1.0	21.3	113	270	228	235	293
Hardness	0.60	19.9	170	126	204	227	333
Dissolved Organic Carbon (DOC) (mg/L)	0.50	0.52	1.00	40.9	33.1	39.2	41.6
pH	0.10	7.44	8.31	8.61	8.39	8.4	8.43
Turbidity	0.10	<0.10	0.27	0.14	2.09	5.42	0.96
Anions and Nutrients (Matrix: Water)	**mg/L**	**mg/L**	**mg/L**	**mg/L**	**mg/L**	**mg/L**	**mg/L**
Bromide	0.005	<0.050	0.0417	<0.250	0.275	0.340	<0.500
Chloride	0.005	2.49	<0.050	33.0	31.7	27.9	28.7
Fluoride	0.020	<0.020	5.35	3.68	3.43	3.29	2.95
Nitrate (as N)	0.005	0.0437	0.022	0.0493	0.789	0.0897	<0.0500
Nitrate + nitrite (as N)	0.005	0.0437	0.0290	0.0493	0.789	0.104	<0.0510
nitrite (as N)	0.001	<0.0010	0.0290	<0.0050	<0.0050	0.0143	<0.0100
sulfate (as SO4)	0.30	1.49	<0.0010	258	395	384	432
Sulfide, total (as S)	0.010	<0.0015	<0.0015	0.0047	0.0047	0.0046	0.0104
Sulfide, total (as H2S)	0.011	<0.0016	<0.0016	0.0050	0.0050	0.0049	0.0111
Total Metals (Matrix: Water)	**mg/L**	**mg/L**	**mg/L**	**mg/L**	**mg/L**	**mg/L**	**mg/L**

Water Sample	Lowest Detection Limit	Dechlorinated Water	Hard Water	Aged OSPW	OSPW	Day 0	Day 15
Aluminum, total	0.0030	0.0244	0.0269	0.0163	0.255 † [a]	0.127 † [a]	0.0059
Antimony, total	0.00010	<0.00010	<0.00010	0.00056	0.00138	0.00148	0.00071
Arsenic, total	0.00010	0.00012	0.00016	0.00156	0.00181	0.00256	0.00101
Barium, total	0.00010	0.00079	0.00106	0.0699	0.160	0.175	0.108
Beryllium, total	0.000100	<0.000100	<0.000100	<0.000100	<0.000100	<0.000100	<0.000100
Bismuth, total	0.000050	<0.000050	<0.000050	<0.000050	<0.000050	<0.000050	<0.000050
Boron, total	0.010	<0.010	<0.010	0.873	1.09	1.16	1.12
Cadmium, total	0.000005	<0.000005	<0.000005	<0.0000100	<0.000015	<0.0000050	<0.0000100
Calcium, total	0.050	7.65	34.8	22.1	45.7	51.5	83.7
Cesium, total	0.000010	<0.000010	<0.000010	0.000022	0.000078	0.000122	0.000024
Chromium, total	0.00050	<0.00050	<0.00050	<0.00050	<0.00050	<0.00050	<0.00050
Cobalt, total	0.00010	<0.00010	<0.00010	0.00030	0.00041	0.00131	0.00041
Copper, total	0.00050	0.00056	0.00060	0.00064	<0.00050	<0.00050	<0.00050
Iron, total	0.010	<0.010	<0.010	<0.010	0.046	0.135	0.143
Lead, total	0.000050	<0.000050	0.000164	<0.000050	<0.000050	0.000053	<0.000050
Lithium, total	0.0010	<0.0010	<0.0010	0.0655	0.0917	0.100	0.101
Magnesium, total	0.0050	0.192	18.3	17.3	21.8	23.8	30.2
Manganese, total	0.00010	0.00042	0.00064	0.00047	0.0211 †	0.0888 †	0.0240 †
Mercury, total	0.0000050	<0.0000050	<0.000005	<0.0000050	<0.0000050	<0.0000050	<0.0000050
Molybdenum, total	0.000050	0.000250	0.000240	0.0310	0.0589	0.0766 [a]	0.0536
Nickel, total	0.00050	<0.00050	<0.00050	0.00468	0.00722	0.00683	0.00468
Phosphorus, total	0.050	<0.050	<0.050	<0.050	<0.050	<0.050	<0.050

Water Sample	Lowest Detection Limit	Dechlorinated Water	Hard Water	Aged OSPW	OSPW	Day 0	Day 15
Potassium, total	0.050	0.217	3.45	8.82	12.9	14.3	14.0
Rubidium, total	0.00020	0.00035	0.00047	0.00721	0.0136	0.0169	0.0109
Selenium, total	0.000050	<0.000050	<0.000050	0.000155	0.000140	0.000120	0.000140
Silicon, total	0.10	1.26	1.30	0.24	5.13	6.04	6.76
Silver, total	0.000010	<0.000010	<0.000010	<0.000010	<0.000010	<0.000010	<0.000010
Sodium, total	0.050	2.46	42.7	198	196	218	212
Strontium, total	0.00020	0.0162	0.165	0.564	0.947	1.01	0.830
Sulfur, total	0.50	<0.50	48.0	92.5	138	152	162
Tellurium, total	0.00020	<0.00020	<0.00020	<0.00020	<0.00020	<0.00020	<0.00020
Thallium, total	0.000010	<0.000010	<0.000010	<0.000010	<0.000010	<0.000010	<0.000010
Thorium, total	0.00010	<0.00010	<0.00010	<0.00010	<0.00010	<0.00010	<0.00010
Tin, total	0.00010	<0.00010	<0.00010	0.00079	<0.00010	<0.00010	<0.00010
Titanium, total	0.00030	<0.00030	<0.00030	<0.00030	<0.00480	0.00280	<0.00060
Tungsten, total	0.00010	<0.00010	<0.00010	0.00068	0.00120	0.00146	0.00033
Uranium, total	0.000010	<0.000010	0.000011	0.00186	0.00269	0.00306	0.00225
Vanadium, total	0.00050	<0.00050	<0.00050	0.00129	0.00562	0.00819	0.00094
Zinc, total	0.0030	<0.0030	<0.0030	0.0056	0.0128	<0.0030	0.0046

Water Sample	Lowest Detection Limit	Dechlorinated Water	Hard Water	Aged OSPW	OSPW	Day 0	Day 15
Zirconium, total	0.00020	<0.00020	<0.00020	<0.00020	0.00036	0.00043	<0.00020
Dissolved Metals (Matrix: Water)	**mg/L**	**mg/L**	**mg/L**	**mg/L**	**mg/L**	**mg/L**	**mg/L**
Aluminum, dissolved	0.0010	0.0241	0.0278	0.0152	0.0017	0.0021	0.0029
Antimony, dissolved	0.00010	<0.00010	<0.00010	0.00057	0.00140	0.00138	0.00070
Arsenic, dissolved	0.00010	<0.00010	<0.00010	0.00166	0.00190	0.00254	0.00114
Barium, dissolved	0.00010	0.00083	0.00129	0.0734	0.169	0.182	0.108
Beryllium, dissolved	0.000100	<0.000100	<0.000100	<0.000100	<0.000100	<0.000100	<0.000100
Bismuth, dissolved	0.000050	<0.000050	<0.000050	<0.000050	<0.000050	<0.000050	<0.000050
Boron, dissolved	0.010	<0.010	<0.010	0.801	0.923	1.04	1.01
Cadmium, dissolved	0.0000050	<0.0000050	<0.000005	<0.0000100	<0.0000150	<0.0000200	<0.0000250
Calcium, dissolved	0.050	8.05	37.8	22.5	42.9	54.3	86.0
Cesium, dissolved	0.000010	<0.000010	<0.000010	0.000024	0.000065	0.000098	0.000023
Chromium, dissolved	0.00050	<0.00050	<0.00050	<0.00050	<0.00050	<0.00050	<0.00050

Water Sample	Lowest Detection Limit	Dechlorinated Water	Hard Water	Aged OSPW	OSPW	Day 0	Day 15
Cobalt, dissolved	0.00010	<0.00010	<0.00010	0.00031	0.00024	0.00052	0.00032
Copper, dissolved	0.00020	0.00052	0.00052	0.00062	<0.00020	<0.00020	<0.00020
Iron, dissolved	0.010	<0.010	<0.010	<0.010	<0.010	<0.010	0.108
Lead, dissolved	0.000050	<0.000050	0.000114	<0.000050	<0.000050	<0.000050	<0.000050
Lithium, dissolved	0.0010	<0.0010	<0.0010	0.0691	0.0890	0.100	0.0990
Magnesium, dissolved	0.0050	0.205	18.3	18.6	23.0	23.4	29.5
Manganese, dissolved	0.00010	0.00036	0.00052	0.00021	0.0108	0.00227	0.00227
Mercury, dissolved	0.0000050	<0.0000050	<0.000005	<0.0000050	<0.0000050	<0.0000050	<0.0000050
Molybdenum, dissolved	0.000050	0.000221	0.000269	0.0321	0.0617	0.0744	0.0537
Nickel, dissolved	0.00050	<0.00050	<0.00050	0.00494	0.00742	0.00620	0.00455
Phosphorus, dissolved	0.050	<0.050	<0.050	<0.050	<0.050	<0.050	<0.050
Potassium, dissolved	0.050	0.217	3.53	9.37	14.2	15.0	14.4
Rubidium, dissolved	0.00020	0.00044	0.00046	0.00783	0.0139	0.0169	0.0108
Selenium, dissolved	0.000050	<0.000050	<0.000050	0.000139	0.000227	0.000205	0.000154

Water Sample	Lowest Detection Limit	Dechlorinated Water	Hard Water	Aged OSPW	OSPW	Day 0	Day 15
Silicon, dissolved	0.050	1.21	1.28	0.222	4.85	5.51	6.49
Silver, dissolved	0.000010	<0.000010	<0.000010	<0.000010	<0.000010	<0.000010	<0.000010
Sodium, dissolved	0.050	2.59	44.0	219	217	217	210
Strontium, dissolved	0.00020	0.0181	0.175	0.603	1.04	1.01	0.847
Sulfur, dissolved	0.50	<0.50	58.6	96.2	142	151	167
Tellurium, dissolved	0.00020	<0.00020	<0.00020	<0.00020	<0.00020	<0.00020	<0.00020
Thallium, dissolved	0.000010	<0.000010	<0.000010	<0.000010	<0.000010	<0.000010	<0.000010
Thorium, dissolved	0.00010	<0.00010	<0.00010	<0.00010	<0.00010	<0.00010	0.00015
Tin, dissolved	0.00010	<0.00010	0.00026	0.00084	<0.00010	<0.00010	<0.00010
Titanium, dissolved	0.00030	<0.00030	<0.00030	<0.00030	<0.00030	0.00032	<0.00030
Tungsten, dissolved	0.00010	<0.00010	<0.00010	0.00066	0.00114	0.00134	0.00031
Uranium, dissolved	0.000010	<0.000010	<0.000010	0.00186	0.00266	0.00286	0.00206
Vanadium, dissolved	0.00050	<0.00050	<0.00050	0.00125	0.00533	0.00691	0.00080
Zinc, dissolved	0.0010	0.0018	0.0022	0.0058	0.0119 [a]	<0.0010	0.0042

Water Sample	Lowest Detection Limit	Dechlorinated Water	Hard Water	Aged OSPW	OSPW	Day 0	Day 15
Zirconium, dissolved	0.00020	<0.00020	<0.00020	<0.00020	<0.00020	<0.00020	0.00025

† Exceeds acute and/or chronic ambient water quality guidelines for the protection of aquatic life from BC Ministry of Environment and Climate Change Strategy.

[a] Exceeds acute and/or chronic environmental quality guidelines for the protection of aquatic life from Canadian Council of Ministers of the Environment.

Table B.6. Summary of hatching success (%) for exposure to OSPW dilutions (n=20), pH-adjusted OSPW dilution (n=18), constructed wetland treatment (Day 0 and Day 15) (n=5 each), and Athabasca River water (n=20) in 2021. Results are presented from onset of experimet (day 1) until day 10 for simplicity. Values are mean ± standard error (S.E.). Aesthetics (*) represent statistical difference compared to the control and letters represent the hypothesis testing method (see note). pH-adjusted 46.0% and 100% OSPW dilutions were statistically compared with unadjusted 46.0% and 100% OSPW dilutions. Dashes represent no available values.

Day	1	2	3	4	5	6	7	8	9	10
Exposure	**Mean ± S.E**	**Mean ± S.E**	**Mean ± S.E**	**Mean ± S.E**	**Mean ± S.E**	**Mean ± S.E**	**Mean ± S.E**	**Mean ± S.E**	**Mean ± S.E**	**Mean ± S.E**
Control	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	5.0 ± 2.0	56 ± 2.4	95 ± 2.0	98.8 ± 1.25	98.8 ± 1.25	98.8 ± 1.25	98.8 ± 1.25
2.20% OSPW	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	8.75 ± 3.75	67.5 ± 1.44	91 ± 5.2	97.5 ± 2.50	97.5 ± 2.50	97.5 ± 2.50	97.5 ± 2.50
4.60% OSPW	0.0 ± 0.0	0.0 ± 0.0	1.25 ± 1.25	6.25 ± 3.75	62 ± 7.6	97.5 ± 1.44	100 ± 0.0	100 ± 0.0	100 ± 0.0	100 ± 0.0
10.0% OSPW	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	1.25 ± 1.25	50.6 ± 5.25	79.9 ± 7.32	95 ± 2.0	95 ± 2.0	95 ± 2.0	95 ± 2.0
22.0% OSPW	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	7.5 ± 3.2	62.5 ± 9.68	87.5 ± 6.29	96 ± 2.4	97.5 ± 2.50	97.5 ± 2.50	97.5 ± 2.50
46.0% OSPW	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	10 ± 2.9	48.8 ± 9.44	90 ± 3.5	96 ± 2.4	96 ± 2.4	96.3 ± 2.39	96 ± 2.4
100% OSPW	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	1.3 ± 1.3	7.6 ± 5.3 * [a]	41 ± 7.3 * [a]	49.9 ± 2.04 * [a]	66.5 ± 2.04 * [a]	67.8 ± 2.04 * [a]	70.5 ± 2.04 * [a]
Adjusted 46.0% OSPW	--	--	--	--	--	--	0.0 ± 0.0 * [a]	97 ± 1.6	98.6 ± 1.39	98.6 ± 1.39
Adjusted 100% OSPW	--	--	--	--	--	--	0.0 ± 0.0 * [a]	97 ± 1.7 * [a]	98.6 ± 1.39 * [a]	100 ± 0.0 * [a]
Control	0.0 ± 0.0	70 ± 17	100 ± 0.0	100 ± 0.0	100 ± 0.0	100 ± 0.0	100 ± 0.0	100 ± 0.0	100 ± 0.0	100 ± 0.0
Day 0 water	0.0 ± 0.0	5.0 ± 5.0 *	95 ± 5.0	95 ± 5.0	95 ± 5.0	95 ± 5.0 *	95 ± 5.0	95 ± 5.0	95 ± 5.0	95 ± 5.0

Day	1	2	3	4	5	6	7	8	9	10
Exposure	**Mean ± S.E**	**Mean ± S.E**	**Mean ± S.E**	**Mean ± S.E**	**Mean ± S.E**	**Mean ± S.E**	**Mean ± S.E**	**Mean ± S.E**	**Mean ± S.E**	**Mean ± S.E**
		[a]								
Day 15 water	0.0 ± 0.0	85 ± 9.6	100 ± 0.0	100 ± 0.0	100 ± 0.0	100 ± 0.0	100 ± 0.0	100 ± 0.0	100 ± 0.0	100 ± 0.0
Control	0.0 ± 0.0	8.0 ± 2.3	93 ± 2.5	100 ± 0.0	100 ± 0.0	100 ± 0.0	100 ± 0.0	100 ± 0.0	100 ± 0.0	100 ± 0.0
Athabasca River water	0.0 ± 0.0	7.0 ± 1.9	95 ± 1.9	100 ± 0.0	100 ± 0.0	100 ± 0.0	100 ± 0.0	100 ± 0.0	100 ± 0.0	100 ± 0.0

[a] Kruskal Wallis and pairwise comparisons, $p<0.05$