EFFECTS OF BITUMINOUS CONTAMINANTS ON FATHEAD MINNOWS (*PIMEPHALES PROMELAS*) OF THE LOWER ATHABASCA RIVER BASIN

SYLVIA S.T. CHOW
BSc NRC, University of British Columbia, 2011

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SYLVIA S.T. CHOW

Date of Defence: December 2nd, 2016

Dr. G. Pyle Professor Ph.D.
Supervisor

Dr. A. Hontela Professor Ph.D.
Thesis Examination Committee Member

Dr. J. Rasmussen Professor Ph.D.
Thesis Examination Committee Member

Dr. S. Kienzle Professor Ph.D.
External Examiner
Department of Geography, University of Lethbridge
Lethbridge, AB

Dr. A. Russell Assistant Professor Ph.D.
Chair, Thesis Examination Committee

Dedication

I dedicate my thesis to my mother and father, two individuals who have shown me the meaning of perseverance: The encouragement, strength and guidance you've provided me in my life and during my project is priceless.
Thank you, mom and dad.

媽, 爸 :
你哋教導我點樣做一個落力, 成功女人,
點樣腳踏實地, 馬死落地行.
多謝.

Abstract

The lower Athabasca River basin of northern Alberta, Canada is situated within bitumen-rich deposits, where oil sands development is a common occurrence. A transplant and chronic exposure experiment between sites containing natural bitumen (NB) and natural bitumen-oil sands area of industry (NB-OSA) was conducted using fathead minnows (*Pimephales promelas*). Reference fish chemosensation and respiration was impaired and lowered, respectively, to levels observed in the fish NB-OSA of industry after having been transplanted to either contaminated sites. Fish previously exposed to contaminants had lower olfactory acuity and respiration rates than reference fish, remaining unchanged after exposure at either site. Fish pre-exposed to contaminants exhibit limited ability to deal with additional stressors compared to reference fish, as observed by the lower survival rates in these bituminous fish populations. This limited stress coping capabilities demonstrate native fish populations do not possess an advantage within the aquatic systems in the basin.

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Respiration:

$$Mass\ corrected\ respiraiton\ rate = respiration\ rate / mass^{0.7}$$

List of Abbreviations

Abbreviation	*Definition*
EOG	Electro-olfactography
EROD	Ethoxyresorufin-*O*-deethylase
FHM	Fathead minnow
L-ala	L-alanine
NAs	Naphthenic acids
NAF	Naphthenic acid fraction
NB	Natural bitumen
NB-OSA	Natural bitumen/oil sands area
OE	Olfactory epithelium
OSA	Oil sands area
OSPW	Oil sands process-affected waters
PAHs	Polycyclic aromatic hydrocarbons
TCA	Taurocholic acid
WAF	Water accommodated fraction
WQG	Water quality guideline
WSF	Water soluble fraction

1 Introduction

Ranking third with respect to global oil reserves following Venezuela and Saudi Arabia, Canada's bituminous sands are an important crude oil resource (Dowedeswell *et al.*, 2010; Bari & Kindzierski, 2015). Known as the McMurray geologic formation, this bituminous oil sands reserve located in northeastern Alberta is found at various depths, 20% of which is accessible less than 75 m from the surface. The remainder of the bitumen-rich oil sands requires underground mining, or *in-situ* mining (Hein & Cotterill, 2006; Alberta Energy Regulator, 2015). Much of the surface-mineable bituminous sand deposit is found in northeastern Alberta, situated in the Lower Athabasca River basin where its tributaries may run through and erode the deposit delivering pulses of bitumen into the aquatic habitat (Conly *et al.*, 2002; Hein & Cotterill, 2006; Jordaan, 2012). It is unclear whether aquatic organisms have undergone local adaptation to the natural and anthropogenic bituminous toxicants associated with the bituminous oil sands in the Lower Athabasca River basin. Answering the question of local adaptation will further the understanding of how aquatic organisms respond to natural and anthropogenic sources of bituminous toxicants from naturally occurring erosional events, on-going industrial mining and refinement, and subsequent reclamation and remediation efforts required of the industry. The goal of this current project is to determine if natural selection has occurred within aquatic populations inhabiting Alberta's oil sands region and to understand how this potential for adaptation can be translated to improvement of industry monitoring programs, and reclamation and remediation efforts.

2 Fish Toxicology and Local Adaptation in the Oil Sands: A Literature Review

Aquatic toxicology and local adaptation in relation to fish in the oil sands will be introduced and reviewed in this section. The current literature review is placed within the context of oil sands development that includes a description of the general geography associated with the area, and the availability of baseline data with regards to natural versus anthropogenic sources of bituminous toxicants. Current monitoring efforts that have been set in place are introduced and discussion will follow on how such efforts can be improved through the incorporation of the concept of local adaptation within the context of field-based aquatic ecotoxicological research methods.

2.1 Context of the Projects

Industry regulations have been set in place to allow for appropriate remediation and reclamation efforts. However, few baseline data distinguishing anthropogenic from natural sources of bituminous toxicants were collected although monitoring efforts began shortly after industrial development started in the late 1960s (Conly *et al.*, 2002). The lack of baseline data diminishes efforts in understanding the ecological risk of bituminous oil sands disturbance as there is no reference point with which to compare effects of toxicants. Through recent paleolimnological, and air quality analyses, the contribution of petrogenic toxicants has been observed to change with expansion of industrial development in the oil sands area (Wang *et al.*, 2014; Elmes *et al.*, 2016).

Currently, 976 million m^3 of oil sands related effluent or tailings is kept within tailings ponds on site within the region (Pramanik, 2016). These tailings are destined for remediation and surface reclamation when the oil sands reserve has less than six years of extractable oil sands remaining (Alberta Energy Regulator, 2015). Because industry-related by-products are contained within these tailings ponds, toxicant deposition and release has recently been focused on potential air-

borne deposition from open-pit mining, upgrading facilities smoke stacks and tailings emissions (Kelly *et al.*, 2009; Kelly *et al.*, 2010; Huang *et al.*, 2016b). Monitoring of the industrial contributions to present toxicant regime in the oil sands has been developing in recent years.

To understand the effects of industry development in the oil sands region, it is important to identify possible sources of oil-sands related toxicants and to acquire an adequate baseline concentration of these toxicants within the ecosystem. Oil sands toxicology primarily focuses on understanding the effects of oil sands process-affected waters and how to reclaim and remediate it for eventual release (Conly *et al.*, 2002; Van der Oost *et al.*, 2003; Hall *et al.*, 2012; Martin *et al.*, 2015). Limited studies have been completed to understand differences between natural and anthropogenic oil sands related toxicants and their effects (Tetreault *et al.*, 2003; Colavecchia *et al.*, 2004).

Baseline data in relation to potential effects of development and natural erosion of bituminous oil sands on the aquatic ecosystem has been recently examined using paleo-limnology techniques where polycyclic aromatic compounds in lake sediments are identified at different depths (Timoney & Lee, 2011; Elmes *et al.*, 2016). Elmes *et al.* (2016) and Hall *et al.* (2012) adapted paleo-limnological techniques to determine the effect of natural erosion and deposition of bitumen in the Slave River Delta and the Peace-Athabasca Delta, respectively. Toxicant concentrations in lake sediment cores associated with bitumen erosion demonstrated that toxicants did not significantly increase in relation to the onset of oil sands development (Hall *et al.*, 2012; Wiklund *et al.*, 2014; Elmes *et al.*, 2016).

Recent studies have examined the potential to distinguish differences between natural and anthropogenic toxicant sources in the oil sands area (OSA) and to determine possible industrial input into the ecosystem (Kelly *et al.*, 2009; Kelly *et al.*, 2010; Dayyani *et al.*, 2016; Huang *et al.*,

2016b). Bitumen toxicants such as polycyclic aromatic hydrocarbons and metals have been measured in airborne deposition from oil sands operations and it is through this primary mechanism of transport that much of the anthropogenic bituminous toxicants are released into the surrounding ecosystem (Zhang *et al.*, 2015a; Zhang *et al.*, 2015b; Gueguen *et al.*, 2016; Huang *et al.*, 2016b). As the detection of the toxicant load from anthropogenic activities in the oil sands area improves, understanding possible local adaptation in aquatic organisms exposed to natural sources of bitumen will further improve our ability to assess ecological risk to such anthropogenic changes occurring in this river basin.

2.2 Monitoring and Environmental Assessment of the Oil Sands

Monitoring and environmental assessment of the oil sands industry have included an environmental toxicology approach to assessing impact of development within the lower Athabasca River basin (Chapman, 2002; Moiseenko, 2008). Many of the peer-reviewed studies focusing on fish-related toxicology in the oil sands emphasize single contaminant laboratory exposures using non-native or ecologically irrelevant species. Studies examining oil sands toxicity emphasise laboratory-based approaches, under the umbrella of environmental toxicology whereby non-native aquatic organisms are used in oil sands-related toxicological research (Chapman, 2002; Moiseenko, 2008; Schmitt-Jansen *et al.*, 2008; Blaise & Gagne, 2009). As such, current research in oil sands toxicology encompasses the environmental toxicology approach, rather than taking an ecotoxicology approach whereby a “big picture” ecosystem-based approach is used to assess environmental impacts (Chapman, 2002). Additionally, understanding the possibility of adaptation or evolution in the populations of aquatic organisms having been consistently exposed to fluvial pulses of bitumen has yet to be tackled in terms of current oil sands toxicity assessment. Improvement of environmental risk assessment of bituminous toxicants will require the

incorporation of such an ecosystem-based approach to environmental toxicology and an in-depth analysis of potential adaptation to bituminous toxicants to enhance understanding of real-world oil sands toxicology.

The importance of comparing the effects of anthropogenic and natural bituminous toxicants associated with the McMurray bitumen formation is crucial for understanding the ecological relevance of environmental assessment within the oil sands region (Schmitt-Jansen *et al.*, 2008). It is through the integration of a field-based approach examining possible evolution that appropriate knowledge of oil sands impact can be assessed on a watershed and ecosystem level (Moiseenko, 2008; Hendry, 2016). Despite the policies in place for the industrial processing of bitumen along with increased environmental attention of the oil sands industry, evidence exists that development has influenced the toxicant input to the surrounding lower Athabasca River basin (Kelly et al. 2009; Kelly et al. 2010). Current toxicological research, although wide ranging, is limited in terms of bringing together different endpoints to achieve an ecosystem-based approach to analysing oil sands toxicology (Moiseenko, 2008; Elshayeb *et al.*, 2009; Puttaswamy & Liber, 2012; Van den Heuvel *et al.*, 2014). Examination for the presence of local adaptation is important to understand the possible role of evolution in the Alberta oil sands. Environmental risk assessment of the oil sands industry can therefore be improved through this incorporation of ecological and evolutionary relevance through a combination of ecosystem- and evolutionary-based approaches to oil sands toxicology.

2.3 Toxicants Associated with the Oil Sands Region

As previously mentioned, bitumen in the Alberta oil sands region is a source of both anthropogenic and natural toxicants. The area around Fort McMurray and extending north passed Fort McKay is a unique area of the Athabasca deposit known as the McMurray formation, as it is

this area where bituminous oil sands are accessible close to the surface (Timoney & Lee, 2011). Due to its proximity to the surface, bituminous oil sands can be accessed through surficial mining development, while natural deposits can be exposed by fluvial erosion. The aquatic ecosystem of the basin has been subjected to pulses of bituminous toxicants introduced through natural fluvial erosion and deposition events within various areas in the watershed (Elmes *et al.*, 2016). Additionally, the surficial and *in-situ* mining of bituminous sands has increasingly developed in the region since the late 1960s, introducing an anthropogenic component to the pre-existing natural fluvial source of bituminous toxicants into the surrounding aquatic ecosystem. Because the aquatic ecosystem of the Lower Athabasca River basin has been exposed to the two natural and anthropogenic sources of bitumen-related toxicants, the potential for local adaptation acting on the aquatic ecosystem exists. By studying the potential for local adaptation, the responses of aquatic organisms to bituminous toxicants can be better understood to improve subsequent and ongoing reclamation and remediation efforts.

Anthropogenic sources of bitumen arise from the surficial mining and upgrading of this resource by industry. Bitumen is extracted from mined oil sands ore through the Clark hot water process (Fong *et al.*, 2004). A resulting bitumen froth floats to the top of the water and is transported for further upgrading (Fong *et al.*, 2004). The froth treatment portion of the extraction process includes the addition of diluents such as naphtha, which helps in further separating the clay and water from the bitumen (Holowenko *et al.*, 2002). The extracted bitumen undergoes further upgrading into synthetic crude oil and is transported to refineries where additional processing occurs to transform the oil into consumable products. Upgrading of the bitumen produces by-products in the form of petrol coke, naphtha, and other associated toxicants. It is during the mining, separation, and upgrading of the bitumen from the ore that potential aerial

deposition may occur (Huang *et al.*, 2016b). Understanding the possible toxic effects of the oil sands by-products on the aquatic ecosystem and developing ways in which these by-products can be made to be safe for release is, therefore, a fundamental part in assessing aquatic toxicity in the oil sands region.

The dominant toxicants associated with erosion of bituminous sands, surficial mining and their associated processing are categorised into two different components: the organic and inorganic fractions (Wei *et al.*, 2015). The inorganic fraction includes salts, dissolved inorganic ions, and metals (Anderson *et al.*, 2012). The organic fraction is composed of the naphthenic acids fraction (NAs) and PAHs (Pourrezaei *et al.*, 2014; Huang *et al.*, 2016b). These toxicants are associated with different stages of bitumen ore extraction and upgrading, but in recent literature, it was demonstrated that aerial deposition into the ecosystem surrounding oil sands operations has been a significant source of polyaromatic compounds and metals (Kelly *et al.*, 2009; Kelly *et al.*, 2010; Parajulee & Wania, 2014). The toxicants of interest for the purpose of this thesis project will be PAHs and metals associated with natural fluvial events and aerial deposition from industrial activities. Other toxicants such as naphthenic acids are detected at low levels (1 mg/L) in riverine systems, but were not currently considered because high concentrations of naphthenic acids are mainly associated with oil sands effluent and are restricted to tailings ponds where aerial transport has not been established to exist (Headley & McMartin, 2004).

2.3.1 Organic Fraction

The organic fraction of oil sands by-products includes hydrophobic polycyclic aromatic hydrocarbons and hydrophilic, water-soluble naphthenic acids fraction (NAs). Both groups of toxicants are petrogenic in origin and occur at measurable concentrations in the aquatic ecosystem (Headley & McMartin, 2004; Jautzy *et al.*, 2015; Mohseni *et al.*, 2015). Of concern is the elevated

toxicity of oil sands process-affected water (OSPW) owing to the concentration of NAs resulting from extraction of bitumen from the ore. Polycyclic aromatic hydrocarbons are also known to be highly toxic and are associated more with the bitumen and the crude oil (Frank *et al.*, 2008; Timoney & Lee, 2011). Many toxicants sampled from aerial deposition included metals and PAHs, whereas NAs and other components of the inorganic fraction are associated more with the toxicity of OSPW (Headley & McMartin, 2004; Mohseni *et al.*, 2015; Huang *et al.*, 2016b).

Polycyclic aromatic compounds are pervasive compounds found in the environment, commonly associated with oil extraction processes in addition to combustion of organic matter or biologic release (Wang *et al.*, 2014; Schuster *et al.*, 2015). Polycyclic aromatic compounds associated with toxicity in the oil sands include alkylated forms of both polycyclic aromatic hydrocarbons and dibenzothiophenes (Schuster *et al.*, 2015). The PAHs associated with bitumen mainly have low molecular weights (whereas PAHs with high molecular weights are produced from pyrogenic or biogenic sources), few aryl rings (with two to three aromatic rings versus four to six for PAHs with high number of rings), and are less hydrophobic and less readily available for biotransformation by aquatic microorganisms (Yunker & Macdonald, 1995; Stout *et al.*, 2015; Ohiozebau *et al.*, 2016).

Understanding the origin of PAHs is important to discern whether they were produced through oil sands operations or through natural wildfires and to understand the bioavailability and toxicity of the PAHs (Hylland, 2006; Kurek *et al.*, 2013; Schuster *et al.*, 2015; Zhang *et al.*, 2015b). Polycyclic aromatic hydrocarbons can be further characterised by their origin: (I) biogenic PAHs are produced by organisms; (II) diagenic PAHs are transformed by sediment and soil processes; (III) petrogenic PAHs are associated with fossil fuels; and (IV) pyrogenic PAHs are products of combustion (Hylland, 2006). Only petrogenic and pyrogenic PAHs are considered for this thesis,

as these two sources contribute much of the PAHs found in Alberta's oil sands region (Hylland, 2006; Pampanin & Sydnes, 2013). Petrogenic PAHs are dominated by the alkylated form of the parent compounds, whereas pyrogenic sources of PAHs primarily consist of the parent compounds (Sauer & Uhler, 1994; Yunker & Macdonald, 1995; Headley *et al.*, 2001; Headley *et al.*, 2002; Wayland *et al.*, 2008). These alkylated PAHs may act as possible markers of petrogenic origin, and knowing the corresponding toxicity and carcinogenicity can add to the ecological risk assessment of the bitumen-based activities associated with the oil sands region (Schuster *et al.*, 2015; Zhang *et al.*, 2015b).

The toxicity of PAHs is dependent on their bioavailability and in turn, is governed by potential degradation that occurs in the aquatic environment. Depending on the category of PAHs, different chemistry and interactions with biotic and abiotic factors will produce differences in toxicity and bioavailability. Petrogenic PAHs are known to exhibit higher bioavailability within aquatic environments as they are not associated or incorporated with particles, exhibit complex interactions with organic and inorganic aqueous colloids, and therefore exhibit greater accumulation in aquatic organisms (Hylland, 2006). The bioavailability of PAHs may also be altered in terms of seasonality, an example of which would be dilution due to spring freshets in the river basin (Kelly *et al.*, 2010; Jautzy *et al.*, 2015; Elmes *et al.*, 2016). The toxicity of the PAHs has also been observed to both increase and decrease through photolytic degradation when certain organisms are exposed to these degraded forms (Hylland, 2006; Giesy *et al.*, 2010; EL-Saeid *et al.*, 2015; Schuster *et al.*, 2015). The predominance of petrogenic PAHs in the oil sands will, therefore, dictate the bioavailability and toxicity of this toxicant.

2.3.2 Inorganic Fraction

The inorganic fraction associated with oil sands processing and refinement include dissolved inorganic ions and metals, and are primarily associated with OSPW (Allen, 2008; Foote, 2012). Peer-reviewed literature examining the effects of dissolved inorganic ions such as Na^+, Cl^-, $SO4^{2-}$, HCO_3^- and ion-mixtures with other bituminous toxicants have shown to have negligible or protective effects, the latter resulting from interactions with HCO_3^- (Van den Heuvel *et al.*, 1999b; Peters *et al.*, 2007; Young *et al.*, 2011; Kavanagh *et al.*, 2012). As OSPW has a higher conductivity than surrounding surface waters, negative impacts on aquatic life including disturbed osmoregulation can accrue (Kavanagh *et al.*, 2011; Anderson *et al.*, 2012). In addition to dissolved inorganic ions, metal contaminants have also been a point of interest for many mining industries such as the oil sands development.

The impacts of metal bioaccumulation and subsequent toxicity of metals on fish have been an ongoing topic of interest as natural resource industries expand (Kamunde and MacPhail 2011; Lapointe et al. 2011). Metals have been primarily associated with OSPW or by-products of upgrading such as coke, but recently a significant source of metals has been associated with aerial deposition from mining and upgrading processes (Kelly *et al.*, 2009; Kelly *et al.*, 2010; Wang *et al.*, 2014; Huang *et al.*, 2016b). Metals such as Cd, Cu, Pb, Hg, Ni, Ag, and Zn were detected in air samples and deposited in the surrounding aquatic ecosystem and were observed to surpass Water Quality Guidelines for the Protection of Aquatic Life established by the Canadian Council of Ministers of the Environment (Conly *et al.*, 2007; Kelly *et al.*, 2009; Kirk *et al.*, 2014; Dayyani *et al.*, 2016; Huang *et al.*, 2016a). Factoring out the variations due to wind directions, sites where bitumen upgrading occurred had elevated levels of Mo, Hg, and V (Kirk *et al.*, 2014; Lynam *et*

al., 2015; Gueguen *et al.*, 2016). The toxicity and effects of exposure to these metals are governed by abiotic factors that will also dictate how biologically available these toxicants are.

Bioavailability of metals and subsequent toxicity are governed by aquatic environmental factors that include temperature, pH, water hardness, and dissolved organic matter (Meyer *et al.*, 1999; Wood & Shelley, 1999; Gueguen *et al.*, 2012). When considering the toxicity of metals in the oil sands, it is important to take these abiotic factors into consideration as different toxicological outcomes may arise from such mixtures effects.

2.4 Fish Aquatic Toxicology in the Oil Sands Region

The impact of oil sands development on the aquatic ecosystem has been extensively examined through the analysis of various aspects of fish health. Techniques used to monitor the effects of the oil sands industry development on aquatic health pertaining to fish include the monitoring of physiology, reproductive success, histology, metabolic rates, performance, toxicity, and survivability (Van den Heuvel *et al.*, 1999a; Farrell *et al.*, 2004; Kavanagh *et al.*, 2009; McNeill *et al.*, 2012; Arens *et al.*, 2015; Leclair *et al.*, 2015; Marentette *et al.*, 2015a; Wang *et al.*, 2015a). Experiments incorporate single or multiple toxicants, where most experiments were conducted within laboratory settings.

A variety of observations, methods and endpoints have been used to determine fish health when exposed to oil sands related toxicants. Exposure to NAs within the laboratory have demonstrated negative effects on fish and may be a result of the membrane-disrupting surfactant effect of the various naphthenic acids molecules on live cells (Nero *et al.*, 2006a; Nero *et al.*, 2006b; Peters *et al.*, 2007; Frank *et al.*, 2008; Marentette *et al.*, 2015a). Membrane disruption, or narcosis, occurs as NAs behave as surfactants having hydrophobic alkyl groups and hydrophilic

moieties, which may interfere with the composition, function, and behaviour of the cell membrane lipid bilayer (Frank *et al.*, 2008). Exposure to PAHs and metals have elicited negative effects on vertebrates, including teleost fish (Stegeman & Lech, 1991; Allen, 2008; Cruz-Martinez & Smits, 2012; Ohiozebau *et al.*, 2016). Endpoints measured in previous literature include disruptions in early life stage development, reproduction, decreases in growth and increased malformations (Colavecchia *et al.*, 2004; Colavecchia *et al.*, 2007; Arens *et al.*, 2015; Marentette *et al.*, 2015b).

2.4.1 Growth, Development and Morphological Changes

Growth, development, and morphological changes have been well documented in fish exposed to a variety of oil sands associated toxicants. Overall growth is negatively affected by exposure to bituminous toxicants (Farwell *et al.*, 2006; Arens *et al.*, 2015). Growth in white suckers (*Catostomus commersonii*) decreased, along with a decrease in gonadal size after exposure to aged OSPW (Arens *et al.*, 2015). In the same study, white suckers showed an increase in growth when exposed to aged oil sands materials that was incorporated into an experimental water body (South Bison Pond) constructed in 1987 using overburden and unextracted oil sands material (Arens *et al.*, 2015). A point of interest is illustrated in the positive relationship between the growth of Japanese medaka (*Oryzias latipes*) and photo-remediation of PAC mixture extracted from oil sands, demonstrating the importance of photo-degradation on PAC toxicity (Farwell *et al.*, 2006). Growth in fathead minnows *(Pimephales promelas*), however, displayed an increase in growth after having been exposed to OSPW for 56 days (Siwik *et al.*, 2000). When fish larvae were reared and exposed to OSPW within laboratory settings, growth increased, but after transferring into the final 21-day in-field mesocosm exposure portion of the experiment, growth in the larvae decreased or was comparable to control (Siwik *et al.*, 2000).

Early life history development has been applied extensively in oil sands toxicology (Colavecchia *et al.*, 2004; Colavecchia *et al.*, 2007; He *et al.*, 2012; Marentette *et al.*, 2015b; Alharbi *et al.*, 2016). Colavecchia *et al.* (2004) compared the toxicities of natural and OSPW affected sediments on the early life stages of fathead minnows through the quantification of mortality, hatching rates, malformations, and growth at different sediment exposure concentrations. Fathead minnow larval growth were reduced following exposure to naphthenic acids fractions after sixteen-days post-hatch (Marentette *et al.*, 2015b). Furthermore, EC50s for total survival and deformities were lower in the early life stages of walleye (*Sander vitreus*) than in fathead minnows (Marentette *et al.*, 2015b).

Studying morphological changes during fish development has been useful in determining the toxicity of exposure to oil sands related toxicants. Following toxicity experiments with PAHs along with co-exposure with UV light, Japanese medaka exhibited increased cranial-skeletal deformities, edema, and heart deformities (Farwell *et al.*, 2006). Increases in ocular defects, craniofacial abnormalities, necrotic tissues, and reduced pigmentation were observed in early life stages of fathead minnows and yellow perch (*Perca flavescens*; Colavecchia *et al.*, 2006, 2007; Peters *et al.*, 2007). Spinal and cardiovascular deformities exhibited a dose-response to increase concentrations of NAs (Peters *et al.*, 2007; Marentette *et al.*, 2015a; Marentette *et al.*, 2015b). With increased exposure to oil sands related compounds, developmental defects and abnormalities rises as well.

2.4.2 Histopathology

Histopathology is a beneficial tool to examine toxicant exposure on fish through the identification of tissue changes in different organs resulting from carcinogenic or toxic effects (Stentiford *et al.*, 2003). Histological samples from yellow perch and goldfish (*Carassius auratus*)

gill and liver were examined after exposure to reclamation ponds in the oil sands area (Nero *et al.*, 2006b). Significant morphological changes observed in fish exposed to oil sands processing material included increased epithelial cell necrosis and increased mucous cell production within gill samples, in addition to the presence of simultaneous cell necrosis, hepatocyte hypertrophy, and nuclear changes within liver samples from both yellow perch and goldfish (Nero *et al.*, 2006b). The number of signs of damage increased in gill tissues of fathead minnows exposed to OSPW as well (Van den Heuvel *et al.*, 2000; Farrell *et al.*, 2004; Nero *et al.*, 2006a).

Histopathological analysis of fish blood has been implemented as an analysis of immunochemical stress observed by the organism. Hematocrit percentage along with red blood cell counts was elevated in fathead minnow and brook sticklebacks (*Culaea inconstans*) following exposure to oil sands-affected materials (OSPMs), which signify increased stress that may eventually influence mortality (Bendell-Young *et al.*, 2000; Farrell *et al.*, 2004). On the other hand, white blood cell and cells associated with immune stress response such as thrombocytes, lymphocytes, leucocytes, and neutrophils were depressed upon exposure to oil sands related toxicants (Farrell *et al.*, 2004; McNeill *et al.*, 2012; Leclair *et al.*, 2013; MacDonald *et al.*, 2013). With increased exposure to oil sands related products, red blood cell percentage and subsequent hematocrit increased, while lymphocytes were decreased owing to the increased stress of exposure (Farrell *et al.*, 2004).

2.4.3 Reproduction

Reproductive success of fish has been shown to be affected by exposure to oil sands process-affected water, with decreases in sex-related hormones, gonad size and secondary sexual characteristics (Lister *et al.*, 2008; Kavanagh *et al.*, 2011; Kavanagh *et al.*, 2012; Van den Heuvel *et al.*, 2012). Sex-related steroid hormones – namely testosterone and 11-ketotestosterone for

males, and 17β-estradiol in females – decreased in fathead minnows, and goldfish that were exposed to aged OSPW in the laboratory (Lister *et al.*, 2008; Kavanagh *et al.*, 2011; Kavanagh *et al.*, 2012). Decreases in plasma sex steroid hormones required for reproduction following exposure to OSPW was found across species including fathead minnows, yellow perch, and white suckers (Van den Heuvel *et al.*, 2012; Kavanagh *et al.*, 2013; Arens *et al.*, 2015).

The size of gonads for males and females exhibited species-dependent differences corresponding to exposure to OSPW or NAs (Van den Heuvel *et al.*, 1999b; Kavanagh *et al.*, 2013). Gonadosomatic index, a measure of gonad size in relation to fish size, was smaller in fish exposed to sites containing OSPW (Kavanagh *et al.*, 2013). A decrease in gonad sizes of male yellow perches was observed after exposure to an experimental lake containing OSPW, while female gonads remained the same (Van den Heuvel *et al.*, 2012). White suckers exposed to experimental lakes containing OSPW displayed a reduction in both female and male gonad sizes by 15% and 30%, respectively (Arens *et al.*, 2015).

Corresponding with the decrease in levels of sex steroid hormones, incidences of secondary sexual characteristics in male fathead minnows exposed to bituminous toxicants have also decreased (Kavanagh *et al.*, 2012; Kavanagh *et al.*, 2013). Kavanagh *et al.* (2012) reported that when fathead minnows were exposed to 5 mg/L and 10 mg/L of NAs extracts – concentrations found in OSPW – for 21 days, male fish exhibited reduced numbers of nuptial tubercles, a secondary sexual characteristic of male fathead minnows. Male secondary sexual characteristics in fathead minnows that include nuptial tubercles and coloration decreased in frequency and intensity after exposure to OSPW (Kavanagh *et al.*, 2012; Kavanagh *et al.*, 2013).

Within the same experiment, fecundity as measured by the number of spawning events and spawning rates were also observed to decrease with increased exposure to NAs extracts (Kavanagh

et al., 2011; Kavanagh *et al.*, 2012). Fecundity decreased to close to zero eggs per female per day in yellow perch exposed to NAs extracted from OSPW, following a 21 day exposure period (Kavanagh *et al.*, 2012). Both the number of eggs produced and the number of spawning events decreased in female fathead minnows exposed to OSPW (Kavanagh *et al.*, 2011). Fecundity of yellow perch increased or remained unchanged after exposure to wetlands experimentally constructed with OSPM (Van den Heuvel *et al.*, 1999b). With increasing concentrations of toxicants or time of exposure to oil sands related toxicants, the success of reproduction decreases in terms of decreased primary and secondary characteristics, hormone levels, and fecundity rates.

2.4.4 Olfactory Acuity

Olfactory acuity or chemosensory capabilities are an important endpoint to consider when analysing the environmental effects of oil sands industrial development and subsequent remediation and reclamation. As olfaction and chemical detection are necessary for aquatic organisms to detect conspecifics, prey, and predators, using such an endpoint in toxicological studies would be a beneficial way to assess toxicity on the level of individual response. Despite this fact, analysis of olfactory acuity within oil sands toxicology is a limited field of research (Blunt, 2014; Sun *et al.*, 2014; Lari *et al.*, 2016b).

Behavioural studies examining toxicological effects of bituminous toxicants have mostly been conducted on invertebrates (Anderson *et al.*, 2012; Lari *et al.*, 2016b). When given a choice of food or control water within a behaviour choice maze, *Daphnia magna* exhibited a significant impairment in food detection after having been exposed to OSPW concentrations of greater than or equal to 5% (Lari *et al.*, 2016b). Olfactory acuity analysis of oil sands toxicity on fish using behavioural bioassays is not readily available within current oil sands peer reviewed literature.

However, another method of quantifying olfactory acuity allows for the measurement of cue detection at the site of sensory reception and has been applied at a limited scale in fish toxicology.

Electro-olfactography (EOG) research in quantifying olfactory acuity in response to oil sands toxicology has been used on a limited scale (Blunt, 2014; Sun *et al.*, 2014). The EOG technique allows for the quantification of chemosensation at the site of cue detection via the sensory neurons of the olfactory epithelium and may be used to complement measurements of higher-level behavioural responses (Hara, 1975; Hamdani & Døving, 2007; Dew *et al.*, 2014). The EOG response of juvenile rainbow trout (*Oncorhynchus mykiss*) displayed strong responses to NAs, and both untreated and ozonated OSPWs, signifying OSPWs as mediators of strong chemosensory responses at the olfactory epithelium (Sun *et al.*, 2014). Rainbow trout exposed to 10% and 1% concentrations of OSPW displayed an impaired response to L-serine (L-ser; an amino acid representing a food cue) after 2 minutes of exposure, followed by recovery after 30 minutes (Sun *et al.*, 2014). Electro-olfactography responses to taurocholic acid (TCA; a bile salt representing a social cue) exhibited signs of impairment at an OSPW concentration of 10%, following a four minute exposure time after which recovery in clean water for 28 minutes improved olfactory acuity (Sun *et al.*, 2014). This impairment in EOG response illustrates that olfactory acuity as measured at the site of chemosensory detection can be impaired by OSPW. The negative effect of toxicants such as metals has been well documented both in the field and in the laboratory tests and this knowledge of fish chemosensation further supports the use of olfaction in oil sands toxicological studies (Azizishirazi *et al.*, 2014; Dew *et al.*, 2014; Azizishirazi & Pyle, 2015; Sakamoto *et al.*, 2016). The mechanism of olfactory impairment, the effects of specific bituminous toxicants on chemosensation, and subsequent ecological outcomes of such impairments are research questions that warrant further investigation.

2.4.5 Swim Physiology

The ability of a fish or any aquatic organism to swim within the environment they inhabit is paramount to their survival, as this ability will dictate their success in avoiding predators, locating food, cover, mate, and maintaining position within a dynamic aquatic habitat. The ability to swim involves the appropriate swimming physiology, which can be broken down and quantified in numerous endpoints such as swimming performance, respiration rate, hematocrit levels, and position or orientation within the water column (Brett, 1964; Houston, 1997; Farrell, 2008). For these reasons, swim physiology is an important endpoint to consider when looking at the effects of bituminous toxicants on fish.

The physiology of swimming in fish has been studied primarily using larval fish, with many negative effects observed following exposure to bituminous toxicants. The effects of exposure to bituminous toxicants on early life stage swimming behaviour of white suckers and fathead minnows demonstrated circular swimming, erratic twitching, lethargy, while maintaining a benthic position within the water column (Colavecchia *et al.*, 2004; Colavecchia *et al.*, 2006). Farrell *et al.* (2004) reported that the critical swimming speed of fathead minnows was lower compared to reference fish after 96 hours of exposure to dyke seepage, a runoff associated with a dyke that retains consolidated tailings water. Hematocrit percentages were also elevated in the group of fish exposed to dyke seepage after the 96 hour exposure period, potentially indicating stress (Farrell *et al.*, 2004). Larval zebrafish (*Danio rerio*) exhibited negligible swimming speeds or distances travelled after exposure to water accommodated fractions (WAF) of diluted bitumen and crude oil, while shelter-seeking behaviours diminished with exposure to 100% WAFs (Philibert *et al.*, 2016). Swimming behaviour indefinitely becomes altered upon exposure to bitumen related toxicants.

Oxygen consumption as a measure of metabolic rate has been used to understand physiological response of fish facing various stressors, but there is a less apparent trend in respiration upon exposure to petroleum-related toxicants (Hopkins *et al.*, 2000; Rajotte & Couture, 2002; Couture & Kumar, 2003; Staub *et al.*, 2004; Fu *et al.*, 2011; Gerger & Weber, 2015; Klinger *et al.*, 2015). Oxygen consumption and corresponding respiration rate as measured by the concentration of oxygen consumed per unit time per unit mass has been an effective measure of metabolic rate, energy expenditure, and physiological stress in various fish species (Beamish & Mookherjii, 1964; Fidhiany & Winckler, 1998; Tatara *et al.*, 2001; Grotan *et al.*, 2012). Waterborne and dietary exposure may explain the discrepancies in respiration rates between different studies. Exposure of chub mackerel (*Scomber japonicas*) to weathered crude oil through aqueous exposures increased the average rate of oxygen consumption after an exposure period of 96 hours (Klinger *et al.*, 2015). A similar increase in oxygen consumption was observed in zebrafish treated with intraperitoneal injections of a standard PAH, benzo-a-pyrene (Gerger & Weber, 2015). However, oxygen consumption and metabolic rates remained unchanged, or decreased upon administration of food contaminated with crude oil related toxicants (Lucas *et al.*, 2016). Generally speaking, impairment of swimming physiology has also been corroborated in other peer-reviewed research examining the toxicity effects of exposure to crude oil or related toxicants (Carls *et al.*, 1999; Mager *et al.*, 2014; Incardona *et al.*, 2015). However, it seems that the mechanisms of toxicity with respect to effects on respiration rate and oxygen consumption differs depending on the route of petroleum-related toxicant exposure. Respiration rate can therefore be an effective tool to assess the effects of bituminous toxicants on fish physiology to determine potential local adaptation in the fish populations of the oil sands region.

2.4.6 Survival

Observations of survival are fundamental in environmental toxicology and have played an important part in shaping the understanding of effects of oil sands development on fish and the aquatic ecosystem. Survival represents the endpoint that summarizes toxicant exposure as it indicates the toxicity of the environmental contaminant and the ability of an individual or population to deal with various environmental stressors (Belarde & Railsback, 2016). Survival is a fundamental driver in the proliferation of a population and plays a key role in the evolution of individuals and populations (Meyer & Di Giuliuo, 2003; Farrell *et al.*, 2008; Sopinka *et al.*, 2014). For these reasons, observation of survival of the fathead minnow populations was used to understand the outcomes of the reciprocal cross-transplant experiment in the present study, and to bring together the physiological endpoints mentioned previously.

To measure toxicity effects of fish exposed to xenobiotic substances in relation to the bituminous oil sands, quantifying survival may be conducted through direct measurements of mortality rates, survival or acute toxicity tests. Acute toxicity of various toxicants to fish has been measured by examining lethal dosages or lethal concentrations such as a 96-hour LC50 toxicant exposures to evaluate toxicity of specific effluents (Gravenmier *et al.*, 2005). Owing to the high concentrations of toxicants and xenobiotics present in OSPW and bitumen, monitoring survival is a useful tool to enumerate toxicological effects on fish.

Monitoring survival is an effective and efficient means to qualify physiological response of fish exposed to bituminous toxicants, where poor survival reflects physiological or metabolic dysfunction. When comparing LC50 values between fathead minnows and white suckers that were exposed to both natural bituminous sediments and OSPW affected-sediments from experimental wetlands, ponds, and lakes where tailings materials have been incorporated, both species

demonstrated lower LC50 values for OSPW sediments, requiring less of the sediment to induce 50% mortality (Colavecchia *et al.*, 2007). Not only did mortality rate of fathead minnows correlated with increased exposure to OSPW – that is, survivability was zero after a 28 day exposure to consolidated tailings – there was significant hematological and gill morphological alterations to the fish after an exposure time of 96 hours (Farrell *et al.*, 2004). Survivability under field conditions has also been monitored in a variety of fish species, to determine the effects of oil sands process-affected water on the given species (Bendell-Young *et al.*, 2000; Cruz-Martinez & Smits, 2012). Exposure of rainbow trout (*Oncorhynchus mykiss*) to naphthenic acids that were either synthetically produced or extracted from OSPW resulted in increased genotoxicity of fish hepatocytes (Lacaze *et al.*, 2014). The same study also suggested that NAs that were higher in molecular weight, and more cyclic were the most genotoxic and that these same highly cyclic naphthenic acids are more resistant to biodegradation (Lacaze *et al.*, 2014). With increasing concentrations of oil sands related toxicants, survival rates have been demonstrated to decrease. Mortality rates will change depending on the type of toxicant, and as such, understanding the mechanism of exposure can help understand the mechanism of toxicity observed in the other physiological endpoints.

2.4.7 Mixtures Effects

As remediation and reclamation is imminent in the future of the oil sands industry, a point of interest is the existence of mixture effects demonstrated among toxicants related to bitumen processing and upgrading (Kavanagh *et al.*, 2012; Gauthier *et al.*, 2014; Gauthier *et al.*, 2016). Studies of mixture effects involving bituminous toxicants demonstrated the protective role of sodium bicarbonate on the effects of NAs on fathead minnow reproductive development and bioaccumulation (Kavanagh *et al.*, 2012). When *Hyalella azteca* were exposed to metal-PAH

mixtures at concentrations present in water quality guidelines, they elicited a more-than-additive effect on mortality, suggesting the complex role of toxicant mixtures on aquatic organisms (Gauthier *et al.*, 2015). The possibility of such mixture effects is important to consider when examining aquatic ecotoxicology in Alberta's oil sands region given the increasing evidence of aerial deposition of PAHs and metals (Kelly *et al.*, 2009; Schuster *et al.*, 2015; Zhang *et al.*, 2015a; Zhang *et al.*, 2015b; Huang *et al.*, 2016b). Mixtures effects plays an important role in informing remediation, reclamation, environmental assessment, and the understanding of the ecological effect of bitumen on the surrounding biological systems.

2.5 Local Adaptation

Adaptation to environmental factors can occur in face of exposure to certain changes in abiotic factors that may induce physiological stress responses or which may otherwise be deemed toxic to a population of organisms. Natural selection in response to environmental factors specific to a habitat occurs when an organism becomes adapted to these parameters through improved survival and reproductive success, and consequently improved fitness (Kawecki & Ebert, 2004). When such adaptation occurs and results in improved fitness in comparison to non-native organisms, local adaptation to the particular environmental factor can occur, given particular factors (Hereford, 2009). For the remainder of this thesis, fitness will be defined as the relative rate of success in reproduction and survival of individuals and its populations through phenotypic or genotypic response to environment factors (Taylor, 1991; Griffiths *et al.*, 2000). Considering the potential for local adaptation, one can apply this theory in the context of oil sands toxicology to understand the effects of bituminous oil sands on aquatic organisms and their ecosystem.

A population of organisms is defined as a group of individuals that are genetically segregated from another group, and are genetically restricted from each other (Kawecki & Ebert, 2004;

Mehner *et al.*, 2011). These populations will still have the potential to mate as speciation has not yet occurred, but it is this reproductive potential that becomes the limiting factor for local adaptation to arise (Kawecki & Ebert, 2004). These populations are genetically isolated from other potential breeding populations by physical or environmental barriers (i.e. heterogeneous habitats) and can no longer mate randomly, leading to adaptive divergence resulting from variation in advantageous genotypes to the habitat type (Mehner *et al.*, 2011; Van Gestel & Weissing, 2016).

Local adaptation occurs only when certain factors within a population is in place, and a population has genotypes that give rise to an advantage within their native site in comparison to other genotypes of other non-native populations (Hereford, 2009; Blanquart *et al.*, 2013). Key factors whereby local adaptation is enhanced and fostered include the inhibition of gene flow among various potential mating populations to maintain genetic polymorphism; variation in genotypic response to certain environmental factors found among different populations which can limit genetic drift; limited selection pressure against genotypic responses to variable environmental factors; constraints on adaptive phenotypic plasticity in order to allow for local adaptation to occur; and a limited difference in resource types between habitat types (Kawecki & Ebert, 2004; Blanquart *et al.*, 2013).

2.5.1 Determining Local Adaptation

The theory of local adaptation has provided a practical approach to understanding adaptation on a scale that is assessable within a small amount of time (Stockwell *et al.*, 2003; Kawecki & Ebert, 2004). Local adaptation differs from the traditional sense of quantifying natural selection through paleontological records of evolution in phenotypes and underlying genotypes that have improved fitness over millennia (Kawecki & Ebert, 2004). Local adaptation may be measured by comparing differences in responses between populations from heterogeneous habitats (Kawecki

& Ebert, 2004). Applying local adaptation theories with respect to aquatic toxicology can, therefore, assist in assessing risks of certain toxicant exposures within a given environment by demonstrating whether particular organisms have adapted to the bituminous toxicant and whether such an adaptation will alter the response of the organism to toxicant exposure.

Comparison of populations can be made in two ways to determine the extent of local adaptation: (i) local or native versus foreign or (ii) home versus away (Kawecki & Ebert, 2004). The home versus away theory emphasizes comparison of the response of a single population within a variety of different habitats; the population will fare the best in its home habitat compared to the other foreign habitats (Kawecki & Ebert, 2004). On the other hand, native versus foreign is the concept in which fitness is assessed and compared between multiple populations within a single habitat (Kawecki & Ebert, 2004). The native population is anticipated to have improved fitness in comparison to a foreign one that was transplanted into the same habitat (Kawecki & Ebert, 2004). In both scenarios, the native or home advantage demonstrates the advantage of local adaptation. However, for the purposes of determining adaptation at the local scale, the native versus foreign theory is argued by Kawecki and Ebert (2004) to be better in illustrating local adaptation as it investigates the underlying divergent evolution needed for local adaptation to occur as comparisons are made within one single habitat type. Contrasted with the home versus away theory, teasing out the mechanism and existence of local adaptation is confounded by heterogeneous environmental factors which overshadows evidence for any divergent evolution (Kawecki & Ebert, 2004). An excellent method in determining local adaptation using the native versus foreign theory is by implementing a reciprocal cross transplant experiment.

Local adaptation through the comparison of fitness between native and foreign has been assessed by implementing reciprocal cross-transplant (RCT) experiments across a range of species

and environmental conditions (Hines *et al.*, 2004; Kawecki & Ebert, 2004; Fraser *et al.*, 2011; Mehner *et al.*, 2011; Westley *et al.*, 2013; Raesaenen & Hendry, 2014; Rolshausen *et al.*, 2015; Ingley & Johnson, 2016). An RCT experiment helps reveal differences among the native and foreign demes in response to the environmental factors within a given habitat, indicating the evolutionary divergence that has occurred (Kawecki & Ebert, 2004). It is expected that the native deme will display the higher fitness within an RCT experiment and hence support the theory of local adaptation (Kawecki & Ebert, 2004).

2.5.2 Phenotypic Plasticity and Local Adaptation

To detect the presence of local adaptation, natural selection that occurs because of this process must be distinguished from adaptive phenotypic plasticity. Phenotypic plasticity is the term used to describe the set of morphological, physiological, and biological capabilities governed by a particular combination of genes that allows for a range in response to fluctuating environmental conditions without having changes to their genetic basis occur (Hendry *et al.*, 2008; Van Gestel & Weissing, 2016). The ability of the individual to respond to certain environmental factors through phenotypic plasticity can result in adaptive, maladaptive, or neutral effects, of which adaptive phenotypic plasticity will be the focus of the current thesis (Hendry, 2016). Adaptive phenotypic plasticity of an individual is conferred as improved fitness governed by phenotypes of a certain genotype given specific habitat characteristics, is often seen to be costlier to the individual and is observed more in habitats having stochastic changes in environmental factors (Crispo, 2008; Hendry, 2016).

Local adaptation occurs as a form of adaptive response and subsequent natural selection to environmental factors of a given habitat (Kawecki & Ebert, 2004; Crispo, 2008; Blanquart *et al.*, 2013). This adaptive divergence in demes manifests as improved fitness of native individuals and

populations when compared to non-native populations (Blanquart *et al.*, 2012; Merila & Hendry, 2014). Unlike phenotypic plasticity, local adaptation to environmental change is a result of change to both genetics and phenotype (Crispo, 2008). Local adaptation is observed in more stable environments where plasticity is not required for survival, therefore favouring local adaptation rather than plasticity alone (DeWitt *et al.*, 1998). Both local adaptation and adaptive phenotypic plasticity are heritable adaptions. Whereas changes to the individual's genetic information does not underpin phenotypic plasticity, an individual's genotype is altered when local adaptation occurs (Crispo, 2008). Given the definitions of both local adaptation and phenotypic plasticity, there are a few methods to determine whether adaptive divergence is a result of local adaptation or adaptive phenotypic plasticity.

Environmental factors and genetic structure of a population will give rise to various ranges of adaptive divergence and by understanding how such speciation occurs, one can compare local adaptation and phenotypic plasticity (Sultan & Spencer, 2002). In the case for finding local adaptation within an RCT experiment, applying statistical comparisons is a way to compare response differences amongst different demes and to discriminate between local adaptation and phenotypic plasticity (Berggren *et al.*, 2016).

Reciprocal cross-transplant experiments have been used to directly infer local adaptation in various species of organisms (Ebeling *et al.*, 2011; Derry *et al.*, 2013; Berggren *et al.*, 2016) under the theory that fitness of populations in a given habitat to which they are familiar will fare better than populations that are new to the same habitat (Kawecki & Ebert, 2004; Hereford, 2009). Comparison of reaction norms, which are the range of phenotypic expression of a given genotype, was used to interpret genotypic and environmental interactions and to demonstrate the involvement of genetic components in potential adaptive responses (Berggren *et al.*, 2016). Interaction effects

between genotype and environment on both hatching success and survival past yolk sac stage were analyzed using pairwise within-family comparisons to evaluate local adaptation in subpopulations of pike (Berggren *et al*., 2016). The genetic influence on reaction norms required for local adaptation to occur was demonstrated as a crossing of reaction norms of demes subjected to native and non-native habitat types (Berggren 2016). Larval pike demonstrated local adaptation to their native habitats through improved hatching success and survival when compared to non-native populations (Berggren *et al.*, 2016). Amphipods (*Gammarus fasciatus*) from habitats with various ion concentrations demonstrated phenotypic plasticity in calcium concentration post-moult and adaptive differences in larval survival, female reproduction, size at maturity, and clutch size, and (Derry 2013). Genetic or maternal effects was observed in survival, fecundity, and growth rate where F1 amphipods with parents from ion-poor habitats had higher success in the respective endpoints regardless of the habitat they were grown and moulted in (Derry 2013). The lack of local adaptation was demonstrated by no interaction between the fitness parameter with the habitat of origin and the habitat for growth. Phenotypic plasticity in calcification was observed as a function of ion concentration in the treatment water, regardless of where *G. fasciatus* was accustomed to (Derry 2013). Lastly, in an RCT experiment using the butterfly bush plant (*Buddleja davidii*), similar comparison of reaction norms such as stem length, biomass and reproduction were analyzed using general mixed effects models to understand potential local adaptation and plasticity in response to three different central European regions or gardens (Ebeling *et al.*, 2011). Lack of local adaptation was demonstrated as limited differences between the three different bush plant populations after examining interaction between population and eco-regions (Ebeling *et al.*, 2011). These examples of a range of local adaptation in various species provide support for the use of

RCT-type experiments to determine local adaptations of fish populations within the Athabasca oil sands region.

2.5.3 Applying Local Adaptation to Examine Oil Sands Toxicology

By adopting an evolutionary approach to studying toxicity of bitumen in the Alberta oil sands region, the present study can clarify the influence of natural and anthropogenic sources of bitumen-related toxicants on populations of fish found in the watershed. A tremendous amount of science has been used to examine the direct impacts of oil sands development on different aspects of the aquatic ecosystem. More specifically, the effects of bituminous toxicants in relation to industrial development and natural toxicant sources on fish physiological, morphological, and biochemical changes has been examined extensively. Local adaptation is a field of research that has been used to examine the effects of various toxicants on the adaptive responses of a wide range of species. However, there has been little exploration into local adaptation of aquatic organisms to the toxicants present in the lower Athabasca River Basin.

Local adaptation can be applied to environmental impacts assessment of anthropogenic activities to understand their effects on ecosystem dynamics (Hendry, 2016). It is, therefore, the goal of this current project to elucidate potential local adaptation in fish populations and further consider the role of adaptation in the response of organisms to certain natural and anthropogenic toxicant regimes demonstrated in northern Alberta's oil sands region. In short, this project can be used to incorporate theories regarding eco-evolutionary dynamics with that of ecotoxicology to bring about more effective ecosystem risk management.

2.6 Summary

Previous studies have demonstrated the negative impacts of bitumen related toxicants on growth, development to reproduction and survival, some of what remains to be answered are questions regarding local adaptation to the toxicants present in the lower Athabasca River Basin regarding physiological changes. Drawing upon previous research within oil sands ecotoxicology related to fish physiology and development, various endpoints and evolutionary concepts of local adaptation to bituminous oil sands will be incorporated in the present study.

3 Scope of Research and Objectives

The aim of this project is to determine whether fish populations inhabiting the oil sands region display local adaptation to the fluvial erosion of bituminous toxicants. This experiment will attempt to test the hypothesis that there will be statistically and biologically significant differences in the tolerance of fish to bitumen toxicants found within tributaries with a range of toxicant availability. Tributaries ranging from naturally occurring bitumen, within natural bitumen and development, and exterior of any surficial sources of bitumen have been considered for the current project. To test the hypothesis, the objectives of this project are (i) to determine whether fish populations pre-exposed to natural bitumen will exhibit a higher tolerance to bitumen than fish that are naïve to bitumen and (ii) to determine if the possible tolerance is a result of local adaptation. Local adaptation will be measured by conducting a transplant experiment using fish populations from tributaries away and downstream of the oil sands area, and from a natural bitumen-bearing stream. Local adaptation will be quantified through the measurement and comparison of physiological responses among fish populations before and after transplantation.

Fathead minnows will be the model species used for this field experiment as they are both ecologically and toxicologically relevant. Not only are fathead minnows an important indicator species used in the field of environmental toxicology, they are also an indigenous fish found within the Lower Athabasca River basin (Scott & Crossman, 1973; Bendell-Young *et al.*, 2000; Ankley & Villeneuve, 2006). Understanding local adaptation would be most effective as fathead minnows are short-lived (1 to 2+ years) and possible adaptive changes can be easily determined as generation time is short (Held & Peterka, 1974; Duffy, 1998). Fathead minnows are fish found in various habitats that range from muddy pools, ponds and lakes to streams and slow-moving rivers (Joynt & Sullivan, 2003). Male fish are highly territorial during the spawning season, and the

individual home-range is generally small (Joynt & Sullivan, 2003). Fathead minnows are found at their northernmost range in the Lower Athabasca River basin (Joynt & Sullivan, 2003). Fathead minnows are generalist in terms of diet which includes algae, zooplankton and benthic organisms. Fathead minnows occupy the prey guild, providing food for predators like the northern pike (*Esox lucius*) and walleye (*Sander vitreus*) within streams and lakes in the Lower Athabasca River basin (Scott & Crossman, 1973; Duffy, 1998; Joynt & Sullivan, 2003). Because it is fundamental for aquatic organisms to have an adequate sense of olfaction and swim capabilities for suitable detection of conspecifics, food items, and predators to occur, endpoints of interest include olfactory acuity, swim physiology and survival. The ability of the minnow to detect food, conspecifics, and predators is fundamental to the success of the forage fish population and its ecosystem, and is an ideal model species in a reciprocal cross-transplant experiment in the oil sands region.

4 Materials and Methods

Fathead minnows were caught using minnow traps set at each location. Fish were enumerated and held within sites for no more than 3 days before being transplanted at the start of the experiment. Ecologically relevant endpoints that were quantified in this study pertained to the forage fish's ability to perform adequately within their ecological niche, which included the measurement of olfactory acuity, and swimming physiology. All procedures using fathead minnows included in the present study were approved by the University of Lethbridge Animal Welfare Committee (protocol #1423) following Canadian Council on Animal Care protocols.

4.1 Study Area & Site Descriptions

All sites were situated in second order tributaries connected to the primary mainstem of the Athabasca River, and all were located within the McMurray Formation of the Athabasca oil sands deposit. Three different sites were included in the transplant experiment: (i) reference tributary, which was outside of surficial bitumen-rich oil sands from the McMurray Formation and the oil sands industrial area; (ii) natural bitumen (NB) site located within the McMurray Formation, but outside of the industrial area; and (iii) natural bitumen/oil sands area (NB-OSA) site which is situated within both natural and anthropogenic sources of surficial bitumen from the McMurray formation and oil sands industry (Figure 1).

The geomorphology of the three sites in terms of substrate was similar, while flow regimes were twice as high in the reference site (Table 1). Substrate found in all three of the sites was dominated with sand and gravel (Table 1). Flow rate was approximately ~ 0.1 m/s for the reference site, 0.09 m/s in the natural bitumen, but was immeasurable in the NB-OSA site due to negligible flow (Table 1). Flow rates were calculated by taking the length of a designated portion of the

tributary and dividing it by the average time it took a floating ping-pong ball to travel from reference to NB-OSA. Measurements were replicated five times, and an average flow rate was calculated. Wetted widths were measured from one side of the tributary that was submerged in water.

Table 1

Tributary name, coordinates, dominant substrate type, average flow rate (± SEM), and wetted width (± SEM) for the reference, natural bitumen (NB), and natural bitumen/oil sands area (NB-OSA) sites. Flow rates were measured in replicates of five, using a ping-pong ball released over a designated length of stream. Flow rate in NB-OSA was too low to be measured using the floatation method (*). Wetted width for the reference site was measured in summer 2014 (**). All other measurements were collected in summer 2015.

Site	**Tributary**	**Coordinates**	**Dominant Substrate**	**Flow Rate (m/s)**	**Wetted Width (m)**
Reference	Halfway Creek	56°30'30.87"N, 111°17'59.46"W	Sand, gravel	0.177 ± 0.089	5.38 ± 0.46**
Natural bitumen, NB	Poplar Creek	56°54'39.38"N, 111°30'13.38"W	Sand	0.0893 ± 0.013	7.21 ± 1.05
Natural bitumen/oil sands area, NB-OSA	Beaver River	57° 6'48.54"N, 111°37'40.74"W	Sand, gravel	n/a*	13.9 ± 0.95

Fish were caught within the three sites, transplanted, and kept within 35.6 cm by 15.2 cm cages constructed from collapsible minnow traps that were tied off at both ends. Chronic exposure at these sites was conducted for 28 days, during which fish were kept within the constructed cages. Daily monitoring included measurements of dissolved oxygen and temperature, in addition to fish health checks and mortality data collection.

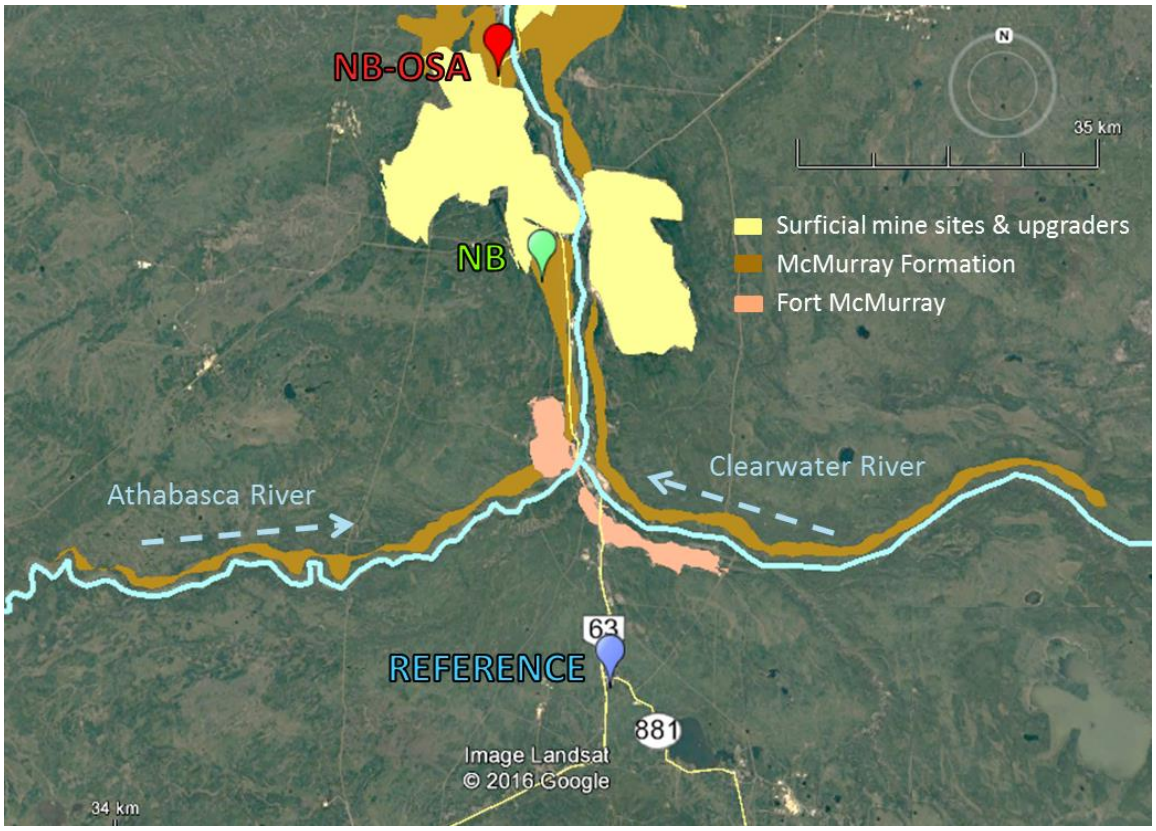

Figure 1

Map of the study area in northeastern Alberta, near the town of Fort McMurray. Shaded areas represent the McMurray geologic formation (brown), surficial mining sites and upgrading facilities (yellow), and the town of Fort McMurray (orange). The three sites in the study include an (i) reference site at Halfway Creek (blue); (ii) natural bitumen (NB) site at Poplar Creek (green); and (iii) a natural bitumen/oil sands area (NB-OSA) site at Beaver River (red). © Google Earth, Landsat Image, 2016.

4.2 Reciprocal Cross Transplant

A reciprocal cross-transplant (RCT) experiment was conducted to determine if local adaptation exist. An RCT experiment consists of transplanting treatment organisms from one site to another and vice versa (Figure 2). A full three-way transplant experiment was planned for the present study, but due to insufficient fish numbers in the reference site, a two-way transplant was conducted using the NB and NB-OSA sites (Figure 2). The number of fathead minnows from the reference site was only enough to allow for transport of reference fish into either of the bituminous

sites; fish were not held at the reference site (dotted blue line in Figure 2). Fish were held at the two sites for 28 days, before and after which endpoints were measured and detailed in the following sections. Fish were monitored and fed daily with TetraFin goldfish flakes.

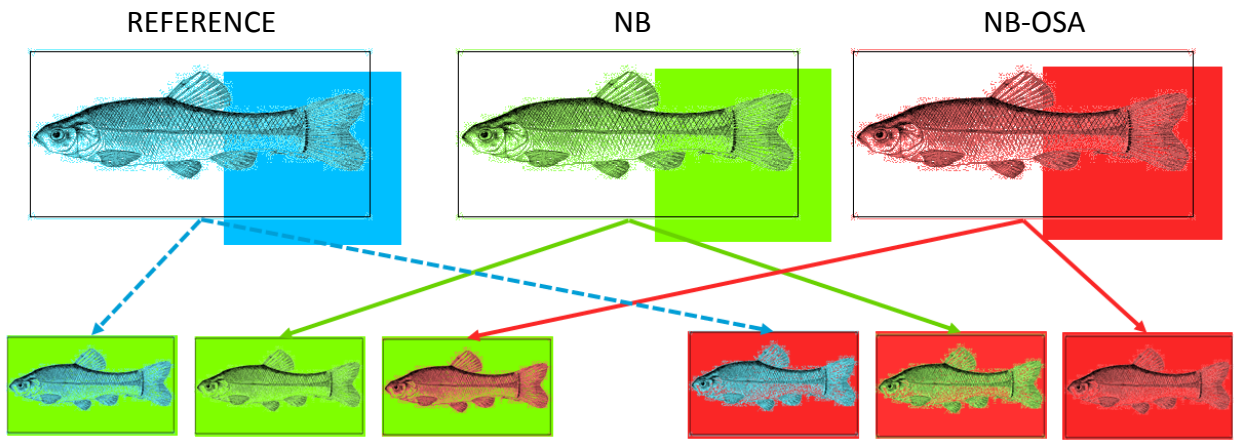

Figure 2
Illustration of a two-way reciprocal cross-transplant between natural bitumen (NB) and natural bitumen/oil sands area (NB-OSA) treatment water site with the corresponding colours: reference site in blue, natural bitumen site in green, and natural bitumen/oil sands area site in red. Colours of the squares represent the treatment water the fish were placed into, and the colour of the fish represents the source of the fish. Natural bitumen fish were kept within their own site and transplanted into the NB-OSA site (solid green arrows), and the same was done for the fish from the NB-OSA site (solid red arrows). Reference fish were placed in both the natural bitumen and natural bitumen/oil sands area sites (dotted blue arrows).

4.3 **Water Quality and Sediment Data**

Daily water quality was measured that included water temperature (^{o}C) and dissolved oxygen (%), and pH, all measured using YSI dissolved oxygen probe (YSI model 85, Ohio, USA) and pH meter (Fisher Scientific, model AP71, Massachusetts, USA). Water samples were collected for trace metal analysis, filtered using a 45 µm filter, placed on ice and preserved with metal grade nitric acid (Fisher Scientific) before analysis at the Lakehead University Nutrient Ecology Laboratory. Water samples were analysed using ICP–MS.

Sediment and water samples were collected in Ziploc bags and dark polyurethane bottles and sent to the Laboratory for the Analysis of Natural and Synthetic Environmental Toxins (LANSET) at the University of Ottawa for analysis of polycyclic aromatic hydrocarbons. Forty-two PAHs were measured which include 17 parent and 25 alkylated PAHs measured from water and sediment samples (n = 3 for each sediment and water samples; Table 2).

Table 2

Parent and alkylated PAHs measured in sediment and water samples from the reference, natural bitumen (NB), and natural bitumen/oil sands area (NB-OSA) tributaries. The reference site is located away from natural bitumen sources and industry development; the natural bitumen site is located within sources of bitumen-rich oil sands; and the natural bitumen/oil sands area site is located downstream from natural bitumen sources and industry development in the Alberta oil sands region.

Parent PAHs	**Alkylated PAHs**
Naphthalene	C1-Naphthalene
Acenaphthylene	C2-Naphthalene
Acenaphthene	C3-Naphthalene
Fluorene	C4-Naphthalene
Phenanthrene	C1-Fluorene
Anthracene	C2-Fluorene
Fluoranthene	C3-Fluorene
Pyrene	C1-Phenanthrene/Anthracene
Benz[a]anthracene	C2-Phenanthrene/Anthracene
Triphenylene + Chrysene	C3-Phenanthrene/Anthracene
Benzo[b]fluoranthene	C4-Phenanthrene/Anthracene
Benzo[k]fluoranthene	C1-Dibenzothiophenes
Benzo[a]pyrene	C2-Dibenzothiophenes
Indeno[1,2,3-cd]pyrene	C3-Dibenzothiophenes
Dibenz[a,h]anthracene	C4-Dibenzothiophenes
Benzo[g,h,i]perylene	C1-Fluoranthene/Pyrene
Dibenzothiophene	C2-Fluoranthene/Pyrene
Retene	C3-Fluoranthene/Pyrene
	C4-Fluoranthene/Pyrene
	C1-Benz[a]anthracene/Chrysene
	C2-Benz[a]anthracene/Chrysene
	C3-Benz[a]anthracene/Chrysene
	C4-Benz[a]anthracene/Chrysene
	C1-Benzofluoranthene/Benzopyrene
	C2-Benzofluoranthene/Benzopyrene

4.4 **Olfactory Acuity**

Olfactory acuity was quantified in terms of electro-olfactography response and behavioural trials. Behavioural trials were conducted to determine avoidance response to fathead minnow conspecific alarm cue, but due to small sample size, details have been included separately in Appendix A.

The measurement of response to olfactory cues at the cellular level was determined using previously described electro-olfactography technique (Green *et al.*, 2010; Azizishirazi *et al.*, 2013; Dew & Pyle, 2014). Fathead minnows were sedated in treatment water mixed with MS222 (200 mg/L) buffered to physiological pH of 7.4 and wrapped with a wet paper towel to prevent desiccation. The minnow was then placed on the EOG rig, while aerated and buffered MS222 (100 mg/L) perfusion water was flushed through gills via a tube during the entire EOG process. The nasal septum of the right naris was excised, exposing the primary olfactory epithelial organ, the olfactory rosette (Laberge & Hara, 2001). Electro-olfactography recording setup follows that of Lari & Pyle (in review) and Green *et al.* (2010).

Olfactory stimuli were prepared using the corresponding treatment water that the individual fish were collected from. Taurocholic acid, FHM conspecific skin cue and L-alanine were used as social and food cues with respective concentrations as 10^{-3} M, 10 cm^2/L, and 10^{-2} M (Table 3).

The stimulus was delivered to the olfactory chamber along with a delivery of a blank of only treatment water. The stimuli and treatment water blank were randomly drawn and then administered to the olfactory chamber for five seconds, with 90 seconds in between each administration. The randomised process was completed for three rounds to account for intra-individual variation and avoid olfactory attenuation to alarm cues. Response to stimuli was

measured as the change in amplitude between the baseline and the peak of the measurement, which was then corrected to the blank, providing the EOG response (mV) to the stimulus.

Table 3
Experimental stimuli used for electro-olfactography (EOG) measurements, including the experimental stimulus' ecological relevance and the concentrations applied in the experiment.

Olfaction Stimuli	Ecological Relevance	Concentration
Taurocholic acid, TCA	social cue	10^{-3} M
Fathead minnow conspecific alarm cue, FHM cue	alarm cue	10 cm^2/L
L-alanine, L-ala	food cue	10^{-2} M

4.5 Hematocrit

The percent red blood cell count was determined by comparing red to white blood cell amount, or hematocrit measurement (Clark *et al.*, 2011). Fish were stunned with a flick to the head and the caudal peduncle was severed as per Animal Welfare Committee approved method of blood sampling and substitution of physical method for euthanasia (protocol #1423). Immediately thereafter, a heparinized capillary tube was used to collect the blood from the severed peduncle and was immediately placed on ice and processed within 2 hours of collection. Microcapillary tubes were spun for three minutes at 10,000 RPM using an International IEC Micro-MB micro-hematocrit spinner (International Equipment Company, Massachusetts, USA; Farrell *et al.*, 2004). The percent red (erythrocytes) was calculated as the ratio of red blood cell to the total blood column. This red blood cell to total blood column ratio was the hematocrit percentage for the given individual (Wells & Pankhurst, 1999).

4.6 Oxygen Consumption

Oxygen consumption by individual fish was measured in a glass respirometer. The respirometer was constructed using a 500-mL glass mason jar, in which a hole was drilled into the

jar lid to allow insertion of a FireStingO_2 fiber-optic oxygen meter (ODO; PyroScience, Aachen, Germany). The ODO meter was fitted with a silicone stopper, creating an airtight seal when inserted into the jar lid hole. Water from respective treatment sites was aerated before placing into the jars. An airtight container housing a motor with an attached magnet acted as a stir plate that spun at a speed of 15 RPM. The Mason jar contained a paper clip to stir the water within and the jar was placed on top of the container. The jar and container were placed within a bucket of treatment water to maintain constant water temperature. Fish were placed into the jar of aerated water and allowed to acclimate for 20 minutes or until calm. The ODO-silicone stopper was placed into the jar and oxygen consumption was measured until fish exhibited signs of stress or until duration approached 20 minutes. Oxygen consumption changes with respect to the mass of the fish, wherein consumption rates decrease with increased mass (Beamish, 1964). As such respiration rates were corrected with a power to 0.7 with the following equation adapted from Beamish and Mookherjii (1964):

$$Mass\ corrected\ respiraiton\ rate = respiration\ rate/mass^{0.7} \qquad \text{Equation (1)}$$

4.7 Statistical Analysis

All statistical analyses were conducted using R (version 3.1.2; R Core Team, 2014) . Normality and homogeneity of variance were calculated using the Shapiro-Wilk's test of normality and the Bartlett's test of homogeneity of variance, respectively. Comparison of pre-treatment data between sources and comparison of before and after treatment of the same source of fish were both conducted using a one-way permutation analysis of variance (1-way permANOVA; Legendre, 2015). Differences in treatment groups and treatment sites were compared using a two-way permutation analysis of variance (2-way permANOVA) to compare the effects and interactions of the treatment site and fish source (Legendre, 2015).

5 Results

5.1 Water Quality

Daily water quality measurements of temperature were similar among the three sites, with temperature observed around 16 ± 1 ºC, while dissolved oxygen was 20% lower in the NB-OSA site compared to the reference and NB sites (Table 4). Hardness was twice as high in the NB and NB-OSA site as the reference site, whereas pH was similar across all three sites (Table 4).

Table 4
Water quality measurements of average temperature (± SD), average dissolved oxygen (± SD), median pH (inter-quartile range, IQR) and average hardness (± SD) for the reference, natural bitumen (NB), and natural bitumen/oil sands area (NB-OSA) experimental sites. The reference site is located away from natural bitumen sources and industry development; the NB site is located within sources of bitumen-rich oil sands; and the NB-OSA site is located within natural bitumen sources and industry development in the Alberta oil sands region.

Site	**Temperature (ºC)**	**Dissolved oxygen (%)**	**pH (median, IQR)**	**Hardness (ppm of $CaCO_3$)**
	n = 11	***n = 11***	***n = 3***	***n = 4***
Reference	15 ± 3	76 ± 7	7.7 (7.7 – 7.6)	140 ± 1
Natural bitumen, NB	17 ± 1	73 ± 4	7.9 (8.0 – 7.8)	270 ± 1
Natural bitumen/oil sands area, NB-OSA	16 ± 1	56 ± 5	7.8 (7.9 – 7.7)	270 ± 1

Total PAH concentrations of both parent and alkylated forms were determined in both sediment and water to delineate PAHs of pyrogenic and petrogenic origin, respectively (Table 5). Total PAH concentrations in sediment and water was highest in the NB site and was lowest at the reference site, but this did not correspond with total alkylated PAH in water. Total alkylated PAH measured in water was higher in the reference site when compared with the natural bitumen/oil sands area site. Total parent polycyclic aromatic hydrocarbons were lower than alkylated PAHs, signifying a higher influence of petrogenic sources of PAH (Table 5). Total parent PAHs followed

the same trend as the total alkylated PAHs: sediment samples displayed a higher concentration of total parent PAHs in the NB site, followed by NB-OSA and the reference sites; total parent PAHs in the water of the NB site was highest of all three sites, where the latter two sites had similar concentrations of total parent PAHs (Table 5). Environment Canada guidelines state that sites are deemed uncontaminated, slightly contaminated, and significantly contaminated following the respective concentrations of Benzo[a]pyrene in sediment: < 100 ng/g; 100 – 1,000 ng/g; 1,000 – 10,000 ng/g (Environment Canada, 1999; Canadian Council of Ministers of the Environment, 2008). As concentrations of PAHs in water are highly variable, and PAHs are adsorbed onto suspended particles, observations of PAHs that exceed theoretical solubility limits indicate that a majority of the PAHs are associated with dissolved organic matter or particulates (Canadian Council of Ministers of the Environment, 2008).

Table 5

Total polycyclic aromatic hydrocarbon (± SEM) for parent and alkylated forms of polycyclic aromatic hydrocarbon in sediment and water samples. Three replicates per water and sediment samples were used in each of the measurements at the reference, natural bitumen (NB), and natural bitumen/oil sands area (NB-OSA) sites within the Alberta oil sands area (OSA).

Site	**Sediment Total Parent PAH (ng/g)**	**Water Total Parent PAH (ng/L)**	**Sediment Total Alkylated PAH (ng/g dry weight)**	**Waters Total Alkylated PAH (ng/L)**
Reference	47.7 ± 4.4	4.8 ± 0.1	260.4 ± 31.7	78.9 ± 1.0
Natural bitumen, NB	207.6 ± 5.4	15.5 ± 0.5	3491.6 ± 86.7	167.1 ± 1.9
Natural bitumen/oil sands area NB-OSA	178.5 ± 39.1	3.6 ± 0.1	2107.6 ± 399.3	50.1 ± 0.6

Copper (Cu) was the trace metal found to be above Alberta Water Quality Guideline (WQG), with concentrations highest in the natural bitumen site (Table 6). Other metals that were measured were well below WQG.

Table 6
Trace metals of interest measured from water samples from the reference, natural bitumen (NB), and natural bitumen/oil sands area (NB-OSA) sites. Dissolved organic carbon was measured from a water sample collected at each site in the summer of 2014, while trace metals were analysed from water samples collected in summer 2015. When unsatisfactory quality control checks for elements existed, the reading was denoted as "not reliable" or "nr".
*Alberta WQG does not exist for vanadium and was adapted from guidelines for livestock protection.

Site	Cu (µg/L)	Ni (µg/L)	V (µg/L)	Zn (µg/L)	Dissolved organic carbon (mg/L)
Alberta WQG for the protection of aquatic life	***7***	***69-120***	***100****	***30***	**--**
Reference	0.9	1	nr	nr	21
Natural bitumen, NB	16	4	nr	nr	37
Natural bitumen/oil sands area, NB-OSA	3	2	nr	nr	18

5.2 Olfactory Acuity

Olfactory acuity was measured by examining response to chemosensory cues using EOG and behavioural trials (Appendix A). Electro-olfactography response to TCA before the 28-day chronic treatment demonstrated an impairment in the olfactory acuity for both the NB and NB-OSA fish in comparison with the reference fish population (Figure 3). Impairment was three times lower for NB and NB-OSA fish populations when compared to the reference fish population signifying an impairment in the detection of TCA in these two treatment groups ($F_{2,10} = 35.9$, $p = 0.02$).

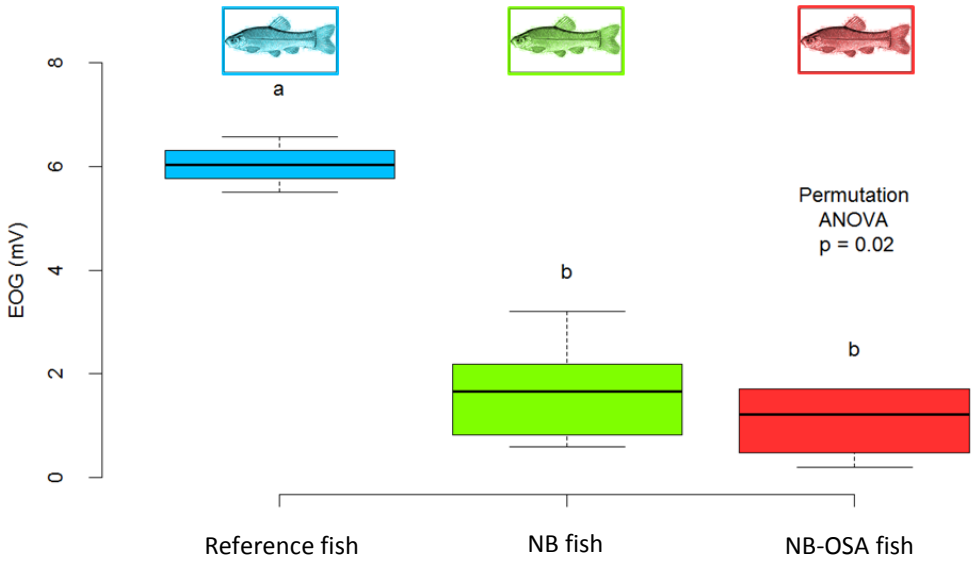

Figure 3

Electro-olfactography (EOG) response to taurocholic acid (TCA) before transplant or treatment. Fish source and treatment water are colour-coded with blue representing reference, green for natural bitumen (NB), and red for natural bitumen/oil sands area (NB-OSA). Colour of the fish represents the source of the fish, while the colour of the rectangle in which the fish are placed represents the exposure water. Different letters represent statistically significant differences between the treatment groups. Pre-treatment fish are enclosed in the same colour box and were measured in the same treatment water as their home-site. Pre-treatment fish are enclosed in the same colour box and were measured in the same treatment water as their home-site. Data are represented as boxplots with the median or 50th percentile as the black bar, 75th percentile is represented as the top of the box and 25th percentile, the bottom of the box, and the upper and lower whiskers represent the maximum and minimum data points.

Olfactory acuity to TCA was impaired in the reference fish population upon exposure within the NB and NB-OSA sites ($F_{2,17} = 167.8$, $p = 0.002$; Figure 4a). When comparing the pre-treatment, and post-transplant data of the olfactory acuity in the NB and NB-OSA fish populations, there was negligible impairment or improvement in TCA olfaction (Figure 4b). Reference fish populations exhibited EOG responses to TCA that were impaired to levels observed in the NB and NB-OSA EOG responses (Figure 4).

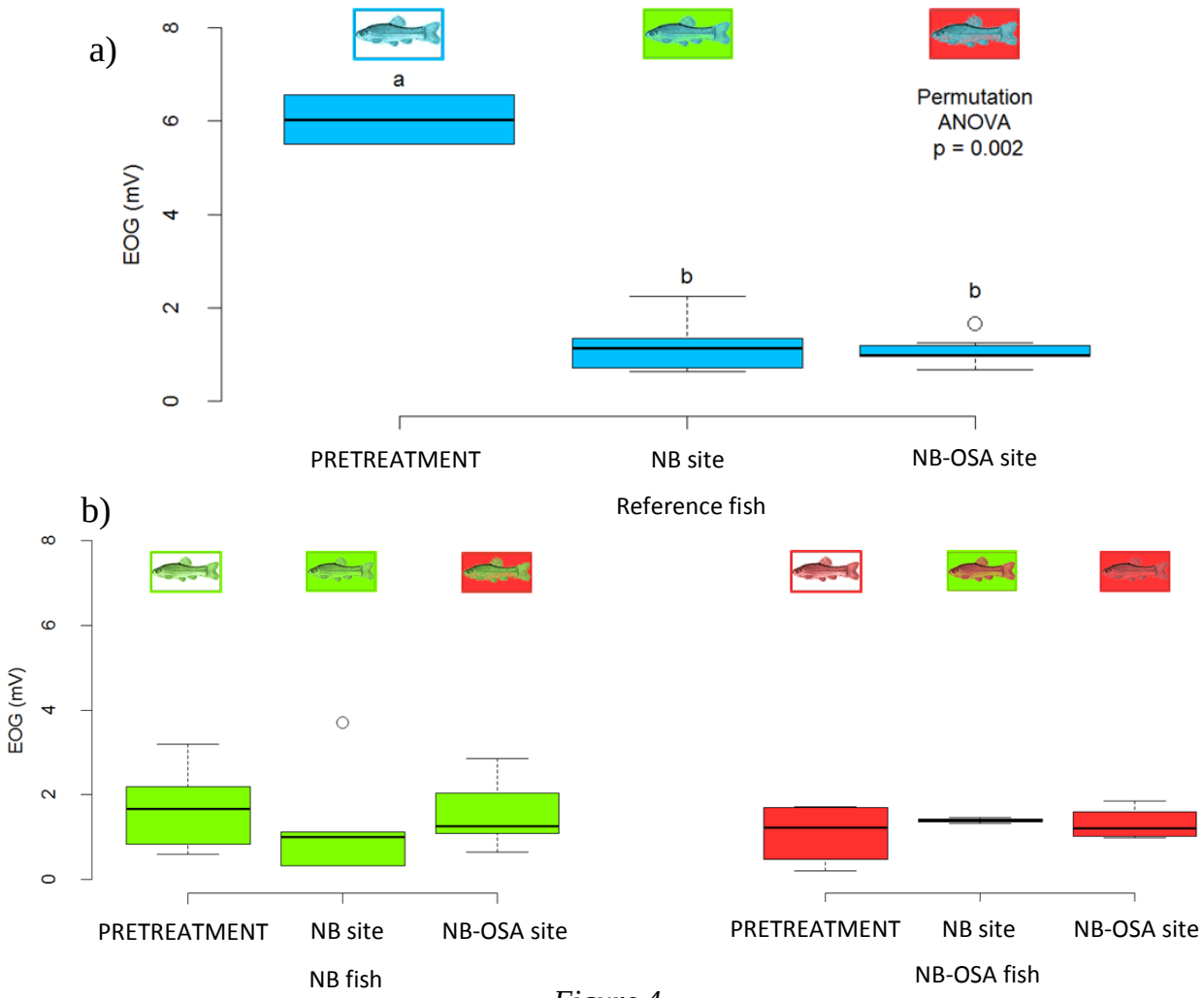

Figure 4

Electro-olfactography (EOG) response to taurocholic acid (TCA) before and after transplant in (a) reference fish population, and (b) natural bitumen (NB, green graphs) and natural bitumen/oil sands area (NB-OSA) fish sources (red graphs). Different letters represent statistically significant differences between the treatment groups. Fish-containing rectangles represent the treatment groups: the colour of the rectangles represents the exposure water and the colour of the fish represents a source of the fish. Details of the boxplots are provided in the caption to Fig. 3.

Olfactory response to FHM conspecific alarm cue before transplant demonstrated a similar trend as described previously for TCA. When EOG responses were compared before transplant occurred, the NB-OSA fish population was impaired by half when compared to the EOG response measured in the reference fish population ($F_{2,10} = 6.33$, $p = 0.03$; Figure 5).

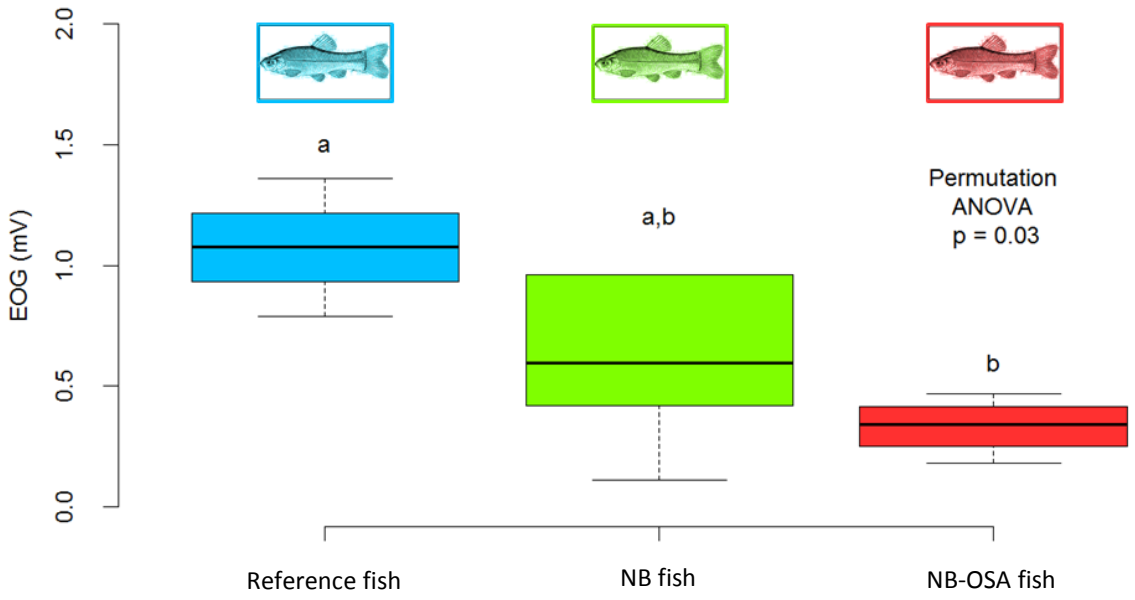

Figure 5

Electro-olfactography (EOG) response to fathead minnow (FHM) conspecific alarm cue before treatment, of the three different treatment fish populations: reference (blue), natural bitumen (NB, green), and natural bitumen/oil sands area site (NB-OSA, red). Treatments are represented within the fish-containing rectangle, where the background colour of the fish represents exposure water, and the colour of the fish represents the source of the fish. Different letters represent statistically significant differences between the treatment groups. Pre-treatment fish are enclosed in the same colour box and were measured in the same treatment water as their home-site. Details of the boxplots are provided in the caption to Fig. 3.

The reference fish population demonstrated a similar trend in impairment in the post-transplant fish having been exposed to either the NB or NB-OSA site with that of the pre-treatment exposure group ($F_{2,17} = 45.9$, $p = 0.001$; Figure 6a). EOG response was impaired in both the natural bitumen ($F_{2,15} = 8.52$, $p = 0.008$) and NB-OSA fish populations ($F_{2,8} = 4.57$, $p = 0.05$) when comparing the post-transplant responses with that of the pre-treatment response to FHM alarm cue (Figure 6b).

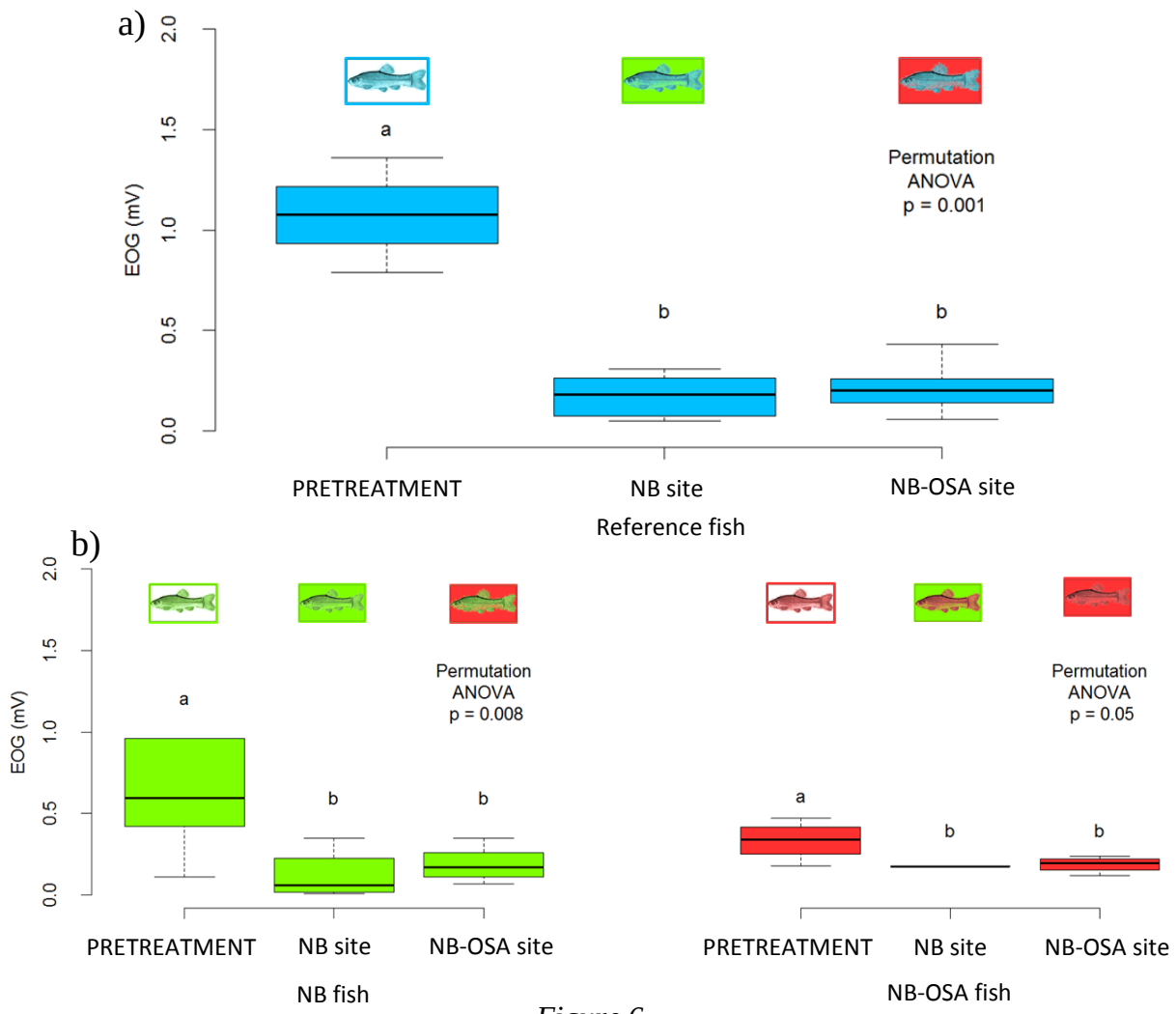

Figure 6

Electro-olfactography (EOG) response to fathead minnow (FHM) conspecific alarm cue before and after exposure. EOG was impaired when compared to before transplant or treatment in the same manner as observed in the natural bitumen (NB) and natural bitumen/oil sands area (NB-OSA) fish populations. Different letters represent statistically significant differences between the treatment groups. Colour of the rectangles represent the exposure water and the colour of the fish represents source of the fish. Pre-treatment fish are enclosed in the same colour box, and were measured in the same treatment water as their home-site. Details of the boxplots are provided in the caption to Fig. 3.

Electro-olfactography response to food cue in the form of L-alanine reveal trends of either impairment and improvement. Fish population from NB-OSA site had impaired EOG responses to L-ala when compared to the reference fish population before any transplant or exposure took

place ($F_{2,10}$ = 12.1, p = 0.009; Figure 7). Natural bitumen fish populations exhibited an EOG response to L-ala at levels that were not different from those of the reference and NB-OSA populations.

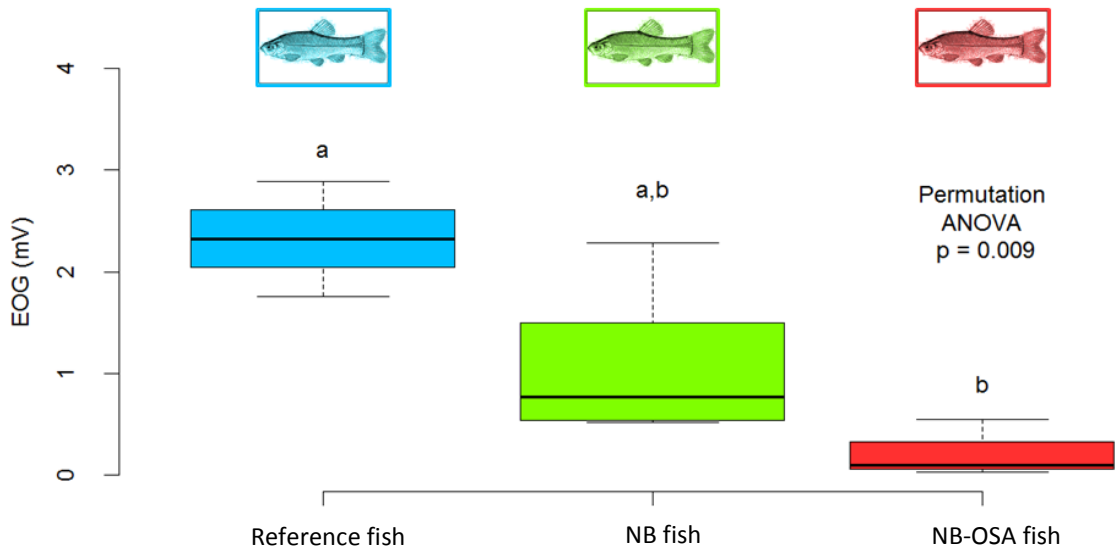

Figure 7

Electro-olfactography (EOG) response to L-alanine (L-ala) in the reference, natural bitumen (NB) and natural bitumen/oil sands area (NB-OSA) fish populations from their homesite before transplant. Responses were measured using water from home-sites. Significant differences are denoted by different letters above the graphs. Pre-treatment fish are enclosed in the same colour box and were measured in the same treatment water as their home-site. Details of the boxplots are provided in the caption to Fig. 3.

Impairment of olfactory detection of L-ala is observed in the reference fish population when pre-treatment olfactory acuity is compared with the responses measured after exposure ($F_{2,15}$ = 50.9, p = 0.001; Figure 8a). Although there is impairment, the chemosensory detection of L-ala in the reference fish population transplanted into the NB waters is more impaired than those transplanted into the NB-OSA site. Rather than impairment, improvement in chemosensory detection of L-ala was observed weakly in the NB fish population ($F_{2,18}$ = 3.38, p = 0.06), while

NB-OSA fish population displayed a stronger improvement trend ($F_{2,8} = 17.4$, $p = 0.008$) after transplant within their native site (Figure 8b).

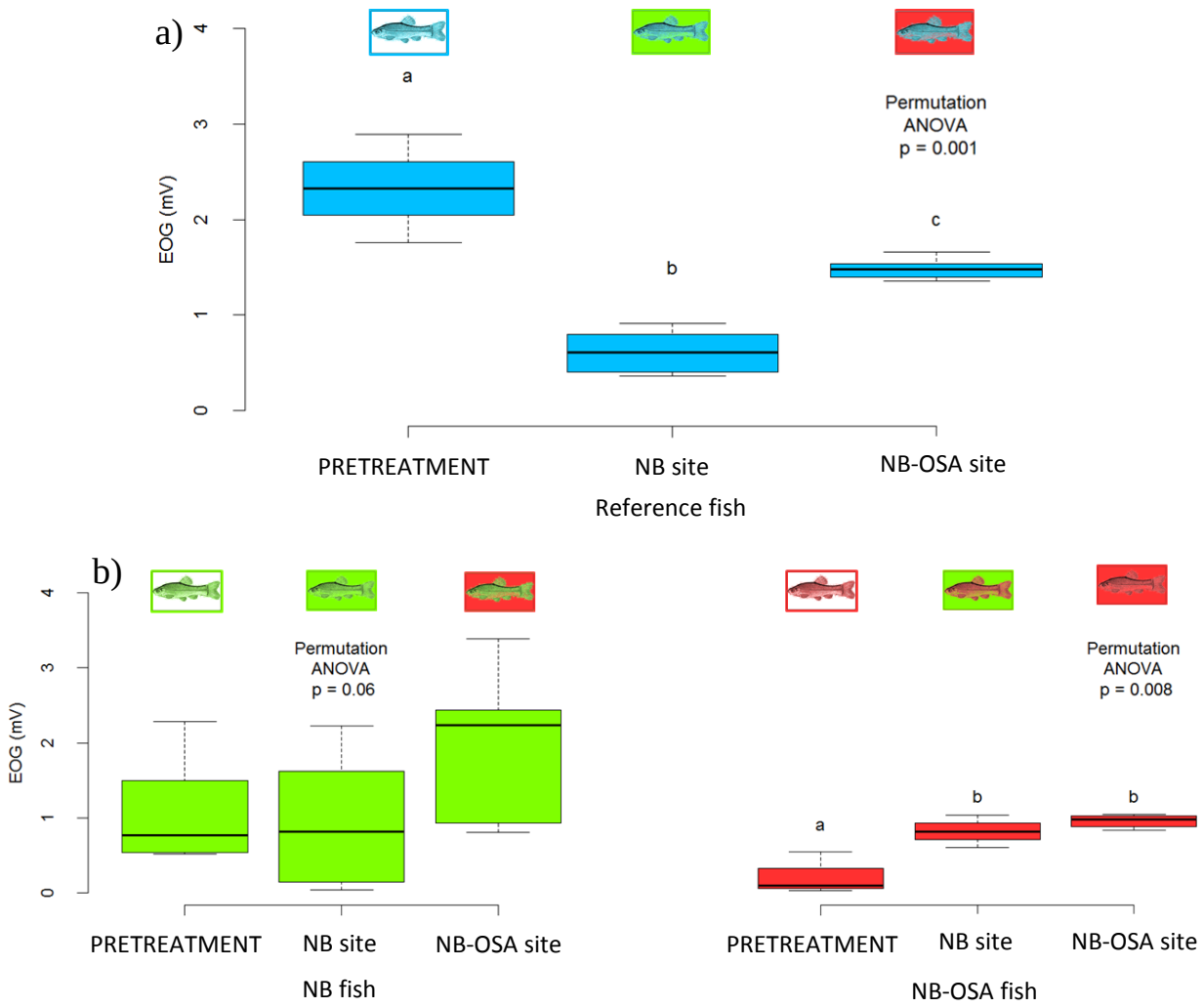

Figure 8

Electro-olfactography (EOG) response to L-alanine (L-ala) food cue before and after transplant for reference, natural bitumen (NB), and natural bitumen/oil sands area (NB-OSA) fish populations. Different letters represent statistically significant differences. Colour of the rectangles represent the exposure water and the colour of the fish represents source of the fish. Pre-treatment fish are enclosed in the same colour box, and were measured in the same treatment water as their home-site. Details of the boxplots are provided in the caption to Fig. 3.

5.3 **Hematocrit**

Hematocrit was only measured in the NB and NB-OSA fish populations. It was not possible to obtain blood samples prior to transplant and experimental exposure for reference fish population, and therefore the post-treatment data for that specific population was not included. The hematocrit remained unaffected in the natural bitumen fish population throughout the tr exposure when compared to data obtained prior to having any transplant or exposure taking place (Figure 9a). Fish from NB-OSA, however, demonstrated a decrease in hematocrit after having been transplanted into the natural bitumen site ($F_{2,10} = 5.84$, $p = 0.02$; Figure 9b). A similar trend towards a decrease in the NB-OSA fish population transplanted into their native site was observed when compared to the pre-treatment data (Figure 9b).

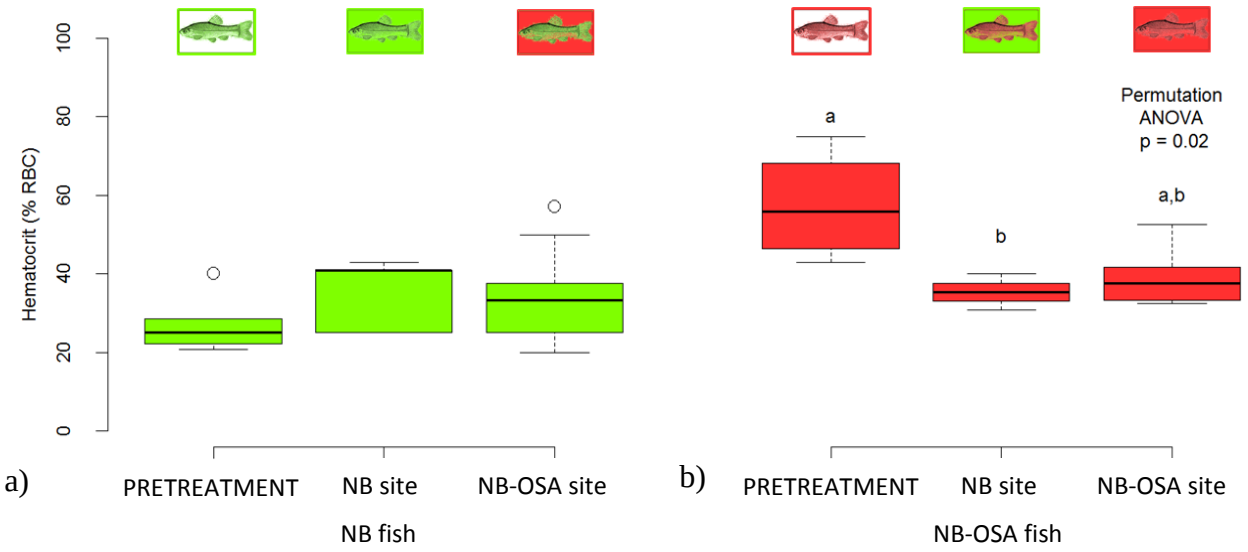

Figure 9

Hematocrit percentage as measured in the a) natural bitumen (NB, green) and b) natural bitumen/oil sands area (NB-OSA, red) fish populations from before and after treatment into natural bitumen and NB-OSA site. Statistically significant differences are represented by different letters. Colour of the rectangles represent the exposure water and the colour of the fish represents source of the fish. Pre-treatment fish are enclosed in the same colour box and were measured in the same treatment water as their home-site. Details of the boxplots are provided in the caption to Fig. 3.

5.4 **Oxygen Consumption**

Respiration rate was lower in the NB and NB-OSA fish population compared with the reference fish population prior to transplant or exposure had begun ($F_{2,18} = 3.00$, $p = 0.05$; Figure 10).

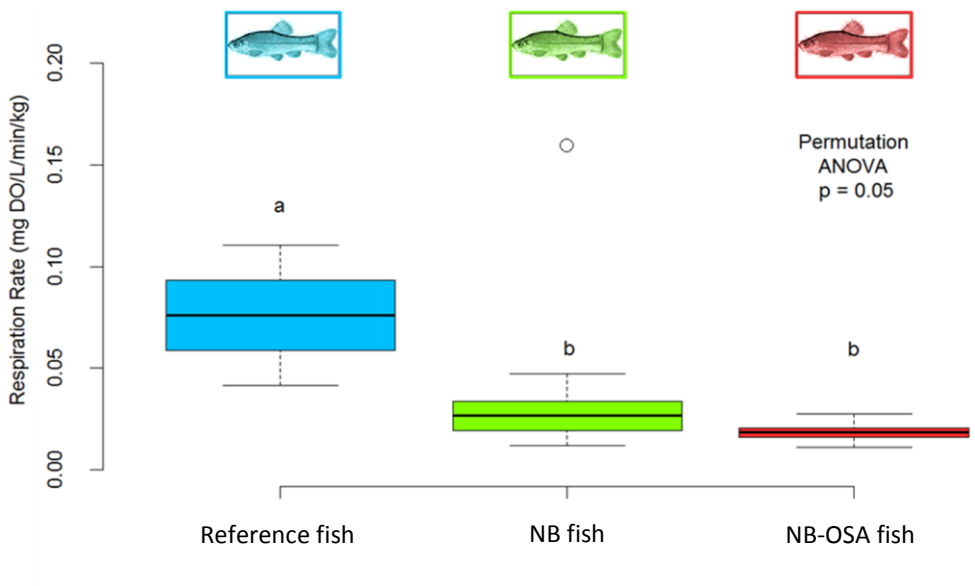

Figure 10

Respiration rates (mg DO/L/min/kg) before transplant or exposure of reference (blue), natural bitumen (NB, green), and natural bitumen/oil sands area (NB-OSA, red) fish populations. Statistically significant outcomes are denoted by the different letters. Pre-treatment fish are enclosed in the same colour box and were measured in the same treatment water as their home-site. Details of the boxplots are provided in the caption to Fig. 3.

After reference fish populations were transplanted and exposed to the NB and NB-OSA sites, respiration rates were impaired to levels observed in the bitumen pre-exposed fish populations ($F_{2,18} = 24.2$, $p = 0.001$; Figure 11a). When examining the respiration rate among the pre-treatment and post-transplant/exposure respiration rates in the NB and NB-OSA fish populations, negligible impairment or improvement was demonstrated after transplantation (Figure 11b).

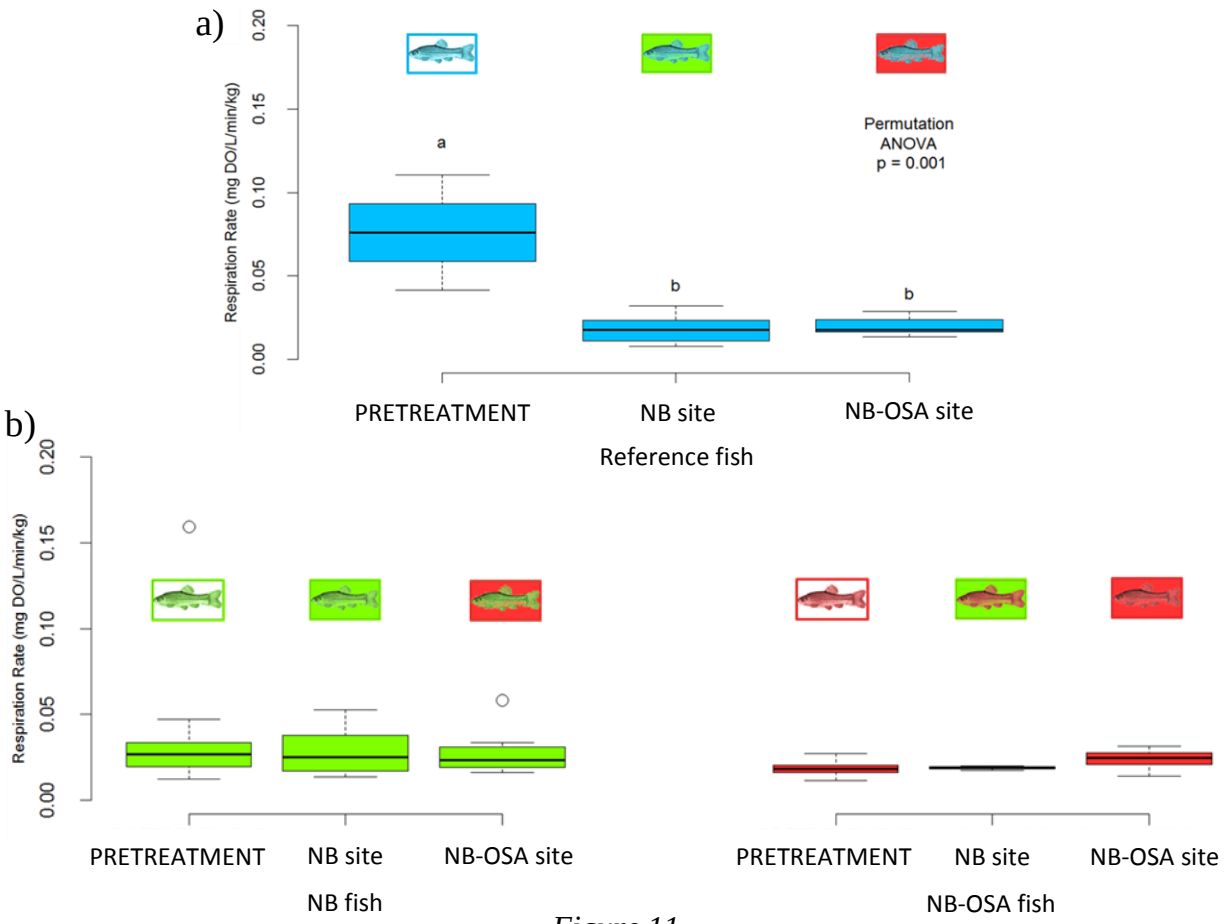

Figure 11

Respiration rate (mg DO/L/min/kg) measured before and after exposure for reference, natural bitumen (NB), and natural bitumen/oil sands area (NB-OSA) fish population and corresponding treatment. Differences between exposures are signified by different letters. Colour of the rectangles represent the exposure water and the colour of the fish represents source of the fish. Pre-treatment fish are enclosed in the same colour box and were measured in the same treatment water as their home-site. Details of the boxplots are provided in the caption to Fig. 3.

5.5 **Survival**

The proportion of survivors in the natural bitumen site and the natural bitumen/oil sands area site was calculated for the 28-day exposure period. Reference fish transplanted to the two toxicant-exposed sites revealed survival rates that were above 90% (Figure 12). Natural bitumen fish population showed a decreasing trend that began on the second day of exposure, to a survival of a

little under 20% (Figure 12). Fish from NB-OSA transplanted into the NB site demonstrated an even steeper rate of decrease, which had reached equilibrium around day 10 at a survival rate of below 10% (Figure 12).

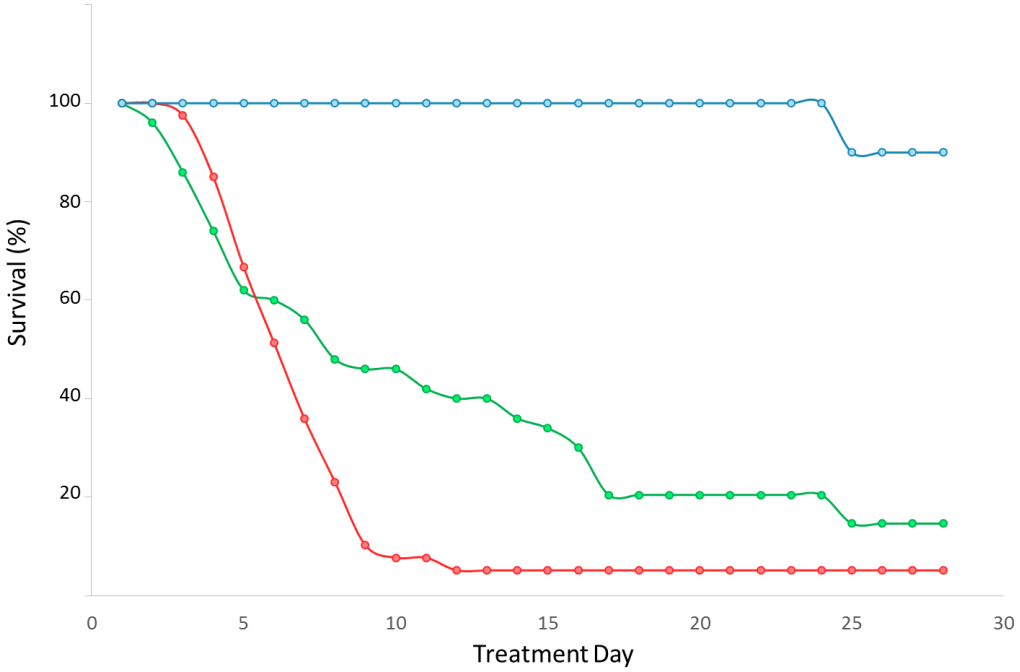

Figure 12

The proportion of survivors of reference, natural bitumen (NB), and natural bitumen/oil sands area (NB-OSA) fish populations held within the natural bitumen waters over the 28-day treatment duration. Data points represent cumulative survivorship at each day. The colour of the line represents the source of the fish, where reference is blue, natural bitumen is green, and natural bitumen/oil sands area is red. Reference fish populations exhibited a survivorship of above 90% throughout the 28-day chronic exposure. The NB and NB-OSA fish, on the other hand, show a high decrease in survivor after the second day, with the NB-OSA population having a higher rate of mortality than the NB fish population.

Natural bitumen fish population transplanted into the NB-OSA site exhibited a similar decrease in survival rate on day 2 of exposure, but steadied off at 50% survival rate (Figure 13). On day 5 of exposure in the NB-OSA site, the NB-OSA fish population presented a similar rate of decrease in survival rates, which remained at 18% after day 17 of exposure (Figure 13).

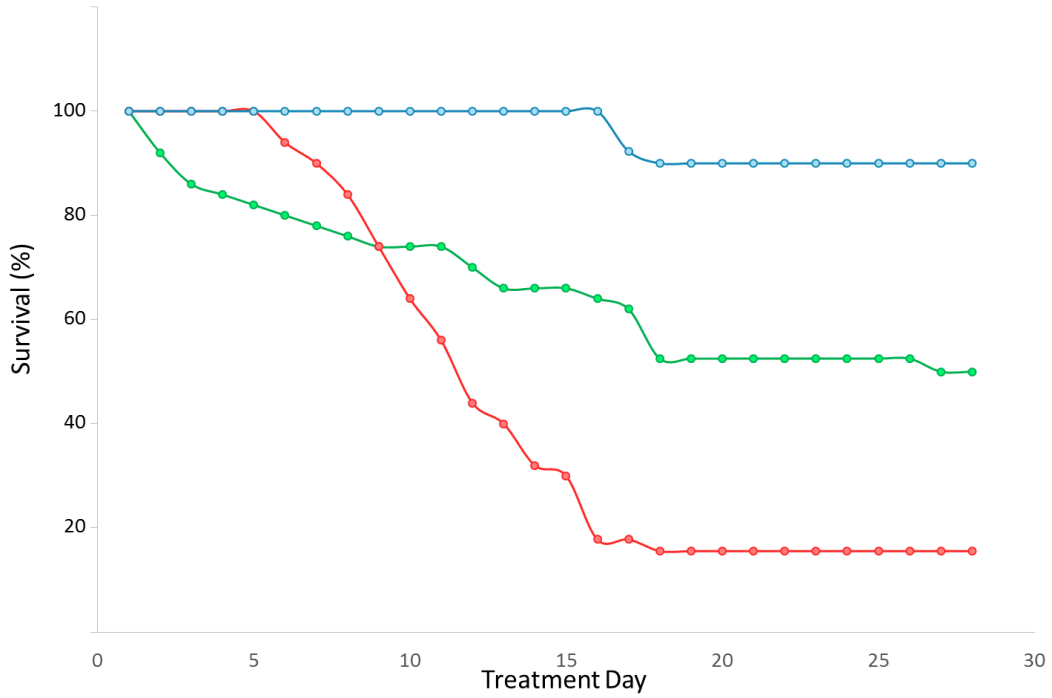

Figure 13

The proportion of survivors of reference, natural bitumen (NB), and natural bitumen/oil sands area (NB-OSA) fish populations transplanted and held within the NB-OSA treatment waters for 28 days. Data points represent cumulative survivorship at each day. Reference fish exhibited a lower survival rate throughout the 28 days, where the proportion of survivors was over 90%. Natural bitumen fish population show a decline in the survivorship after day 1, with survival rates below 50%. The NB-OSA fish population demonstrated a steeper and greater decrease in the number of survivors after the fifth day, with under 20% of the fish population surviving to the full treatment day.

6 Discussion

A chronic two-way transplant experiment was conducted to answer the question of whether local adaptation to natural bituminous contaminants existed in the fish populations from the Alberta oil sands region. Having inhabited these water bodies for millennia, and being subjected to intermittent fluvial pulses of naturally sourced bituminous toxicants, localized adaptation within these populations seemed plausible. Current literature related to bituminous toxicants and xenobiotics has not included concepts of local adaptation, despite the existence of local adaptation research pertaining to exposure to petroleum products and other xenobiotics (Ownby *et al.*, 2002; Meyer & Di Giuliuo, 2003; Claireaux *et al.*, 2013; Rolshausen *et al.*, 2015).

It should be noted that water quality was similar among the three sites throughout the exposure period, with the exception of dissolved oxygen in the NB-OSA site. Dissolved oxygen measured in the NB-OSA site was approximately 50%, or 20% less than that of the reference and the NB sites. Despite this difference in dissolved oxygen, dissolved oxygen did not appear to affect the endpoints measured in the three populations. Furthermore, given the fact that fathead minnows are highly tolerant to hypoxic conditions, such a small decrease in dissolved oxygen should not be intolerable.

6.1 Olfactory Acuity

The impairment that was observed in the reference fish populations before and after transplant in the current study reflect the olfactory acuity impairment demonstrated in previous studies examining metals as chemosensory inhibitors. Impairment of olfactory acuity at the OE was evident in the NB-OSA fish population, and to a lesser extent in the natural bitumen fish populations before any transplant occurred, which may be explained by pre-exposure to toxicants

in the treatment water (Figures 3, 5, 7). Additionally, the reference fish populations displayed similar trends of impairment after transplant into non-native waters (Figure 4a, 6a, 8a). The impairment that was observed in the natural bitumen and natural bitumen/oil sands area fish populations before and after treatment, and the similar impairment demonstrated by the reference fish populations upon transplant alone indicate that treatment water from the NB and NB-OSA sites have imposed a constraint on fish olfaction. If the EOG impairment was due to a caging or transplant effect, the lowered EOG response in the pre-exposed fish populations would not demonstrate similar EOG response before any transplant or caging occurred. The impairment of the reference fish populations exposed to the treatment waters are similar to the impairment observed in previous studies examining metal impairment of EOG response (Baldwin *et al.*, 2003; Sandahl *et al.*, 2006; Bilberg *et al.*, 2011).

Chemosensory detection of olfactory cues in fish that were acutely exposed to metal-contaminated waters displayed impairment in olfactory acuity comparable to what was observed in the present research (Hara *et al.*, 1976; Baldwin *et al.*, 2003; Sandahl *et al.*, 2006; Pyle & Mirza, 2007; Mirza *et al.*, 2009; Green *et al.*, 2010; Dew *et al.*, 2014). Chemosensory detection of alarm cues was impaired in both laboratory and field behavioural experiments utilising fathead minnows and Iowa darter (*Etheostoma exile*), respectively, exposed to copper and nickel contaminated waters (McPherson *et al.*, 2004; Carreau & Pyle, 2005). Metal-mediated impairment of olfactory acuity to various cues measured using EOG has been observed in fathead minnows, yellow perch, Pacific salmonids, crucian carp (*Carassius carassius*) and Eurasian perch (*Perca fluviatilis)* (Baldwin *et al.*, 2003; Sandahl *et al.*, 2006; Bilberg *et al.*, 2011; Azizishirazi *et al.*, 2013; Dew *et al.*, 2014; Azizishirazi & Pyle, 2015). Olfactory detection to cues such as TCA, L-alanine, L-serine, L-arginine, L-aspartic acid, L-leucine, and pheromones were all impaired upon exposure

to copper, nickel, silver nanoparticles, or metal mixtures (Baldwin *et al.*, 2003; Mirza *et al.*, 2009; Bilberg *et al.*, 2011; Azizishirazi *et al.*, 2013; Dew *et al.*, 2014). Such impairments in higher-level physiological responses from exposures provide support for potential metal-mediated impairment of overall olfactory acuity in the present study.

The mechanism of impairment as measured by and observed as a behavioural endpoint was not successfully carried out in the current study and conclusions of behavioural impairment cannot be made. Because the present study was limited in sample size, subsequent behavioural data were inconclusive as a large sample size is required to limit variability in behavioural data. Behavioural data has been included as a separate appendix (Appendix A). The mechanism of chemosensory detection by olfactory sensory neurons may be impaired as a result of environmental or physiological changes. However, documentation of such impairment in relation to exposure to oil sands related toxicants has been limited. The presence of olfaction-impairing xenobiotics can disable detection of cues either at the source of sensory detection or elsewhere along the chain of sensory processing (Laberge & Hara, 2001; Mirza *et al.*, 2009). Observations of EOG can only distinguish impairment at the site of sensory detection, and therefore only observations of toxicity at this site were detected.

There was limited evidence for local adaptation from the transplant experiment when examining EOG response of the natural bitumen and natural bitumen/oil sands area fish populations. The olfactory response of NB and NB-OSA fish populations to TCA was not impaired or improved, while olfactory acuity to L-ala exhibited a limited improvement in detection (Figures 4b-c, 6b-c, 8b-c). Impairment or improvement of the endpoint of interest would have indicated local adaptation to the two different toxicant regimes at these sites (Hendry, 2016), but this is not the case for EOG response to TCA in these two fish populations residing within the oil sands

region (Figure 4). On the other hand, the EOG response to L-ala in the NB-OSA fish populations displayed a trend towards improvement after transplant into both native and non-native waters, which may be a sign of improved stimulation of the ciliated sensory neurons of the olfactory epithelium upon transplantation regardless of treatment water (Figure 8; Dew *et al.*, 2014). Improvement of olfactory response to L-ala was only observed in the natural bitumen fish transplanted into the NB-OSA treatment water, which may indicate plasticity in the olfactory capabilities rather than local adaptation as olfaction was more impaired in the native home-site rather than in the non-native site (Figure 8b). General improvement in olfactory acuity towards L-ala was observed in Mirza *et al.* (2009) examined the effects of metal contamination on EOG response of juvenile yellow perch. However, most peer-reviewed literature has observed an impairment in olfactory detection of L-ala upon exposure to metal-contaminated treatments (Hara *et al.*, 1976; Sveinsson & Hara, 2000; Green *et al.*, 2010; Bilberg *et al.*, 2011; Azizishirazi *et al.*, 2013; Dew *et al.*, 2014). The EOG response to TCA and L-ala may be a sign of differences in physiological impairment of the various sensory neurons located within the olfactory epithelium (Dew *et al.*, 2014).

Detection of FHM conspecific alarm cue at the olfactory epithelium displayed impairment in all three fish populations after transplant. The impairment in EOG response observed in the reference, NB, and NB-OSA fish populations when comparing before and after transplant does not correspond to the elevated EOG response to conspecific alarm cues previously measured in juvenile yellow perch from metal-contaminated lakes (Mirza *et al.*, 2009). Metal versus PAH contamination may play a role in this difference in EOG response, as the metal concentrations in the current study was not as large of a concern than was total PAHs, where the latter may have presented unknown effects on olfactory acuity (Table 3; Table 4). Despite this contradiction, the

impairment in EOG response to FHM conspecific alarm cue observed in the three fish populations agrees with the observed responses to TCA and L-ala in the naïve reference fish populations, and with previous EOG data of reference fish having been placed into contaminated waters (Azizishirazi *et al.*, 2013).

Impairment of chemosensory detection measured by a lowered EOG response was observed at copper concentrations of as low as 3 µg/L to 10 µg/L in laboratory settings, while in-field copper contaminants ranging from 8.6 µg/L to 14 µg/L elicited an impairment in chemosensation (Sandahl *et al.*, 2006; Mirza *et al.*, 2009; Green *et al.*, 2010; Azizishirazi *et al.*, 2013). These concentrations reconcile with the copper concentrations observed in both the natural bitumen water (16 µg/L) and the natural bitumen/oil sands area water (3 µg/L), and may be used to explain the impairment observed in the transplanted reference fish populations and the inherent impairment of olfaction in natural bitumen and natural bitumen/oil sands area fish (Table 6). Impairment may be explained by difference in metal concentrations in the present study, but the effect of PAHs as an odorant or inhibitor of chemosensation in fish has yet to be studied.

Differences in total PAHs are evident among the three sites, but do not fully explain the EOG impairment data observed in the present experiment. Total PAH concentrations are highest in both water and sediment samples from the natural bitumen site, which can be used to support the impaired EOG responses observed in all fish populations exposed to this treatment water (Table 5). The concentrations of total PAHs in the NB-OSA and reference sites are similar in water samples, which cannot explain what is observed in the EOG responses. A clear conclusion of total PAHs' effects on olfactory acuity cannot be made and the effects of PAHs as a potential odorant or inhibitor of chemosensory acuity warrants further investigation.

A more thorough examination of chemosensory needs to include olfactory acuity recovery, mechanism of impairment, and treatment exposure period variability to understand the different aspects of chemosensory acuity of fish in the Alberta oil sands region. It has been demonstrated that exposure to metal contaminated waters for a duration of 24 hours will elicit olfactory impairment in yellow perch (Azizishirazi *et al.*, 2013). Rainbow trout demonstrated impairment in olfactory acuity to cues upon only less than two minutes of exposure to oil sands process-affected waters (Sun *et al.*, 2014). Chronic exposure may be an adequate amount of time for fish to acclimate to environments, choosing acute exposure times may help elucidate the physiological response of the fish olfactory impairment immediately following transplant. Secondly, recovery of olfactory acuity occurs upon introduction into uncontaminated or reference waters (Sandahl *et al.*, 2006; Azizishirazi *et al.*, 2013; Blunt, 2014; Azizishirazi & Pyle, 2015). In the present study, transplant into reference site may help clarify the role of recovery in these forage fish subjected to various levels of bituminous toxicants. Lastly, olfactory acuity as measured by EOG can only illustrate the effect of the treatment; the consequences of impairment resulting in altered behavioural patterns cannot be explained. Without an adequate behavioural assay to support the EOG responses measured, it is difficult to translate the impairment that is observed at the site of cue detection (olfactory epithelium of the rosette) to an actual impairment of higher biological responses (for example, avoidance or attraction behaviour). Future eco-toxicological field studies examining olfactory acuity should consider a shorter length of exposure, additional reference water treatment, and appropriate behavioural trials.

Local adaptation is not the case for fathead minnow populations of the Lower Athabasca River basin, as it was not observed in the EOG endpoint measured in the present study. Potential local adaptation gave promise for the ability of aquatic populations to withstand certain stressors

that are placed on the individual and its population (Ownby *et al.*, 2002; Schulte, 2007; Fraser *et al.*, 2011; Westley *et al.*, 2013; Rolshausen *et al.*, 2015). It is possible that with long-term exposure to contaminated sites, chemosensory acuity may be interrupted to the point where disruption of signal processing between the site of cue detection to higher level biological responses occurs, which may later be translated into poor individual and population growth and survival (Mirza *et al.*, 2009). The ecological consequences of reclamation, remediation, and continued mining within the Alberta oil sands region may therefore indirectly affect aquatic organism richness and diversity through impairment in olfaction. The potential to ameliorate such contaminant-driven impairment in olfaction, however, has been demonstrated in field and laboratory, where recovery of chemosensory acuity occurred after depuration in uncontaminated water following exposure to metal contamination and OSPW (Sun *et al.*, 2014). The possible recovery of chemosensory function, therefore, exists when examining chemosensation in fish from the oil sands region.

6.2 **Hematocrit**

Hematocrit measured before and after transplant in the current study did not change in the same manner as has been observed in previous ecotoxicology studies. Hematocrit shows possible signs of stress through the increase in red blood cells. Stress response as measured using hematocrit was not clear in the natural bitumen and natural bitumen/oil sands area fish following transplant, as the hematocrit remained the same or decreased after 28 days of exposure (Figure 9). Differences in hematocrit can be observed when comparing the pre-treatment hematocrit levels between natural bitumen and natural bitumen/oil sands area fish populations: percent red blood cells is higher in the NB-OSA fish population than in the NB fish population. We may conclude that NB-OSA fish before transplant exhibited a higher stress level than natural bitumen fish populations (Figure 9). The increase in hematocrit in the NB-OSA fish population before transplant, when

compared to the NB fish population, is comparable to measurements of hematocrit of other fish exposed to toxicants.

The increase in red blood cells and related cell types has been reported in peer-reviewed literature examining the impacts of bituminous toxicants on fish physiology. Fathead minnows and brook stickleback exhibited increased hematocrit percentages when transferred and held within wetlands containing oil sands process-affected materials (Bendell-Young *et al.*, 2000). Fathead minnows that were exposed to a variety of mixtures that included consolidated tailings, and tailings pond seepage waters, demonstrated increased hematocrits by 20% after 96-hour exposure (Farrell *et al.*, 2004). After exposure to the water-soluble fraction of crude oil, juvenile Caspian roach (*Rutilus caspicus*) displayed negligible changes in hematocrit measurements, but the oxygen-carrying capacity of the blood as measured by a decrease in pO_2 and an increase in pCO_2 were apparent (Lari *et al.*, 2016a). The maintenance of hematocrit levels as observed in the natural bitumen fish population pre-treatment and after transplant may require exploration into other aspects of haematology to decipher potential changes in blood composition (Hudson *et al.*, 2008; Kammerer *et al.*, 2010; Lari *et al.*, 2016a).

Possible decrease in hematocrit of the NB-OSA fish population is inconsistent with the stress response of increase in red blood cell production (Leclair *et al.*, 2013). Reduced hematocrit and red blood cell count have been demonstrated in rats directly exposed to crude oil (US Environmental Protection Agency, 2011), which may be used to explain possible effects of PAHs on the hematocrit results presently observed. If NB-OSA fish populations were stressed, energy may have been conserved or re-allocated from the production of red blood cells to conserve energy reserves (Lowe & Davison, 2005). The reallocation of energies may be an explanation as to why hematocrit levels in the natural bitumen/oil sands area fish population decreased in comparison to

pre-treatment data. Because the hematocrit for reference was unavailable for the present study, suppression or elevation of hematocrit resulting from exposure to treatment water cannot be ascertained. Hematocrit alone may not be an adequate tool in determining stress or oxygen carrying capacity potential and other aspects of haematology that were not measured should be considered.

Hematocrit measurements obtained in the present study may potentially be explained by the oxygen concentrations at each site. Environmental oxygen concentrations were higher in the natural bitumen site than in the natural bitumen/oil sands area site by a factor of 20%, which may account for the difference in response of the NB-OSA fish population quantified by the hematocrit information. Hematocrit decreased in the NB-OSA fish after transplant into both native and non-native waters, which may demonstrate the plasticity of this endpoint in response to the stress of transplant. Hematocrit measurements should be accompanied by other haematological methods of quantifying changes expressed through blood composition after exposure to toxicants. By examining respiration rates in the same fish populations, the justification for differences in physiological scope may become apparent.

6.3 Oxygen Consumption

Respiration rates observed in the reference fish population decreased after transplant to levels that were comparable to the fish pre-exposed to bituminous toxicants (Figure 11a). The lowered oxygen consumption rates observed in the present project agree with observed respiration rates in fish exposed to crude oil and related toxicants (Christiansen *et al.*, 2010; Gerger *et al.*, 2015; Incardona *et al.*, 2015; Lucas *et al.*, 2016). Respiration rates in polar cod (*Boreogadus saida*) were impaired after exposure to water-soluble fractions (WSFs) of crude oil (Christiansen *et al.*, 2010). Cod acutely exposed to WSF for 60 minutes displayed a large decrease in oxygen consumption rates (Christiansen *et al.*, 2010). After exposure to treatment waters dosed with a

standard AhR agonist, beta-naphthoflavone, zebrafish respiration rates significantly decreased in comparison to fish in the control treatment (Gerger *et al.*, 2015). A similar decrease in oxygen consumption was observed in zebrafish exposed to both intraperitoneal injection and aqueous exposures to benzo-a-pyrene (Gerger & Weber, 2015). When juvenile herring (*Clupea pallasi*) were exposed to effluent from oiled gravel columns, lowered metabolic rate in relation to oxygen consumption was also observed (Incardona *et al.*, 2015).

The decreased oxygen consumption may be a result of exposure to either or both bituminous toxicants (e.g., PAHs) and trace metals found in the natural bitumen and natural bitumen/oil sands area waters (Table 5; Table 6). Various fish species exhibit a similar decrease in respiration rates resembling the present observations following introduction to trace metal contaminated waters (Rajotte & Couture, 2002; Couture & Kumar, 2003; Pistole *et al.*, 2008; Peles *et al.*, 2012; Mager *et al.*, 2014). Fathead minnows and emerald shiners (*Notemigonus crysoleucas*) displayed lowered oxygen consumption rates following exposure to copper (Peles *et al.*, 2012). However, different effects on respiration rates occurred for various fish populations, which highlights the need for population-level characterization of respiration rates (Peles *et al.*, 2012). Yellow perch exposed to copper and cadmium displayed negative effects on respiration rate and activity levels, where copper had more of an effect on respiration rate as it inhibits specific enzymatic pathways required for respiration (Rajotte & Couture, 2002; Couture & Kumar, 2003). Lowered metabolic rate or no change in metabolic rate were observed in fathead minnows exposed to copper, cadmium, and high salinities (Pistole *et al.*, 2008). However, changes in oxygen consumption were dependent on the length of exposure to the contaminant (Pistole *et al.*, 2008).

Respiration rates in the natural bitumen and NB-OSA populations (Figure 11b) remained unaffected after transplant and lowered compared to reference fish prior to transplant has been

observed in previous studies of petrol-related toxicants and trace metals. Oxygen consumption did not change in lake chubsuckers (*Erimyzon sucetta*) following exposure to sediments taken from coal-ash polluted sites (Hopkins *et al.*, 2000). When sheepshead minnow (*Cyprinodon variegatus*) were exposed to sediments obtained from a coal-fired power generator settling basin, oxygen consumption did not differ from control after 375 days (Rowe, 2003). Similar lack of effects on respiration was observed in mosquito fish (*Gambusia holbrooki*) after exposure to mercury (Hopkins *et al.*, 2003). Since the NB and NB-OSA fish populations were consistently exposed to either bituminous toxicants and/or metals, respiration rates may be limited by morphological change of the gills.

The decrease in respiration observed in the fish pre-exposed to bituminous toxicants and reference fish transplanted into treatment sites may be explained by toxicant-governed gill damage. Thickened interlamellar epithelial layer, increased chloride cells, and apoptosis and eventual gill tissue aneurysms were observed in yellow perch introduced into experimental lakes with consolidated oil sands tailings (Van den Heuvel *et al.*, 2000). Likewise, fathead minnows displayed similar gill abnormalities and cell proliferation when exposed to experimental lakes containing OSPW (Kavanagh *et al.*, 2013). Fasulo *et al.* (2012) exposed ornate wrasse (*Thalassoma pavo*) to crude oil for 48, 96, and 192 hours and demonstrated similar gill tissue malformations, with increased mucous cell and increased lifting of the epithelial cell layer with increased duration of exposure. The same gill morphological abnormalities were observed in various fish species exposed to petrol-related compounds and toxicants (Agamy, 2013b, 2013a). The gill tissue degeneration observed in these previous studies may be used to explain the decrease in oxygen consumption rates shown in the current transplant study.

Although gill histopathology was not examined in the present project, the lowered oxygen consumption observed in reference fish following transplant into the treatment sites may be potentially explained by increased gill abnormalities as observed in previous studies examining petrol-related toxicants. Because oxygen consumption in the post-transplant reference fish was lowered to levels observed in the fish populations pre-exposed to bituminous toxicants, gill damage can also explain the lowered oxygen consumption in the NB and NB-OSA fish populations. Furthermore, gill damage in these fish populations familiar to bituminous toxicants can explain the lack of effect transplant on oxygen consumption.

Local adaptation was not demonstrated in the current respiration rate data. Fish populations having been pre-exposed to bituminous toxicants exhibited respiration rates that were unaffected before and after transplant, whereas the reference fish naïve to such toxicants demonstrated a substantial decrease. Decreased oxygen consumption may be due to atypical responses to stresses associated with transplant, changes in toxicant exposure or may be related to age or other characteristics of these populations not explicitly examined in the present study (Calow & Forbes, 1998; Fidhiany & Winckler, 1998; Svecevicius *et al.*, 2005; Sardella & Brauner, 2008). The lowered and lack of change in respiration rates in the three fish populations may point towards various ways fish deal with the additional stressors resulting from transplant, caging and exposure to treatment waters.

6.4 **Survival**

Survival after reciprocal transplant decreased only in the natural bitumen and natural bitumen/oil sands area fish populations (Figure 12 and 13). The impaired survival observed in these NB and NB-OSA fish supports the idea that these fish possess a limited ability to deal with multiple stressors. The effects of naturally sourced bituminous toxicant on different fish species

are further supported by the observations in the present reciprocal transplant experiment. The increased mortality observed in the natural bitumen and natural bitumen/oil sands area fish populations may be explained by the exposure to the bituminous treatment waters.

Lowered survival may be explained by the bituminous toxicants limiting the ability of fish populations to cope with additional stressors. The physiological constraints observed previously in oxygen consumption, and hematocrit percentage indicate the potential limitation that pre-exposure to bituminous toxicants have imposed upon the natural bitumen and natural bitumen/oil sands area fish populations. Lowered hematocrit percentages and decreased oxygen consumption rates showed that the fish pre-exposed to bituminous toxicants were already coping with stressors found within their environment. The inability of these fish to handle the additional stressors of transplant and handling may have translated into the observed trends in survivorship following transplant (Figure 12 and 13).

Fish can respond to stressors both in the short and long term, and differences in responses result from the variation of mechanisms each individual has to handle stresses (Karakach *et al.*, 2009). Survival may be affected as further stressors are introduced because an individual's abilities to deal with stressors is limited, constrained by environmental factors, and may be inhibited by additional factors and costs such as energetic requirements (Farrell *et al.*, 2008; Karakach *et al.*, 2009; Martinez *et al.*, 2016). The two fish populations pre-exposed to bituminous toxicants had to deal with both short-term stresses of transplant into new environments, in addition to the long-term stress of exposure to contaminants (Karakach *et al.*, 2009). Trade-offs in prioritizing certain responses to cope with and survive in face of these additional pressures result in limited survival, evident in past and present studies (Power, 1997; Cucco *et al.*, 2012; Killen *et al.*, 2013). Multiple stressor effects on fish can decrease growth, reproduction, and survivorship (Sardella *et al.*, 2007;

Sardella & Brauner, 2008; Raicevich *et al.*, 2014). Because the reference fish were not subjected to bituminous toxicants previously, these fish did not have to compensate for long-term exposure to these toxicants. The concepts of multiple stressors and potentially limited stress-coping methods can therefore be used to explain what is observed in the survivorship in the natural bitumen and natural bitumen/oil sands area fish populations.

Reference fish populations displayed limited change in survival during chronic exposure to both the natural bitumen and natural bitumen/oil sands area site. The high survival rate of the reference fish potentially demonstrates the population's ability to maintain adequate physiological response in light of new and additional stressors. Reference fish were previously exposed to very little bitumen-related toxicants, and as such had a larger range of resources and mechanisms to deal with additional stressors. Additionally, impairment in oxygen consumption was observed in the reference fish populations only after transplant into the treatment waters. The range of these reference fish's physiological stress response may have been large enough for the fish to deal with the additional stressors of caging, transplant and handling, producing mechanisms allowing them to survive for the 28-day exposure (Figure 12 and 13). Fish from the reference tributary exhibit only short-term stress responses to the immediate transition into toxicant-containing environments, eliciting mechanisms that can respond to and compensate for the multiple stressors associated with the transplantation (Power, 1997). Energy, previously in reserve, is used to respond to and initiate compensatory mechanisms (Karakach *et al.*, 2009) and it is quite possible that energy reserves in the reference fish allowed for a larger range of physiological responses with which to deal with additional stressors, and therefore limiting mortality as presently observed.

Local adaptation in terms of survival within native habitats was not observed in the NB or NB-OSA fish populations upon transplant and chronic exposure. Natural bitumen/oil sands area

and natural bitumen fish populations both exhibit decreased respiration in accordance with a lowered survival. The observation and possible correlation of altered physiological measures with that of impaired survival may indicate the limited stress-coping mechanisms that these fish possess resulting from pre-exposure to stressors within their home-sites. The observations seen in the summary of all the endpoints in addition to the survivorship suggests that physiological limitations exist in fish populations pre-exposed to bituminous toxicants rather than local adaptation.

6.5 **Local Adaptation**

Local adaptation or tolerance to bituminous toxicants was not evident in the present study in any of the endpoints that were measured following the reciprocal cross transplant. The lack of local adaptation may be due to various ecological reasons such as impacts of stress, and degree of toxicant exposure or selective divergence. It has been demonstrated that stressful conditions are not conducive to local adaptation (Blanquart *et al.*, 2012; Falk *et al.*, 2012; Rolshausen *et al.*, 2015). Guppies (*Poecilia reticulata*) subjected to different crude oil exposures did not exhibit high degrees of local adaptations in the field and in laboratory transplant experiments (Rolshausen *et al.*, 2015). The lack of adaptation to crude oil for this particular species may be a result of the lack of strong divergent selection characteristic of local adaptation or that the selection pressure from toxicant exposure is both large and acts upon small ecosystems in which diversity and richness are reduced to a large enough extent to hinder occurrence of local adaptation (Rolshausen *et al.*, 2015). Other factors that may have diminished the strength of local adaptation in the present study in addition to gene flow between the three populations may include limited genetic diversity within each of the populations, lack of selection pressure on genotypes in response to variable environmental factors, lack of constraints on adaptive phenotypic plasticity, or large difference in resource types between habitats (Kawecki & Ebert, 2004).

Factors considered in the present study design were to limit the constraints of local adaptation. These factors included ensuring fish species displayed limited dispersion, and obstructed gene flow among populations. The populations were assumed to be separate because the tributaries were separated by more than 50 km, with barriers that hindered access to the main stem of the Athabasca River. Although the distances between tributaries were considered far, given the small home-range of fathead minnows and the presence of barriers, flood events may occur that could increase the chance of gene flow among the three different tributaries. However, despite the possibility of migration and gene flow, local adaptation has been previously observed to occur with moderate to high rates of gene flow within a population (Garant *et al.*, 2007; Fitzpatrick *et al.*, 2015).

Because genetic analyses in the present study was not included, the lack of local adaptation observed could not be further supported by genetic evidence. Underlying genetic information is selected for and altered only with regards to local adaptation and as such, genetic markers may be used to discern local adaptation from phenotypic plasticity (Ackerman *et al.*, 2013; Milano *et al.*, 2014; Wang *et al.*, 2015b). Examining allele frequencies at particular parts of the genome, such as single nucleotide polymorphisms (SNPs), is an example of how adaptive changes such as local adaptation can be detected through genetic analyses (Fraser *et al.*, 2011; Milano *et al.*, 2014; Fraser *et al.*, 2015). Genetic evidence for the lack of local adaptation or for genetic barriers among these populations can therefore be the next step to confirm the observations of the present transplant experiment.

7 Conclusion

The major outcome of the present transplant experiment demonstrated the lack of tolerance and local adaptation in fish populations exposed to natural and anthropogenic bituminous toxicants. Because the fish population examined did not demonstrate any local adaptation, but exhibited signs of physiological impairment, studies utilizing wild fish from uncontaminated populations are representative of wild conspecifics from the Athabasca basin chronically exposed to bituminous toxicants. Although local adaptation was not observed through comparisons of physiological responses, genetic evidence for this lack of local adaptation should be the next step to confirm the lack of adaptation before assuming such a position when assessing the ecological risk of oil sands industry.

Local adaptation did not occur among fish from the three study sites because these fish are most likely from the same population. Although the small-bodied fathead minnow is a territorial fish and unlikely to migrate more than a few tens of metres from its territory and measures were taken to ensure physical separation (as previously mentioned), genes can flow freely through demes wherever there is contiguous water and no significant barrier to reproduction. Gene flow among the three sites eliminates any possibility for local adaptation. Future studies should verify this conclusion by conducting a thorough genetic analysis on fish from each of the three sites.

Because local population adaptation was not observed in this study, fish from the two contaminated sites showed signs of physiological stress relative to those from the reference site. Moreover, fish from the natural bitumen site were equally stressed as those from the oil sands area site. The incomplete reciprocal cross-transplant design (specifically, the lack of contaminated fish transplanted in the uncontaminated site) precluded drawing conclusions on contaminant effects, *per se*. However, fish from the two contaminated sites showed reduced survival in cages regardless

of site, whereas fish from the reference site survived well at both contaminated sites. This result suggests that fish from the contaminated sites were likely suffering physiological duress owing to their long-term exposure to bituminous toxicants, compared to fish from the clean site. The simple stress of transplantation and caging was enough to reduce survival in these fish. Fish from the reference sites were much more tolerant of additional stresses related to transplanting and caging. Therefore, fish from contaminated sites show lower resilience to additional stresses compared to those from uncontaminated sites

Knowing that fish exposed to bituminous toxicants have a limited ability to cope with additional stressors can help inform and improve ecological risk assessment of oil sands development, remediation, and reclamation. The inability to endure additional stressors typical of contaminated fish in this study, can greatly affect the aquatic ecosystem. Results from this study may translate to other species—particularly, small-bodied forage fish (mainly cyprinids). Moreover, data collected using non-native fish to assess toxicity of natural and anthropogenic sources of bituminous toxicants can be translated to ecological risk assessment efforts of native fish populations in the oil sands region. By understanding the sensitivity of fish populations inhabiting contaminated environments, remediation efforts can be developed to optimize their ability to protect natural fish populations.

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9 Appendix A – Olfactory Acuity: Behavioural Assay

Olfactory acuity is an important sense for aquatic organisms to detect conspecific individuals, food, and predator events. Behaviour is used to assess the olfactory acuity at the organismal level, used to corroborate the olfactory acuity measured at the cellular level using the EOG technique with that of the physiological response. To understand the physiological response of fathead minnows to conspecific alarm cues and to bridge the olfactory acuity that was measured at the cellular level, avoidance behaviour of fathead minnows (FHM) exposed to conspecific alarm cue was assessed.

9.1 Introduction

Avoidance behaviours of fathead minnows to conspecific alarm cue were measured to provide an assessment of higher-level biological response upon exposure to the natural bitumen and downstream tributaries. Being categorised under the super-family Ostariophysan, fathead minnows produce an alarm substance when the epidermis is broken. Other individuals of the population recognise this as a predator alarm cue and will elicit an avoidance behaviour (Dew *et al.*, 2014). Behaviour bioassay was used in the present transplant experiment to assess the fish populations' chemosensory ability to detect conspecific alarm cues.

9.2 Methods

An I-maze constructed using a trough (69 cm x 14 cm x 16 cm; L x W x H) was used to determine avoidance behaviour as previously described (Dew *et al.*, 2014). Conspecific alarm cues were made fresh daily, using skin extracts prepared from fish from the same site with the same treatment water (Dew *et al.*, 2014). All behaviour trials were completed behind a visual barrier and recorded on a high pixel camera connected to a PC computer. Fish were placed into the acclimation zone and allowed to acclimatise for 20 minutes or until calm. Tubes connected to syringes were taped to either ends on the length of the maze and a 10-mL volume of control treatment water and fathead minnow alarm cue was administered at either ends. Fish avoidance behaviour was monitored and recorded every 10 seconds for 8 minutes (Dew *et al.*, 2014). The total amount of time spent in both the alarm cue and control cue arm were totalled and statistically compared as explained below.

All statistical analyses were conducted using R (version, 3.1.2, R Development Core Team, 2012). Normality and homogeneity of variance were calculated using the Shapiro-Wilk's test of normality and the Bartlett's test of homogeneity of variance, respectively. Time spent in each arm were compared using a permutation two-sample t-test, as the dataset did not meet the assumption for equal variance (Legendre, 2015).

9.3 Results

Trends toward avoidance were seen, but there were no clear signs of avoidance due to insufficient sample numbers. For this reason, comparison among the treatment sites was not further conducted. There was a slight trend toward avoidance in the reference fish measured after exposure in the natural bitumen site (Figure A15; permutation two-sample t-test, $p = 0.10$), but significant differences in the time spent in the control cue and alarm cue arms were not observed in the pre-treatment, downstream-transplanted reference fish population (Figure A15). Avoidance behaviour was observed in the natural bitumen fish populations both before and after transplant, but only the pre-treatment (permutation two-sample t-test, $p = 0.001$) behaviour data and the downstream-

transplanted group (permutation two-sampled t-test, p = 0.02) exhibited a significant difference between the control cue and the alarm cue (Figure A15b). or the entire downstream fish population (Figure A15c).

9.4 Discussion

The trends towards avoidance or attraction to alarm cue is unclear in the present reciprocal transplant since the sample sizes were insufficient to observe significant differences. Avoidance behaviour to alarm cues has been used extensively across species to monitor the effects of stress or stressors on chemosensory acuity (McPherson *et al.*, 2004; Pollock *et al.*, 2005; Sandahl *et al.*, 2006; Mirza *et al.*, 2009; Palm & Powell, 2010; Tierney *et al.*, 2010; Dew *et al.*, 2014). With increasing exposures to toxicants, impairment of chemosensory detection was demonstrated by a lack of avoidance behaviour when alarm cue was present (McPherson *et al.*, 2004; Carreau & Pyle, 2005; Ferrari *et al.*, 2006; Mirza *et al.*, 2009; Azizishirazi *et al.*, 2014). This avoidance is observed most apparently in the natural bitumen fish population, but less so in the reference and NB-OSA fish populations. With larger sample sizes, the variation observed currently may have been limited and a clearer picture drawn from the behaviour data.

Behavioural trials did not show clear trends in avoidance, but bituminous toxicants have been shown to elicit behavioural impairment. Behavioural bioassays examining the effects of water accommodated fractions of diluted bitumen on larval zebrafish (*Danio rerio*) demonstrated that 60% WAF concentrations elicited impaired shelter-seeking behaviour (Philibert *et al.*, 2016). With this knowledge, it can only be assumed that avoidance behaviour would be impaired upon exposure. Behaviour measurements is an endpoint that exhibit much large amount of variation and as such requires as an adequate number of replicates to overcome this inherent variability (Alonzo, 2015). Subsequently, the sample sizes were inadequate to overcome the variation in avoidance behaviour before and after exposure, conclusions pertaining to impairment or improvement in avoidance of alarm cues cannot be presently drawn.

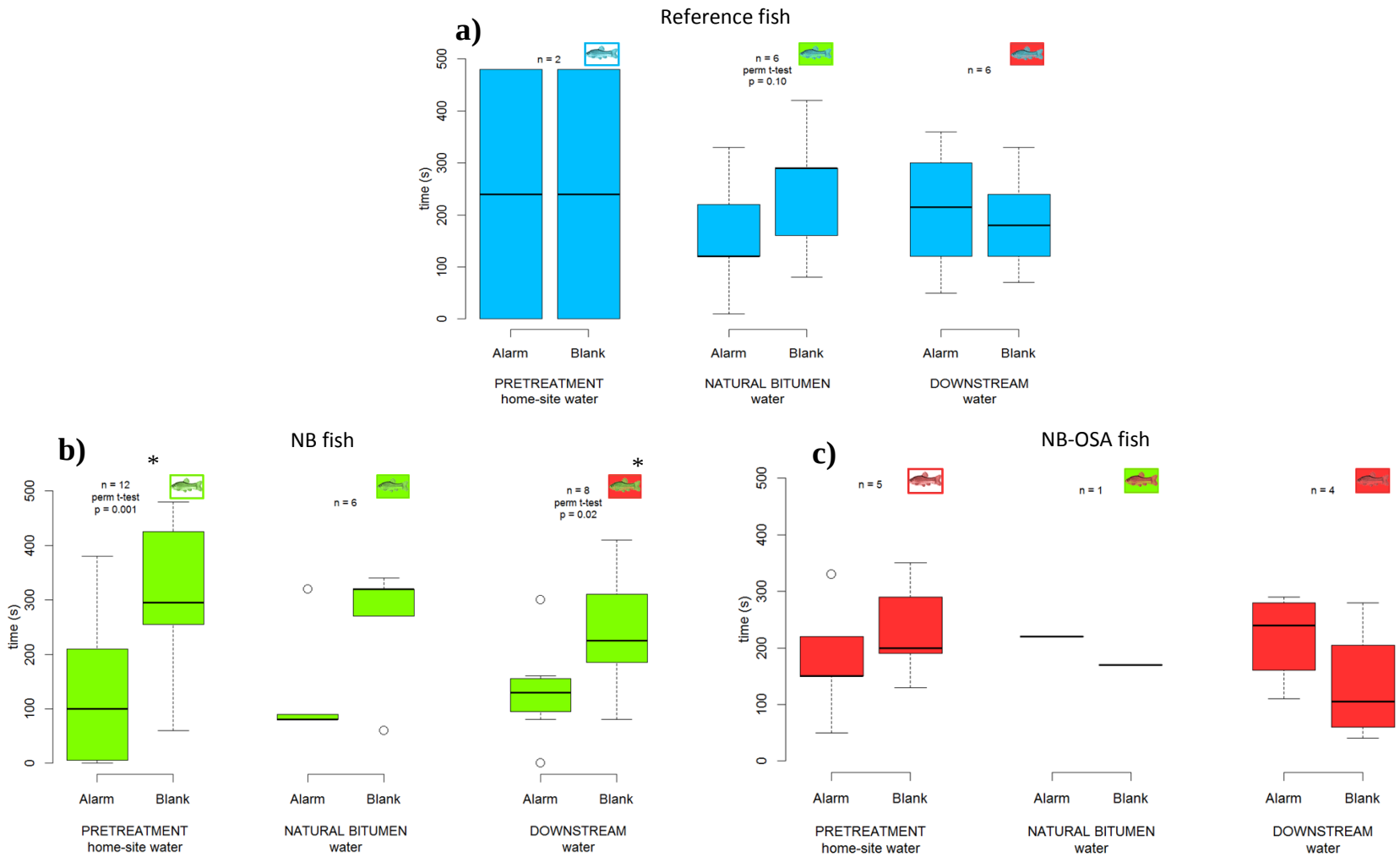

Figure A14

Avoidance behaviour bioassay results divided up by fish populations originating from the (a) reference, (b) natural bitumen (NB), and (c) natural bitumen/oil sands area (NB-OSA) tributary sites. Avoidance relationship in the reference and NB-OSA populations was weak (a, c). Avoidance response was observed only in (b) the natural bitumen fish population before transplant (permutation t-test, p = 0.001), and in the natural bitumen fish transplanted into the NB-OSA treatment water (permutation t-test, p = 0.02). Significant differences are denoted by an asterisk.

10 Appendix B – Toxicant Biotransformation

Various methods have been implemented to quantify toxicant exposure in studies examining exposure to oil sands related toxicants. The measurement of cytochrome P4501A (CYP4501A) monooxygenase enzyme activity through the induction of ethoxyresorufin-O-deethylase (EROD) serves as a biological marker of exposure to PAHs (Munkittrick *et al.*, 1995; Flammarion & Garric, 1999; Van der Oost *et al.*, 2003; Colavecchia *et al.*, 2006, 2007; Ohiozebau *et al.*, 2016). Toxicants such as PAHs act as agonists to the aryl hydrocarbon receptor which then initiates the mechanistic chain in which the CYP4501A gene is activated, the production of corresponding enzyme is induced, and the toxicant is bio-transformed into components for further transformation, degradation, and expulsion from cells (Mohammadi-Bardbori, 2014). Ethoxyresorufin-O-deethylase induction via CYP4501A activation in fish cells in the presence of PAHs has been shown to be highly indicative of contaminant presence within the aquatic environment (Whyte et al. 2000). The use of EROD as a biological marker of exposure to bituminous toxicants can therefore be an effective tool to help understand how fish will respond to different exposures of PAHs associated with natural and anthropogenic bitumen sources.

10.1 Introduction

Cytochrome P4501A activity and corresponding EROD activity have been observed to increase with increasing exposure to oil sands sourced PAHs. Cytochrome P4501A activity was observed to increase as quantified by Ah receptor-mediated activity after *in vitro* exposure of cell lines to OSPW derived NAs (Leclair *et al.*, 2015). White suckers stocked into water bodies with varying concentrations of aged OSPW exhibited elevated CYP4501A activity as measured by increased hepatic EROD activity (Arens *et al.*, 2015). Immunohistochemistry measurements of CYP4501A expression in the eye, liver, and kidney of fish were also observed to increase after exposure to tailings ponds sediments (Colavecchia *et al.*, 2007). Liver EROD activity increased as well in early life stage white suckers after having been exposed to natural and anthropogenic sources of bitumen-affected sediments (Colavecchia *et al.*, 2006).

Determining biotransformation products of PAHs is another way in which PAH exposure in fish is estimated. The level of toxicant exposure across the oil sands area can be determined through bile concentrations of bio-transformed PAH products (Ohiozebau *et al.*, 2016). Polycyclic aromatic hydrocarbon metabolite concentrations in bile increased after rainbow trout (*Oncorhynchus mykiss*) were exposed to NAs and OSPW, and is suggested that metabolite concentrations may not be a good indicator of PAH exposure as demonstrated by the lack of hepatic EROD activity measured after exposure to OSPW (Leclair *et al.*, 2013). Despite variation in concentrations of bio-transformed PAH products, previous studies indicate that EROD activity is an appropriate measure of exposure to bitumen-sourced PAHs and can help further comprehend routes/mechanisms of exposure by demonstrating activity of the cytochrome P4501A enzyme that is a product of toxicant detection and initiation of the toxicant biotransformation pathway associated with the aryl hydrocarbon receptor.

10.2 Methods

Liver samples were collected from each fish, placed within Corning^{tm} threaded cryogenic vials (Fisher Scientific), immediately frozen in liquid nitrogen, and stored in liquid nitrogen in-field for later analysis. Liver samples were then transported back to the University of Lethbridge and stored at -80°C. Fish gut and gills were removed and placed into cryogenic vials separately,

while the body was placed into sterile Whirl-Paks (Nasco). Gut, gills, and body were flash frozen on dry ice, stored at -20°C.

Exposure to PAHs was examined by studying the CYP14501A enzyme toxicant metabolism activity, which was quantified by determining ethoxyresorufin-*O*-deethylase (EROD) activity in the liver samples that were collected and stored at -80°C. Hepatic EROD activity was measured using a CYP4501A1 EROD Activity Reagent Kit and in accordance with the corresponding microplate instructions (IKZUS Research Sea Environment^{TM}, Alessandria, Italy). All samples and homogenates were kept on ice. Liver samples were defrosted on ice, and weighed, followed by addition of the kit's phosphate homogenization buffer at 100 µL per 1 mg of sample. The homogenate was then centrifuged at 10,000 RPM at 5°C for 25 minutes to obtain the post-mitochondrial supernatant (PMS, or S9 fraction). The supernatant was transferred into another centrifuge tube on ice, which was then used for EROD analysis. Ethoxyresorufin-O-deethylase activity was measured by resorufin fluorescence measured fluorometrically using a kinetic assay of 30-second intervals for 9 minutes with excitation and emission wavelengths of 535 nm and 595 nm, respectively. Protein concentrations for the samples were then analysed using the Bradford Protein Assay kit (Bio-Rad, California, USA) where protein concentrations were measured photometrically as per the Bradford method. EROD activity was calculated using the following equation:

$$EROD\ activity = \frac{average\ EROD\ rate_{sample}\ /\ mg\ protein_{sample}}{average\ EROD\ rate_{standard}\ /\ \ pmol\ resorufin_{standard}} \qquad \text{Equation (1)}$$

The denominator in equation (1) represents the fluorescence emission of 1 pmol of resorufin, which is the slope of the resorufin standard curve. EROD activity had units of pmol of resorufin/min/mg of protein.

10.3 **Results**

Toxicant exposure, as quantified by CYP1401A enzyme activity through its metabolic role in the ethoxyresorfun-*O*-deethylase biotransformation, was measured in fathead minnow liver samples before and after transplant and experimental exposure. Ethoxyresorfun-*O*-deethylase levels remained at the same levels for each of the three fish populations measured before any transplant or exposure occurred (Figure B15). Both the reference and natural bitumen fish populations exhibited negligible impairment or improvement in toxicant metabolism in either of the treatment sites. An increase in EROD activity, however, was observed in the downstream fish that were transplanted and exposed to the natural bitumen site $F_{2,42} = 3.74$, $p = 0.001$; Figure B16). Sample sizes ranged from 3 to 12. The natural bitumen fish population did not demonstrate any impairment or enhancement of toxicant metabolism after the transplant and 28-day chronic exposure, as EROD activity remained the same among the pre-treatment and post-treatment data. When compared with the pre-treatment data, EROD activity levels were elevated in the downstream fish population that were transplanted into the natural bitumen site and did not change after transplant and containment within the downstream waters ($F_{2,10} = 4.42$, $p = 0.05$). Reference and natural bitumen fish populations did not demonstrate any change in EROD levels in native or non-native sites after transplant and chronic exposure in the corresponding natural bitumen and downstream site (Figure B17).

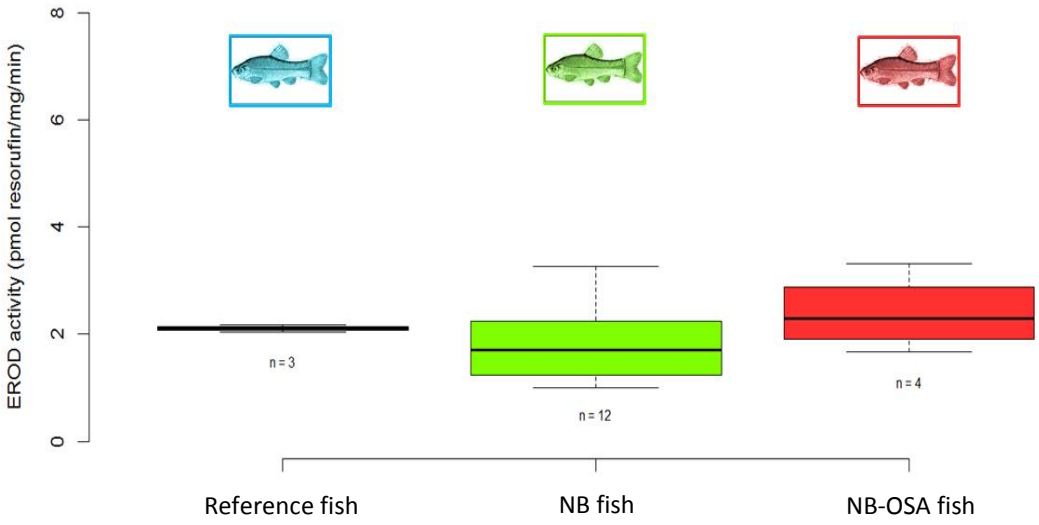

Figure B15

Pre-treatment hepatic ethoxyresorfuin-O-deethylase (EROD) activity data in reference (blue), natural bitumen (NB, green), and natural bitumen/oil sands area (NB-OSA, red) fish populations, before transplant or exposure. Sample size of the hepatic livers obtained and analysed are included under each of the graphs. Pre-treatment fish are enclosed in the same colour box and were measured in the same treatment water as their home-site. Data are represented as boxplots with the median or 50th percentile as the black bar, 75th percentile is represented as the top of the box and 25th percentile, the bottom of the box, and the upper and lower whiskers represent the maximum and minimum data points.

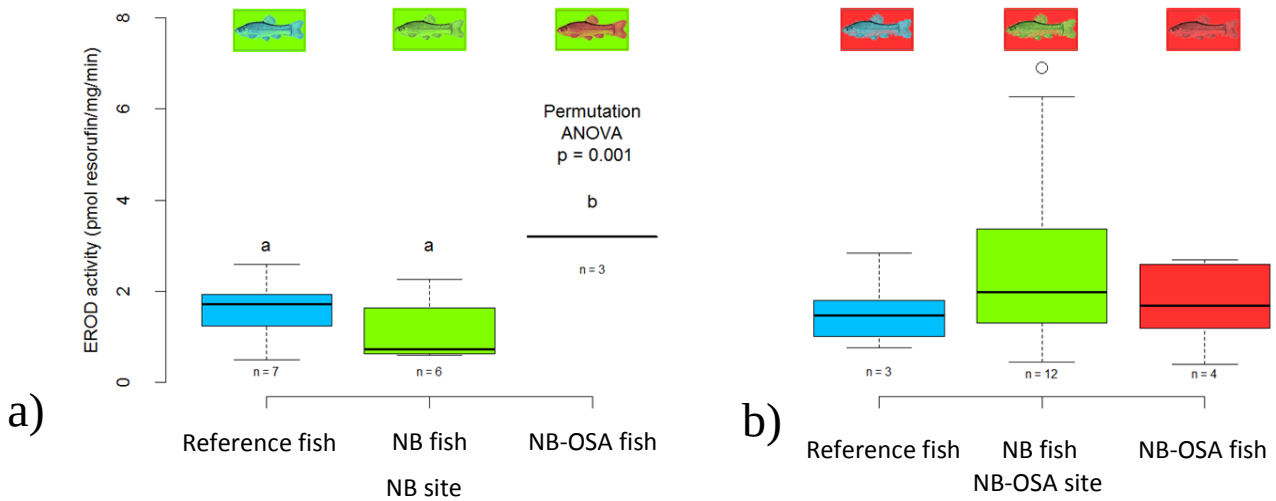

Figure B16

Hepatic EROD activity grouped by (a) natural bitumen (NB) site and (b) natural bitumen/oil sands area (NB-OSA) treatment waters, after exposure for 28 days. Differences in results are denoted with a different letter. The background colour of the rectangle the fish is in represents the treatment water that the endpoint was measured in. Boxplot details are provided in the caption of Fig. 3.

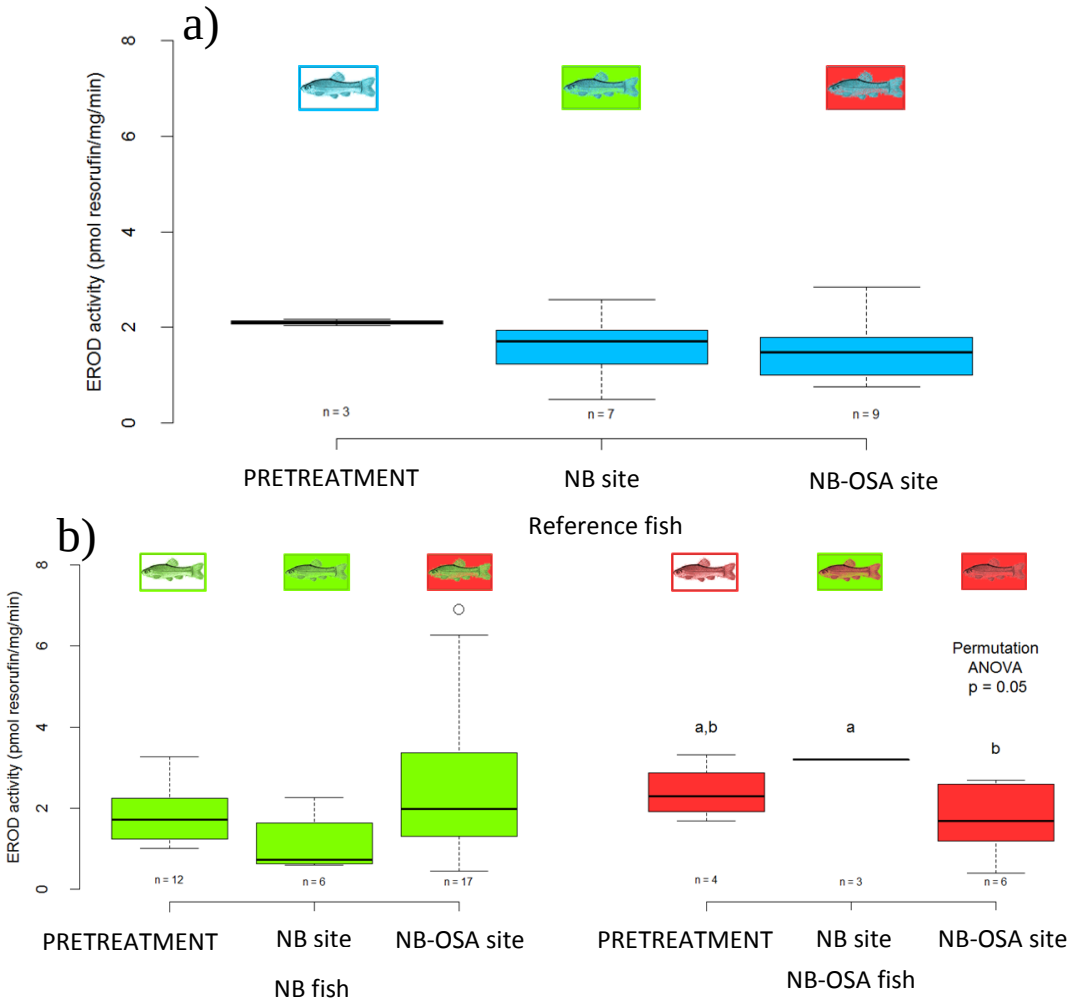

Figure B17

Hepatic EROD levels measured in the (a) reference and (b) natural bitumen (NB) and natural bitumen/oil sands area (NB-OSA) fish populations compared before and after exposure. Pre-treatment fish are enclosed in the same colour box and were measured in the same treatment water as their home-site. The background colour of the rectangle the fish is in represents the treatment water that the endpoint was measured in. Boxplot details are provided in the caption of Fig. 3.

10.4 **Discussion**

Quantification of toxicant metabolism was used to indicate the presence of biologically available xenobiotic, and as an assessment of toxicant biotransformation abilities before and after exposure. Ethoxyresorufin-*O*-deethylase has been used extensively in peer-reviewed literature as a measure of the cytochrome P4501A enzyme biotransformation activity upon exposure to xenobiotics such as PAHs in various aquatic and terrestrial organisms (Munkittrick *et al.*, 1995; Whyte *et al.*, 2000; Cruz-Martinez *et al.*, 2015). The EROD bioassay has also been used

extensively in relation to oil sands research and has been shown to increase in activity in fish exposed to xenobiotics (Munkittrick *et al.*, 1995; Van der Oost *et al.*, 2003; Sarkar *et al.*, 2006; Balk *et al.*, 2011; Mohammadi-Bardbori, 2014; Cruz-Martinez *et al.*, 2015). The measurement of EROD activity within liver samples was analysed before and after transplant in the present project to understand whether fathead minnow populations differed in their ability to metabolise xenobiotics given prior absence or presence of bitumen exposure.

Xenobiotics such as polycyclic aromatic hydrocarbons act as agonists to the aryl hydrocarbon receptor (AhR) of the cell (Fragoso *et al.*, 1998; Abrahamson *et al.*, 2007; Mohammadi-Bardbori, 2014). The binding of PAHs to AhR initiates the cytochrome P4501A gene activation chain, inducing the production of the CYP4501A enzyme subsequently used in the biotransformation of these agonistic xenobiotics thereby reducing toxicity (Figure B18; Billiard *et al.*, 2002; Billiard *et al.*, 2006; Mohammadi-Bardbori, 2014; Alharbi *et al.*, 2016). CYP4501A enzyme activity can be quantified through the reduction of ethoxyresorufin substrate to the fluorescent resorufin compound otherwise known as the ethoxyresorufin-*O*-deethylase, or EROD activity (Munkittrick *et al.*, 1993; Munkittrick *et al.*, 1995; Whyte *et al.*, 2000). With increased exposure to agonistic xenobiotics such as PAHs, CYP4501A enzyme concentrations and corresponding EROD activity increase making for a useful field and laboratory bioassay to assess exposure to toxicants such as PAHs (Whyte *et al.*, 2000; Vehniainen *et al.*, 2012; Mohammadi-Bardbori, 2014).

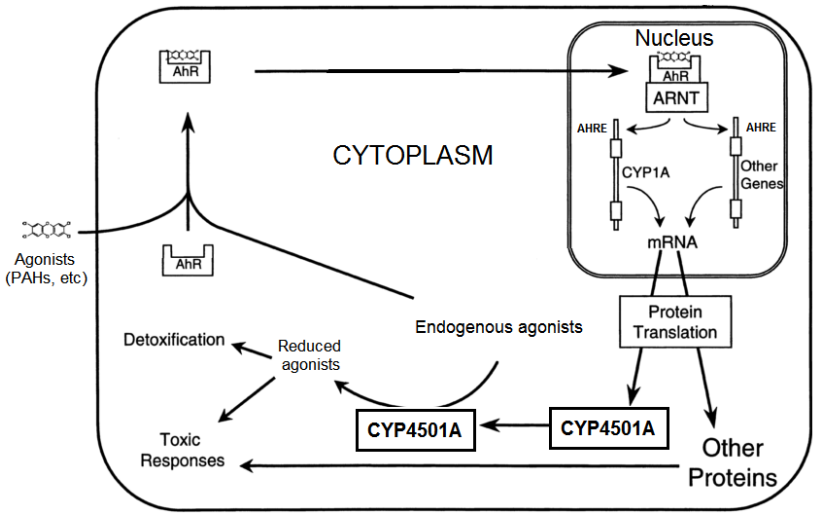

Figure B18

Activation of biotransformation enzyme, cytochrome P450-1A, within the cell. Xenobiotics such as polycyclic aromatic hydrocarbons act as agonists to the aryl hydrocarbon receptor within the cytoplasm of the cell. The AhR with PAH bound to its receptor site, undergoes further binding in the nucleus with its corresponding dimerization partner, the aryl hydrocarbon receptor nuclear translocator. The AhR-ARNT transcription factor complex binds to a specific part of the DNA, the AhR-xenobiotic responsive element (AHRE). The binding to the AHRE region initiates CYP4501A enzyme gene activation, used for reduction of the toxicant leading to further detoxification or toxic responses (Whyte *et al.*, 2000; Celander, 2011; Mohammadi-Bardbori, 2014). Diagram redrawn and adapted from Whyte *et al.* (2000) and Mohammadi-Bardbori (2014).

Cytochrome P4501A and EROD activity has been observed frequently in understanding PAH biotransformation associated with elevated alkyl PAHs in oil sands process-affected waters (OSPW), in bituminous affected habitats, and in crude oil influenced environments (Fragoso *et al.*, 1998; Van den Heuvel *et al.*, 1999a; Meyer *et al.*, 2002; Tetreault *et al.*, 2003; Colavecchia *et al.*, 2007; Turcotte *et al.*, 2011; McNeill *et al.*, 2012; Arens *et al.*, 2015). Yellow perch hepatic EROD levels were highest when sampled from experimental ponds with oil sands process-affected materials (OSPM) with activities that were 9.6-fold and 16.2-fold higher in males and females, respectively (Van den Heuvel *et al.*, 1999a). EROD levels measured in yellow perch from lakes containing natural bitumen were found to have intermediate activity (Van den Heuvel *et al.*, 1999a). Similar increases in hepatic EROD activity were observed in white suckers having experimentally transplanted into reference lakes and experimental ponds containing OSPM, with 2.2 to 4.0 times higher activity levels than natural reference lake (Arens *et al.*, 2015). Juvenile rainbow trout (*Oncorhynchus mykiss*) after having been exposed to water accommodated fraction (WAF) of crude oils displayed 60 times higher EROD activity than mineral oil and water controls (Ramachandran *et al.*, 2004). Ramachandran *et al.* (2004) also observed an increase in EROD activity relative to increasing concentrations of WAF, which elicited a decrease in activity at the highest concentration most likely due to liver damage. EROD activity after exposure to oil sands related toxicants increased in various species, however, this was not observed in the current reciprocal cross-transplant experiment.

EROD levels measured in the liver samples of reference, natural bitumen and natural bitumen/oil sands area fish populations demonstrated little to know change before or after transplant. Incongruent with current literature is the pre-treatment EROD levels that were measured, which did not change between the three different populations, as downstream and natural bitumen fish populations were expected to have higher CYP4501A activity than reference fish populations (Figure B17).

Total PAH levels determined from water and sediment samples is a good indicator of why there was a lack of CYP4501A induction in the present study. The natural bitumen tributary displayed the highest concentrations of parent and alkylated PAHs when compared to both the reference and natural bitumen/oil sands area tributaries (Table 5). Such a discrepancy may be explained by the presence of natural bitumen expulsions within this tributary (Figure B19). The presence of this higher concentration of PAHs in the natural bitumen water and sediments can explain the elevated EROD levels in downstream fish that were transplanted into the natural bitumen water (Figure B16 and B17). Certain PAHs with low molecular weights (fluoranthene and benzo[a]pyrene) have been observed

Figure B19
Release of bituminous material at the natural bitumen site, Poplar Creek near Fort McMurray, Alberta, Canada. Release of such material was observed frequently in the tributary, and were only present at this site. Photo: Sylvia Chow, 2015.

to inhibit EROD levels (Willett *et al.*, 2001; Pathiratne & Hemachandra, 2010; Traven *et al.*, 2013) and may also be an explanation for a limited CYP4501A activity in the present study as these low molecular weight PAHs were found in both the natural bitumen and downstream sites. Furthermore, the lack of EROD induction may be explained by the fact that exposure occurred during the late summer periods, where streamflow was not as high resulting in an inconsistent concentration of toxicants within the site. Concentrations of PAHs have been shown to change with fluvial events such as high floods during spring freshets and this can be an explanation for the lack of EROD induction observed in the fish populations before transplant (Timoney & Lee, 2011; Jautzy *et al.*, 2015; Elmes *et al.*, 2016).

The lack of EROD activity measured in the reference fish population after exposure may be explained by variables such as specificity of AhR receptors, agonist mixtures effect, and species and individual differences in CYP4501A induction (Fragoso *et al.*, 1998; Alharbi *et al.*, 2016). Firstly, differences in the affinities of various agonists of AhR will encourage variation in the amount of CYP4501A protein produced and a corresponding change in EROD level (Hahn & Stegeman, 1994; Fragoso *et al.*, 1998). Induction of EROD activity was 2 to 200 times lower when measured in trout exposed to retene than to dioxins, and is thought to be due to the binding affinities of the latter agonist and its ability to activate AhR (Fragoso *et al.*, 1998; Durieux *et al.*, 2012). As such, fish populations from the three tributaries may exhibit differences in EROD levels due to the decreased or increased activation affinities of the different PAHs at the two treatment sites. Secondly, the presence of different persistent organic pollutants and other water quality factors may further encourage or inhibit binding affinity to AhR (Billiard *et al.*, 2002; Billiard *et al.*, 2006; Bittner *et al.*, 2009; Gauthier *et al.*, 2014; Alharbi *et al.*, 2016). Co-exposures of retene (a pyrogenic PAH) with OSPW elicited an increase in the expression of CYP4501A protein and increased pericardial edema when exposed to the mixture, rather than when exposed to the toxicants singularly (Alharbi *et al.*, 2016). Inhibition of EROD activity has also been shown to occur in the presence of metals, dioxins, and other chemicals (Ortiz-Delgado *et al.*, 2008; Durieux *et al.*, 2012). With exposure to estrogenic compounds, CYP4501A activity and EROD levels demonstrate inhibited activity after exposure activity inducing compounds (Cionna *et al.*, 2006; Kirby *et al.*, 2007; Spearow *et al.*, 2011). Lastly, species differences and the naivety of individuals to toxicants may play a role in the EROD levels that presently observed (Vehniainen *et al.*, 2012; Ohiozebau *et al.*, 2016). Post-microsomal supernatant of eight fish species exhibited differences in measured EROD levels as a result of natural variations that occur among species and among individual fish (Vehniainen *et al.*, 2012). Ohiozebau *et al.* (2016) measured products of PAH biotransformation in fish across trophic levels and from different regions in the oil sands region and found that fish of lower trophic levels exhibited higher amounts of products of biotransformation where such products were found highest in goldeye (*Hiodon alosoides*). Differences existing in fish at different trophic levels is evident when examining different trophic levels, and may be dependent on the method and the species that is used to determine toxicant exposure (Ohiozebau *et al.*, 2016). Accounting for differences at the individual and among species level along with the influence of mixtures effects and differing affinities of PAHs, the unresponsiveness of EROD levels in reference fish populations may be explained.

After having been exposed to the treatment waters for 28 days after transplant, EROD levels were found to be the same as that of the pre-treatment data in all but one treatment (Figure B17). The lack of EROD level may be a result of the prolonged exposure and inadequate hepatic sampling regime. Constant exposure to pulp mill effluent demonstrated elevated EROD levels

throughout an exposure of 32 days (Fragoso *et al.*, 1998). However, EROD levels have been measured to peak after the first few hours or days of exposure to PAHs (Yuen & Au, 2006; Abrahamson *et al.*, 2007). With constant exposure to AhR agonists from pulp mill effluent, CYP4501A enzyme activity was continuously induced in rainbow trout (*Oncorhynchus mykiss*) hepatic samples, but decreased to levels that were comparable to control EROD levels after 4 days of exposure (Fragoso *et al.*, 1998). Slimy sculpin (*Cottus cognatus*) exposed in the laboratory to sediments sampled from various locations of the Steepbank River displayed significantly higher hepatic EROD activity after exposure to development-influenced sediments for 4 days (Tetreault *et al.*, 2003). However, hepatic EROD levels decreased after the four days (Tetreault *et al.*, 2003). Similar trends in decreases in EROD induction have been observed in various species after a certain amount of exposure time (Munkittrick *et al.*, 1995; Arcand-Hoy & Metcalfe, 1999) and this may be the case for the present pre-treatment observations, where natural bitumen fish populations exposed to constant levels of PAHs will acquire a lowered level of EROD activity. As the liver samples were collected at the end of 28 days, rather than a few days after exposure, elevation of EROD levels may not have been captured in the current study design.

Ethoxyresorufin-O-deethylase levels measured before and after reciprocal transplant did not agree completely with peer-reviewed literature. There was no clear evidence of local adaptation and evidence for toxicant biotransformation is unclear. Bioassay activity and CYP4501A enzyme induction may be better suited to understand immediate toxicant exposure or a different sampling regime that include seasonal and site variations. A different measurement of toxicant exposure such as metabolite concentration (Beyer *et al.*, 2010), gene expression (Alharbi *et al.*, 2016) and protein concentrations (Durieux *et al.*, 2012) may be an improvement in measuring exposure in fish species that have resided within environments subjected to constant exposure to xenobiotics. Current EROD data may be affected by the presence of confounding factors as mentioned above, which should be considered in future reciprocal transplant studies examining toxicant biotransformation.