Ecotoxicology and Environmental Safety 84 (2012) 254–261

Contents lists available at SciVerse ScienceDirect

Ecotoxicology and Environmental Safety

journal homepage: www.elsevier.com/locate/ecoenv

Immunological impacts of oil sands-affected waters on rainbow trout evaluated using an in situ exposure

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ARTICLE INFO

Article history:
Received 29 May 2012
Received in revised form
11 July 2012
Accepted 12 July 2012
Available online 9 August 2012

Keywords:
Fish
Immune
Oil sands
Polycyclic aromatic hydrocarbon
Naphthenic acids
Antibody
Leukocyte
In situ

ABSTRACT

Rainbow trout were exposed in situ to oil sands-affected waters for 21 d, either with or without an immune stimulation using inactivated *Aeromonas salmonicida*. Three aquatic systems were utilized for the experiment: a pond containing oil sands tailings capped with approximately 3 m of natural surface water, a second pond where unextracted oil sands materials were deposited in the watershed, and a reservoir receiving Athabasca River water as a reference caging location. The three systems showed a gradient of oil sands-related compounds, most notably, total naphthenic acids were highest in the system containing tailings (13 mg/L), followed by the system influenced by unextracted oil sands (4 mg/L), followed by the reference cage location (1 mg/L). Biochemical and chemical measures of exposure in rainbow trout showed the same trend, with the tailings-influenced system having the highest hepatic EROD activity and elevated bile fluorescence measured at phenanthrene wavelengths. Trout caged in the tailings-influenced location had significantly fewer leukocytes and smaller spleens as compared to the reference fish, though liver size and condition factor were unaffected. Fish in the tailings-influenced waters also demonstrated increased fin erosion, indicative of opportunistic infection. The trout exposed to tailing-influenced waters also showed a significantly decreased ability to produce antibodies to the inactivated *A. salmonicida*. Given the complexity of the exposure conditions, exact causative agents could not be determined, however, naphthenic acids, polycyclic aromatic hydrocarbons and pH correlate with the immunotoxic effects while elevated salinity or metals seem unlikely causes.

1. Introduction

While the immune system of fishes is fundamental to survival, the impact of environmental contamination on immunological function has received relatively little attention. This is despite increasing occurrence of opportunistic disease being widely recognized as an indicator of polluted environments (Noga, 2000). Disease in the form of fin erosion has been observed to be associated with a wide array of pollution sources including pulp and paper (Khan, 2006; Lindesjöö and Thulin, 1990), municipal effluents (Cross, 1984), PCB and metal contamination (McCain et al., 1992), and petroleum (Moles and Norcross, 1998). Viral disease triggered by chemical contamination was also suggested as the likely cause of the pacific herring collapse after the Exxon Valdez oil spill (Marty et al., 1998). An increased incidence of fin erosion and viral lesions was previously observed in yellow perch exposed to oil sands-affected waters in experimental systems (van den Heuvel et al., 2000). There are also anecdotal claims by subsistence fisherman on the Athabasca River of elevated levels of lesions on fishes potentially associated with oil sands activities (Tenenbaum, 2009).

The oil sands of Northern Alberta has disturbed over 600 km^2 of boreal forest (Royal Society of Canada, 2010). Various industry and government data indicate that it requires from 2 to 4.5 barrels of water to produce one barrel of oil from the oil sands. This water, largely used in the extraction process, will become contaminated with a suite of oil sands-related chemicals including naphthenic acids (cyclic and acyclic saturate carboxylic acids), polycyclic aromatic hydrocarbons, and elevated levels of major ions (sodium, sulphate, bicarbonate and chloride; Royal Society of Canada, 2010). While the industry currently operates under a zero discharge policy, this process-affected water and tailings must ultimately be incorporated back into the landscape. Thus, the exposure of aquatic biota to some level of oil sands related chemical contamination is inevitable.

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http://dx.doi.org/10.1016/j.ecoenv.2012.07.016

Recently, attention has also been drawn to the potential for aquatic contamination from atmospheric sources (Kelly et al., 2010).

This study forms part of a larger research program designed to link the previous observations of oil sands-associated opportunistic diseases in wild fishes to a laboratory model. In this context, a caging study is able to incorporate the same exposure regime as previous studies with wild fish and utilize a model species in order to validate the use of that species as a surrogate. A validated laboratory model would then be used for hypothesis-driven examination of causative agents, and determining thresholds of chemical contaminants for immunological effects. In situ exposures using fishes represent an excellent linkage between the laboratory and the field in that laboratory model species can be used in a field setting. In this case, the rainbow trout, for which a significant number of immunological tools exist, can be adapted to measure responses that are not yet possible to evaluate with wild species. In an immunological context, using a species that has adapted in culture to tolerate stress due to handling is also beneficial. Two other principle advantages of in situ techniques are the certain knowledge of the location and duration of exposure that cannot always be ascertained with wild fishes (Oikari 2006).

The hypothesis of this research was that exposure to oil sands-affected waters would cause impaired immune response in rainbow trout. This hypothesis was examined through in situ exposure of rainbow trout for 21 d in three bodies of water with varying levels of oil sands-related chemical contamination. Trout were co-exposed to pathogen-associated molecular patterns (PAMPs) via an injection of formalin-inactivated *Aeromonas salmonicida* (*A. salmonicida*) or a phosphate buffered saline (PBS) control. Leukocyte counts, organ size, cortisol, and the ability to form antibodies in response to the antigen challenge were the primary immune endpoints. Water chemistry, hepatic CYP1A activity and bile fluorescence were evaluated to characterize exposure.

2. Materials and methods

2.1. Experimental design

Three water bodies, Demonstration Pond, South Bison Pond, and Mildred Lake were used for the caging experiments in this study. The Demonstration Pond was constructed in 1993 and is a 3 ha pond constructed at the Syncrude Canada Ltd. oil sands lease located on the Athabasca oil sands deposit north of Fort McMurray, Alberta. The pond contains 70,000 m^3 of tailings capped with 70,000 m^3 of natural surface water to a depth of 2.9 m above the tailings–water interface. This pond is a small-scale version of an 'end-pit' lake proposed as a reclamation option (FTFC, 1995). The second experimental pond, the South Bison Pond, is a 6 ha pond formed in 1987 as a drainage basin in an area designated to be reclaimed as bison pasture on the south portion of the oil sands lease. The basin, and the area surrounding the pond, received overburden (any material removed from above the oil sands deposit) and lean oil sands not suitable for extraction. The clays within the redeposited material were saline/sodic in nature and contained bituminous material. Surface capping of the surrounding basin with a peat/soil mixture, and planting of pasture in the area surrounding the South Bison Pond was completed in 1996. Mildred Lake is a modified natural lake, located on the Syncrude Canada Ltd. mining lease. Beginning in 1975, Mildred Lake (151 ha) was modified from its natural state into a reservoir for extraction plant water pumped from the Athabasca River. Rainbow trout were caged in the three water bodies for 21 d. At the initiation of the experiment, trout received an injection of either formalin-inactivated *A. salmonicida*, or PBS. All trout were sacrificed at 21 d.

2.2. Fish caging and sampling

Rainbow trout, mean (SEM) length 17.7 (0.2) cm and weight 65.1 (1.6) g, were obtained from Ackenberry Trout Farm in Camrose, Alberta. Trout were an all female strain derived from Trout Lodge (Sumner, WA, USA) strain eggs. Fish were transported from Camrose to the caging locations north of Fort McMurray using an aerated tanker truck (approximately 5 h travel), whereupon fish were immediately placed in the experimental cages in the three water bodies on August 20, 2010. Cages were constructed using a wood frame with a hinged lid. The length, width and height of the cage below water was 1.44 m for a total volume of 3.0 m^3. The stocking density of trout in the cages was 1.4 kg/m^3 (66 trout per cage). Cage mesh was 6.4 mm plastic Vexar mesh fastened to the frame with fencing staples. An additional layer of chicken wire was fastened over the Vexar mesh as a predator deterrent. The cages were supported on all sides by modular floating docks (CanDock, Sherbrooke, Canada). This support was linked to shore by a floating dock extension to allow sufficient depth such that the cage floor was above the bottom substrate. This supporting dock structure was fastened to shore using steel stakes, and was also anchored on all four corners. Trout were fed ad libitum with commercial trout food every second day.

Rainbow trout were injected with either inactivated *A. salmonicida* at a dose of 10^9 particles/kg, or with PBS on August 23rd and 24th, 2010. Treatments were differentiated by the use of two colors visual implant elastomer tags (Northwest Marine Technologies, Seattle, USA) implanted in the transparent skin approximately 2 mm posterior of the eye. Trout were sampled on September 13th and 14th in the same site order that they were injected initially. A total of 16 trout were removed from the cage and bled immediately (approximately 5–10 min) from the caudal vein using a 3 mL syringe with a 2.5 cm, 23 gauge needle for cortisol analysis. Those trout were placed on ice, and the remainder of sampling conducted at the conclusion of bleeding. The remaining trout were sampled sequentially. Blood was immediately placed in either a heparinized microtainer (BD) to obtain plasma for cortisol analysis, or an unheparinized microtainer for serum for antibody determination. A dilution of heparanized blood was made using Dacie's solution for total leukocyte counting, and a standard blood smear was made for differential leukocyte counts.

Trout were sacrificed by a blow to the head, weighted, fork length recorded, and inspected for any external abnormalities or lesions. An index for fin erosion was recorded for each fish. The index used a ranking between 0 and 4, with 0 representing perfect fins with no signs of erosion, 1 representing fins with damage or erosion that had previously healed, 2 representing minor active erosion restricted to one fin, typically the caudal fin, 3 representing substantial active erosion on most or all fins, and a 4 indicating severe fin erosion with protruding cartilage and at least 50% of the caudal fin area destroyed. Liver and spleen were weighed and liver, gall bladder, spleen and head kidney were snap frozen in liquid nitrogen and ultimately transferred to -80 °C pending analysis.

2.3. Water chemistry

Temperature, dissolved oxygen, conductivity and pH was recorded at the bottom of the trout cages every second day throughout the experimental exposure using a YSI MDS 650 multi-parameter water quality meter equipped with a model 600QS sonde (YSI Inc. Yellow Springs, OH, USA). Total naphthenic acids in water were quantified by Fourier transform infrared spectroscopy following dichloromethane extraction of water (Jivraj et al., 1995) as described by Holowenko et al. (2001). Due to only one to three naphthenic water chemistry samples collected each year, 2008–2010 data was pooled in order to increase sample size and better reflect natural variability.

2.4. Bile metabolites

Bile samples were diluted by a factor of 100 in distilled water then filtered via 13 mm polypropylene syringe filters (0.45 μm pore size; Pall, Ville St. Laurent, Canada) into glass autosampler vials. To perform the analysis, a Varian Prostar model 240 HPLC pump, a model 410 autosampler, and a model 363 fluorescence detector were employed. Separations were conducted with a 150 × 4.6 mm Varian Microsob-MV C18 column at a flow rate of 1 mL per minute at 35 °C column temperature. Solvent elution profile started at 5% acetonitrile (Caledon Laboratories, Georgetown, Canada) and 95% HPLC grade water (Caledon). These ratios were changed to 98% acetonitrile and 2% water gradually over a 25 min period with this ratio being held until the end of the run (5 min). Excitation and emission wavelengths used were that of phenanthrene (256 nm and 380 nm, respectively). Phenanthrene wavelengths were chosen because previous studied had shown the oil sands bile metabolite signature to be strongest at those wavelengths (van den Heuvel et al., 1999b). The PAH equivalent concentration was then derived by summing the area of all metabolite peaks that eluted after the first 3 min of the run and dividing by the slope of a phenanthrene (Sigma) standard curve.

2.5. 7-Ethoxyresorufin-O-deethylase

Hepatic mixed function oxygenase (MFO) enzyme activity was estimated in post-mitochondrial supernatant (PMS). EROD activity was determined using a modification of the fluorescence platereader technique outlined by van den Heuvel et al. (1995). Livers were homogenized in a cryopreservative buffer (0.1 M phosphate, 1 m MEDTA, 1 mM dithiothreitol, and 20% glycerol, pH 7.4) and centrifuged at 9000 × g to obtain the PMS. The EROD reaction mixture contained 0.1 M HEPES buffer pH 7.8 (Sigma, St. Louis, MO, USA), 5.0 mM Mg_2+, 0.5 mM NADPH (Sigma), 1.5 M 7-ethoxyresorufin (Sigma) and about 0.5 mg/mL of PMS protein. EROD activity was determined as a final endpoint after reaction

termination with acetonitrile on a fluorescence plate reader (Bio-Tek FLx800) with 528-nm excitation and 590-nm emission filters. Protein content was estimated from fluorescamine fluorescence (390-nm excitation, 460-nm emission filters) against bovine serum albumin standard (Sigma).

2.6. Cortisol

Cortisol was measured in duplicate according to the radioimmunoassay methods of McMaster et al. (1992). Plasma samples were thawed and steroid hormones were extracted with diethyl ether. Cortisol was obtained from Sigma and purified cortisol antibody from Acris (Germany). Tritiated cortisol was obtained from GE healthcare products (Amersham, Buckingshire, UK).

2.7. Total and differential leukocyte counts

Total leukocyte counts were made within 24 h of sampling. Leukocyes in whole blood diluted in modified Dacie's solution were counted in duplicate using an improved Neubauer hemocytometer and leukocyte number was calculated as cells per mL of whole blood. For differential white blood cell counts, blood smears were made with whole blood directly from the syringe dropped onto slides and allowed to air dry. Slides were fixed in methanol for 10 min and stained with May-Grunwald-Giemsa stain for differential white blood cell counts (minimum of 100 leukocytes counted), which were expressed as percentage of total cells counted.

2.8. A. salmonicida antibody ELISA

ELISA for the detection of *A. salmonicida*-specific antibodies in trout serum were performed according to methods adapted from Köllner and Kotterba (2002). ELISA plates were coated with inactivated *A. salmonicida* at 4 °C overnight. Plates were blocked with protein free blocking buffer (Pierce, Rockford, IL, USA) for 1 h at 20 °C, washed three times with a PBS and 0.05% tween washing buffer. Single samples of trout sera from all experimental fish were diluted 1:10 in washing buffer and subsequent 1:1 dilutions were made for a 8-point antibody titer. Non-specific adsorption was prevented by competitive blocking of fish sera with 5% skim milk according to methods of Kim et al. (2007). Diluted sera were incubated for 1 h at 4 °C and washed three times with washing buffer. To detect bound trout antibodies the plates were incubated for 1 h at 4 °C with monoclonal mouse–anti trout immunoglobulin M antibody (mab 4C10). After washing three times with PBS/T plates were incubated with goat anti-mouse IgG/IgM-peroxidase conjugate (Pierce, Germany) for 1 h at 4 °C. Plates were washed another three times and developed using SigmaFAST OPD substrate (Sigma) for 30 min. Reaction was stopped using 3 N HCl and plates were read using an ELISA micro-plate reader at 490 nm. The antibody titer was determined as being the first dilution with an absorbance greater than twice the mean skim milk-blocked blank value. The relative quantity of non-specifically binding IgM was evaluated by subtracting a non-skim milk-blocked blank (non-specific binding) from a skim milk-blocked serum control.

2.9. Statistics

Fin erosion indices were evaluated by a non-parametric means test. All other statistics were evaluated using analysis of variance following testing for normality (normal probability curves) and homogeneity of variance (Levene's and Brown-Forsythe tests) with appropriate transformations where those assumptions were not met. Body weight, liver weight and spleen weight were analyzed using analysis of covariance (ANCOVA) with logarithmically transformed values using length (weight) or body weight (liver, spleen), plus the categorical treatment variables. Somatic data were expressed as indices for presentation purposes using the least square means and covariate means from the ANCOVA. For exposure indicators and immune parameters a full factorial design comprised of the exposure pond and *A. salmonicida* challenge was evaluated using ANOVA. The Demonstration and Bison Pond groups were subsequently compared to the Mildred Lake reference group using Dunnett's post-hoc test. For leukocytes, the percentage of each population was expressed as a proportion of the total identified leukocyte population only, thus factoring out the variable effects of contamination with other cells types such as erythrocytes. For leukocyte number in blood, percentages of differential counts were multiplied by the total white blood cells counts to obtain absolute counts of each sub-population. All statistics were performed in STATISTICA version 8.0 using an experiment-wise alpha of 0.05.

3. Results

3.1. Water chemistry

The experimental water bodies reflected a gradient of oil sands contamination (Table 1). Mildred Lake was generally the least impacted as measured primarily by conductivity and naphthenic acids, followed by the South Bison Pond and the Demonstration Pond. While the Demonstration Pond had higher levels of naphthenic acids, the conductivity was similar to the Bison Pond. Despite similar conductivities, the major ions in the ponds were substantially different as Bison Pond was dominated by sodium sulphate, with chloride being a less prominent anion, whereas the Demonstration Pond was dominated by sodium chloride and sodium bicarbonate/carbonate (see van den Heuvel et al., 2012). The influence of the latter ion was apparent in the pH, for which Demonstration Pond often exceeded 9.5.

Chemical and biochemical indicators of exposure were elevated in the Bison and Demonstration Ponds. Bile phenanthrene equivalent concentration was two-fold and six-fold increased over Mildred Lake in the Bison Pond and Demonstration Pond, respectively (Fig. 1A). Only the Demonstration Pond bile fluorescence was significantly elevated over the Mildred Lake bile metabolite values. Liver hepatic EROD activity was two-fold and three-fold increased over Mildred Lake in the Bison Pond and Demonstration Pond, respectively (Fig. 1B). Both the Demonstration Pond and Bison Pond EROD activity was significantly induced as compared to Mildred Lake trout EROD activity.

3.2. Mortality, external lesions and physiological condition

There was only a single mortality in the Mildred Lake caged fish (1.5%), two mortalities in the Bison Pond cage (3%), and eight mortalities in the Demonstration Pond group (14%) during the 21 d exposure period. Mortalities in the Demonstration Pond were often characterized by opaque or bulging eyes. Upon sampling, the Demonstration Pond trout also showed increased levels of external lesions in the form of active fin erosion (Table 2). The Demonstration Pond fin erosion index was significantly higher than Mildred Lake. There was no statistically significant effect of *A. salmonicida* injection on fin erosion index (data not shown). There was no statistically significant difference in weight as it covaries with length between the *A. salmonicida* and PBS groups, nor between the exposure groups (Table 2). While fish were fed every second day, examination of stomachs upon necropsy revealed that most trout were also eating invertebrates at all three caging locations. There was a statistically significant interaction between *A. salmonicida* injection and exposure groups for liver size as it covaries with weight (Table 2). The liver size in the Bison Pond trout increased with *A. salmonicida* injection, the liver size in Mildred Lake trout remained stable, whereas the Demonstration Pond trout liver size decreased with *A. salmonicida* injection. Spleen size as it covaries with weight was

Table 1
Water quality characteristics (SEM, *n*).

Cage location	Temperature (°C)	Conductivity (μS/cm)	Dissolved oxygen (%)	pH	Naphthenic acids (mg/L)
Mildred Lake	16.0 (0.4, 10)	300 (1, 7)	97.0 (1.0, 7)	8.18 (0.01, 7)	1.0 (0.2, 7)
South Bison Pond	15.4 (0.4, 10)	1818 (3, 7)	75.4 (3.1, 7)	8.03 (0.03, 7)	4.6 (0.4, 5)
Demonstration Pond	15.3 (0.5, 10)	1692 (10, 8)	110.0 (2.5, 8)	9.55 (0.03, 8)	13.0 (0.4, 8)

found to be significantly lower in the Demonstration Pond than Mildred Lake with no effect of *A. salmonicida*.

3.3. Immunological endpoints and cortisol

There was an overall decrease in total leukocyte counts in the Demonstration Pond as compared to Mildred Lake (Fig. 2). There was no significant ANOVA main effect due to *A. salmonicida* injection, indicating that *A. salmonicida* did not stimulate leukocyte numbers after 21 d. The same pattern of exposure water-related effects was observed for lymphocytes as these represent the majority of the leukocyte population (Table 3). There was a statistically significant elevation in Bison Pond trout thrombocytes counts as compared to Mildred Lake, but no *A. salmonicida* effect. Granulocytes were elevated in the Demonstration Pond as compared to Mildred Lake, but again there were no *A. salmonicida* effects. There were no exposure group or *A. salmonicida*-mediated effects on monocytes. Analysis of plasma cortisol showed statistically significant effects of both cage location and *A. salmonicida* injection (Fig. 3). However, when *A. salmonicida* groups were evaluated separately, only the PBS group showed a statistically significant elevation of cortisol levels in the Demonstration Pond group (Fig. 3). Evaluation of cortisol concentrations as compared to the order of fish sampling revealed no correlation, indicating that fish were bled before the onset of the cortisol response.

Demonstration Pond trout had significantly lower levels of serum *A. salmonicida* antibodies as compared to Mildred Lake, while Mildred Lake and Bison Pond showed similar antibody titers (Fig. 4). The mean (SEM, *n*) relative quantity of non-specifically binding IgM (no skim milk treatment of samples) as indicated by the optical density at 490 nm was 0.28 (0.07, 19), 0.23 (0.08, 19), 0.11 (0.03, 18) for Mildred Lake, Bison Pond, and Demonstration Pond trout, respectively. While these data followed the same pattern as *A. salmonicida* antibody production, there were no statistical differences. There were no statistically significant correlations between A.s. antibody production and either cortisol, or total leukocyte count.

4. Discussion

Rainbow trout caged in oil sands-affected waters showed the initial stages of opportunistic disease as indicated by increased fin erosion and an increase in granulocytes in blood. That disease was

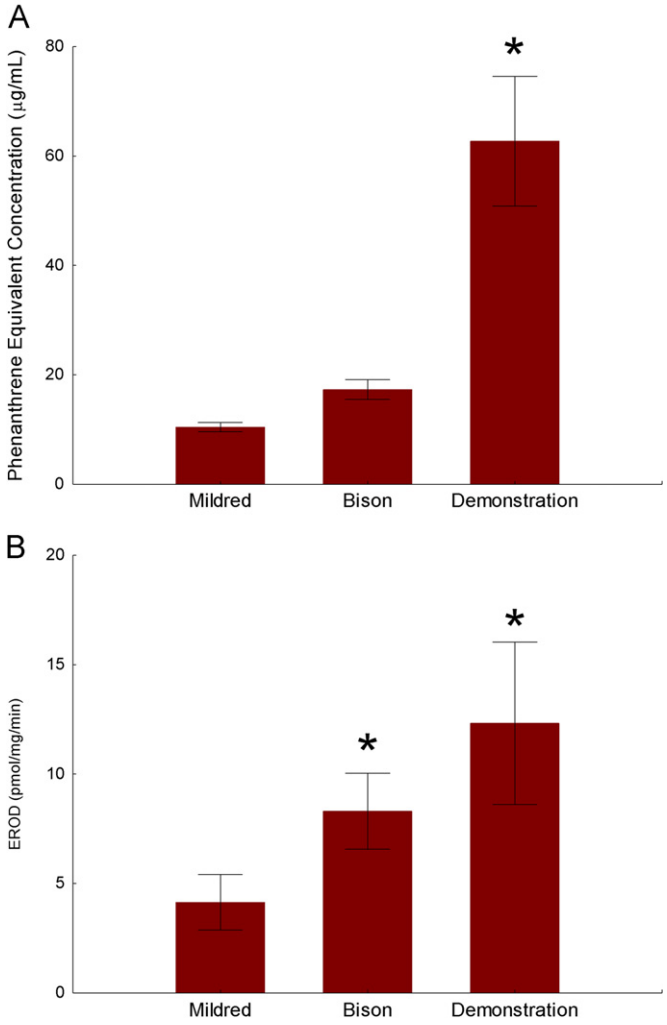

Fig. 1. Mean (A) biliarly phenanthrene equivalent concentration as determined by HPLC-fluorescence, and (B) hepatic 7-ethoxyresorufin-O-deethylase activity in rainbow trout caged at three oil sands locations for 21 d. Asterisks indicates statistically significant difference at $p < 0.05$. Error bars are the standard errors of the means.

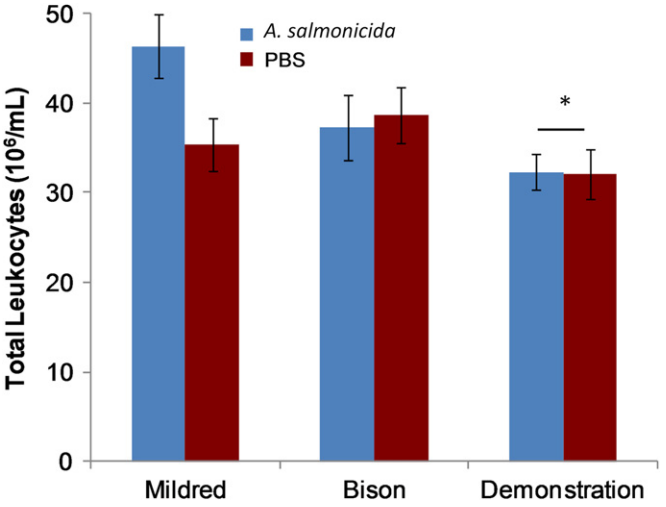

Fig. 2. Mean total leukocyte count for rainbow trout caged at three oil sands location and injected with either inactivated *A. salmonicida*, or phosphate buffered saline (PBS). Asterisks indicate statistically significant difference at $p < 0.05$ for the pond water exposure as compared to the Mildred Lake group. Error bars are the standard errors of the means.

Table 2
Mean (SEM, *n*) rainbow trout fin erosion index and somatic indices.

Cage location	Fin erosion index	Condition factor	Liver somatic index	Spleen somatic index
Mildred Lake	0.53 (0.09, 40)	1.13 (0.02, 40)	1.29 (0.05, 40)	0.180 (0.010, 40)
South Bison Pond	0.63 (0.09, 40)	1.15 (0.02, 40)	1.28 (0.05, 40)	0.166 (0.09, 39)
Demonstration Pond	1.33 (0.10, 40)*	1.15 (0.02, 39)	1.12 (0.05, 39)	0.147 (0.008, 39)*

* Significant difference from the Mildred Lake reference caged trout at $p < 0.05$.

Table 3
Mean (SEM., *n*) differential leukocytes counts. All values are in millions of cells per mL of blood.

Exposure	Challenge	Lymphocytes	Thrombocytes	Granulocytes	Monocytes
Mildred Lake	PBS	32.6 (3.0, 19)	1.1 (0.5, 19)	1.9 (0.5, 19)	0.4 (0.1, 19)
	A.s.	42.7 (3.5, 19)	1.8 (0.6, 19)	1.3 (0.3, 19)	0.6 (0.3, 19)
South Bison Pond	PBS	33.8 (3.1, 17)	2.7 (0.7, 17)*	1.6 (0.6, 17)	0.5 (0.2, 19)
	A.s.	32.9 (2.9, 19)	3.5 (0.9, 19)*	0.8 (0.2, 19)	0.1 (0.1, 19)
Demonstration Pond	PBS	27.4 (2.1, 17)*	2.3 (0.8, 17)	1.9 (0.5, 17)*	0.4 (0.2, 17)
	A.s.	27.5 (2.0, 17)*	1.5 (0.5, 17)	2.8 (0.6, 17)*	0.6 (0.2, 17)

*Statistically significant difference in exposure site as compared to Mildred Lake.

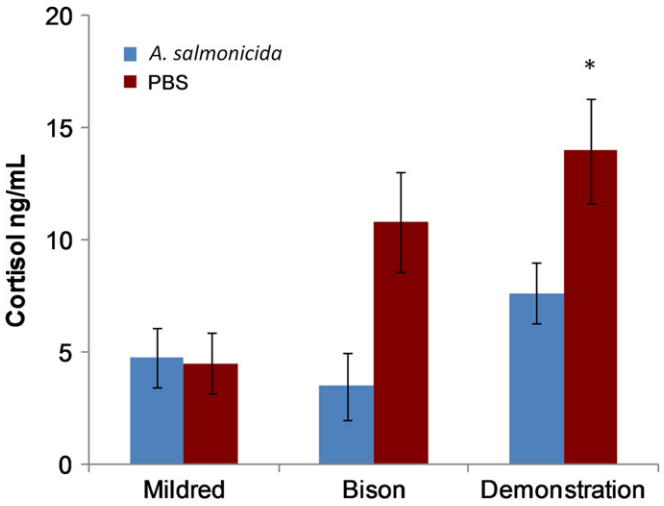

Fig. 3. Mean plasma cortisol levels for rainbow trout caged at three oil sands location and injected with either inactivated *A. salmonicida*, or phosphate buffered saline (PBS). Asterisks indicates statistically significant at $p < 0.05$. Error bars are the standard errors of the means.

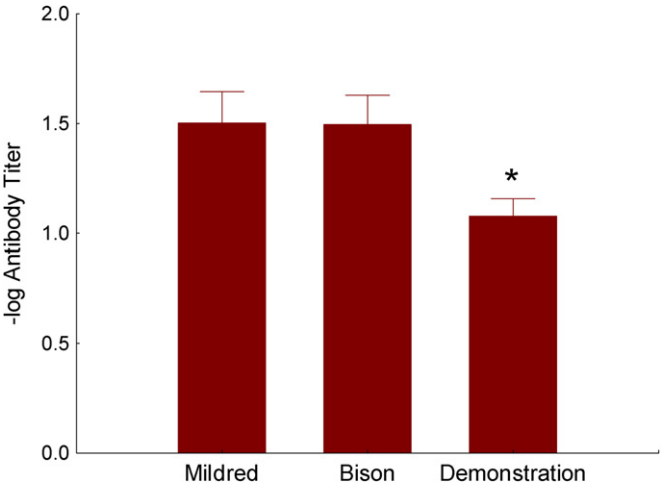

Fig. 4. Mean *Aeromonas salmonicida* antibody titer for rainbow trout caged at three oil sands location and injected with inactivated *A. salmonicida*. Asterisks indicates statistically significant difference at $p < 0.05$ with the *A. salmonicida* and PBS groups evaluated independently. Error bars are the standard errors of the means.

accompanied by reduced spleen size and decreased lymphocytes counts. Trout showed a reduced ability to produce antibody in response to *A. salmonicida*. There was also some low-level stress as indicated by cortisol levels in the pond with the highest organic contamination. Increased exposure to naphthenic acids, high pH, and PAHs derived from bitumen was indicated by elevated water concentrations of naphthenic acids and pH, increased liver CYP1A activity, and bile fluorescence at phenanthrene wavelengths. Total dissolved solids per se did not seem to be strongly related to the observed effects.

The results of this study are consistent with research performed with yellow perch in the same experimental sites. In those earlier studies, perch were released into the pond environment for periods of at least three months. Despite increased energy storage and reproductive development (van den Heuvel et al., 1999a), yellow perch exhibited fin erosion and skin lesions (van den Heuvel et al., 2000). The skin lesions have been subsequently identified by sequencing of the major capsid protein as being caused by the lymphocystis disease virus (Palmer et al., 2012). While the earlier studies of the 1990s showed that the Bison Pond had more severe disease effects, recent replication of those experiments show more severe effects in the Demonstration Pond (Hogan et al., 2011). This observation was consistent with increases in naphthenic acids and pH in the Demonstration Pond due to release of tailings pore water, and decreases in naphthenic acids in the Bison Pond (van den Heuvel et al., 2012). However, pH in the Bison Pond was not elevated for the region in studies from the mid 1990s and thus was not considered a potential causative factor at that time (van den Heuvel et al., 1999a,2000, 2012).

Given the more recent effects observed in perch in the Demonstration Pond, rainbow trout in situ bioassay appears to be a suitable surrogate for perch responses though other species exposed to these waters do not show an equally acute response. Fathead minnows (*Pimephales promelas*), present in the South Bison and Demonstration Pond virtually since their inception, have not been reported to show overt indications of external disease (Siwik et al., 2000; Kavanagh et al., 2011). Another cyprinid species, the white sucker (*Catostomus commersonii*) was recently introduced to the experimental systems and while it does not show increased fin erosion or evidence of viral diseases, a visible hemorrhaging was observed, largely in the fins (Arens et al., 2011).

The elevation of bile fluorescence and measures of CYP1A activity was also consistent with previous studies. This was previously observed in yellow perch (van den Heuvel et al., 1999b) and has also been seen in white sucker (Arens et al., 2011). The bile fluorescence response was elevated as measured at phenanthrene wavelengths, consistent with the presence of three ring aromatic structures. The precise identity of the bile metabolites has not been determined and due to the elevated levels of naphthenic acids, that also have fluorescent characteristics at similar wavelength pairs, the presence of PAH metabolites in the bile has not yet been absolutely confirmed. Recently, Rowland et al. (2011) reported aromatic naphthenic acids in oil sands-affected water, though it remains unknown how much these could contribute to bile fluorescence. Naphthenic acids are not highly bioaccumulative, but have been observed to be taken up into fish tissue from water (Young et al., 2007, 2011). However, as structures that cannot attain a planar conformation, naphthenic acids

would not be expected to induce CYP1A. A recent study has confirmed this as rainbow trout injected with up to 100 mg/kg of naphthenic acid mixture purified from oil sands tailings water did not show increased bile phenanthrene equivalent concentration or elevated hepatic EROD activity (MacDonald et al., submitted for publication). PAHs with a high degree of alkyl substituents have been observed in tailings under the Demonstration Pond capping layer (Madill et al., 1999), and have been measured in invertebrates in the Demonstration and South Bison Pond (Wayland et al., 2008). Sediment and insects from the Demonstration Pond and South Bison Pond showed elevated levels of C1–C4 substituted anthracence/phenanthrenes, fluorenes, chrysenes, and dibenzothiophenes with concentrations of the parent molecules being approximately two orders of magnitude lower than that alkyl substituted congeners (Wayland et al., 2008). The C3 and C4-substituted PAHs were generally more abundant than the C1 and C2 substituted forms. While alkyl-substituted PAHs are not well-studied in general, the C4 alkyl phenanthrene retene, a biotransformation product of abietane resin acids in pulp mill biosolids and wastewater has received considerable study and is a potent inducer of CYP1A activity and produces fluorescent metabolites in bile (Fragoso et al., 1999; Hogan et al., 2010). The dibenzothiophene parent structure was found to be inhibitory of β-naphthoflavone-induced CYP1A activity (Wassenberg et al., 2005) but does not likely fluoresce strongly at phenanthrene wavelengths. However, alkylated dibenzothiophenes with substituents in the 2,3,7 or 8 position are more likely to bind to the Ah receptor than would the parent molecule and could also elicit some of the observed effects.

Rainbow trout exposed to pond waters showed indication of a reduced ability to respond to antigen and to resist opportunistic disease. It is not possible from the current results to determine if that observation was due to a specific toxicant acting on the immune system, or due to the effects of chronic stress. As fish are highly susceptible to stress, a successful caging experiment must reduce the methodological influences of stress on the experimental organisms in order to best interpret the influences of water exposure (Oikari et al., 2006). Cortisol value for the reference fish from Mildred Lake were under 5 ng/mL, indicating relatively unstressed fish (Gesto et al., 2008). However, there did appear to be some low level stress in the PBS-injected Demonstration Pond trout. While increases in caging density alone can cause 10-fold higher cortisol levels that what was observed in the present study (Oikari, 2006), this was not likely the case as density was identical between all three test cages. While every effort was made to quickly bleed the trout for cortisol analysis, the onset of cortisol production is rapid in rainbow trout (Hogan et al., 2010) and we cannot absolutely rule out some effect of sampling stress. Lister et al. (2008) also found elevated levels of cortisol in caged goldfish associated with elevated levels of oil sands contamination. Cortisol is a major regulatory hormone of the immune system and stress is well known to be associated with opportunistic diseases in fishes. It could be speculated co-administration of *A. salmonicida* with pond water exposure led to a temporally complex inflammatory response, and as cortisol is an important regulatory hormone of the immune response, some suppression of cortisol release may have occurred at the late stage of the inflammatory response. Cortisol can lead directly to reduced lymphocyte counts as it has been observed to induce apoptosis in B-cells (Weyts et al., 1998). While the term immunotoxic is not well defined, one definition is that the primary site of toxicant action is lymphatic tissue. It is difficult from the present study to know whether toxicants acted directly on immune cells, or whether a secondary cortisol effect led to immune modulation. However, a number of compounds, including PAHs are known to directly affect tissues of the immune system (Reynaud and Deschaux, 2006) and trout leukocytes are known to possess the Ah receptor making them responsive to PAHs (Nakayama et al., 2008).

Trout in the in situ exposures were exposed to a number of chemical stressors that may have the ability to directly alter immune function. The known potential stressors include naphthenic acids, PAHs, elevated major ions, metals and pH. Metals have also been implicated as potential toxicants in the region (Kelly et al., 2010). A number of metals, As, Ba, Cu, Fe, Ni, Mn, Cd, and Hg were detectable in the Demonstration and Bison Pond, but did not differ more than two-fold from far field reference sites and were thus considered to be within background for Alberta (van den Heuvel et al., 2012). The only divalent metal found to be increasing in the Demonstration Pond was Zn and that only marginally exceeded the guideline for the protection of aquatic life for Zn of 30 mg/L (van den Heuvel et al., 2012). Only the metals B and Li, indicative of regional clays, have increased markedly over time as tailings in the Demonstration Pond consolidate but remain under water quality guidelines (van den Heuvel et al., 2012). Thus, we have found no substantial evidence that metals are in any way associated with the observed biological results from this study. In this study, the Demonstration Pond and South Bison Pond had similar total dissolved solids, yet quite different effects on the trout immune system. The species used for oil sands studies (perch, rainbow trout) are known to be highly adaptable to salinity, thus salinity seems an unlikely factor influencing the changes observed. Fish in culture have been observed to elicit fin erosion in response to high pH, and the dominance of unionized ammonia may play a role in this (Bosawowsky and Wagner, 1994). Due to the scale of the pond environment, and levels of ammonia documented as being lower than 0.1 mg/L (van den Heuvel et al., 1999a), the involvement of ammonia is unlikely. Elevated pH cannot be eliminated as a potential cause based on this trout study alone. However, while elevated pH is unique to the Demonstration Pond, studies from the 1990s showed a high incidence of disease in yellow perch stocked into the South Bison Pond (van den Heuvel et al., 2012). The pH of that pond at the time was 8.1, similar to the current study, and within normal range for the region (van den Heuvel et al., 2012). This is suggestive that a common causative agent other than pH may have been responsible for the disease effects in yellow perch. Over the period of study of fish exposure in these experimental ponds, naphthenic acids have doubled in the Demonstration Pond and decreased significantly in the South Bison Pond (van den Heuvel et al., 2012). While PAH concentrations have not been specifically evaluated, the present study would suggest that these have also become higher in the Demonstration Pond based on bile fluorescence and CYP1A activity. Thus, PAH concentrations likely follow the trend of naphthenic acid concentrations as both are tailings-derived organics. Yellow perch studies suggest that disease in this species best correlates with the organic constituents as disease was most severe in the South Bison Pond in the 1990s (van den Heuvel et al., 2000) but has subsequently become much more prevalent in the Demonstration Pond (Hogan et al., 2011). Trout in the present study also were exposed to both PAHs and naphthenic acids, so neither can be ruled out as causative agents in the present study.

Limited study has been directed at the potential of naphthenic acids to cause immune suppression. An early study showed that naphthenic acids stimulated leukocyte production at 6 d of exposure and depressed production at 21 d (Dokholyan and Magomedov, 1984). A study using waterborne exposure of goldfish to commercially available naphthenic acids showed that the ability to resist parasite infection was increased at 1 week exposure but decreased at 12 weeks exposure, and that naphthenic acids were associated with increases in pro-inflamatory cytokines at higher doses (Hagen et al., 2012). In a mammalian

study, immunologic function on mouse bone marrow cells exposed to commercial naphthenic acids showed a reduction in phagocytosis associated with alteration in pro-inflammatory cytokines (Garcia-Garcia et al., 2011). As the chemical nature of commercial naphthenic acids is substantially different that what is found in oil sands-affected waters (Grewer et al., 2010), the ability to extrapolate the aforementioned laboratory studies to the field is unknown. A recent study examine the immunological potency of naphthenic acids extracted and purified from aged oil sands tailings water injected into rainbow trout at 100 mg/kg. This study showed some initial reduction of circulating leukocytes at 5 d, but at 21 d exposure a stimulation of leukocytes was observed (MacDonald et al., submitted for publication). This is consistent with the above study in that an inflammatory response appeared to be induced by naphthenic acids. However, this pattern of response was inconsistent with what was observed in the rainbow trout caged in the present study as pond exposures significantly reduced leukocyte numbers. A further inconsistency is that the exposure to purified oil sands-derived naphthenic acids did not induce CYP1A or cause elevated bile fluorescence in the exposed trout, strongly suggesting that PAHs are an additional chemical factor that must be considered in oil sands immune toxicity.

Certain PAHs have been clearly shown to be potent immunotoxicants and their immunotoxic effects in fishes has been recently reviewed (Reynaud and Deschaux, 2006). However, effort has been directed at the study of immunological impacts of only a narrow subset of PAHs. The main compounds examined include Benzo[a]pyrene, dimethylbenzanthracene, and methylcholanthrene. Those PAHs studied are also known to be strongly mutagenic/carcinogenic. There is evidence of a relationship between carcinogenicity and immune toxicity as the DNA-binding dimethylbenzanthracene 3,4-dihydrodiol 1,2-epoxide is considered to be the immunosuppressive metabolite of this compound. This has led to the hypothesis that mechanisms of immunotoxicity for PAHs are mediated by a genetic mechanism. The expression of the DNA damage sensor p53 in response to dimethylbenzanthracene supports the DNA-binding hypothesis (Gao et al., 2008).

There is only sparse information on the toxicity and immunotoxicity of alkyl PAHs similar to those found in oil sand-associated material. A study examining the model alkylated phenanthrene, retene found that it was not a potent immunotoxicant, but instead had a modest stimulatory effect on the immune system (Hogan et al., 2010). However, studies with of rainbow trout embryos to retene have shown fin erosion (Billiard et al., 1999). If genotoxicity is indeed required for immunotoxicity, many alkyl PAHs may not be expected to be potent immunotoxicants in fishes, as in the case of retene, the primary oxidative metabolites are on the alkyl substituents, not ring oxidations (Tabash, 2003). Thus, diol-epoxide metabolites suspected of both mutagenicity and immune toxicity are less likely in many alkyl PAHs. Such diol-epoxide metabolites also require a bay, or bay-like region in the PAH structure. While many of the substituted anthracenes, phenanthrenes, fluorenes present in oil sands-associated materials do not have this characteristic, chrysenes do, and may be of interest as potential immunotoxicants.

In addition to the immune/disease effects observed here, oil sands-affected waters have also been shown to demonstrate effects on fish reproductive development (Kavanagh et al., 2011 van den Heuvel et al., 2012) and on fish embryo survival and deformities (Colavecchia et al., 2006; Peters et al., 2007). Water quality criteria do not yet exist for naphthenic acids or other compounds associated with oil sands. However, regardless of the causative agent, this study and others consistently suggest that significant physiological effects are manifest in fishes when naphthenic acids exceed 10 mg/L, as measured by Fourier transform infrared spectroscopy (van den Heuvel et al., 1999a, 2012). This concentration can serve as a rough guideline as to safe levels in the aquatic environment even though there is no evidence that these compounds are causative of the observed effects.

As rainbow trout is very amenable to laboratory studies, we would conclude that based on the observations, this species is an adequate surrogate for the native yellow perch. The present trout study alone demonstrated significant disease and immune effects similar to those observed in yellow perch (van den Heuvel et al., 2000). Based on the trout caging study, naphthenic acids, PAHs, and pH all remain as potential causative agents. However, when this study is evaluated in the context of the yellow perch studies conducted since 1995, and one assumes that these effects are caused by common substances, naphthenic acids and PAHs come out as the factors consistent with all elevated disease observations.

Acknowledgments

This work was supported by Syncrude Canada, Suncor Energy, Shell Albian Sands, Total E&P Canada, and Canadian Natural Resources Limited under the auspices of Canadian Oil Sands Network for Research and Development, by an NSERC CRD grant held by MRV. The authors wish to thank Samantha Tavener, Terry van Meer, Michael MacKinnon, Warren Zubot, Coleen Campbell, Chris Beierling, and Richard Kavanagh for their assistance.

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