Universidade de Aveiro Departamento de Biologia
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Efeitos da exposição de betume em ecossistemas aquáticos nas *Oil sands*: uma abordagem ecotoxicológica integrada

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Tese apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Doutor em Biologia, realizada sob a orientação científica da Doutora Susana Patrícia Mendes Loureiro (Professora auxiliar com agregação do Departamento de Biologia da Universidade de Aveiro).

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o júri

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palavras-chave

Betume, erosão em encostas de rios, contaminação natural, elutriados, água e sedimentos, mesocosmos.

resumo

Os depósitos de *oil sands* (ou areias petrolíferas) do Atabasca, situados no Norte de Alberta, Canadá, são uma mistura natural de betume, areia, argila e outros minerais. O betume é um tipo de petróleo extremamente viscoso que é extraído e posteriormente tratado para produção de gasolina, gasóleo e outros produtos compostos por hidrocarbonetos. O depósito natural das *oil sands* do Atabasca é uma fonte de *stressores* químicos e físicos para os rios da região que desaguam/fluem sobre esses depósitos. O *stress* físico causado pelo betume nos organismos aquáticos é resultado dos processos de erosão das encostas dos rios, enquanto o stress químico é resultante dos contaminantes derivados do betume que são depositados nos rios através da sua erosão. Para entender os efeitos ecológicos induzidos pela pressão antropogénica da utilização do betume na exploração petrolífera, é necessário identificar e avaliar quais os efeitos adjacentes a uma contaminação natural, originada pela erosão natural do betume. Com isto, o objetivo principal desta tese consistiu em avaliar/analisar os possíveis efeitos ecotoxicológicos associados aos processos de erosão do betume nos rios. Para isso, foram realizados uma série de ensaios ecotoxicológicos, utilizando invertebrados aquáticos bentónicos e pelágicos expostos a betume recolhido das encostas de rios que correm sobre depósitos de betume. Esta exposição foi efetuada de forma direta, misturando o betume no sedimento ou através de elutriados obtidos deste betume. Verificou-se que estes organismos são sensíveis à presença de elutriados de betume recolhido nas encostas dos rios Steepbank e Ells, afetando diversos parâmetros vitais de espécies modelo como o pequeno crustáceo planctónico *Daphnia magna, a bactéria Vibrio fischeri,* o caracol de água doce *Physa acuta* e a planária *Dugesia tigrina*. Quando o betume foi incorporado no sedimento, induziu toxicidade na espécie *Chironomus riparius*, que respondeu negativamente à sua presença, revelando assim a sua sensibilidade ao betume. Verificaram-se reduções do seu tamanho larvar, aumento do seu tempo à emergência, redução da sua emergência total e uma diminuição de peso dos adultos.

Utilizando uma abordagem mais complexa, um ensaio de mesocosmos foi efetuado com uma comunidade natural de invertebrados recolhida no Rio Mau (Portugal). A incorporação do betume no sedimento induziu efeitos negativos e maioritariamente observados na redução da abundância de *Ephemera* sp. e *Chironomus* sp que se alimentam essencialmente de partículas finas de sedimento onde o betume está incorporado, levando assim a efeitos tóxicos nos organismos. O uso de amostras de betume e respetivos elutriados, em combinação com o uso de uma série de organismos aquáticos utilizados em diferentes tipos de ensaios, forneceram uma abordagem mais realista e holística, na avaliação dos efeitos dos processos naturais que levam à entrada de betume em rios da área de estudo. Estes resultados enfatizam a necessidade de estudar mais aprofundadamente a influência da entrada do betume nos rios, diferenciando assim, os possíveis efeitos causados por processos naturais e os efeitos causados pela pressão antropogénica.

keywords

Natural bitumen, weathering of river banks, natural contamination, elutriates, background toxicity; water and sediments; mesocosms.

abstract

The Athabasca oil sands deposits in northern Alberta, Canada, are a naturally occurring mixture of bitumen, sand, clay and other minerals. Bitumen, which is a heavy and extremely viscous oil, is mined and then subsequently refined to produce gasoline, diesel and other hydrocarbon-based products. Moreover, the naturally occurring Athabasca Oil sands deposits are a source of both physical and chemical stressors to local rivers that flow through the deposit. Physical stress to aquatic biota from natural bitumen results from hillslope erosion processes and slumping of material into the rivers, while chemical stress arises from bitumen-derived contaminants entering the waters. To fully understand the ecological and cumulative effects of oil sands mining activities on aquatic ecosystem water quality and associated biological structure and function, there is a need to evaluate the effects of naturally occurring bitumen in the aquatic environment. Within this, the main objective of this thesis was to evaluate the possible ecotoxicological effects associated with the slumping of river bank material (i.e., oil sands deposit that naturally enters the river systems through fluvial geomorphological processes). For that, a series of inter-related laboratory ecotoxicological assays were conducted using benthic and pelagic aquatic invertebrates exposed to oil sands material collected from four different sources in regional rivers. Organisms were sensitive to the presence of oil sands elutriates, especially to elutriates generated from bitumen material collected in the banks of the Steepbank and Ells river, with effects on the life traits of *Daphnia magna*, *Physa acuta, Vibrio fischeri,* and *Dugesia tigrina*. When bitumen is mixed with sediment, it was also toxic to *Chironomus riparius,* which responded negatively revealing to be sensitive to bitumen samples with more bitumen content in sediments with a decrease in their body size, a delay on the emergence time, reduced total emergence and a decrease in the weight of imagoes. On a more complex approach, bitumen negatively affected natural communities collected from Rio Mau (Portugal) by reducing the number of *Ephemera* sp. and *Chironomus* sp. that feed on fine sediment particles. The use of solid bitumen samples and elutriates from those samples, combined with a suite of representative species and different experiments provides a comprehensive and holistic approach to assess effects of oil sands materials arising from bank erosion-related processes, emphasising the need to discriminate natural processes from mining-related activities of surface and groundwater contamination in oil sands areas.

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Chapter 1: General Introduction

1- General introduction

1.1 - The oil sands

Oil sands - also known as tar or bituminous sands - are sand deposits impregnated with dense, viscous petroleum called bitumen (Figure 1.1). Oil sands are comprised of a loose mixture of sand, clay, water and bitumen, a highly viscous, heavy crude oil composed of a complex mixture of hydrocarbons, hetero-organics, and metals (Colavecchia et al., 2004; Colavecchia et al., 2006) (Figure 1.2).

Figure 1.1 – Natural bitumen attached to sediment found in the Athabasca oil sands. This bitumen is collected, separated from sands and finally produce oil.

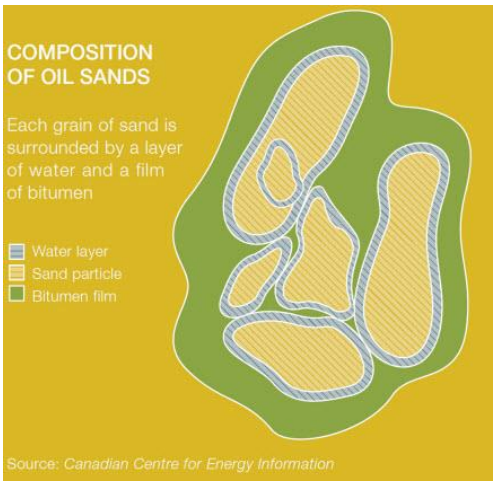
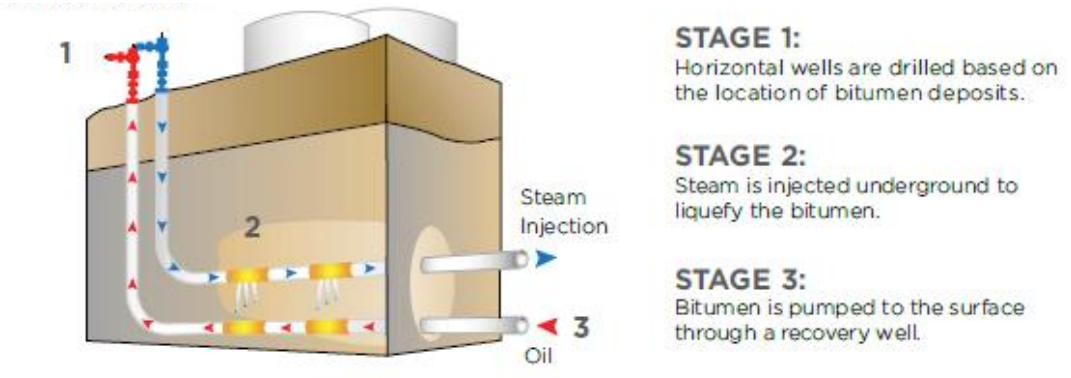


Figure 1.2 – Representative scheme of an oil sands particle, with sand mixed with bitumen and water. Source: Canadian Centre for Energy Information.

Canadian oil sands were formed millions of years ago when the area was covered by a warm tropical sea, and the death and deposit of compressed marine creatures on the seafloor formed liquid rock oil. During the Rocky Mountains formation process, the tectonic plates shifted and made the oil be in contact with the sands. In the beginnings, bitumen was used as gum to waterproof First Nations canoes, but with the discovery of its economic potential, these oil sands were defined as the largest potential oil field in the world. To this day, the development of Canadian oil sands is an ongoing challenge.

Nowadays, in these Canadian oil sands, the bitumen is found within 70 meters of the surface, but the majority is found deeper underground, shallow enough to permit open-pit mining. Therefore, depending on the depth of the deposit, two different approaches are used to mine/extract the crude bitumen from the oil sands: 1) by open-pit mining of the shallow deposits, and 2) in-situ recovery using extraction methods of the deeper oil (Figure 1.3). In 2017, 57% of the oil sands production is from in situ methods and 43% by open-pit mining (CAPP, 2018).

In-situ recovery method



Open-pit mining

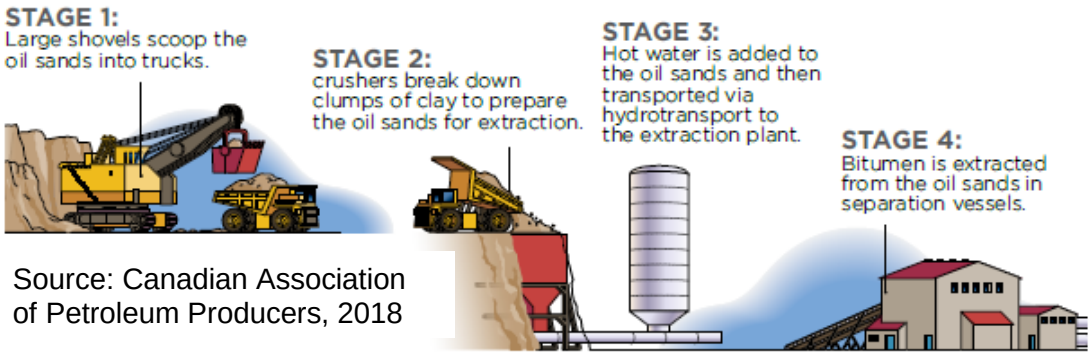


Figure 1.3 - Representative scheme of the two different methods of extraction: In situ recovery method, where steam is generated to heat the bitumen underground, allowing it to flow to the surface; Open-pit mining, where sticky bitumen is separated from sand and clay using heated water**.**

In the province of Alberta, Canada, there are four major deposits of oil sands: Athabasca, Peace River, Cold Lake, and Wabasca (Figure 1.4). One of the largest Cretaceous oil-sands deposits in the world is in the Athabasca Basin (Quagraine et al., 2005) and despite the impact of devastating wildfires in 2016, the production of crude bitumen increased slightly from 2.53 million in 2015 to 2.65 million barrels per day in 2017. Since 2008, the production of crude bitumen increased, and it is related to the expansion of oil sands projects that are already in production (Alberta Ministry of Energy, 2016; CAPP, 2018).

Source: Government of Alberta

Figure 1.4 - Oil sands in Alberta region, Canada**.**

1.2 - Environmental impacts

1.2.1 - Anthropogenic sources of contamination

At the same time that oil sands exploitation development raised, the concerns about the associated environmental impacts also increased. Environmental concerns in these Canadian Oil sands relies mainly on the industrial activities, resulting in landscape disturbance and habitat loss for natural species (Kelly et al., 2010; Kurek et al., 2013). This anthropogenic pressure increased the concern about the potential environmental impacts on air quality, water use, tailings wastewater production, groundwater contamination, and habitat disturbances in the surrounding terrestrial and aquatic ecosystems (Dowdeswell et al., 2011; He et

al., 2012; Kelly et al., 2010). As an example, studies revealed that anthropogenic emissions of airborne particles increase the concentrations of soluble and particulate polycyclic aromatic hydrocarbons (PAH) in waters and snow (Kelly et al., 2010; Kelly et al., 2009). Also, the surface mining activities will release wind-blown dust that will potentially contaminate other subjacent areas. Surface deposition of contaminants that were then spread by air and are often washed into aquatic systems by run-off, increasing the contaminants concentrations in the regional rivers. Surface mining also induces land disturbance, causing loss of habitat and native species (Parajulee and Wania, 2014).

One of the main concerns is related to the storage of large volumes of oil sands process-affected waters (OSPWs). In the process of crude oil extraction, large volumes of OSPWs are produced and stored in tailing pounds containing residual bitumen, organic acids, and PAHs, which have been shown to be acutely and chronically toxic to aquatic organisms (Lari et al., 2017; Nero et al., 2006; Yergeau et al., 2012). It is estimated that these tailings ponds contain 840 million m^3 of OSPW (Energy Resources Conservation Board, 2015). However, OSPW is currently not approved for release back into the receiving environment due to the Canadian “zero discharge policy” (Government of Canada, 2015a; Government of Canada, 2015b).

1.2.2 - Natural sources of contamination

Despite the impact on surrounding ecosystems induced by multiple anthropogenic sources in the oil sands area, natural sources of contamination are prone by themselves to negatively impact the aquatic systems in this Canadian oil sands region (Barton and Wallace, 1979; Gerner et al., 2017; Headley and McMartin, 2004). The mainstream Athabasca River and its tributaries (e.g., the Clearwater, Ells, Steepbank, Muskeg, and Firebag Rivers) over geological time have eroded into and are flowing through naturally exposed oil sands deposits (Figure 1.5). Such proximity and contact of the river flow to natural bitumen provides opportunities for continuous direct and indirect loading of bitumen through

streambed and bank erosion processes (Barton and Wallace, 1979; Headley and McMartin, 2004). These processes lead to an increased potential risk of exposure to PAHs, Naphthenic acids (NAs) and other contaminants (e.g., metals) that are naturally present in the bitumen (Gerner et al., 2017; Headley et al., 2001; Headley and McMartin, 2004). Also, episodic flood events that occur in the region, may transport and deposit relatively larger volumes of oil-sand derived sediment along the rivers and after that, pose an exposure risk to the aquatic systems due to the potential high load of associated contaminants and related physical habitat disturbance (Conly et al., 2002).

Figure 1.5 – Steepbank River is cutting through bitumen deposits. Top left corner represents the eroded margins or Steepbank River with an asphalt-like pavement, where we can walk through the “road” of bitumen. Top right corner represents a broader view of the eroded margin in the Steepbank River. The bottom left represents an oil sheen in the interface between the bituminous margins of the river and the river, releasing the oil material into the stream. The bottom right represents a rock collected in the bottom of the river, covered by sticky bitumen but also colonized by specific species.

Due to the composition of eroded bitumen, poor settling and long-range transport are expected, alongside with a longitudinal increase in Polycyclic Aromatic Compounds (PACs) concentration from upstream to downstream of rivers (Droppo et al., 2018). Consequently, when bitumen enters into freshwaters, it is expected that:

a) part of the eroded bitumen is immediately washed into the water;

b) the remaining natural eroded bitumen will settle on the top sediment of the river;

c) the fine-grained part is transported through the river to downstream areas.

The following example (Figure 1.6) is representative of the mentioned natural erosion that leads to the input of contaminants in freshwaters. The scheme refers to pictures from a river slump in the Steepbank river (56°57.71'N 111°8.932'W) over different periods of time, clearly demonstrating the erosion process occurring and the consequent entrance of bitumen in Steepbank river. After the winter of 2012, slumping material brought to the river fresh bitumen which nearly choked off the channel between the two margins. Through the months, material from the slump had been eroded by the river flow, widening the channel, and removing much of the material at the base of the slump (June 2014). These events strengthen the main objective of this thesis, studying the effects induced by this natural riverine eroded bitumen to the aquatic biota.

Due to the overlapping of both natural and anthropogenic contaminations in these Canadian oil sands region, difficulties in the characterization of each type of contamination source are a fact, and a topic of concern (Hein and Cotterill, 2006).

June 2012

During the winter of 2011/12, there was new slumping which brought down fresh bitumen material which nearly choked off the channel. Attention to how narrow the channel is.

July 2012

By July, considerable material from the slump had been eroded away by river flow.

September 2012

By September, more slump material had been eroded away and the channel had become considerably wider.

August 2013

By August 2013, erosion had widened the channel further and removed much of the material at the base of the slump.

June 2014

Note the quantity of the material from the most recent slumping at this location (winter 2011/12) has now been eroded by the river flow.

Figure 1.6 – The schematic figure is representing the erosion of the river slump in the Steepbank river (56°57.71'N 111°8.932'W) over different periods of time. Between June 2012 and June 2014, severe erosion processes widened the channel, removing much of the material at the base of the slump.

1.3 - Constituents of bitumen

The composition of bitumen-derived substances entering the aquatic environment is complex and highly variable. The major components are the hydrocarbons, where PAHs, NAs and other polar organic chemicals are comprised and inorganic salts and different levels of metals (Gerner et al., 2017; Kelly et al., 2010).

Due to their high hydrophobicity, PAHs are generally related to the suspended particles, being usually present mainly within the sediment and to a less extent in the water column (Gerner et al., 2017). PAHs with high molecular weight (4- to 7-ring) are known to be carcinogenic and mutagenic, presenting low solubility and volatility, comparing with the lower molecular weight PAHs (2- and 3-ring), being more volatile but conferring strong acute toxicity to aquatic biota (Gill and Robotham, 1989).

NAs, are naturally occurring organic acids in bitumen in a complex mixture consisting of alkyl-substituted cyclo-aliphatic carboxylic and acyclic aliphatic (paraffinic or fatty) acids (Headley and McMartin, 2004). NAs could enter in water systems through natural or anthropogenic sources and their levels ranging from generally below 1 mg/L in Alberta rivers to concentration as high as 110 mg/L in tailing pounds containing OSPWs (Headley and McMartin, 2004). The different components of NAs are classified by their structures and the number of carbon atoms in the molecule, presenting a general formula $C_mH_{2m+z}O_2$ where m represents the carbon number and z the number of lost hydrogen atoms in the process, where structures become more compact. Z value varies from 0 in saturated linear hydrocarbon chains, to -2 in monocyclic naphthenic acids, -4 in bicyclic, -6 in tricyclic and so on, and z= -4 is reported as mainly present in oil sands tailing ponds (Headley and McMartin, 2004).

The toxicity of NAs to aquatic biota is often related to their surfactant characteristics. NAs are known to be the most significant environmental contaminants and toxic components in oil sands natural deposits and OSPWs in tailing ponds, especially the low molecular weight NAs (Rogers et al., 2002).

As occurs typically in oil exploration areas, metals are also a relevant group present in bitumen and toxicologically significant. Bioavailability of metals and consequent toxicity depends on a few abiotic factors such as pH, water hardness, and the availability of organic ligands (Gerner et al., 2017; Hein and Cotterill, 2006).

It is also important to refer that in natural bitumen samples all these contaminants mentioned before are in a mixture, with consequent interactions that could synergistically affect the aquatic organisms.

1.4 – Natural contamination related studies

Knowing the importance that studying the natural effects of bitumen/oil sands material have in the aquatic systems, it is imperative the perception of the "background toxicity" in the oils sands area for a correct and accurate risk assessment study of the oil sands development.

One of the first studies demonstrating that oil sands natural processes were affecting the aquatic biota was conducted by Barton and Wallace (1979), reporting the inherent risks of natural weathered bitumen in the oil sands zone towards freshwaters. That work found a reduction in biodiversity in areas where waters are influenced by bitumen formations. After that, on an attempt to discriminate between potential effects of natural and anthropogenic sources of contamination, some field studies have been conducted. Using the fish species *Cottus cognatus* and *Semotilus margarita* collected from 1) reference areas, 2) from areas with naturally occurring oil sands-related compounds (natural loading of oil sands material) and 3) from areas adjacent to surface mining activity, the authors found altered baseline biochemical parameters (Ethoxyresorufin-O-deethylase (EROD) activity) in fish residing in the natural oil sands deposit when compared to the

same fish species residing within reference conditions, increasing these alterations with the proximity to areas with mining activities (Tetreault et al., 2003a; Tetreault et al., 2003b). Also, Colavecchia et al. (2004) found that exposure to natural bitumen in early life stages of *Pimephales promelas* altered their survival rate, hatching success, an increase of malformations and effects on size and increase of deformities (e.g., edemas, hemorrhages, and spinal malformations). Later, Colavecchia et al. (2006) exposed white sucker *Catostomus commersoni* eggs and larvae to sediment containing natural bitumen and wastewater sediments and found that both sediments impaired the development and survival of both life stages. Although the eggs hatched earlier, there was an increase in mortality, malformations and retarded larval growth, possibly affecting the fitness and stability of fish populations. Biochemical alterations can be related to the observed deformities in the embryos and larvae after exposure to those waters that were in contact with oil sands material (Colavecchia et al., 2007). Slimy sculpin (*Cottus cognatus*) and pearl dace (*Margariscus margarita*) exposed to waters that flow through bitumen deposits revealed a reduction in steroid production and increase in EROD activity when compared with values reported for reference sites. Lacaze et al. (2014) conducted a different approach, evaluating the potential genotoxic effects of OSPW and Oil Sands Leaching Waters (OSLW), simulating the natural release of contaminants from oil sands using a laboratory extraction of the oil shore sands (naturally formed). Results revealed that OSPW led to a significantly higher DNA damage than OSLW in the rainbow trout's hepatocytes (assessed by comet assay), whereas OSLW (with natural source) revealed to be genotoxic to the exposed fish.

1.5 - The objective of the thesis

Given the complexity of the potential sources, magnitude and duration of oil sands-derived sediments entering riverine ecosystems in the Athabasca region, there is a need for a more accurate assessment of related exposure risks to aquatic organisms. It is essential to define baseline conditions and ascertain the contribution of naturally derived contaminants to the aquatic ecosystem, additional to any anthropogenic contamination sources. A key knowledge gap has been

related to understanding both the magnitude and significance of the toxicological and ecological effects to aquatic organisms when exposed to naturally occurring bitumen. Since few studies reported the levels of contaminants in waters and few presented adverse effects on the aquatic biota in waters influenced by natural bitumen deposits, the present thesis will add relevant ecotoxicological data to those previous studies.

Up to now, those studies related to natural contamination in oil sands only addressed effects on collected organisms from the naturally contaminated, or by exposing laboratory organisms to collected contaminated sediments from rivers. The study presented in this thesis is the first where natural bitumen material was collected in the field (never exposed to water), taken to the laboratory, and the effects of that material were addressed using ecotoxicological assays. This was carried out directly, by mixing solid bitumen to the sediment, or indirectly by performing elutriates from the solid bitumen samples. Within this, even losing some environmental realism, it is guaranteed that we are addressing the effects of the bitumen input exclusively, into streams.

Considering the knowledge gap regarding the effects of eroded bitumen when in waters, this thesis aims to reply to the following questions:

1) Is the parental natural bitumen material affecting the aquatic biota?

2) Are the natural bitumen contaminants released to the water phase? If yes, are the contaminants available for aquatic biota?

3) Are the natural bitumen samples homogeneous regarding their chemical composition and potential toxicity?

4) The possible toxicity of natural eroded bitumen will change with the eventual liberation of oil sands sediment-bound contaminants when in the water?

5) How the organisms respond to bitumen attached to sediment and when diluted/dissolved in waters?

7) Should we take into consideration the natural background toxicity when evaluating the anthropogenic effects in this oil sands region?

For that, ecotoxicological assays are presented, using both elutriate, simulating the dissolution of natural weathered bitumen into rivers, and contaminated sediment exposures, simulating the attaching of bitumen to the sediments, and a battery of organisms on an attempt to find responses to the raised questions.

1.6 - Study area

In this specific area, the Athabasca River flows northward and ends up in Lake Athabasca forming a net of rivers and tributaries. More specifically, the present study was conducted collecting natural bitumen from four different locations in the slumping areas of the three rivers in the Athabasca oil sands in Alberta, Canada. Two samples were collected in the banks of the Steepbank River: SP (56°58'47.3"N 111°17'53.0"W) and STB (56°59'55.1"N 111°24'12.1"W) (Figure 1.7). The other two samples were collected in the slumping area of the Ells River (ELLs - 57°16'49.0"N 111°42'17.0"W) and in the banks of the Athabasca mainstream (ATB - 58°12'03.6"N 111°22'48.0"W), where bitumen is present in the form of “bitumen balls” (Figure 1.8). In the sampling rivers, streambeds that resembled asphalt-like pavement were observed, with visible oil sheens in water. Nevertheless, vegetation and aquatic organisms (macroinvertebrates and fish) were present and visible near all sampling points. For about 100km, bitumen deposits are exposed to natural forces that can lead to an input of bitumen in the aquatic system.

Figure 1.7 – Bags of sediment were collected from the base of the slump at Steepbank river. The sediment sampling locations were selected aiming that the slumped material had never been in contact with river water. Same procedures were used in the Ells River.

Figure 1.8 – Collection of bitumen material in the form of “bitumen balls.” In that specific area, natural bitumen samples were collected in the margins of the Athabasca River after suffering physical forces that made them end up in the Athabasca River.

1.7 – Test organisms

In the present thesis, different levels of organisms were used to assess the effects of bitumen on aquatic biota into streams.

The cladoceran *Daphnia magna* is a freshwater species commonly used as a standard bioindicator in a variety of ecological studies. This species presents a short life cycle, is easy to culture in the laboratory, and could be maintained in high populations in relatively low volumes of media. This zooplankton grazer is a widely used species in ecotoxicology studies, with well-defined standardized protocols to assess the toxicity of a broad range of contaminants and physical threats (OECD, 2004a; OECD, 2012). Is considered one of the most sensitive organisms to toxic chemicals, occupying a central position in the lentic food chain (Naddy et al., 2007). *Daphnia magna* was already used to test the toxicity of oil sands process-affected water (OSPW), confirming their high toxicity to aquatic organisms (Lari et al., 2017).

Vibrio fischeri is a Gram-negative bacterium found globally in marine environments (Madigan and Martinko, 2005). This marine bacterium produces luminescence by expressing the lux operon, a small cluster of genes found in several of the Vibrionaceae, and it also has symbiotic associations, with sepiolid squids (Ruby et al., 2005). Due to the bioluminescence activity, this bacterium is often used in ecotoxicological assays, usually measuring the inhibition of bioluminescence as a response to the presence of stressors. *Vibrio fischeri* was also previously tested to assess the toxicity of OSPWs (Meshref et al., 2017; Pourrezaei et al., 2014)

Physa acuta belongs to the monophyletic taxonomic family Physidae and, as other aquatic pulmonated gastropods, could take oxygen from the water or even from the air, breathing via a lung-like pulmonary cavity within the mantle (Dillon 2000; Thorp and Covich 2010). This species is worldwide distributed, in both lotic and lentic freshwater ecosystems, which confers some tolerance to rough environments (Thorp and Covich 2010). It is a commonly used species in ecotoxicological assays assessing the effects of contaminants in freshwaters. Up

to now, there is no information regarding their responses to oil sands-related studies.

Planarians, organisms that are typically found in ponds and rivers, are carnivores species that exhibit a negative phototaxis behavior. Planarians are invertebrates that employ motile cilia for locomotion and have been studied extensively over the last century for their regenerative properties and were more recently established as a model species for stem cell biology. It is not yet considered a model species in ecotoxicology, but due to their regeneration capacities, *Dugesia tigrina* (asexual planarian) was a useful organism to assess the effects of naturally eroded bitumen into rivers.

Chironomidae (Insecta, Diptera), non-biting midges, are opportunistic tube-dwelling detritivores (Pinder, 1986). These organisms are ubiquitous, dominating the benthic communities of lotic and lentic environments (Armitage et al., 1995). *Chironomus riparius,* that was used in the present study, is widely used when studying sediment toxicity (Ankley et al., 1994) assessing the effects of contaminants into the sediment. Also, the contaminated liquid medium could also be tested, using *C. riparius* as test species, with established standardized protocols for both tests (OECD, 2004b; OECD, 2004c).

1.8 - Outline of the thesis

The present thesis is divided into 7 chapters. This first chapter aims to give a general introduction, the second to sixth constitute the description of the experimental material of the thesis and the seventh chapter overviews a general discussion obtained from the thesis.

Chapter 2 - Entitled “Assessing the acute and chronic toxicity of exposure to naturally occurring Oil sands deposits to aquatic organisms using *Daphnia magna*,” is the first of a series of studies where the effects of natural riverine bitumen on biota are evaluated by using elutriates. Here, the extraction methodology is described, along with cycles of elutriate extraction were performed to observe the effects of time in the toxicity of dissolved natural bitumen.

Chapter 3 - Entitled “Effects of natural bitumen in the aquatic environment of the Athabasca Oil sands: an ecotoxicological approach”, following the findings in chapter 2, the possible heterogeneity in composition and toxicity from different samples was evaluated, adding three different natural bitumen samples collected in different rivers to the already studied, and consequent exposure to *Daphnia magna*, *Physa acuta,* and *Vibrio fischeri*.

Chapter 4 - Entitled “Oil sands bitumen elutriates affect the life traits of *Dugesia tigrina* (Planaria),” emphasizes the usefulness of freshwater planarians and endpoints such as locomotion and head regeneration in ecotoxicological assays and the need to discriminate natural background toxicity in surface and groundwaters within the Oil sands area from mining-related activities.

Chapter 5 - Entitled “Contaminated sediment with natural Oil sands bitumen impaired the *Chironomus riparius* life-history under laboratory condition,” chapter 5 differentiate the toxicity induced by bitumen present in sediment and from elutriates exposure, using four different natural bitumen samples exposed to Chironomus species.

Chapter 6 - Entitled “Changes in macroinvertebrate communities caused by exposure to Oils sands bitumen in mesocosms,” here a mesocosm experiment was carried out, on an attempt to predict the possible effects of natural bitumen collected from the banks of the Ells river to a benthic community collected from a natural river.

1.9 - References

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Chapter 2: Assessing the acute and chronic toxicity of exposure to naturally occurring oil sands deposits to aquatic organisms using *Daphnia magna*.

Assessing the acute and chronic toxicity of exposure to naturally occurring oil sands deposits to aquatic organisms using *Daphnia magna*.

2.1 – Abstract

Canada has the third-largest global oil reserves located in the Cretaceous sandstone oil-sands deposits found in three regions within the provinces of Alberta and Saskatchewan. In the Athabasca region in north-eastern Alberta, the oil sands, which are a loose mixture of sand, clay, water and a viscous form of petroleum referred to as bitumen, are located at or near the surface making open-pit mining viable. In addition, the Athabasca River and its tributaries flow through these oil sands deposits, thereby receiving bitumen-associated complex hydrocarbons, hetero-organics, and metals through natural fluvial erosional and weathering processes. A key knowledge gap has been related to understanding both the magnitude and significance of the toxicological and ecological effects to aquatic organisms when exposed to naturally occurring bitumen. Using the *Daphnia magna* model system, the present study assessed the ecotoxicological effects of exposure to three bitumen-elutriate treatments that simulated a range of fluvial/weathering exposure conditions. All three elutriate treatments were found to impair daphnids reproduction and growth (length) after a 21-day exposure period. Neonates collected from the chronic tests (F1) were further tested for sensitivity to the reference substance potassium dichromate ($K_2Cr_2O_7$), and a decrease in their sensitivity was observed. Also, F1 daphnids from all elutriate exposures were used in reproduction tests using a clean, reference medium to attain potential generational effects, and significant decreases in neonate production were still observed. Comparison of the three cycles used to generate elutriates, after three washing cycles of extraction daphnids mortality increased, possibly revealing that contaminants will be released in time. This work advances our understanding of the possible effects of natural bitumen exposure on riverine aquatic ecosystems in the Alberta oil sands region and provides valuable baseline information for future environmental monitoring program design and related focused research.

Key-words: Oil sands elutriates, cycles of extraction, *Daphnia magna* reproduction output, weathered bitumen, recovery.

2.2 – Introduction

One of the largest global oil sands deposits is located in the Athabasca River Basin, north-eastern Alberta, Canada (Quagraine et al., 2005). Oil sands are comprised of a loose mixture of sand, clay, water and bitumen, a highly viscous, heavy crude oil composed of a complex mixture of hydrocarbons, hetero-organics, and metals (Colavecchia et al., 2004). Despite the impact of devastating wildfires in 2016, production of crude bitumen increased slightly from 2.53 million in 2015 to 2.65 million barrels per day in 2017, with an increased production since 2008 related to the expansion of oil sands projects that are already in production (Alberta Ministry of Energy, 2016). The mining and upgrader developments associated with oil sands extraction has increased the concern about the potential environmental impacts on air quality, water use, tailings wastewater production, groundwater contamination, and habitat disturbances in the surrounding terrestrial and aquatic ecosystems (Dowdeswell et al., 2011; He et al., 2012; Kelly et al., 2010). Petroleum-related hydrocarbons and other associated contaminants can occur naturally or be released to the environment by anthropogenic activities related to mining the bitumen containing ore and through its upgrading at associated facilities (Conly et al., 2002; Gerner et al., 2017). Several studies have revealed that the end-product from the water used to separate bitumen from oil sands, most often referred to as oil sands process water (OSPW), is both acutely and chronically toxic to a range of aquatic organisms (e.g., Bartlett et al., 2017; Frank et al., 2008; Hersikorn et al., 2010; Lari et al., 2017; Marentette et al., 2015; Morandi et al., 2016; Raine et al., 2017; Raine et al., 2018; Scarlett et al., 2013). It is estimated that tailings ponds contain 840 million m^3 of OSPW on industrial leases (Energy Resources Conservation Board, 2015). However, OSPW is currently not approved for release back into the receiving environment due to the "zero discharge policy" (Government of Canada, 2015a; Government of Canada, 2015b).

In the Athabasca oil sands area, the mainstem Athabasca River and its tributaries (e.g., the Clearwater, Ells, Steepbank, Muskeg, and Firebag Rivers) over geological time have eroded into and are flowing through naturally exposed oil sands deposits. Such proximity and contact of the river flows to the oil sands deposit provides opportunities for continuous direct and indirect loading of bitumen through streambed and/or bank erosion processes (Barton and Wallace, 1979; Headley and McMartin, 2004). This leads to an increasing potential risks of exposure to associated Polycyclic Aromatic Hydrocarbons (PAHs), Naphthenic acids (NAs) and other contaminants (e.g., metals) that are naturally present in the bitumen (Headley et al. 2001; Headley and McMartin, 2004; Gerner et al., 2017). Episodic flood events that also occur in the region may transport and deposit relatively larger volumes of oil-sand derived sediment along the rivers and after that pose an exposure risk to the aquatic systems due to the potential high load of associated contaminants and related physical habitat disturbance (Conly et al., 2002).

Recent studies by Droppo et al. (2018) examining sediment transport and dynamics in the Steepbank River, Alberta, found that suspended fluvial sediments from the McMurray oil sands formation and associated Polycyclic Aromatic Compounds (PACs) could be transported long distances and had associated oil coatings resulting in hydrophobic effects. Suspended sediment concentrations were found to progressively increase downstream in the catchment with increasing discharge, and the associated PACs concentrations and chemical signatures were not homogenous across the geological formation with both petrogenic and pyrogenic (i.e., forest fire related) characteristics evident.

Given the complexity of the potential sources, magnitude and duration of oil sands-derived sediments entering riverine ecosystems in the Athabasca region, there is a need for a more accurate assessment of related exposure risks to aquatic organisms. It is essential to define baseline conditions and ascertain the contribution of naturally derived contaminants to the aquatic ecosystem, additional to any anthropogenic contamination sources (Headley et al., 2001).

To date, only a limited number of studies have been conducted assessing the ecotoxicological effects to aquatic organisms of exposure to natural oil sands sediments. Tetreault et al. (2003) reported changes in biochemical parameters in fish residing in the natural oil sands deposit when compared to the same fish species residing within reference conditions outside of the deposit. Moreover, the adverse effects increased with the proximity to areas having active mining activities. In a laboratory exposure study, Colavecchia et al. (2004) reported significant hatching alterations in early life stages of the fathead minnow *Pimephales promelas*, with increased mortality, reduced body size, and larval deformities when exposed to either natural and anthropogenic-derived oil sands material. In a subsequent study, Colavecchia et al. (2006) found that exposure to oil sands sediments impaired the eggs and larval development and survival of the white sucker *Catostomus commersoni*.

As emphasized by Vaajasaari et al. (2002), the complementary use of ecotoxicological assays with the chemical analysis is crucial for a better and a more accurate risk assessment study where it was concluded that chemical analyses alone were not sufficient to predict the environmental effects of petroleum products in soils. Our study focuses on the effects that compounds extracted from naturally weathered bitumen through a simulated weathering process (simulating the weathering of bitumen into the rivers) will face when in aquatic streams, using the same approach of Vaajasaari et al. (2002) combining ecotoxicological assays and chemical analysis.

Using an integrated experimental approach that considers environmental, chemical, toxicological and ecological conditions and responses, this study evaluates the potential acute and chronic ecotoxicological effects of naturally occurring oil sands sediments in aquatic systems using the standardized *Daphnia magna* test system. Treatment elutriates were produced in a manner to simulate the input of newly exposed oil sands bank sediments into the aquatic receiving environment and subsequent natural erosion/weathering processes. A range of life history endpoints was evaluated for *D. magna* assessing survival, reproduction, F1 generation health status, and recovery from exposure. Results from this study will

be used to further inform oil sands monitoring and research program designs aimed at improving the capability to discriminate the environmental risks and effects of exposure to natural versus anthropogenic-derived oil sands contaminants in aquatic environments.

2.3 – Material and methods

2.3.1- Bitumen Source Material

Oil sands bitumen samples were collected in June 2014 from the Steepbank River (east side of the Athabasca River), Alberta, Canada (56° 58.754′ N 111° 17.902′ W), approximately at the interface between the Clearwater and McMurray oil sands geological formations, and outside the zone of influence of oil sands mining/development (Tetreault et al. 2003), although the Steepbank river has extensive open-pit mining in its lower half and is, therefore, representative of a disturbed basin. Samples were taken from three local sites (approx. 5 meters apart) from the bank of the river (Figure 2.1). The three collections were treated as independent samples (SP1, SP2, SP3) and not used to create a composite sample due to their distinct physical properties (i.e., differing proportional aggregates of sand, clays, and bitumen), which made it difficult to generate a homogeneous pooled sample using mechanical mixing methods. The three bitumen samples were packed in separate food grade plastic bags under refrigerated conditions and stored at 4° C until shipping to the Department of Biology, University of Aveiro, Portugal where they arrived in July 2014.

Figure 2.1 - Natural bitumen sample collected in June 2014 at the Athabasca Basin (Coordinates: 56° 58.754′ N 111° 17.902′ W), on the banks of the Steepbank River,

Alberta, Canada, approximately at the interface between the Clearwater and McMurray geological formations.

2.3.2 – Elutriates extraction

Upon arrival, the bitumen samples were stored at 4° C at the University of Aveiro until use for elutriating extraction. Elutriates were produced following van Gestel et al. (2001) and Loureiro et al. (2005) who outlined protocols to ensure reliable, reproducible and representative extraction methods for use in acute or sublethal bioassays from polluted soils.

Elutriate extraction was performed using a 1:2 ratio (solid:liquid / 400g of natural bitumen: 800 ml of ASTM per extraction process), mixing bitumen with artificial moderate hard water (ASTM, 1980). The mixture was shaken in the dark for 24 h on a benchtop orbital shaker and afterward placed in 50 mL *Falcon* tubes for centrifugation (45 min, at 3220g). After this procedure, the supernatant from each sample was stored at 4° C until testing, never exceeding a one-week holding time for testing fresh elutriates.

Three extraction cycles were performed to simulate the continuous washing/weathering of oil sands bitumen entering a river through natural bank erosional processes. After the collection of the supernatant from a 1st extraction cycle (Elutriate 1), the remaining pellet was used for a new cycle of extraction (2nd cycle – Elutriate 2), using the same methodology as previously described for the 1st cycle. This procedure was repeated to obtain the 3rd elutriate cycle (Elutriate 3).

Also, an elutriate control was used to check for a possible turbidity effect on daphnids performance. While elutriates were performed using natural riverine bitumen samples, LUFA 2.2 elutriates (control elutriate) were performed using the natural standard sandy-loam soil LUFA 2.2 (Speyer, Germany) and the turbidity was visible to the unaided eye of LUFA 2.2 elutriates.

2.3.3 – Experimental Design and Ecotoxicological *Daphnia magna* bioassays

2.3.3.1 – Experimental Design

Figure 2.2 outlines the overall experimental design of the elutriate exposure study. Elutriates were produced for each field sample of oil sands bank sediments (SP1, SP2, SP3), with three elutriate treatment levels as described above. Acute and chronic ecotoxicological tests using the K6 clone of *Daphnia magna* Straus (originally from Antwerp, Belgium) on each elutriate combination as described below. The K6 clone has been kept in laboratory culture for more than 10 years at the Department of Biology, University of Aveiro, Portugal. Cultures were maintained in ASTM moderated-hard-water medium, on a controlled temperature regime (19 °C- 21 °C) at 16 h light–8 h dark photoperiod. Daphnids in cultures were fed every two days with *Raphidocelis subcapitata* (formerly known as *Pseudokirchneriella subcapitata*) at a concentration of $3x10^5$ cells/ml and supplemented with an organic extract (Marinure seaweed extract, supplied by Glenside Organics Ltd.) (Baird et al., 1989). For testing, neonates used were selected from the 3rd to 5th broods.

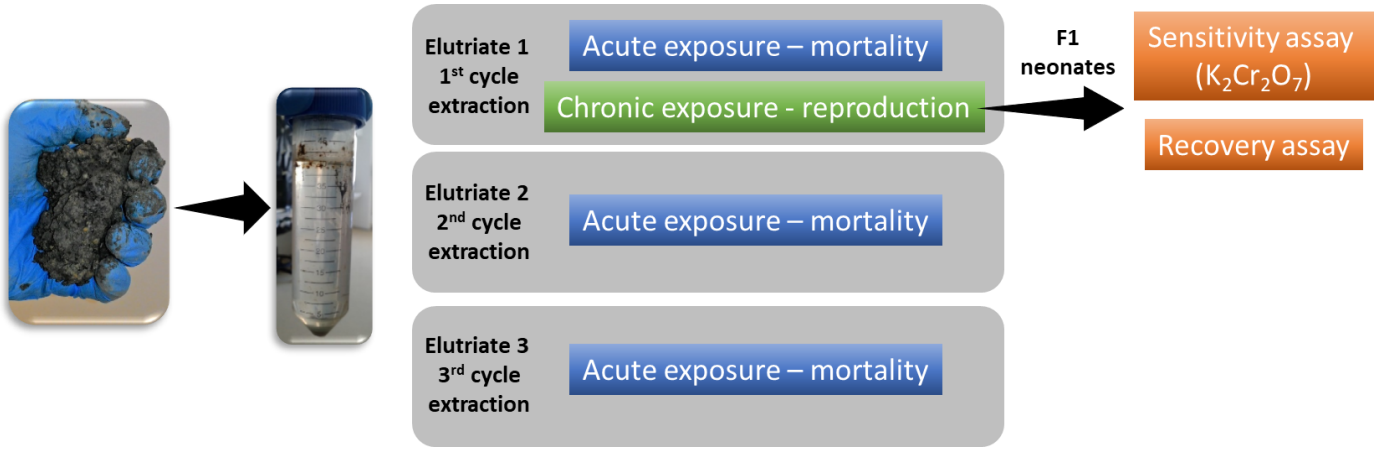

Figure 2.2 – Experimental design for the ecotoxicological evaluations for the three elutriate extraction cycles derived from naturally occurring oil sands bitumen samples from the Steepbank River, Alberta (SP1, SP2, SP3). A recovery/sensitivity bioassay was also conducted using only the first extraction cycle (Elutriate 1) for all three sampling sites.

2.3.3.2 – Acute tests – *Daphnia magna* Immobilisation test

Non-diluted elutriates (100% of elutriate) were used in a ratio of 5 animals per replicate, in a set of 5 replicates per treatment, at 20±1 °C with a 16:8h light:dark photoperiod, following the immobilization test OECD 202 guideline (OECD, 2004) (Figure 2.2). Negative control of ASTM and an elutriate control collected from natural LUFA 2.2 soil (following the same procedure for elutriate extraction described above, using LUFA 2.2 instead of bitumen samples mixed with ASTM) were used in the acute toxicity tests. To check the immobilization of daphnids when in the presence of Oil sands elutriates, each replicate had 50 mL of the respective media (controls and pure elutriates), and daphnids were observed after 24h and 48h of exposure, where the number of immobilized organisms was recorded. No food was provided during the test.

2.3.3.3 – Chronic tests – reproduction of *Daphnia magna*

The reproductive capacity of *D. magna* was evaluated during the exposure to elutriates generated from the 1st extraction cycle only (Figure 2.1), for all three bitumen samples (SP1, SP2, SP3). The rationale for performing the chronic reproductive tests only using the elutriates from the 1st extraction cycle (Elutriate 1) was based on: 1) the requirement for large volumes of elutriates for the chronic assay, with an every other day media renewal, 2) the limited quantity of natural bitumen available for testing (considering transport from Canada to Portugal), 3) the 2nd cycle extraction (Elutriate 2) showed similar acute toxicity responses as observed for Elutriate 1, and 4) the finding that Elutriate 3 produced a significantly high acute toxicity, which precluded its use in the 21 day chronic exposure reproductive tests (see results section).

Each elutriate treatment had 10 replicates, with one neonate each (<24h old; 3rd to 5th brood), negative control of ASTM and an elutriate control of LUFA 2.2 soil complemented the control treatments. Organisms were fed daily with *R. subcapitata* (3x10^5 cells/ml, plus organic extract) under a 16:8 h light:dark photoperiod regime at 20±1 °C and were exposed in a final volume of 50 ml. The test ran for 21 days and elutriates were replenished every two days along with the controls, according to the OECD 211 guideline (OECD, 2012). During the test,

survival and the total number of neonates were recorded, along with parental body length (mm), measured from the head to the insertion of the anal spine under a stereomicroscope, after the 21 days of exposure.

2.3.3.4 – Sensitivity test and recovery of F1 generation daphnids

To assess the health status of the produced neonates (generation F1), organisms from the 5th brood (< 24h old) were collected from the chronic test (section 2.3.3) and were exposed to a range of $K_2Cr_2O_7$ concentrations (0.3 – 3.0 mg/kg) for 24h, using similar methodologies as described above (2.3.3.3), with immobilized organisms being recorded (Figure 2.2). Neonates from the 5th brood were selected as this generation of neonates coincided with the reproductive cohort arising after 21-day chronic exposure. $K_2Cr_2O_7$ is used as a reference substance following the OECD 202 guideline (OECD, 2004) as an international ring-test used to assess fitness in daphnids. F1 neonates from all exposures (from both negative and positive controls, plus elutriates from SP1, SP2, SP3) were also used in a reproduction test, exposed to ASTM media to assess the recovery of neonates in clean medium (Figure 2.2). The test procedures were similar to those described above for the reproduction test. The number of neonates produced was recorded.

2.3.4 – Elutriate Chemical Analysis

Chemical analysis for metals, PAHs and NAs only in the SP3 bitumen sample and respective elutriate from the 1st cycle of extraction, since Elutriate 1 from SP3 sample induced the strongest chronic effects on daphnids, with a stronger reduction in total number neonates produced with decreased daphnid growth. This was confirmed for the three samples where a similar acute ecotoxicity output was obtained for the first and second cycles of extraction, but not for the third cycle. Also, considering the financial costs involved in the analysis of a high number and type of chemicals, only one bitumen sample and respective elutriate (1st cycle-SP3) was characterized.

The SP3 natural bitumen sample and respective elutriate were analyzed for metals and Naphthenic Acids (NAs) (InnoTech Alberta, Canada) and PAHs (AXYS Analytical Services Ltd, Canada). NAs aqueous solutions were analyzed using HPLC-Orbitrap-MS in water samples, previously adjusted to pH≈2, spiked with international (Dodecanoic acid-d23) and extracted by automated solid phase extraction. Compound characterization and quantification were performed using liquid chromatography coupled to Orbitrap mass spectrometer. NAs were analyzed in solid bitumen samples with a previous liquid/liquid extraction with an alkaline solution before analysis using HPLC-Orbitrap-MS. Metals were analyzed by Inductively Coupled Plasma Mass Spectrometry (ICP-MS) (Elan DRC-II with ESI SC-8XC high throughput FAST autosampler). Before analysis, digestion of samples was performed using an Ethos UP with the Maxi44 rotor (Milestone Inc). PAHs were analyzed by gas chromatography-mass spectrometry (GC-MS), through the AXYS Method MLA-02.

2.3.5 – Statistical analysis

Data normality and equality of variances were tested by the Shapiro-Wilk and Levene´s tests, respectively. t-tests were performed to assess statistical differences between the ASTM control and the elutriate control LUFA 2.2 treatments. One-way ANOVA followed by a multiple comparison procedure (Tukey Test) were used to test for significant differences ($p<0.05$) in immobilization, total number of neonates per female, and length of daphnids among the three (SP1, SP2, and SP3) treatment groups, controls, and recovery tests. The sensitivity of daphnid exposure was assessed by calculating the concentration that caused 50% of survival on the daphnid population (LC_{50}) using probit analysis (MINITAB 14).

2.4 - Results and discussion

2.4.1 – Ecotoxicology of elutriates

The turbidity visible to the unaided eye of LUFA 2.2 elutriates did not affect the performance of *Daphnia magna* (t-test; $p>0.05$), achieving one of the validation criteria in elutriates extraction.

No significant acute lethality was observed in F0 daphnids exposed for 48h to elutriate 1 of SP1, SP2 and SP3 bitumen samples (One-way ANOVA; $p>0.05$) (Figure 2.3-A). Elutriate 2 from the 2nd cycle was also found to have similar levels of toxicity to F0 individuals exposed in elutriate 1 (Figure 2.3-B). In contrast, a significant decrease was observed in daphnids survival when exposed to elutriate (Tukey test, $p<0.05$) (Figure 2.3-C), suggesting that ongoing erosional/weathering processes can be responsible for the eventual liberation of oil sands sediment-bound contaminants at levels toxic to aquatic organisms. Chronic toxicity tests performed using Elutriate 1 extracts from the three sample sites revealed significant impairment in daphnids reproduction during a 21d exposure and reduction in the total neonates produced per female (F1 neonates) (Figure 2.4; Tukey test; $p<0.05$). The ANOVA results also revealed statistical differences in all three field samples compared with both the ASTM and Lufa 2.2 exposure controls ($p<0.05$). Both SP1 and SP2 samples differed significantly from SP3, which had a significantly lower total number of neonates per female (Figure 2.4; Tukey test, $p<0.05$). Daphnid growth (measured as body length) was found to significantly differ from the control Lufa 2.2 elutriate exposure for all three sites (SP1, SP2, SP3), while only SP3 differed from all other samples and controls (Figure 2.5; Tukey test, $p<0.05$).

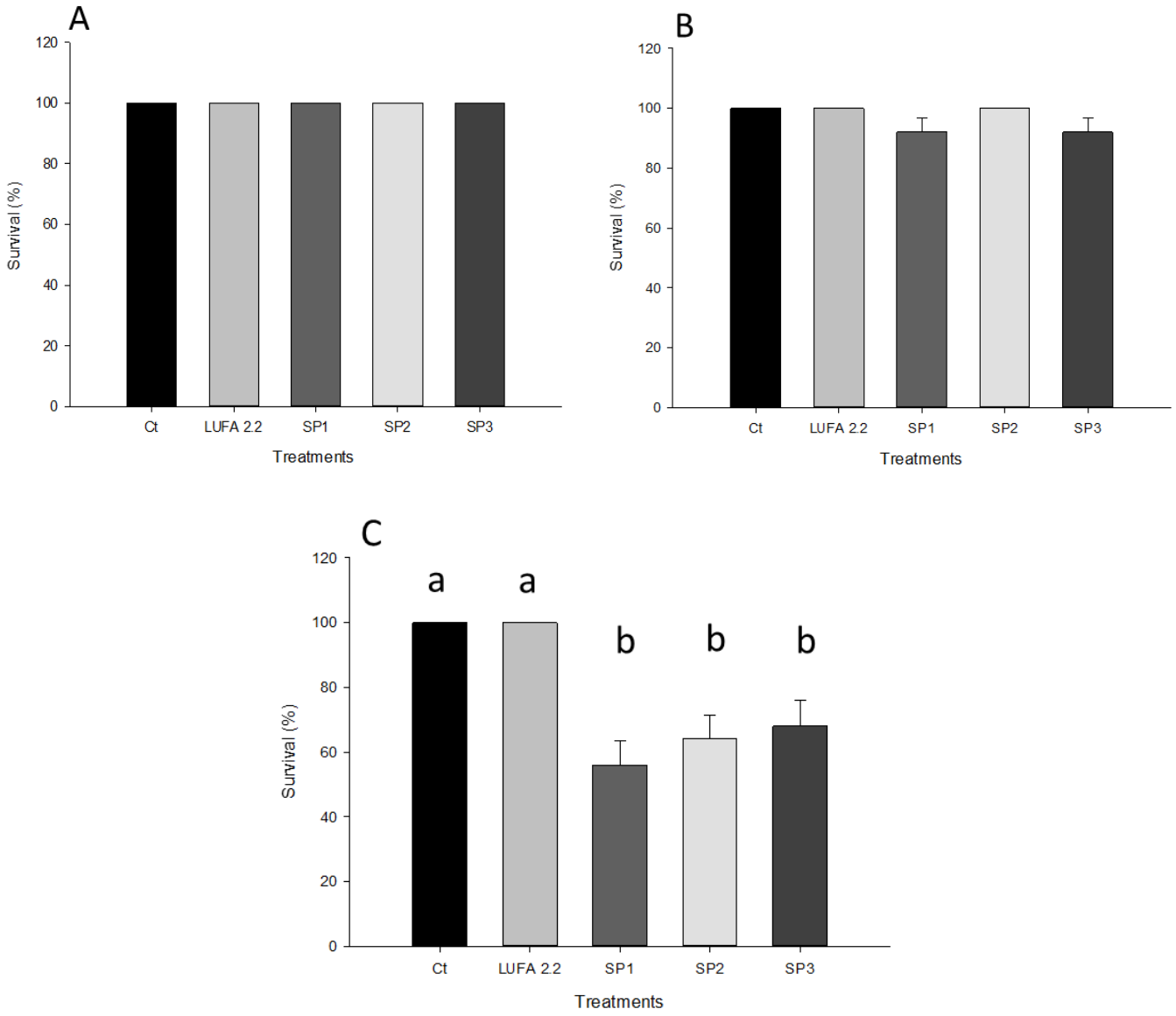

Figure 2.3 – Mean survival (%) and associated standard error of *Daphnia magna* exposed for 48h to elutriates produced from naturally occurring oil sands bitumen samples from the Steepbank River, Alberta (SP1, SP2, SP3). Elutriates were produced after (A) one extraction cycle, (B) two extraction cycles and (C) three extraction cycles. Ct represents the ASTM and Lufa 2.2 the control elutriates. Analysis of variance showed no significant differences among treatments for Cycle 1 (A) and Cycle 2 (B); however significant variation among treatments and controls were observed for Cycle 3 (C). A post hoc Tukey test showed the controls (a) differed significantly from the bitumen elutriates (b) ($p<0.05$), although no differences were observed among the sample sites.

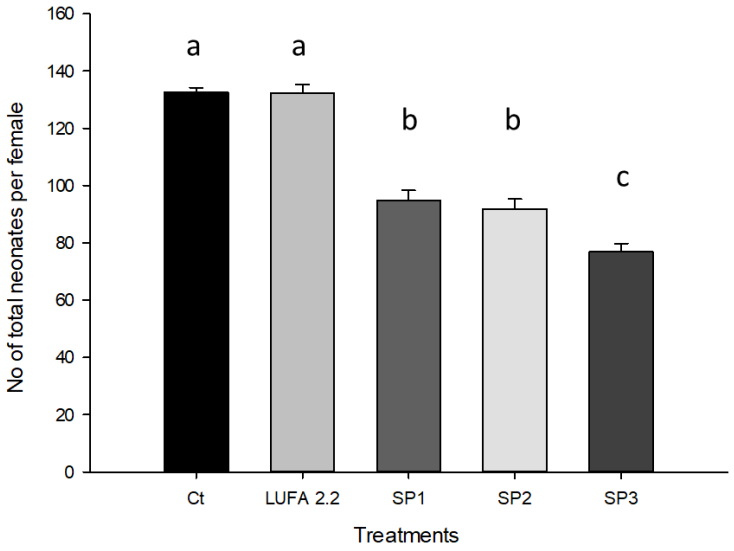

Figure 2.4 –Reproduction output (mean total number of neonates per female with standard error) of *Daphnia magna* exposed for 21 days to elutriates produced from the first extraction cycle of naturally occurring oil sands bitumen samples from the Steepbank River, Alberta (SP1, SP2, SP3). Ct represents the ASTM and Lufa 2.2 the control elutriates. Analysis of variance showed significant differences among control and bitumen elutriate samples ($p<0.05$). treatments A post hoc Tukey test showed the controls (a) differed significantly from the bitumen elutriates (b,c), and SP3 showed significantly lower neonate production (c) than the other two bitumen sites (b).

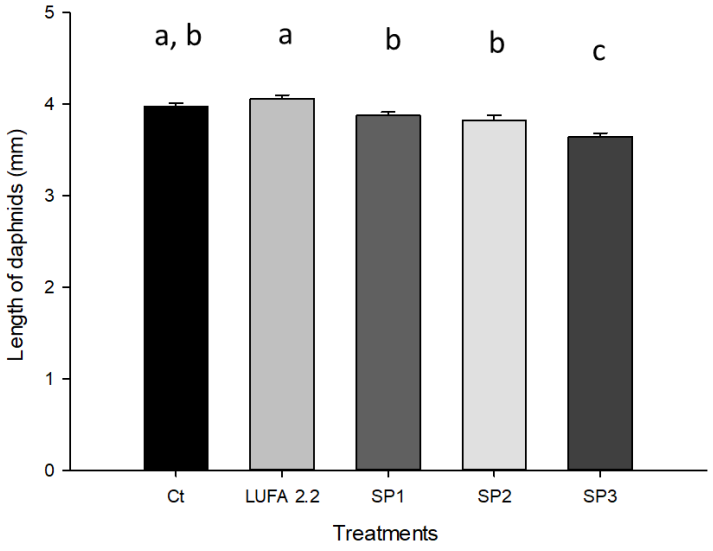

Figure 2.5 – Length of parental *Daphnia magna* (mm) after a 21-day exposure to elutriates produced from the first extraction cycle of naturally occurring oil sands bitumen samples from the Steepbank River, Alberta (SP1, SP2, SP3). Ct represents the ASTM and Lufa 2.2 the control elutriates. Analysis of variance showed significant differences among control and bitumen elutriate samples ($p<0.05$). A post hoc Tukey test showed the controls (a) differed significantly from the bitumen elutriates (b,c), although the Ct control was not significantly different from SP1 and SP2. SP3 showed significantly lower parental lengths (c) than the other two bitumen sites (b).

In the sensitivity test with the reference substance $K_2Cr_2O_7$, F1 neonates collected from parental daphnids exposed to natural bitumen elutriates were more sensitive than those from the two controls (ASTM and LUFA 2.2 soil elutriates) Comparing the LC_{50}s, all the observed values were in the recommended range provided by the OECD (2004) guideline (0.6 mg/L to 2.1 mg/L). Nevertheless, the observed LC_{50}s of daphnids from both controls (1.59 and 1.84 mg/L) were more than twice the LC_{50}s of the daphnids pre-exposed to oil sands elutriates (0.63 – 0.65 mg/L). This suggests that after exposure to oil sands material, daphnids were more sensitive than the ones not pre-exposed to the treatment material.

After the 21 days of exposure, daphnids from the 5th brood in the reproduction assay were subsequently used in the reproduction recovery tests (using ASTM media only). While a slight recovery was observed regarding the total neonates produced per female (Figure 2.6), statistical differences were still found between pre-exposed organisms and controls ($p<0.05$).

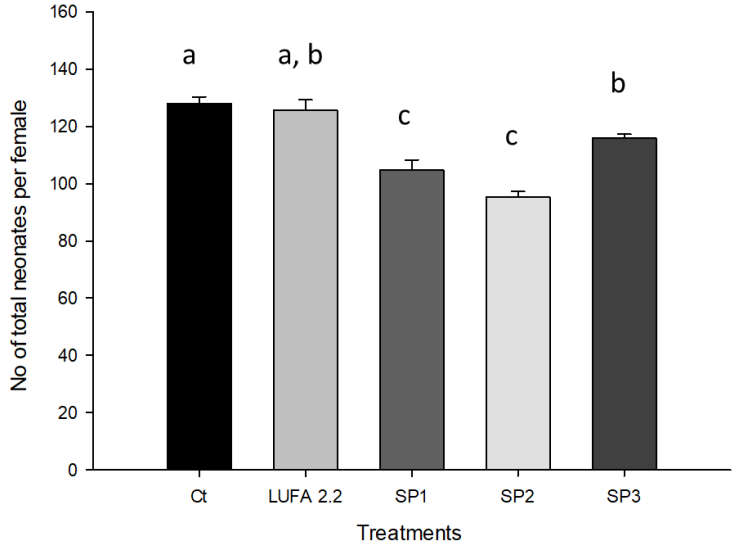

Figure 2.6 – Reproductive output (mean total number of neonates per female and standard error) of F1 *Daphnia magna* during the recovery test for 21 days in ASTM media. Organisms were pre-exposed to Ct, Lufa 2.2, SP1, SP2, and SP3 elutriates. Analysis of variance showed significant differences among control and bitumen elutriate samples ($p<0.05$). A post hoc Tukey test showed the controls (a) differed significantly from the bitumen elutriates (b,c), although the Lufa 2.2 control was not significantly different from SP3. The SP1 and SP2 treatments showed significantly lower reproductive output (c) than SP3 (b).

2.4.2 – Chemical analysis of natural bitumen samples and respective elutriates

Table 2.1 summarizes the metal content of both solid bitumen samples and respective elutriates from SP3. Comparisons between the measured levels of metals and the LC_{50}s reported by the US EPA (United States Environmental Protection Agency) database revealed none of the individual metals had higher concentrations than the respective LC_{50}s.

Table 2.2 summarizes the concentrations of the different PAHs found in the SP3 bitumen sample and respective elutriate. Similar to metals, none of the measured PAHs had higher concentrations than the respective LC_{50}s. Total naphthenic acid concentrations of 28.6 µg/L in the elutriate were measured and 33.4 µg/g the solid in the bitumen samples, respectively.

Despite the presence of generally low concentrations of single chemical compounds in elutriate and natural bitumen sample, combined effects derived from the presence of different metals, PAHs and NAs could pose a cumulative exposure risk to aquatic organisms, as observed in the effects on daphnids presented in this study. In this case, synergism or multiplicative effects may be present, and the interaction of chemicals in the organisms may be occurring.

Table 2.1 – Metal concentration levels obtained from: the SP3 bitumen sample (SP3 – solid) and respective elutriate (SP3 – elutriate (first cycle), collected from the Steepbank River, Alberta, Canada; LC_{50} for 48h *D. magna* exposures from the US EPA database.

	SP3 - elutriate (µg/L)		**SP3 – Solid sample (µg/g)**	***Daphnia magna* 48 h LC50 (EPA database)**
	Total	Dissolved	Total	
Aluminium	7.8	0.75	133000	38646.42
Antimony	0.577	0.569	1.05	15
Arsenic	0.386	0.381	21.7	5815
Barium	27.3	26.8	552	410
Beryllium	0.030	0.030	3.62	-
Bismuth	0.023	0.023	0.485	-
Boron	1740	1730	250	166666
Cadmium	0.082	0.081	0.247	101.50
Calcium	256000	255000	30900	-
Chloride	22000	21600	415	-
Chromium	0.04	< 0.1	85.5	292.50
Cobalt	0.272	0.067	34.1	2506.94
Copper	4.35	3.77	70.3	9.4
Iron	15.4	1.4	116000	10884.17
Lead	0.080	0.054	29.4	1815.00
Lithium	261	261	125	3942
Manganese	2.95	0.58	723	26193.11
Molybdenum	1.90	1.86	2.12	320150.00
Nickel	12.4	11.7	83.1	2257.90
Selenium	3.73	3.68	2.07	750
Silver	0.022	0.021	0.743	5.84
Strontium	5150	5060	376	128465
Thallium	0.0642	0.0634	0.810	61.00
Thorium	0.0493	0.0487	15.7	-
Tin	0.032	0.031	2.00	32020
Titanium	1.23	0.80	3340	-
Uranium	1.16	1.14	3.54	-
Vanadium	0.31	0.28	168	2381.45
Zinc	8.3	7.65	162	1100.00

Table 2.2 – PAHs content present in elutriates tested (SP3 – elutriate) and solid samples that originated elutriates (SP3 – solid), analyzed by gas chromatography mass spectrometry (GC-MS). Toxicity values were calculated as median of 48h *D. magna* toxicity studies available in the US EPA database. <D.L – above the detection limited;

	SP3 - elutriate (ng/L)	**SP3 - Solid (ng/g)**	***Daphnia magna* 48 h LC_{50} (EPA database)**
Naphthalene	22.7	102	2160
Acenaphthylene	<D.L.	<D.L.	2567
Acenaphthene	<D.L.	<D.L.	2097
2-Methylfluorene	<D.L.	<D.L.	-
C2 Phenanthrenes/Anthracenes	2.15	40.3	-
Fluorene	1.92	<D.L	430
Phenanthrene	7.49	40	658.2
Anthracene	<D.L.	<D.L.	55.6
C1 Phenanthrenes/Anthracenes	2	<D.L	-
Fluoranthene	2.34	13.7	105.7
Pyrene	1.39	21	1820
Benz[a]anthracene	0.412	<D.L	-
Chrysene	1	17.8	1023
Benzo[b]fluoranthene	<D.L.	<D.L.	>1024
Benzo[j,k]fluoranthenes	<D.L.	<D.L.	-
Benzo[e]pyrene	<D.L.	<D.L.	-
Benzo[a]pyrene	<D.L.	<D.L.	-
Perylene	0.778	71.3	-
Dibenz[a,h]anthracene	<D.L.	<D.L.	-
Indeno[1,2,3-cd]pyrene	<D.L.	18.1	-
Benzo[ghi]perylene	<D.L.	14	-
2-Methylnaphthalene	10.3	90.8	-
1-Methylnaphthalene	5.37	55.7	-
C1-Naphthalenes	15.7	146	-
Biphenyl	11	67.6	2014
C1-Biphenyls	9.59	86.1	-
C2-Biphenyls	34.5	33.8	-
C2-Naphthalenes	23.6	<D.L.	-
1,2-Dimethylnaphthalene	<D.L.	<D.L.	-
2,6-Dimethylnaphthalene	1.87	<D.L	-
C3-Naphthalenes	9	34.1	-
2,3,6-Trimethylnaphthalene	0.972	<D.L.	-
2,3,5-Trimethylnaphthalene	<D.L.	<D.L.	-
C4-Naphthalenes	1.67	50.3	-
C1-Acenaphthenes	<D.L.	<D.L.	-
C1-Fluorenes	2.84	<D.L.	-
1,7-Dimethylfluorene	<D.L.	<D.L.	-
C2-Fluorenes	2.66	<D.L.	-
C3-Fluorenes	6.47	<D.L.	-
Dibenzothiophene	0.737	<D.L.	-
C1-Dibenzothiophenes	<D.L.	<D.L.	-

	SP3 - elutriate (ng/L)	SP3 - Solid (ng/g)	*Daphnia magna* 48 h LC_{50} (EPA database)
2/3-Methyldibenzothiophenes	<D.L.	<D.L.	-
C2-Dibenzothiophenes	1.5	52	-
2,4-Dimethyldibenzothiophene	<D.L.	<D.L.	-
C3-Dibenzothiophenes	3.96	118	-
C4-Dibenzothiophenes	4.9	360	-
3-Methylphenanthrene	1.17	<D.L.	-
2-Methylphenanthrene	0.828	<D.L.	-
2-Methylanthracene	<D.L.	<D.L.	-
9/4-Methylphenanthrene	<D.L.	<D.L.	-
1-Methylphenanthrene	<D.L.	<D.L.	-
3,6-Dimethylphenanthrene	0.255	<D.L	-
2,6-Dimethylphenanthrene	<D.L.	<D.L.	-
1,7-Dimethylphenanthrene	0.356	8.54	-
1,8-Dimethylphenanthrene	<D.L.	<D.L.	-
C3-Phenanthrenes/Anthracenes	1.1300	24.7	-
1,2,6-Trimethylphenanthrene	<D.L.	<D.L.	-
Retene	<D.L.	21.8	-
C4-Phenanthrenes/Anthracenes	3.5700	91.7	-
C1-Fluoranthenes/Pyrenes	3.5100	52.8	-
3-Methylfluoranthene/Benzo[a]fluorene	1.6300	<D.L	-
C2-Fluoranthenes/Pyrenes	4.1500	139	-
C3-Fluoranthenes/Pyrenes	<D.L.	48.9	-
C4-Fluoranthenes/Pyrenes	<D.L.	49.4	-
C1-Benzo[a]anthracenes/Chrysenes	0.8100	35.3	-
5/6-Methylchrysene	<D.L.	<D.L.	-
1-Methylchrysene	<D.L.	9.42	-
C2-Benzo[a]anthracenes/Chrysenes	0.7420	68.6	-
5,9-Dimethylchrysene	<D.L.	<D.L	-
C3-Benzo[a]anthracenes/Chrysenes	<D.L.	13.5	-
C4-Benzo[a]anthracenes/Chrysenes	<D.L.	<D.L.	-
C1-Benzofluoranthenes/Benzopyrenes	<D.L.	<D.L.	-
7-Methylbenzo[a]pyrene	<D.L.	<D.L.	-
C2-Benzofluoranthenes/Benzopyrenes	<D.L.	36	-
1,4,6,7-Tetramethylnaphthalene	<D.L.	<D.L.	-

2.4.3 – Summary

As reported by Conly et al. (2002), Headley et al. (2001), Headley and McMartin (2004), Gerner et al. (2017), the discrimination between natural and anthropogenic sources of contamination in the oil sands area is difficult but crucial to objectively defining ecological baseline conditions against which ecological change can be compared. The continuous fluvial erosion of the naturally occurring bitumen deposits in the oil sands region has occurred over long periods of time.

Consequently, these riverine systems and the organisms that live in the rivers and streams have been and are continuously exposed to low levels (and to different doses) of hydrocarbons and metals through water and/or sediment exposure (Barton and Wallace, 1979).

Within this study, we aimed to assess the effects that a natural load of bitumen into aquatic biota and chemical analysis clearly indicated that contaminants were released from the bitumen samples to the elutriates. Despite the presence of low concentrations of each one of the contaminants in the elutriate samples, the effects on daphnids were clearly observed, reducing their reproductive capacity and their recovery from exposure to oil sands elutriates, when in the clean medium. This happened due to the possible synergistic effects that contaminants could induce to the exposed biota. A total sum of different levels of metals, PAHs and NAs (and other contaminants that were not covered by the present study) was able to induce effects on daphnids, demonstrating the extreme importance of the ecotoxicological assays, that with a combined approach with chemical analyses, provided a more complete approach to the effects of bitumen elutriates into aquatic organisms.

Elutriates produced from three cycles (72h shaking) were found to be more toxic than elutriates generated from one cycle of extraction (24h shaking). This shows that contaminants are released with time, which could be problematic when we are dealing with samples that suffer long periods of washing forces by the river flow. This result opens an opportunity to go forward and explore more in detail how these contaminants are inducing strong effects to the biota with the increase in cycles of extraction.

Heterogeneity of toxicity was also stated in this work. Even when the three samples distanced 5 meters apart, different toxicities were observed from the SP3 sample to the other two samples used. This highlights the complexity when evaluating the effects of bitumen into streams since bitumen collected from three close locations induced different toxicities in exposed daphnids. The obtained result was not previously expected. The preliminary idea was to test a composite sample with the three different samples. Due to the difficulties to generate a

homogeneous pooled sample, three different samples were tested, and the results should be used in future designing monitoring programs. Due to this difference in toxicity, chemical analysis for metals, PAHs and NAs only in the SP3 bitumen sample and respective elutriate from the 1st cycle of extraction.

Working in the Steepbank River, Droppo et al. (2018) revealed a longitudinal increase in PAC concentrations from upstream to downstream, with an increase during high flow periods when the erosive forces are the greatest, and the overland flow contribution is high. The present study complements Droppo et al. (2018) by providing new insights regarding the potential ecotoxicological effects of freshly eroded streambank bitumen. Both acute and chronic toxicological responses were observed in *Daphnia magna* when individuals were exposed to bitumen-derived elutriates that simulated fluvial erosional processes. Moreover, chronic, long-term exposure to the complex mixture of chemical compounds (polyaromatic hydrocarbons, metals) contained in the bitumen elutriates were shown to result in potential population-level effects, as highlighted by the low recovery by the F1 individuals and lower growth rates of adults (Figure 2.4 and 2.5).

This study contributes further to previous work of Tetreault et al. (2003) who assessed the physiological responses of the sentinel fish species *Cottus cognatus* and *Semotilus margarita*, collected from reference areas, from areas with naturally occurring oil sands-related compounds (natural loading of oil sands material) and from areas adjacent to surface mining activity. Results from that study revealed altered baseline biochemical parameters (Ethoxyresorufin-O-deethylase (EROD EROD) activity) in fish residing in the natural oil sands deposit when compared to the same fish species residing within reference conditions, increasing these alterations with the proximity to areas with mining activities. In other related studies, Colavecchia et al. (2004) found that exposure to sediments within natural oil sand deposits from the Athabasca River sediments in early life stages of *Pimephales promelas* altered their survival rate, hatching success, an increase of malformations and effects on size and increase of deformities (e.g., edemas, hemorrhages, and spinal malformations). Colavecchia et al. (2006) exposed white

sucker *Catostomus commersoni* eggs and larvae to river sediment containing natural bitumen and wastewater pound sediments and found that both sediments impaired the development and survival of both life stages. Although the eggs hatched earlier, there was an increase in mortality, malformations and retarded larval growth, possibly affecting the fitness and stability of fish populations.

The results from this study reinforce the need for longer-term multi-generational chronic ecotoxicological tests to provide more realistic and conclusive information regarding population-level responses and resulting cascading ecological effects arising from exposure to naturally occurring bitumen sources in the Alberta oil sands region. Defining ecological baseline conditions in the oil sands region must be informed on having a more detailed understanding of the geographic extent and magnitude of effects that exposure to natural oil sands deposits and related fluvial sediments have on aquatic biota.

2.5 – Acknowledgments

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Chapter 3: Effects of natural bitumen in the aquatic environment of the Athabasca Oil sands: an ecotoxicological approach

Effects of natural bitumen in the aquatic environment of the Athabasca Oil sands: an ecotoxicological approach

3.1 - Abstract

The Athabasca oil sands deposits in northern Alberta, Canada, are a naturally occurring mixture of bitumen, sand, clay and other minerals. Bitumen is a dense and extremely viscous oil, which is mined and then subsequently refined to produce gasoline, diesel and other hydrocarbon-based products. Moreover, the naturally occurring Athabasca oil sands deposits are a source of both physical and chemical stressors to the local rivers that flow through the bitumen deposits. Physical stress on aquatic biota from natural bitumen results from hillslope erosion processes and slumping of material into the rivers, while chemical stress arises from bitumen-derived contaminants entering the waters. To fully understand the ecological and cumulative effects of oil sands mining activities on the water quality of the surrounding aquatic ecosystem and associated biological structure and function, it is essential to evaluate the effects of the natural bitumen in the aquatic ecosystem. The primary objective of this study was to evaluate the potential ecotoxicological effects associated with the slumping of river bank bitumen (i.e., oil sands deposits that naturally enters the river system through fluvial geomorphological processes). A series of laboratory ecotoxicological assays were conducted using aquatic organisms from different trophic levels (*Daphnia magna*, *Physa acuta,* and *Vibrio fisheri*). All these model organisms were exposed to elutriates produced from natural bitumen collected from four different sources from the regional rivers: two different locations at the Steepbank river (SP and STB samples), one from the Ells river (ELLs sample) and another from the Athabasca River (ATB sample). All ecotoxicological results were complemented with a chemical analysis of metals, naphthenic acids (NAs) and polycyclic aromatic hydrocarbons (PAHs) to understand the possible effects that this natural bitumen may induce when in contact with the aquatic systems. All tested organisms responded negatively to the presence of STB and ELLs samples through the

exposure to elutriates prepared from each sample. Low toxicity was also perceived regarding SP samples. These results are in accordance with the chemical analysis, where higher levels of PAHs and NAs were detected in the ELLs sample. In summary, tests revealed that oil sands material negatively affected the organisms, under laboratory exposures, especially in the samples with higher contents of NAs and PAHs.

Key-words: natural bitumen, weathering of river banks, oil sands natural contamination, oil sands elutriates, background toxicity.

3.2- Introduction

Athabasca oil sands are one of the four natural oil sands deposits in northern Alberta, Canada, an area rich in natural oil, representing over 174 billion barrels of crude oil reserve, producing 2.65 million barrels/day in 2017, and projecting to produce 4.09 million barrels/day in 2035 (CAPP, 2018). These oil sands are a naturally occurring mixture of bitumen, sand, clay and other minerals (Barton and Wallace, 1979) where bitumen is a heavy and extremely viscous oil that, in refineries, and after some treatment produces gasoline and diesel among other products. Hence, these Athabasca oil sands are a source of bitumen and bitumen-derived contaminants, that will possibly reach the aquatic environment.

Mining or associated upgrading facilities are the primary input source of contaminants into rivers and tributaries in oil sands, with industrial activities associated with surface mining (Open-pit mining) and u**p**grading of bitumen actively also contributing to a significant landscape disturbance and potential habitat loss (Kelly et al., 2010; Kurek et al., 2013). Also, the extraction process of crude oil from bitumen produces large volumes of process-affected waters that are stored in tailing pounds containing residual bitumen, naphthenic acids, and Polycyclic Aromatic Hydrocarbons (PAHs), which have been shown to be acutely and chronically toxic to aquatic organisms (Lari et al., 2017; Nero et al., 2006; Yergeau et al., 2012).

Despite this possible impact on surrounding ecosystems induced by anthropogenic sources in the oil sands area, natural sources of potential

contamination are also possible. Local rivers and tributaries flow through bitumen deposits and are consequently exposed to bitumen and bitumen derived components (Colavecchia et al., 2004; Conly et al., 2002; Gerner et al., 2017; Yergeau et al., 2012) with a new load of fresh bitumen material entering in the systems by erosion of the slumping areas. Thus, bitumen, which is rich in PAHs, metals and Naphthenic acids (NAs), could have a negative impact on the aquatic organisms in consequence of its release into the aquatic systems (Barton and Wallace, 1979; Colavecchia et al., 2004; Conly et al., 2002; Gerner et al., 2017). Due to the overlapping of both natural and anthropogenic potential sources of contaminations in the region, characterization of each type of contamination source remains a priority (Hein and Cotterill, 2006).

Few studies have tried to define and characterize the effects that natural constituents of oil sands can pose to aquatic biota. In the attempt to provide baseline information regarding the health of fish from sites within the Athabasca oil sands deposits, Tetreault et al. (2003) collected *Cottus cognatus* and *Semotilus margarita* from the Ells and Steepbanck Rivers to evaluate the influence of naturally occurring oil sands-related compounds in the fishes' reproductive output. Results revealed altered biochemical baseline parameters in the small sentinel fish species from the oil sands area compared to fish collected in reference areas, outside the oil sands area, proving that the natural background toxicity influenced fish physiology. Also, laboratory studies have demonstrated that the presence of sediment contaminated with natural loads of bitumen negatively affected the early life stages of fish (Colavecchia et al., 2004; Colavecchia et al., 2006; Colavecchia et al., 2007).

Therefore, there is a need to provide more information about the effects that natural input of bitumen and bitumen-related materials may have on aquatic systems and organisms, to accurately derive ecological risk due to oil sands mining-related activities. This will enable to establish the inherent toxicity driven by natural processes as well as define the background levels to discriminate anthropogenic level effects (Wrona et al., 2011).

This study aimed to evaluate the effects that natural erosion processes in oil sands banks can pose to aquatic organisms, with the entrance of bitumen into the aquatic systems. The approach employed was based on the one described in Chapter 2, using elutriates (liquid extracts) produced from naturally occurring bitumen collected from the Athabasca oil sands area (with no industrial activity) to perform a battery of aquatic bioassays. Furthermore, this study was performed to confirm if the previously detected toxicity by one sample collected from the banks of the Steepbank River would also be observed in different aquatic organisms and exposed to samples collected from different locations in the Athabasca oil sands area and to what extent. Therefore, natural bitumen was collected from four different locations within the oil sands area: two sampling sites in the Steepbank River (SP and STB), one in the Ells River (Ells) and one in the mainstream of the Athabasca River (ATB). The generated elutriates were used in ecotoxicological assays using the cladoceran *Daphnia magna*, the pond snail *Physa acuta* and the marine bacteria *Vibrio fischeri*, assessing multiple endpoints that will contribute to a better perception of the effects of the natural load of bitumen in aquatic systems

3.3 – Material and methods

3.3.1 – Study area and sample collection

Natural bitumen was collected from four different locations in three rivers in the Athabasca oil sands in Alberta, Canada. Two samples were collected in the banks of the Steepbank River: SP (56°58'47.3"N 111°17'53.0"W) and STB (56°59'55.1"N 111°24'12.1"W), being the SP sample used in the Chapter 2. The other two samples were collected in Ells River (ELLs - 57°16'49.0"N 111°42'17.0"W) and in the banks of the Athabasca mainstream (ATB - 58°12'03.6"N 111°22'48.0"W), where bitumen is present in the form of “Bitumen balls”. The texture, color, odor, and visual characteristics differed between all samples. The samples were packed into a heavy gauge, food-safe plastic bags, placed in coolers and sent to the Department of Biology, University of Aveiro, in Portugal. Samples were stored at 4° C till testing.

3.3.2 – Elutriates extraction

The elutriate extraction followed the procedure described in Chapter 2, adapted from Loureiro et al. (2005). Each collected natural bitumen sample (solid:liquid / 400g of natural bitumen: 800 ml of liquid medium per extraction process) was mixed in a 1:2 ratio (solid:liquid). For daphnids, American Society for Testing and Materials (ASTM) was used as liquid medium, liquid phase MICROTOX® diluent for *Vibrio fischeri* and Artificial Pond Water (APW) (Naylor et al., 1989) for the snail tests. The solid bitumen and liquids were shaken in a benchtop orbital shaker for 24h in the dark. Afterward, samples were placed in 50 mL *Falcon* tubes and centrifuged for 45 min at 3220g. The supernatant was then collected and stored at 4° C until the tests were performed, never exceeding one week.

3.3.3 – Test species

Daphnia magna Straus Clone K6 was obtained from laboratory cultures at the Department of Biology, University of Aveiro, Portugal. Daphnids were maintained in ASTM moderated-hard-water medium, under a controlled temperature of 20±1°C and 16 h light–8 h dark photoperiod cycle. The medium was renewed three times per week and daphnids fed with the algae *Raphidocelis subcapitata* (formerly known as *Pseudokirchneriella subcapitata*) at a concentration of $3x10^5$ cells/mL. Also, the medium was supplemented with an organic extract (Marinure seaweed extract, supplied by Glenside Organics Ltd.) (Baird et al., 1989). Neonates prevenient from the 3rd to 5th broods were used in each test.

The pond snails *Physa acuta* were obtained from the same laboratory and maintained in 6-L glass aquaria, with a maximum of 50 snails per aquarium with 3 L of APW. Snails were fed *ad libitum* three times a week with ground fish food (Tetramin®). Cultures were maintained in a temperature-controlled room of 20±1° C, with a 16 h light-8 h dark photoperiod, the pH was kept above 7.9±0.3 (since acidic environments cause shell fractioning), and continuous aeration (dissolved oxygen above 8 mg/L). Culture media was partially renewed twice a week and entirely renewed once a week. New snail cultures were initiated from newly hatched offspring using egg masses deposited by adult snails and collected into 1-L glass vials.

The luminescent bacteria *Vibrio fisheri* was purchased from Azur Environmental (Azur Environment Ltd, 1998) and used in a MICROTOX equipment.

3.3.4 – Ecotoxicological bioassays with oil sands elutriates

3.3.4.1 – *Daphnia magna* exposed to elutriates

To assess the effects of natural bitumen elutriates in daphnids' life traits, standardized immobilization tests (OECD, 2004) were conducted, exposing daphnids, with less than 24h and between the 3th to 5th brood, to a series of dilutions (6, 12.5, 25, 50, 75 and 100%) of each elutriate (ATB, STB, and ELLs), using 5 replicates per treatment and 5 neonates per replicate. The SP elutriate was not used as its' toxicity to daphnids has been already reported in Chapter 2, and it will be used for comparison with results from the other elutriates. The lethal concentration inducing 50% mortality (LC_{50} - number of immobilized daphnids) were also calculated (see Statistical analysis section). After analyzing the results of the immobilization tests, standardized chronic assays (OECD, 2012) were also performed using one daphnid per replicate (10 replicates per treatment) with less than 24h, exposed to oil sands elutriates with different dilution factors. Considering the different acute toxicity levels of each elutriate, in the chronic assays daphnids were exposed to: a) 6, 12.5, 25, 50, 75 and 100% of the ATB elutriate, b) 5, 10, 30, 40, 50% of the STB elutriate and c) 0.5, 1.5, 2.5, 5, 15 and 25% of the ELLs elutriate. At the end of each reproduction test, each mother daphnid was measured (body length (mm), excluding the anal spine) using a stereomicroscope.

For both acute and chronic exposures, a negative control treatment with ASTM was prepared along with an extra control, set up with elutriates extracted from the natural soil LUFA 2.2 in ASTM (in a similar extraction ration of 1:2 as previously described), to check the possible turbidity effect on daphnids' performance, considering their filter feeding activity. Both experiments were conducted under standardized controlled conditions, using a 16h/8h light: dark photoperiod and a temperature of 20±1° C.

3.3.4.2 – *Physa acuta* assays

To evaluate the effects of oil sands extracts in snails, egg masses of *Physa acuta* were exposed to the four oil sands elutriates (SP, ATB STB, and ELLS). At the beginning of the test, the number of eggs (embryos) per egg mass (aged 2 to 4 days old) were counted using a stereomicroscope. Tests were conducted using one egg mass per replicate, in a total of 5 replicates per treatment, using a series of dilutions of 6, 12.5, 25, 50, 75 and 100% for each elutriate. Each exposure was set up in 50-mm-diameter Petri dishes with approximately 15 mL of the test solution, and a full medium renewal was performed every other day during the testing period (semi-static test). Each exposure was carried out until the control reached 90% hatching success (13d). The number of alive and dead snails plus the number of alive and dead embryos were checked daily. At the end of the test (day 13), the percentages of alive and dead snails and alive and dead embryos were calculated. The LC_{50} (cumulative mortality including snails and embryos) and the effective concentration inducing 50% effect (EC_{50}) on hatching success (alive and dead snails included) were also calculated (see Statistical analysis section). All tests were conducted in a temperature-controlled room (20±1° C), with a 16:8-h light: dark photoperiod.

3.3.4.3 – MICROTOX® basic test

To evaluate the effects of oil sands elutriates in the bacteria *V. fischeri*, Microtox® bioassays were conducted using different dilutions of each elutriate prepared with the liquid phase diluent, following the procedure described in the “81.9% Basic test” (screening test) protocol (Azur Environment Ltd, 1998). The protocol defines 81.9% as the maximum concentration of exposure, and a series of dilutions were prepared to derive a dose-response curve of the bacteria’s bioluminescence inhibition exposed to each elutriate. Bacterial luminescence was measured after 5 and 15 min of exposure using the Microtox M500 Toxicity Analyzer. The EC_{50} regarding the effects on the inhibition of the bioluminescence was also calculated. The effects on the bioluminescence of the bacteria *V. fischeri* was also evaluated in the presence of LUFA 2.2 elutriates, considering possible interferences that turbidity may have upon bioluminescence measurements. Results were presented as a percentage of bioluminescence inhibition.

3.3.5 – Chemical analysis

Chemical analysis for metals, NAs (by InnoTech Alberta, Canada) and PAHs (by AXYS Analytical Services Ltd, Canada) were carried out for both natural bitumen and respective elutriate for all samples (SP, ATB, STB, ELLs). NAs in elutriates were analyzed using HPLC-Orbitrap-MS, with pH previously adjusted to ≈2, spiked with international Dodecanoic acid and extracted by automated solid phase extraction. Chemical characterization and quantification were performed using liquid chromatography coupled to Orbitrap mass spectrometer. NAs were analyzed in solid bitumen samples with a previous liquid/liquid extraction with an alkaline solution before analysis using HPLC-Orbitrap-MS. Metals in elutriate samples were analyzed by Inductively Coupled Plasma Mass Spectrometry (ICP-MS), being the digestion of samples accomplished using an Ethos UP with the Maxi44 rotor (Milestone Inc). The ICP-MS system used for the analysis was the Elan DRC-II with ESI SC-8XC high throughput FAST autosampler. Regarding the analysis of PAHs, an extraction procedure by gas chromatography-mass spectrometry (GC-MS), through the AXYS Method MLA-02 was conducted.

3.3.6 – Statistical analysis

Both ASTM control and LUFA 2.2 elutriates were compared using a *t-test* for each daphnids' assay. Data normality and equality of variances were tested by the Shapiro-Wilk and Levene´s tests, respectively. If data were not normally distributed and/or the variances were not homogenous, and data transformation did not correct it, a Kruskal–Wallis One Way Analysis of Variance on Ranks was performed. In the daphnids tests, possible statistical differences in survival, reproduction, and length were analyzed by one-way analysis of variance (ANOVA), followed by post hoc multiple comparison Dunnett's Method whenever significant differences were obtained. The LC_{50} was calculated by probit analysis (MINITAB 14). The same approach was used for the snails' tests, where statistical differences in hatching success and survival were evaluated through a One-way ANOVA, followed by a Dunnett's Method or Dunn's test if a Kruskal–Wallis One Way Analysis of Variance on Ranks was performed. The EC_{50} for hatching

success was also calculated using a sigmoidal (logistic, 3 parameter) equation and the LC_{50} for cumulative mortality (dead snails and embryos) was calculated by probit analysis (MINITAB 14). For the MICROTOX® tests, the EC_{50} for bioluminescence inhibition of the bacteria was calculated using a sigmoidal (logistic, 3 parameter) equation.

3.4 – Results

3.4.1 – Elutriate composition

SP elutriates presented a higher metal content (∑metals: 285239 µg/L), followed by ATB (78003 µg/L), STB (58751 µg/L), and ELLs (64746 µg/L) elutriates. On an attempt to see which metals are at concentrations that may pose some concern, measured values in samples were compared with the maximum allowed by the Water Quality for the Protection of Aquatic Life and by the Soil Quality for the Protection of Environmental and Human Health from the Canadian Council of Ministers of the Environment (CCME). As shown in Table 3.1, the elutriates had concentrations of metals higher than the maximum allowed by CCME, for aluminum, copper, iron, lead, and selenium.

Taking into consideration the 16 priority PAHs defined by the U.S. Environmental Protection Agency, ELLs elutriates had the highest ∑PAHs content (499.35 ng/L), followed by STB elutriates (70.32 ng/L), and finally SP (37.25 ng/L) and ATB (35.67 ng/L) elutriates with similar PAHs contents. Pyrene and Benzo[a]pyrene concentrations were high in the ELLs elutriate, with values above the maximum allowed by CCME (Table 3.2).

NAs content followed the pattern observed for PAHs, with total values of 28.6, 663, 3330 and 5630 µg/L for SP, ATB, STB and ELLs samples, respectively. Also, the dominant presence of low molecular NAs in ELLs and STB elutriates was noted, compared with the presence of higher molecular NAs in SP and ATB elutriates (Table 3.3).

3.4.2 – *Daphnia magna* exposed to oil sands elutriates

Statistical differences were observed in the immobilization of daphnids exposed to 75, and 100% of the ATB and STB elutriates, while for the ELLs elutriate these differences compared to the control were already observed at the 50% dilution (Figure 3.1; Dunnett's test, $p<0.05$). Even though statistical differences were found for the three elutriates, mortality in STB and ELLs elutriates reached 90%, while the ATB elutriate induced only 10-20% mortality in daphnids after 48h. LC_{50} values of 69.3 and 40.3% were obtained for the STB and ELLs elutriates respectively (Table 3.4).

The chronic exposures results revealed statistical differences at low concentrations of the STB and ELLs elutriates, with a significant reduction in the total number of neonates per female already with only 5 and 1.5% of elutriates, respectively, compared to the control (Dunnett's test, $p<0.05$) (Figure 3.2). Consequently, almost all concentrations of STB and ELLs elutriates decreased the reproduction capacity of daphnids (Dunnett's test, $p<0.05$). The ATB elutriate dilutions did not affect the total number of neonates after 21 days of exposure (ANOVA $p=1.65$) (Figure 3.2).

Table 3.1 - Metal content of natural bitumen samples from SP, ATB, STB, and ELLs, collected from local rivers of the oil sands area in Alberta, Canada, and respective elutriates. Dissolved and total values are presented for elutriates. The right-side column presents the maximum level allowed by the Water Quality for the Protection of Aquatic Life and by the Soil Quality for the Protection of Environmental and Human Health from the Canadian Council of Ministers of the Environment (CCME). n.d.- not determined; values in bold highlight values above or close to the limit established by the CCME.

	SP elutriate (µg/L)		**ATB elutriate (µg/L)**		**STB elutriate (µg/L)**		**ELLs elutriate (µg/L)**		**SP solid (µg/g)**	**ATB solid (µg/g)**	**STB solid (µg/g)**	**ELLs solid (µg/g)**	**Maximum allowed by CCME (µg/L)**
	Total	Dissolved	Total	Dissolved	Total	Dissolved	Total	Dissolved	Total	Total	Total	Total	
Aluminium	7.8	0.75	8.5	0.85	**835**	**90.5**	**4040**	**2070**	133000	7630	10300	38400	**100**
Antimony	0.577	0.569	0.790	0.780	0.181	0.179	0.426	0.420	1.05	0.514	0.092	0.968	n.d.
Arsenic	0.386	0.381	2.05	1.94	0.896	0.611	1.74	1.09	21.7	23.5	2.24	3.02	5
Barium	27.3	26.8	48.6	46.8	26.4	24.8	60.6	41.8	552	131	28.3	107	n.d.
Beryllium	0.030	0.030	0.009	< 0.009	0.044	0.012	0.766	0.330	3.62	1.14	0.327	1.39	n.d.
Bismuth	0.023	0.023	0.005	0.005	0.007	0.007	0.108	0.046	0.485	0.065	0.025	0.13	n.d.
Boron	**1740**	**1730**	46.0	45.4	22.5	22.2	420	372	250	27.2	12.0	70.2	**1500**
Cadmium	0.082	0.081	0.019	0.019	0.010	0.010	0.061	0.019	0.247	0.309	0.025	0.091	0.09
Calcium	256000	255000	67500	66900	43500	43200	16300	14400	30900	16000	1270	2000	n.d.
Chloride	22000	21600	9950	9930	13700	13500	38200	32700	415	463	228	356	n.d.
Chromium	0.04	< 0.1	< 0.03	< 0.1	0.80	0.1	7.15	3.3	85.5	15.5	6.80	27.7	8.9
Cobalt	0.272	0.067	0.942	0.042	0.831	0.612	5.72	2.21	34.1	16.3	3.58	13.2	n.d.
Copper	**4.35**	**3.77**	1.05	0.98	1.38	1.36	**11.5**	**5.19**	70.3	12.1	2.8	11.2	**2**
Iron	15.4	1.4	72.4	2.8	**501**	**118**	**5190**	**1410**	116000	42700	6000	16300	**300**
Lead	0.080	0.054	0.084	0.013	0.665	0.186	**12.6**	**5.10**	29.4	9.41	4.71	11.7	**1**
Lithium	261	261	4.29	4.23	8.65	8.54	60.1	53.7	125	11.8	13.8	90.0	n.d.
Manganese	2.95	0.58	175	95.6	10.3	10.3	57.7	32.0	723	578	50.5	677	n.d.
Molybdenum	1.90	1.86	1.12	1.11	1.52	1.50	1.59	1.57	2.12	3.96	3.55	2.19	73
Nickel	12.4	11.7	1.15	1.14	7.33	6.67	14.7	7.12	83.1	47.3	35.3	36.7	25
Selenium	**3.73**	**3.68**	0.40	0.40	0.41	0.34	**1.01**	0.69	2.07	1.07	0.33	0.57	**1**
Silver	0.022	0.021	0.003	0.002	0.017	0.005	0.147	0.073	0.743	0.469	0.137	0.315	0.25
Strontium	5150	5060	182	180	110	108	240	238	376	101	23.8	74.9	n.d.
Thallium	0.0642	0.0634	0.0269	0.0264	0.0312	0.0308	0.0708	0.0501	0.810	0.235	0.118	0.238	0.8
Thorium	0.0493	0.0487	0.0090	0.0089	0.0741	0.0482	7.03	3.00	15.7	10.2	4.32	7.27	n.d.
Tin	0.032	0.031	< 0.003	< 0.003	0.023	0.007	0.164	0.159	2.00	0.466	0.226	1.10	n.d.
Titanium	1.23	0.80	0.94	0.62	16.4	6.95	86.9	85.8	3340	2090	799	1800	n.d.
Uranium	1.16	1.14	0.529	0.519	0.060	0.057	1.43	0.901	3.54	2.53	0.800	1.16	15
Vanadium	0.31	0.28	2.45	2.19	1.97	0.76	14.4	7.09	168	74.2	67.3	66.9	n.d.
Zinc	8.3	7.65	4.3	3.73	4.9	3.35	9.9	5.20	162	45.5	12.2	32.6	30

Table 3.2 - PAHs content (analyzed by gas chromatography mass spectrometry (GC-MS)) in natural bitumen present in the SP, ATB, STB and ELLs samples, collected from local rivers of the oil sands area in Alberta, Canada, and respective elutriates. The right-side column presents the maximum level allowed by the Water Quality for the Protection of Aquatic Life and by the Soil Quality for the Protection of Environmental and Human Health from the Canadian Council of Ministers of the Environment (CCME). <D.L. – below detection limit; n.d.- not determined.

	SP elutriate (ng/L)	ATB elutriate (ng/L)	STB elutriate (ng/L)	ELLs elutriate (ng/L)	SP solid (ng/g)	ATB solid (ng/g)	STB solid (ng/g)	ELLs solid (ng/g)	Maximum allowed by CCME (ng/L)
Naphthalene	22.7	22.9	25.1	23.2	102	113	105	93.9	1100
Acenaphthylene	<D.L.	<D.L.	<D.L.	<D.L.	<D.L.	<D.L.	<D.L.	<D.L.	n.d.
Acenaphthene	<D.L.	<D.L.	<D.L.	<D.L.	<D.L.	<D.L.	<D.L.	<D.L.	5800
2-Methylfluorene	<D.L.	<D.L.	<D.L.	<D.L.	<D.L.	<D.L.	<D.L.	<D.L.	n.d.
C2 Phenanthrenes/Anthracenes	2.15	<D.L.	9.45	110	40.3	546	2580	2070	n.d.
Fluorene	1.92	1.350	1.57	2.18	<D.L.	<D.L.	<D.L.	<D.L.	300
Phenanthrene	7.49	5.08	4.88	12.6	40	<D.L.	<D.L.	<D.L.	400
Anthracene	<D.L.	<D.L.	<D.L.	<D.L.	<D.L.	<D.L.	<D.L.	<D.L.	12
C1 Phenanthrenes/Anthracenes	2	<D.L.	<D.L.	24.2	<D.L.	<D.L.	<D.L.	<D.L.	n.d.
Fluoranthene	2.34	1.85	2.58	23.4	13.7	<D.L.	296	166	40
Pyrene	1.39	2.06	9.34	**95.6**	21	<D.L.	1720	752	**25**
Benz[a]anthracene	0.412	<D.L.	<D.L.	<D.L.	<D.L.	<D.L.	<D.L.	<D.L.	18
Chrysene	1	2.43	15.2	186	17.8	1070	3030	1330	n.d.
Benzo[b]fluoranthene	<D.L.	<D.L.	4.78	46.3	<D.L.	417	700	356	n.d.
Benzo[j,k]fluoranthenes	<D.L.	<D.L.	<D.L.	6.87	<D.L.	<D.L.	<D.L.		n.d.
Benzo[e]pyrene	<D.L.	<D.L.	8.16	114	<D.L.	619	1200	721	n.d.
Benzo[a]pyrene	<D.L.	<D.L.	<D.L.	**22.6**	<D.L.	227	411	205	**15**
Perylene	0.778	<D.L.	19.6	88.6	71.3	1190	1080	488	n.d.
Dibenz[a,h]anthracene	<D.L.	<D.L.	<D.L.	13.9	<D.L.	<D.L.	165	95.9	n.d.
Indeno[1,2,3-cd]pyrene	<D.L.	<D.L.	2.54	19.1	18.1	207	228	101	n.d.
Benzo[ghi]perylene	<D.L.	<D.L.	4.33	47.6	14	341	495	275	n.d.
2-Methylnaphthalene	10.3	10.7	9.41	15.3	90.8	125	95.9	90.1	n.d.
1-Methylnaphthalene	5.37	5.18	5.03	7.66	55.7	64.7	51.9	57.3	n.d.
C1-Naphthalenes	15.7	15.9	14.4	22.9	146	190	95.9	90.1	n.d.
Biphenyl	11	11.5	9.81	9.78	67.6	51.4	49.4	43.8	n.d.
C1-Biphenyls	9.59	6.47	8.49	10.9	86.1	111	94.3	76.3	n.d.
C2-Biphenyls	34.5	23.5	34.5	22.7	<D.L.	<D.L.	<D.L.	<D.L.	n.d.
C2-Naphthalenes	23.6	22.30	15.5	24	<D.L.	<D.L.	<D.L.	<D.L.	n.d.
1,2-Dimethylnaphthalene	<D.L.	<D.L.	<D.L.	<D.L.	<D.L.	<D.L.	<D.L.	<D.L.	n.d.
2,6-Dimethylnaphthalene	1.87	<D.L.	<D.L.	2.71	<D.L.	<D.L.	<D.L.	<D.L.	n.d.
C3-Naphthalenes	9	6.52	18.2	26.4	<D.L.	<D.L.	<D.L.	<D.L.	n.d.
2,3,6-Trimethylnaphthalene	0.972	<D.L.	2.19	3.32	<D.L.	<D.L.	<D.L.	<D.L.	n.d.
2,3,5-Trimethylnaphthalene	<D.L.	<D.L.	<D.L.	<D.L.	<D.L.	<D.L.	<D.L.	<D.L.	n.d.
C4-Naphthalenes	1.67	2.67	23	50.7	50.3	56.2	861	1010	n.d.
C1-Acenaphthenes	<D.L.	<D.L.	<D.L.	<D.L.	<D.L.	<D.L.	<D.L.	<D.L.	n.d.
C1-Fluorenes	2.84	2.48	3.36	9.59	<D.L.	107	469	134	n.d.
1,7-Dimethylfluorene	<D.L.	<D.L.	<D.L.	<D.L.	<D.L.	<D.L.	<D.L.	<D.L.	n.d.
C2-Fluorenes	2.66	<D.L.	5.91	49.3	<D.L.	551	2360	518	n.d.
C3-Fluorenes	6.47	11.6	48.7	283	<D.L.	1760	6890	3250	n.d.
Dibenzothiophene	0.737	0.947	<D.L.	2.88	<D.L.	<D.L.	<D.L.	<D.L.	n.d.
C1-Dibenzothiophenes	<D.L.	<D.L.	<D.L.	4.62	<D.L.	<D.L.	445	<D.L.	n.d.

	SP elutriate (ng/L)	ATB elutriate (ng/L)	STB elutriate (ng/L)	ELLs elutriate (ng/L)	SP solid (ng/g)	ATB solid (ng/g)	STB solid (ng/g)	ELLs solid (ng/g)	Maximum allowed by CCME (ng/L)
2/3-Methyldibenzothiophenes	<D.L.	<D.L.	<D.L.	<D.L.	<D.L.	<D.L.	445	512	n.d.
C2-Dibenzothiophenes	1.5	2.75	27.7	257	52	880	5000	3090	n.d.
2,4-Dimethyldibenzothiophene	<D.L.	<D.L.	<D.L.	<D.L.	<D.L.	<D.L.	<D.L.	<D.L.	n.d.
C3-Dibenzothiophenes	3.96	6.94	68.6	1470	118	13700	22900	13900	n.d.
C4-Dibenzothiophenes	4.9	10.3	172	1440	360	34700	45000	24700	n.d.
3-Methylphenanthrene	1.17	<D.L.	<D.L.	9.54	<D.L.	<D.L.	<D.L.	<D.L.	n.d.
2-Methylphenanthrene	0.828	<D.L.	<D.L.	<D.L.	<D.L.	<D.L.	<D.L.	<D.L.	n.d.
2-Methylanthracene	<D.L.	<D.L.	<D.L.	<D.L.	<D.L.	<D.L.	<D.L.	<D.L.	n.d.
9/4-Methylphenanthrene	<D.L.	<D.L.	<D.L.	14.7	<D.L.	<D.L.	<D.L.	<D.L.	n.d.
1-Methylphenanthrene	<D.L.	<D.L.	<D.L.	<D.L.	<D.L.	<D.L.	<D.L.	<D.L.	n.d.
3,6-Dimethylphenanthrene	0.255	<D.L.	<D.L.	11.4	<D.L.	<D.L.	310	173	n.d.
2,6-Dimethylphenanthrene	<D.L.	<D.L.	<D.L.	<D.L.	<D.L.	<D.L.	198	144	n.d.
1,7-Dimethylphenanthrene	0.356	<D.L.	<D.L.	8.4	8.54	<D.L.	256	193	n.d.
1,8-Dimethylphenanthrene	<D.L.	<D.L.	<D.L.	<D.L.	<D.L.	<D.L.	<D.L.	<D.L.	n.d.
C3-Phenanthrenes/Anthracenes	1.13	1.89	22.4	590	24.7	2590	11000	8420	n.d.
1,2,6-Trimethylphenanthrene	<D.L.	<D.L.	<D.L.	15.4		<D.L.	<D.L.	<D.L.	n.d.
Retene	<D.L.	<D.L.	<D.L.	<D.L.	21.8	<D.L.	<D.L.	<D.L.	n.d.
C4-Phenanthrenes/Anthracenes	3.57	20.3	256	1510	91.7	25600	57000	28200	n.d.
C1-Fluoranthenes/Pyrenes	3.51	7.77	86.1	896	52.8	4920	16900	7390	n.d.
3-methylfluoranthene/Benzo[a]fluorene	1.63	<D.L.	10.5	134	<D.L.	<D.L.	2680	1310	n.d.
C2-Fluoranthenes/Pyrenes	4.15	14.8	154	1950	139	16300	37900	19400	n.d.
C3-Fluoranthenes/Pyrenes	<D.L.	9.1	154	1600	48.9	21800	42400	20700	n.d.
C4-Fluoranthenes/Pyrenes	<D.L.	4.07	76.7	1120	49.4	8730	22200	7650	n.d.
C1-Benzo[a]anthracenes/Chrysenes	0.81	2.52	35.6	394	35.3	5530	9130	4230	n.d.
5/6-Methylchrysene	<D.L.	<D.L.	2.15	48.4	<D.L.	523	1020	660	n.d.
1-Methylchrysene	<D.L.	<D.L.	2.19	17.7	9.42	486	453	187	n.d.
C2-Benzo[a]anthracenes/Chrysenes	0.742	4.16	87.3	530	68.6	10100	17900	7470	n.d.
5,9-Dimethylchrysene	<D.L.	<D.L.	22.2	124	<D.L.	2120	3680	1600	n.d.
C3-Benzo[a]anthracenes/Chrysenes	<D.L.	0.918	35	198	13.5	6440	10400	4950	n.d.
C4-Benzo[a]anthracenes/Chrysenes	<D.L.	<D.L.	11.2	60.9	<D.L.	835	2120	758	n.d.
C1-Benzofluoranthenes/Benzopyrenes	<D.L.	<D.L.	34.1	326	<D.L.	3480	5200	2970	n.d.
7-Methylbenzo[a]pyrene	<D.L.	<D.L.	<D.L.	45.9	<D.L.	330	523	307	n.d.
C2-Benzofluoranthenes/Benzopyrenes	<D.L.	0.995	19.7	171	36	2560	3710	2100	n.d.
1,4,6,7-Tetramethylnaphthalene	<D.L.	<D.L.	1.81	6.67	<D.L.	<D.L.	205	193	n.d.

Table 3.3- Percentage of each Naphthenic acid constituent in the SP, ATB, STB and ELLs elutriates, generated by bitumen samples collected from different local rivers of the oil sands area in Alberta, Canada, analyzed by HPLC-Orbitrap-MS.

	SP (%)	ATB (%)	STB (%)	ELLs (%)
$C_5H_{10}O_2$ (Z = 0; DBE = 1)	0	0	0	0
$C_6H_{12}O_2$ (Z = 0; DBE = 1)	0.01	0	0	0
$C_7H_{12}O_2$ (Z = -2; DBE = 2)	0	0	0	0
$C_7H_{14}O_2$ (Z = 0; DBE = 1)	0	0	0	0
$C_8H_{14}O_2$ (Z = -2; DBE = 2)	0.02	0.04	0.12	0
$C_8H_{16}O_2$ (Z = 0; DBE = 1)	1.16	0	0.03	0
$C_9H_{14}O_2$ (Z = -4; DBE = 3)	0.01	0	0	0
$C_9H_{16}O_2$ (Z = -2; DBE = 2)	0.03	0	0	0
$C_9H_{18}O_2$ (Z = 0; DBE = 1)	0.82	0.03	0.04	0
$C_{12}H_{18}O_2$ (Z = -6; DBE = 4)	0	0.22	0	0.26
$C_{12}H_{20}O_2$ (Z = -4; DBE = 3)	0	1.55	0	2.74
$C_{12}H_{22}O_2$ (Z = -2; DBE = 2)	0.08	0.57	0	1.87
$C_{12}H_{24}O_2$ (Z = 0; DBE = 1)	1.48	0.04	0	0.14
$C_{13}H_{16}O_2$ (Z = -10; DBE = 6)	0.23	3.15	1.83	0.88
$C_{13}H_{18}O_2$ (Z = -8; DBE = 5)	0	0.44	0	0.18
$C_{13}H_{20}O_2$ (Z = -6; DBE = 4)	0.16	1.24	0	1.81
$C_{13}H_{22}O_2$ (Z = -4; DBE = 3)	0	0	0.02	6.84
$C_{13}H_{24}O_2$ (Z = -2; DBE = 2)	0.15	0.62	3.68	2.49
$C_{13}H_{26}O_2$ (Z = 0; DBE = 1)	0	0.02	0.02	0.18
$C_{14}H_{16}O_2$ (Z = -12; DBE = 7)	0	0.44	0	0.16
$C_{14}H_{18}O_2$ (Z = -10; DBE = 6)	0	3.23	0	1.35
$C_{14}H_{20}O_2$ (Z = -8; DBE = 5)	0.26	1.52	0	0.94
$C_{14}H_{22}O_2$ (Z = -6; DBE = 4)	3.33	3	1.71	5.05
$C_{14}H_{24}O_2$ (Z = -4; DBE = 3)	1.13	5.44	3.78	9.8
$C_{14}H_{26}O_2$ (Z = -2; DBE = 2)	0.23	0.51	1.64	2.33
$C_{14}H_{28}O_2$ (Z = 0; DBE = 1)	2.16	0.14	0.22	0.16
$C_{15}H_{14}O_2$ (Z = -16; DBE = 9)	0.02	0	0	0
$C_{15}H_{16}O_2$ (Z = -14; DBE = 8)	0.04	0	5.12	0
$C_{15}H_{18}O_2$ (Z = -12; DBE = 7)	1.41	1.65	0	1.29
$C_{15}H_{20}O_2$ (Z = -10; DBE = 6)	1.57	5.6	3.93	2.28
$C_{15}H_{22}O_2$ (Z = -8; DBE = 5)	0.91	2.3	0.31	1.8
$C_{15}H_{24}O_2$ (Z = -6; DBE = 4)	2.41	4.13	6.44	6.56
$C_{15}H_{26}O_2$ (Z = -4; DBE = 3)	1.46	4.02	2.21	6.81
$C_{15}H_{28}O_2$ (Z = -2; DBE = 2)	0.23	0.45	2.32	1.3
$C_{15}H_{30}O_2$ (Z = 0; DBE = 1)	0.92	0.1	0.19	0.08
$C_{16}H_{14}O_2$ (Z = -18; DBE = 10)	0.03	0	0	0
$C_{16}H_{16}O_2$ (Z = -16; DBE = 9)	0.52	0.36	0	0.21
$C_{16}H_{18}O_2$ (Z = -14; DBE = 8)	0.56	0	8.57	0.32
$C_{16}H_{20}O_2$ (Z = -12; DBE = 7)	5.1	4.82	0.44	2.8
$C_{16}H_{22}O_2$ (Z = -10; DBE = 6)	2.7	5.7	1.87	2.58

	SP (%)	ATB (%)	STB (%)	ELLs (%)
$C_{16}H_{24}O_2$ (Z = -8; DBE = 5)	1.93	2.29	0.28	2.28
$C_{16}H_{26}O_2$ (Z = -6; DBE = 4)	4.16	4.85	4.17	5.2
$C_{16}H_{28}O_2$ (Z = -4; DBE = 3)	1.54	2.85	5.33	3.5
$C_{16}H_{30}O_2$ (Z = -2; DBE = 2)	0.7	0.3	0.15	0.14
$C_{16}H_{32}O_2$ (Z = 0; DBE = 1)	0.59	0	0	0
$C_{17}H_{18}O_2$ (Z = -16; DBE = 9)	1.29	0.3	7.51	0.42
$C_{17}H_{20}O_2$ (Z = -14; DBE = 8)	1.97	0.13	2.82	0.28
$C_{17}H_{22}O_2$ (Z = -12; DBE = 7)	7.89	5.88	1.34	3.5
$C_{17}H_{24}O_2$ (Z = -10; DBE = 6)	2.93	4.01	2.89	2.34
$C_{17}H_{26}O_2$ (Z = -8; DBE = 5)	2.26	1.73	2.45	1.58
$C_{17}H_{28}O_2$ (Z = -6; DBE = 4)	2.64	2.34	3.01	2.04
$C_{17}H_{30}O_2$ (Z = -4; DBE = 3)	0.73	0.99	1.22	0.87
$C_{17}H_{32}O_2$ (Z = -2; DBE = 2)	0	0.11	0.16	0.13
$C_{17}H_{34}O_2$ (Z = 0; DBE = 1)	0.05	0	0	0
$C_{18}H_{20}O_2$ (Z = -16; DBE = 9)	1.29	0.17	4.28	0.26
$C_{18}H_{22}O_2$ (Z = -14; DBE = 8)	3.46	0.38	2.06	0.87
$C_{18}H_{24}O_2$ (Z = -12; DBE = 7)	6.92	4.99	0.38	2.96
$C_{18}H_{26}O_2$ (Z = -10; DBE = 6)	2.21	2.42	1.02	1.49
$C_{18}H_{28}O_2$ (Z = -8; DBE = 5)	1.76	1.03	0.92	0.7
$C_{18}H_{30}O_2$ (Z = -6; DBE = 4)	1.27	0.91	0.6	0.52
$C_{18}H_{32}O_2$ (Z = -4; DBE = 3)	0.45	0.3	0.22	0.18
$C_{18}H_{34}O_2$ (Z = -2; DBE = 2)	0.53	0.13	0.09	0.05
$C_{18}H_{36}O_2$ (Z = 0; DBE = 1)	0.26	0.12	0.05	0
$C_{19}H_{20}O_2$ (Z = -18; DBE = 10)	0.77	0.1	0.94	0.23
$C_{19}H_{22}O_2$ (Z = -16; DBE = 9)	1.03	0.04	1	0
$C_{19}H_{24}O_2$ (Z = -14; DBE = 8)	3.21	0.39	0.23	0.52
$C_{19}H_{26}O_2$ (Z = -12; DBE = 7)	3.9	3.29	1.45	1.6
$C_{19}H_{28}O_2$ (Z = -10; DBE = 6)	1.25	1.26	0.96	0.65
$C_{19}H_{30}O_2$ (Z = -8; DBE = 5)	0.64	0.38	0.21	0.18
$C_{19}H_{32}O_2$ (Z = -6; DBE = 4)	0.37	0.28	0.1	0.11
$C_{19}H_{34}O_2$ (Z = -4; DBE = 3)	0.09	0.1	0.04	0.04
$C_{19}H_{36}O_2$ (Z = -2; DBE = 2)	0	0	0	0
$C_{19}H_{38}O_2$ (Z = 0; DBE = 1)	0.01	0	0	0
$C_{20}H_{22}O_2$ (Z = -18; DBE = 10)	0.52	0.08	0.23	0.08
$C_{20}H_{24}O_2$ (Z = -16; DBE = 9)	0.73	0.07	0.02	0.04
$C_{20}H_{26}O_2$ (Z = -14; DBE = 8)	2.09	1.02	0.47	0.42
$C_{20}H_{28}O_2$ (Z = -12; DBE = 7)	2.11	1.59	1.02	0.69
$C_{20}H_{30}O_2$ (Z = -10; DBE = 6)	0.81	0.57	0.27	0.2
$C_{20}H_{32}O_2$ (Z = -8; DBE = 5)	0.24	0.12	0.04	0.04
$C_{20}H34O2$ (Z = -6; DBE = 4)	0.09	0.08	0.02	0.03
$C_{20}H_{36}O_2$ (Z = -4; DBE = 3)	0	0.03	0	0.02
$C_{20}H_{38}O_2$ (Z = -2; DBE = 2)	0	0	0	0
$C_{20}H_{40}O_2$ (Z = 0; DBE = 1)	0.08	0.01	0	0

	SP (%)	ATB (%)	STB (%)	ELLs (%)
$C_{21}H_{24}O_2$ (Z = -18; DBE = 10)	0.32	0.03	0	0
$C_{21}H_{26}O_2$ (Z = -16; DBE = 9)	0.42	0.05	0.02	0
$C_{21}H_{28}O_2$ (Z = -14; DBE = 8)	0.84	0.61	0.19	0.16
$C_{21}H_{30}O_2$ (Z = -12; DBE = 7)	0.5	0.43	0.19	0.13
$C_{21}H_{32}O_2$ (Z = -10; DBE = 6)	0.11	0.12	0.04	0.03
$C_{21}H_{34}O_2$ (Z = -8; DBE = 5)	0.03	0.02	0	0.01
$C_{21}H_{36}O_2$ (Z = -6; DBE = 4)	0.02	0.03	0	0.01
$C_{21}H_{38}O_2$ (Z = -4; DBE = 3)	0	0.01	0	0
$C_{21}H_{40}O_2$ (Z = -2; DBE = 2)	0	0	0	0
$C_{21}H_{42}O_2$ (Z = 0; DBE = 1)	0	0	0	0
$C_{22}H_{32}O_2$ (Z = -12; DBE = 7)	0.15	0.09	0.03	0.02
$C_{22}H_{34}O_2$ (Z = -10; DBE = 6)	0.06	0.04	0	0
$C_{22}H_{36}O_2$ (Z = -8; DBE = 5)	0	0	0	0
$C_{22}H_{38}O_2$ (Z = -6; DBE = 4)	0	0	0	0
$C_{22}H_{40}O_2$ (Z = -4; DBE = 3)	0	0	0	0
$C_{22}H_{42}O_2$ (Z = -2; DBE = 2)	0	0	0	0
$C_{22}H_{44}O_2$ (Z = 0; DBE = 1)	0	0	0	0
$C_{23}H_{32}O_2$ (Z = -14; DBE = 8)	0.16	0.07	0.02	0.02
$C_{23}H_{34}O_2$ (Z = -12; DBE = 7)	0.03	0.03	0	0
$C_{23}H_{36}O_2$ (Z = -10; DBE = 6)	0	0	0	0
$C_{23}H_{38}O_2$ (Z = -8; DBE = 5)	0	0	0	0
$C_{23}H_{40}O_2$ (Z = -6; DBE = 4)	0	0	0	0
$C_{23}H_{42}O_2$ (Z = -4; DBE = 3)	0	0	0	0
$C_{23}H_{44}O_2$ (Z = -2; DBE = 2)	0	0	0	0
$C_{23}H_{46}O_2$ (Z = 0; DBE = 1)	0	0	0	0
$C_{24}H_{36}O_2$ (Z = -12; DBE = 7)	0	0	0	0
$C_{24}H_{38}O_2$ (Z = -10; DBE = 6)	0	0	0	0
$C_{24}H_{40}O_2$ (Z = -8; DBE = 5)	0	0	0	0
$C_{24}H_{42}O_2$ (Z = -6; DBE = 4)	0	0	0	0
$C_{24}H_{44}O_2$ (Z = -4; DBE = 3)	0	0	0	0
$C_{24}H_{46}O_2$ (Z = -2; DBE = 2)	0	0	0	0
$C_{24}H_{48}O_2$ (Z = 0; DBE = 1)	0.01	0	0	0
$C_{25}H_{38}O_2$ (Z = -12; DBE = 7)	0	0	0	0
$C_{25}H_{40}O_2$ (Z = -10; DBE = 6)	0	0	0	0
$C_{25}H_{42}O_2$ (Z = -8; DBE = 5)	0	0	0	0
$C_{25}H_{44}O_2$ (Z = -6; DBE = 4)	0	0	0	0
$C_{25}H_{46}O_2$ (Z = -4; DBE = 3)	0	0	0	0
$C_{25}H_{48}O_2$ (Z = -2; DBE = 2)	0	0	0	0
$C_{25}H_{50}O_2$ (Z = 0; DBE = 1)	0.01	0	0	0

Table 3.4 –Concentrations of Aluminum, Iron, Copper, and Lead (µg/L) that are equivalent to the EC_{50} and LC_{50} levels (calculated based on the % of dilution) calculated for *Daphnia magna*, *Physa acuta* and *Vibrio fisheri* exposed to the STB and ELLs elutriates, produced from the natural bitumen samples. * Concentrations above the maximum level allowed by the Canadian Council of Ministers of the Environment (CCME); maximum levels reported at the bottom of the table.

		STB elutriate		**ELLs elutriate**			
		Al (µg/L)	Fe (µg/L)	Al (µg/L)	Fe (µg/L)	Cu (µg/L)	Pb (µg/L)
Daphnia magna	LC_{50} STB (69.30%)	578.6*	347.2*	-	-	-	-
	LC_{50} ELLs (40.30%)	-	-	336.5*	201.9	4.63*	5.07*
Physa acuta	EC_{50} STB (49.20%)	410.82*	246.49	-	-	-	-
	LC_{50} STB (92.40%)	771.54*	462.92*	-	-	-	-
	EC_{50} ELLs (46.30%)	-	-	1870.5*	2402.97*	5.32*	5.83*
	LC_{50} ELLs (68.92%)	-	-	2783.56*	3576.94*	7.92*	8.68*
Vibrio fischeri	EC_{50} STB (25.60%)	213.76*	128.25	-	-	-	-
	EC_{50} ELLs (11.43%)	-	-	461.77*	593.21*	1.31	1.44*
	Max. CCME level	100	300	100	300	2	1

Nevertheless, the size of the adults was significantly smaller at the higher % of elutriate tested for each elutriate exposure (STB, ELLS, and ATB) compared to the control (Dunnett's test, $p<0.05$) (Figure 3.3). Results from immobilization, reproduction, sensitivity, and recovery of daphnids exposed to the SP elutriate are reported in Chapter 2.

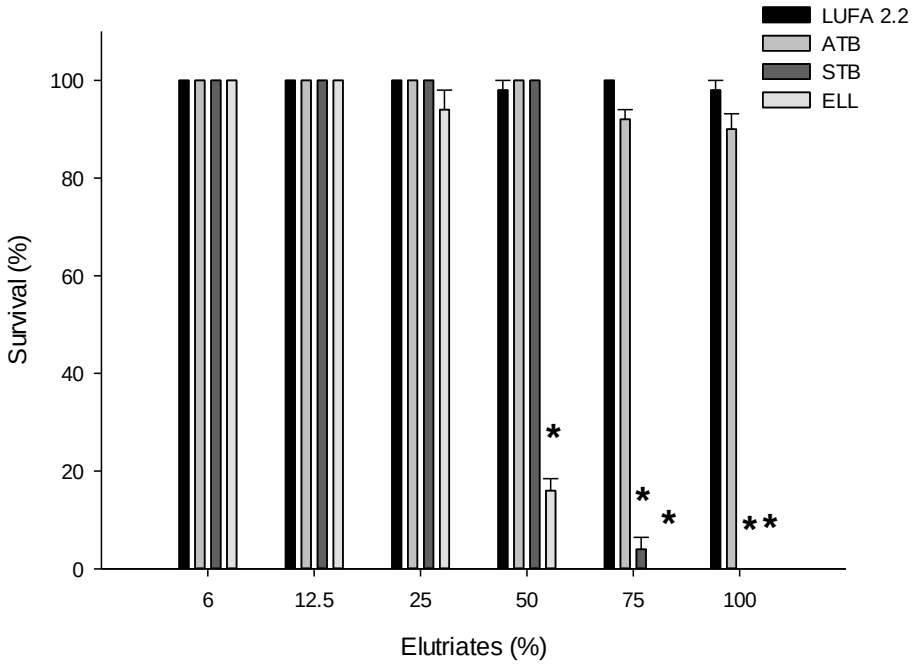

Figure 3.1 – Survival of *Daphnia magna* exposed for 48h to different dilutions of elutriates produced from oil sands bitumen (ATB, STB, and ELLs samples) and Lufa 2.2 natural soil. All elutriates were produced with ASTM as the liquid medium. Data are presented as the average number of live daphnids with standard error. * shows significant differences compared with the control ($p < 0.05$ Dunnett's test).

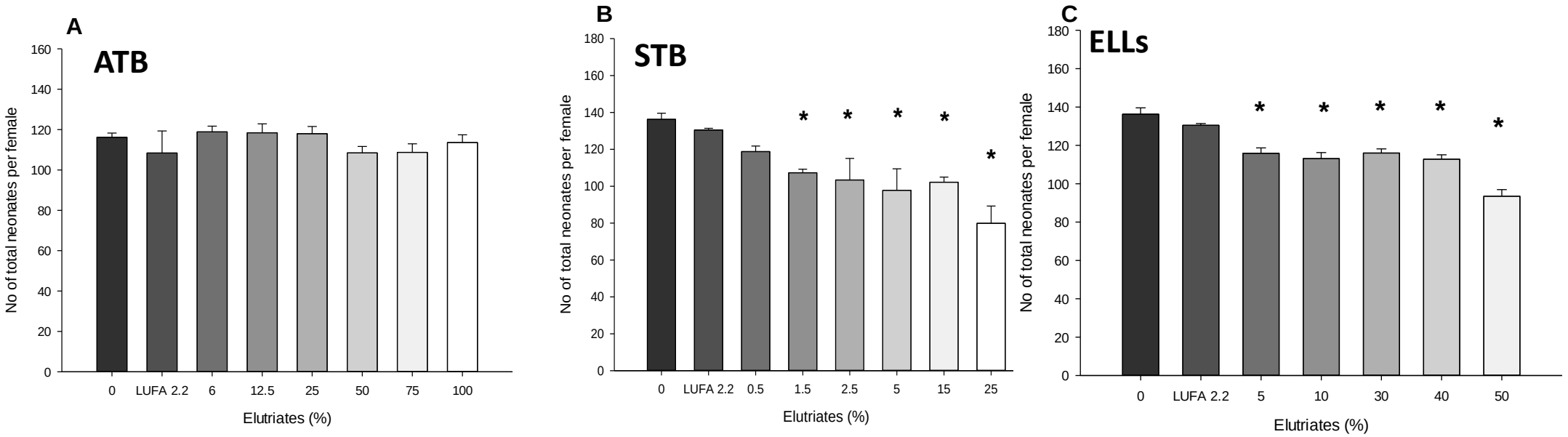

Figure 3.2 – Reproduction output of *Daphnia magna* exposed for 21 days to the different elutriates produced from natural bitumen samples collected from local rivers of the oil sands in Alberta, Canada: A) ATB; B) STB and C) ELLs. Data are presented as the mean number of total neonates per female with standard error. * shows significant differences compared to the control ($p < 0.05$ Dunnett's test).

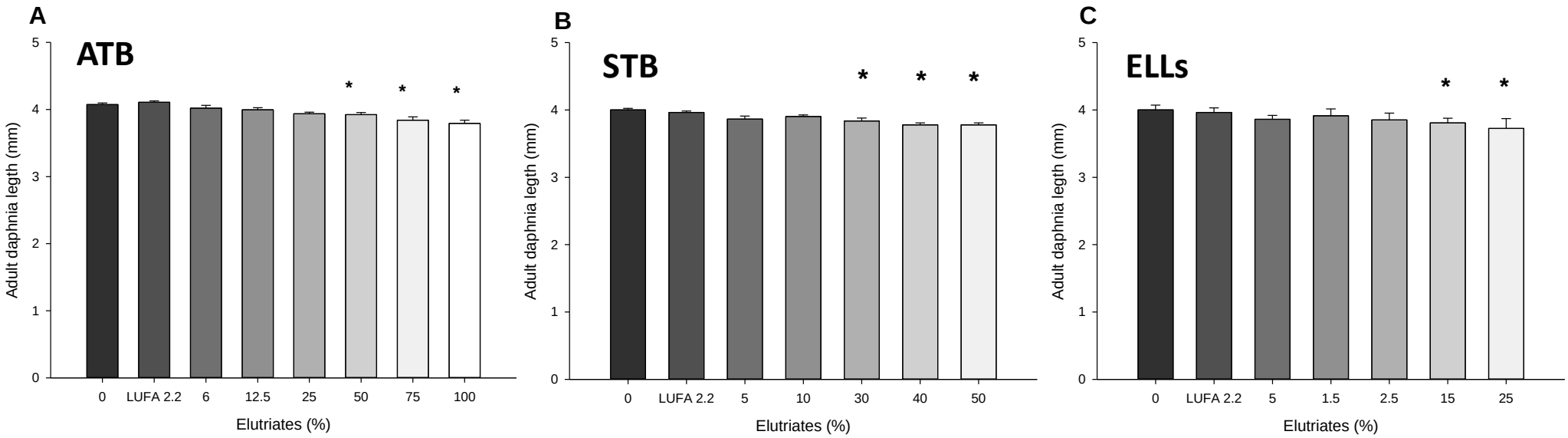

Figure 3.3 - Length of parental *Daphnia magna* exposed for 21 days to the different elutriates produced from natural bitumen samples collected from local rivers of the oil sands in Alberta, Canada: A) ATB; B) STB and C) ELLs. Data are presented as the average of daphnids length with standard error. * shows significant differences compared to the control ($p < 0.05$ Dunnett's test).

3.4.3 – *Physa acuta* exposed to oil sands elutriates

Considering the different elutriates used, snails responded differently, revealing different patterns of toxicity. No mortality and no significant effects were observed in the hatching success of the snails after 13 days of exposure to the SP elutriate (Figure 3.4-A1 and A2; Kruskal–Wallis one-way ANOVA, H=11.51, df=6, p=0.074). The same absence of significant differences for hatching success (Kruskal–Wallis one-way ANOVA, H=7.017, df=6, P=0.319) and mortality (Kruskal–Wallis one-way ANOVA, H=5.200, df=6, p=0.518) was observed when snails were exposed to the ATB elutriate (Figure 3.4-B1 and B2). In contrast, when snails were exposed to the STB elutriate, a statistically significant reduction in snails' hatching success occurred for egg masses exposed to 6, 50, 75 and 100% of elutriate (Figure 3.4-C1; Dunnett's test, $p<0.05$), with a calculated EC_{50} value of 49.2% (Table 3.4). Also, mortality increased exponentially as elutriate concentration increased, with statistically significant differences observed at 75 and 100% of the STB elutriate (Dunnett's test, $p<0.05$), where mortality was above 75% in the maximum elutriate concentration (Figure 3.4-C2) and a calculated LC_{50} value of 92.4% was obtained (Table 3.4). The Ells elutriate induced a complete dose-response curve on snails' hatching success, with statistical differences at 75% and hatching success being close to 0% when exposed to the elutriate in its pure state, i.e., 100% (Figure 3.4-D1; Dunnett's test, $p<0.05$), with a calculated EC_{50} value of 46.3% (Table 3.4). A statistical significant increase in mortality was observed when snails were exposed to 75 and 100% of the ELLs elutriate (Figure 3.4-D2; Kruskal–Wallis one-way ANOVA, H=26.776, df=6, $p<0.001$, Dunn's test, $p<0.05$), with a calculated LC_{50} of 68.92% (Figure. 3.4-D2) (Table 3.4).

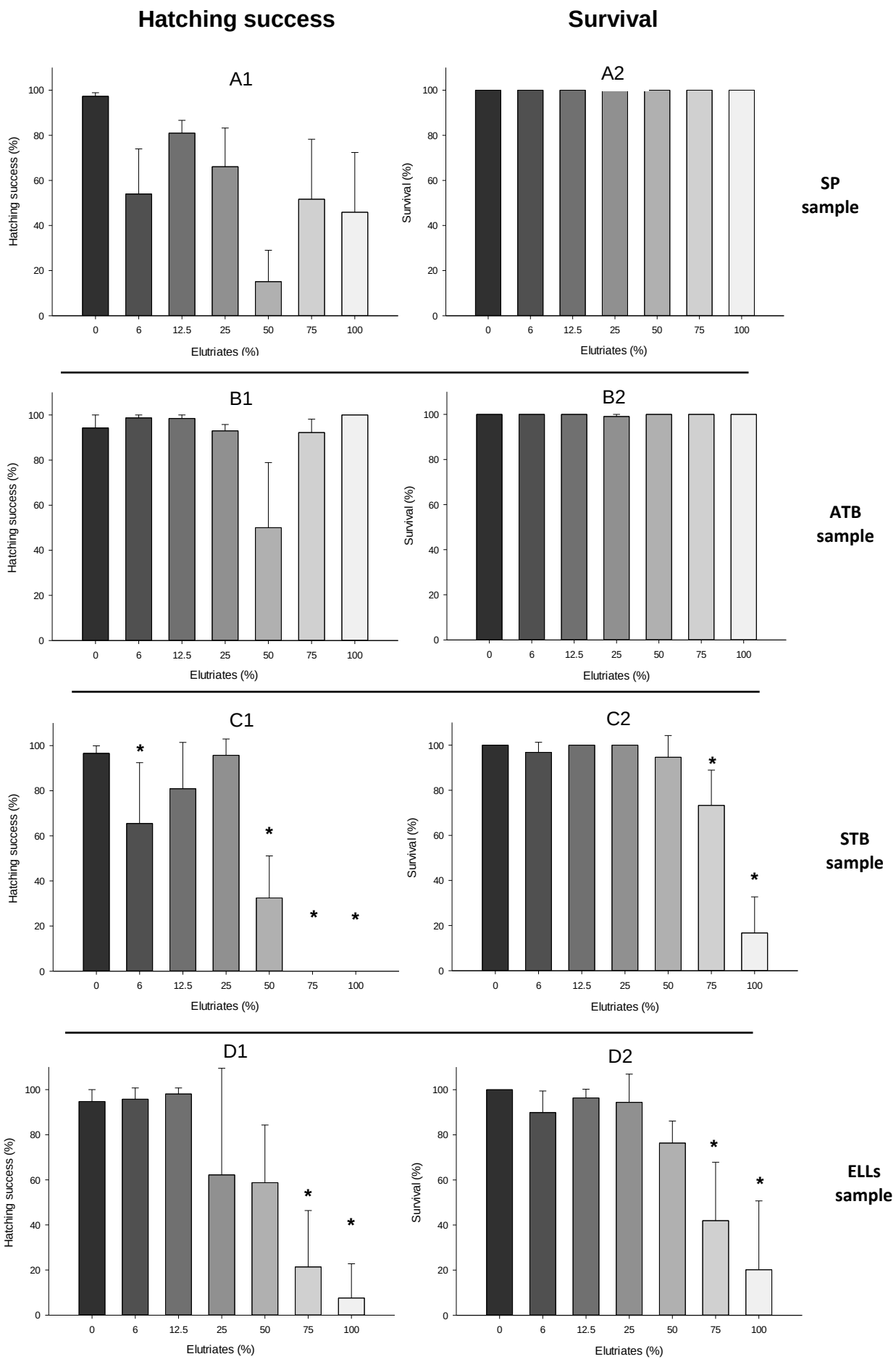
Hatching success
Survival
A1
A2
B1
B2
C1
C2
D1
D2
Hatching success (%)
Survival (%)
Elutriates (%)
0
6
12.5
25
50
75
100
SP sample
ATB sample
STB sample
ELLs sample

Figure 3.4 – Hatching success (includes alive and dead snails – left graphs – number 1) and survival (includes alive and dead embryos - right graphs) of *Physa acuta* exposed for 13 days to the different elutriates produced from natural bitumen samples collected from local rivers of the oil sands in Alberta, Canada: SP (A1 and A2), ATB (B1 and B2), STB (C1 and C2), and ELLs (D1 and D2). Data are presented as average with standard error. * shows significant differences compared to the control ($p < 0.05$ Dunnett's test for samples and Dunn's test for samples).

3.4.4 – *Vibrio fischeri* exposed to oil sands elutriates

Due to similar results in both times of exposure assessed for the bioluminescent measured, only the results of the 15 minutes are provided. The bioluminescence of *V. fischeri* was inhibited in the presence of the STB and ELLs elutriates (Figure 3.5). In both cases, a full dose-response curve was obtained, with EC_{50} values of 25.6% for the STB elutriate and 11.43% for the ELLs elutriate (Table 3.4). For the remaining elutriate samples (SP and ATB) and LUFA 2.2, no EC_{50} value was calculated, since the bioluminescence inhibition of the bacteria did not reach 50%.

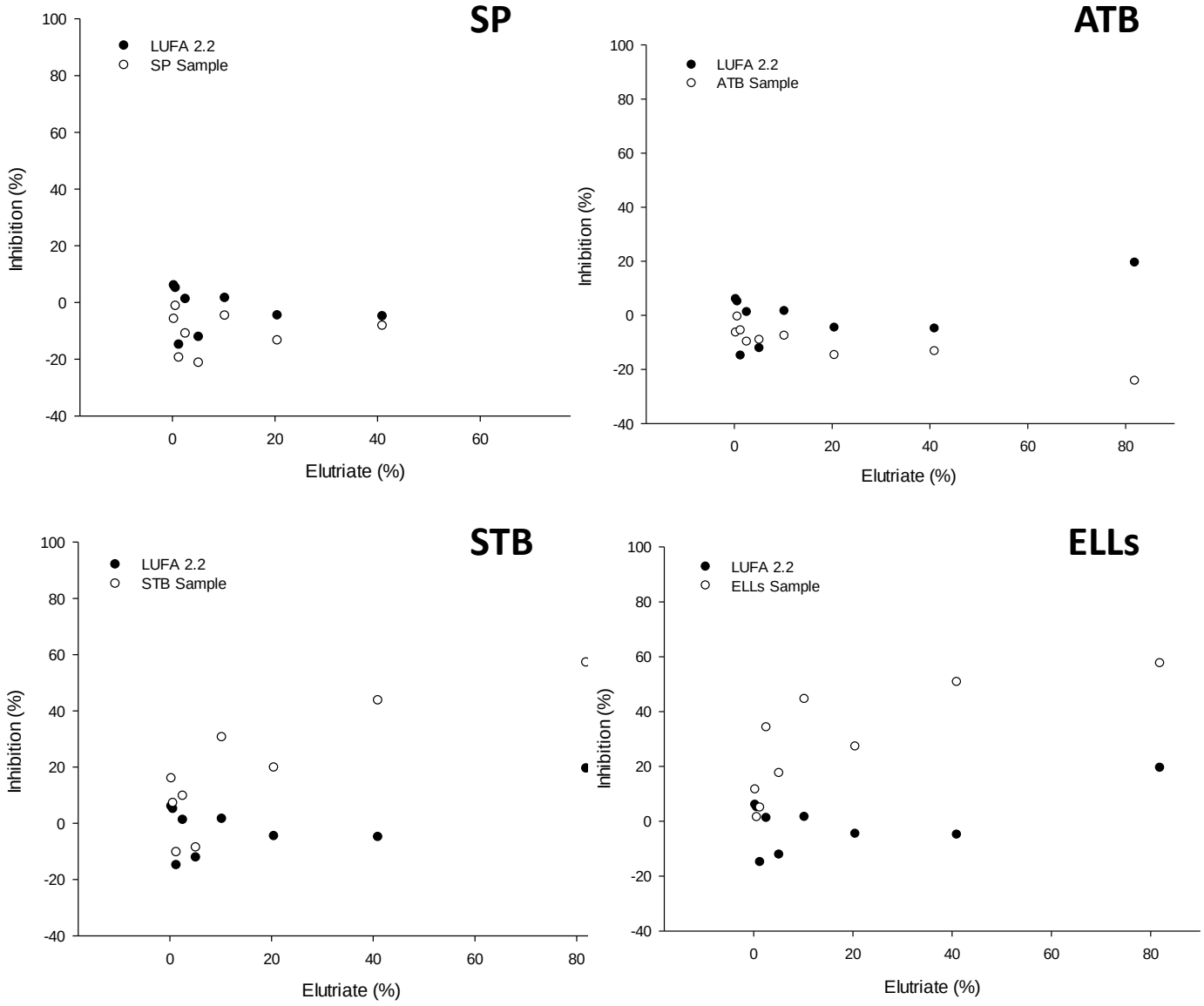

Figure 3.5 – Luminescence inhibition of the marine bacteria *Vibrio fischeri* after 15 min of exposure to a series of dilutions of Lufa 2.2 soil and a series of dilutions of each elutriate produced from natural bitumen samples collected from local rivers of the oil sands in Alberta, Canada: SP, ATB, STB, and ELLs. Data are presented as a percentage.

3.5 - Discussion

On the line of the studies where the effects of the natural load of fresh bitumen into rivers (from natural processes) are reported, the present study aimed to predict the effects of weathered bitumen in water by exposing aquatic invertebrates in

laboratory conditions to liquid extracts produced from bitumen. The ecotoxicological assays revealed different toxicities in the studied organisms, with SP and ATB elutriates inducing low to no effects on all organisms, compared to the significant effects of the STB and ELLs elutriate exposures. Consequently, the present study highlighted the fact that the toxicity observed in aquatic organisms is not spatially homogeneous when considering different rivers. Samples presented different compositions of metals, PAHs and NAs and consequently, their toxicity to the aquatic biota was different. This heterogeneity of oil sands natural bitumen is an important factor that should be highlighted and analyzed carefully when discussing the inherent toxicity of natural bitumen samples in rivers. These oil sand materials contain bitumen in different quantities and qualities, and the content of the bitumen will be the primary factor of toxicity to organisms.

Alongside with the ecotoxicological results, chemical analyzes also revealed differences in the composition of the four collected bitumen samples. The total sum of the metals analyzed revealed that the SP elutriate contains 3.6 to 4.8 times higher metal contents than the remaining elutriates (3.6 times the ATB, 4.4 times the ELLs, and 4.8 times the STB sum of the metals analyzed). The elutriates produced presented concentrations of metals higher than the maximum allowed by the CCME, such as aluminum, copper, iron, lead, and selenium. In the STB sample, Aluminum and Iron were above the maximum allowed by CCME by 8 and 1.6 times, respectively. The ELLs elutriate had Aluminum, Iron, Copper and Lead above the CCME limit for aquatic freshwater streams: 4.8 times above for Aluminum; 10.3 times above for Iron; 7.8 times above for Copper and 12.6 times above for Lead. The SP elutriate also presented metals above limit: Boron 1.16 times above, Copper 2.17 times above, and Selenium 3.73 times above. Contrarily, the ATB elutriate did not reveal any metal concentration above the maximum allowed by CCME. Thus, the high levels of Aluminum, Iron, Copper, Lead and Selenium present in the ELLs elutriate probably contributed to the high toxicity observed comparatively to the ATB, STB, and SP elutriates.

Interestingly, for the more toxic samples (STB and ELLs), when concentrations of metals were calculated for the LC_{50} and EC_{50} of the different organisms (in Table

3.1 concentrations are related to the pure elutriate) in general, at the equivalent concentration of the LC_{50}s and EC_{50}s are still above the maximum that is recommended by the Canadian guidelines for good quality of freshwaters. Even knowing that this relation is not straightforward, this is a simple exercise that could provide us an idea that even at the concentrations that induces 50% of mortality (or effects), the predicted concentrations of metals are still above the CCME guidelines.

The chemical analysis of PAHs showed clear differences between the four elutriates. In the Ells elutriate, pyrene was present in concentrations almost 4 times higher and Benzo[a]pyrene 1.5 times higher than the maximum allowed by the CCME. These high levels of pyrene and benzo[a]pyrene found in the ELLs elutriate can be directly linked to the high toxicity observed in the studied organisms since both PAHs are known to be very toxic to aquatic organisms (Ikenaka et al., 2013) and benzo[a]pyrene is also known to be a carcinogen. This emphasizes the need to identify these compounds in the environment and predict their effects (Christensen and Zhang, 1993). STB, ATB, and SP elutriates did not reveal any PAHs above the maximum allowed by the CCME for freshwater streams even though STB ∑16PAHs was 2 times higher than SP ∑16PAHs and ATB ∑16PAHs.

An increase of NAs content in the 4 elutriates was observed as following: SP<ATB<STB<ELLs, with the ELLs elutriate having 200, 8 and 1.7 times more NAs content than SP, ATB, and STB elutriates, respectively. The NAs levels could be one of the key factors responsible for the toxicity observed in the aquatic organism studied. NAs were previously considered as the most contributing contaminant to the toxicity observed in the aquatic biota using Oil Sands Process-affected Waters (OSPWs), although more recent studies reported that more classes of organic compounds also contributed (Morandi et al. 2015). It is interesting to note that the ELLs elutriate, that induced higher toxicity, presented 4 times less total NAs levels than the lowest value reported in the literature for NAs in OSPWs, a water originated in the extraction of bitumen from oil sands deposits, ranging between 20 and 120 mg NAs L^{1} (Holowenko et al., 2002; Headley and

McMartin, 2004). Literature also states that higher proportions of lower molecular weight compounds in a NAs mixture correlate with greater toxic response in various organisms (Frank et al., 2008, Swigert et al., 2015). This is in accordance with the results obtained in the present study, where higher proportions of lower molecular weight compounds in the STM and ELLs elutriates induced higher effects on the studied organisms

Although exposure and related effects most commonly rely on chemical analysis of individual chemical compounds, bitumen contains a mixture of chemicals that can have a more significant impact than predicted from a one-by-one chemical assessment. The presence of several chemicals in a mixture may increase (or decrease) the toxicity compared to the predicted from the individual compounds, which limits the interpretation accuracy of the chemical analysis. Only the joint perception of chemical analysis and ecotoxicological tests can provide more accurate information about the real effects that complex mixtures, such as bitumen-related materials, pose to aquatic organisms, and then be used in risk assessment studies.

As referred before, the ELLs elutriate was, in general, more toxic to daphnids, snails, and bacteria, which comes in agreement with the higher levels of PAHs and NAs observed for the ELLs elutriate. *Daphnia magna* was the most sensitive organism, where a significant reduction in the total neonates per female was observed when exposed to only 1.5% of the ELLs elutriate (Figure 3.1C). The total number of neonates produced per female also significantly decreased when daphnids were exposed to only 5% of the STB elutriate. On the other hand, the ATB elutriate caused no effects on the number of neonates produced per female. Snails were also sensitive to the presence of the STB and ELLs elutriates, with a severe reduction in hatching success and increased mortality. The bioluminescence of the bacteria (*V. fischeri*) was also significantly inhibited when exposed to the STB and ELLs elutriates, contrarily to when exposed to the SP and ATB elutriates where the inhibition was very low.

In the field, Polycyclic Aromatic Compounds (PAC) concentrations vary in rivers throughout the year. Droppo et al. (2018) reported a longitudinal increase in PAC

concentrations from upstream to downstream in the Steepbank River, with an increase during high flow periods, when the erosive forces are higher, and the overland flow contribution is high. Taking into consideration these variables, it is difficult to state if the elutriates used in the present study mimic exactly the real processes of bitumen washing. Nevertheless, the levels of PAHs and metals measured in the elutriates produced were equivalent to those present in rivers that flow through natural bitumen deposits (Colavecchia et al., 2004; Colavecchia et al., 2006; Colavecchia et al., 2007; Gerner et al., 2017). The elutriates extraction methodology was explored in Chapter 2, where the methodology was validated and was efficient in the extraction of contaminants through solid materials into a liquid matrix. The experiments reported in the present study highlight those conclusions since the same procedure was successfully applied in more three Oil sands solid samples prevenient from different locations. Comparing the natural bitumen samples collected in the field and the respective elutriates, the same pattern of contaminants was observed between the four different samples (solid and liquid). Based on that, and assuming the premise that elutriates are a good tool to assess the effects of natural bitumen in freshwater streams, the concentrations where effects were observed are still above of what is defined as good quality water and lead us to conclude that the present concentrations in real scenarios could pose a risk to the aquatic biota.

Knowledge on the effects that natural bitumen can induce in aquatic systems is scarce but extremely important in order to understand the “background toxicity” of oil sands areas and for an accurate risk assessment oh such areas. To fill this gap, previous studies collected fish and water and/or sediment from oil sands areas to observe the potential toxic effects caused by natural oil sands deposits (Colavecchia et al., 2004; Colavecchia et al., 2006; Colavecchia et al., 2007; Tetreault et al., 2003). Water collected from the oil sands area and naturally exposed to loads of fresh bitumen material revealed to be toxic to the early-life stages of fathead minnow and white sucker, when exposed in laboratory conditions (Colavecchia et al., 2004; Colavecchia et al., 2006), with increasing mortality of embryos, decreasing and delaying of hatching and abnormal embryonic development. Biochemical alterations were also related to the abnormal

development of the embryos and larvae observed (Colavecchia et al., 2007). Slimy sculpin (*Cottus cognatus*) and pearl dace (*Margariscus margarita*) exposed to waters that flow through bitumen deposits revealed a reduction in steroid production and increase in Ethoxyresorufin-O-deethylase (EROD) activity when compared with values reported for reference sites (Tetreault et al., 2003). Lacaze et al. (2014) evaluated the potential genotoxic effects of OSPW, residual waters that are produced when bitumen is isolated from sand and clay and then stored in tailing ponds, and Oil Sands Leaching Waters (OSLW), simulating the natural release of contaminants from oil sands using a laboratory extraction of the oil shore sands. Results revealed that OSPW led to a significantly higher DNA damage than OSLW in the rainbow trout's hepatocytes (assessed by comet assay), whereas OSLW revealed to be genotoxic to the exposed fish. Although the elutriates prepared in the present study followed a different methodology than the used to obtain OSLW, no information is provided in the study of Lacaze et al. (2014) about the origin of the natural samples.

The toxicity observed in *D. magna*, *P. acuta* and *V. fischeri* are in line with the previous results, emphasizing the importance of leachates/elutriates as reliable tools to evaluate the effects of oil sands material in aquatic organisms. A more recent study analyzed PAHs, metals, and NAs of an oil sands area and also the invertebrate community present from 2010 up to 2012, on a yearly basis (Gerner et al., 2017). The high PAHs concentrations obtained along the years were linked with a reduction in the aquatic invertebrate community. In the same study, the SPEAR (SPEcies At Risk) approach was applied, revealing alterations in the structure of the invertebrate community, with an increase in the physiological sensitivity and a delay in the time to the next generation for the most common species present in the oil sands area.

Further studies in the Oil Sand area should also be prioritized for a better understanding of the background toxicity, using different approaches with natural bitumen samples collected from different locations and other types of organisms (e.g., sediment organisms).

3.6 – Conclusion

The present study highlights the need to take into consideration the impact that background toxicity of oil sands areas can induce to aquatic organisms and that should also be included when performing risk assessment of mining-related activities and oil sands areas, namely in the Oil sands areas in Alberta, Canada, due to its' high economic impact. Effects on aquatic organisms depend on the quantity and quality of the bitumen that enters the rivers, which will consequently influence the presence of contaminants (such as metals, PAHs and NAs). Bitumen collected in the banks of the Ells river produced the most toxic elutriate, which is in line with the higher presence of contaminants, especially of NAs. Alongside, the heterogeneity of toxicity and chemical composition of different bitumen samples was evident and a topic for further discussion. Although this study represents a simulation of the washing process of bitumen, the results obtained are evidence of how bitumen alone can induce adverse effects on aquatic organisms present in Oil Sands areas aquatic systems.

Further studies in the Oil Sands areas of Alberta, Canada, should also be prioritized for a better understanding of the background toxicity, using different approaches with natural bitumen samples collected from different locations and other types of organisms (e.g., sediment organisms).

3.7 – Acknowledgments

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Chapter 4: Oil sands bitumen elutriates affect the life traits of *Dugesia tigrina* (Planaria).

Oil Sands bitumen elutriates affect the life traits of *Dugesia tigrina* (Planaria)

4.1 - Abstract

In Canadian Oil sands, Athabasca River and tributaries flow through bitumen deposits (McMurray formation) and consequently receive bitumen and bitumen-associated contaminants mainly from erosion processes. Ecological effects of a naturally occurring input of bitumen to the aquatic environment need to be assessed to achieve knowledge on a toxicity related background. In this work, laboratory ecotoxicological assays were conducted using the freshwater planarian *Dugesia tigrina*, to evaluate the toxicity of two different elutriates collected from natural bitumen samples from the banks of two representative oil sands rivers (Ells and Athabasca). Exposure to Ells River elutriates caused high levels of mortality, reduced locomotion and lead to a delay in head regeneration after decapitation in planarians. Contrarily, Athabasca River elutriates were not toxic to *D. tigrina*, evidencing the heterogeneity regarding the toxicity induced by natural bitumen samples from different areas within this Oil Sands. The present study emphasizes the usefulness of freshwater planarians in the effect assessment of environmental derived samples, by having sensitive measurable traits (e.g., locomotion and head regeneration) that were able to highlight natural background toxicity.

Keywords: natural-weathered bitumen; regeneration; behavior; bitumen elutriate; locomotion.

4.2. Introduction

Athabasca Oil sands are one of the world's largest reserves of oil, draining an area of 140,200 km^2 in Alberta, Canada. Here, bitumen can be found at 75 m of the surface and it is open pit mined for later production of oil and diesel (Conly et al., 2002). Alberta rivers and tributaries flow through bitumen deposits and inputs of natural bitumen in these streams can occur due to erosion and weathering of river banks, snow melting or by water level rising. The effects of this natural input of

bitumen to aquatic biota is a topic of discussion due to the possible ecological impacts on natural populations (Berry et al. 2017; Droppo et al. 2018; Gerner et al. 2017).

Few studies already reported the effects of natural contamination in this area, mainly induced by the direct contact between water and bitumen. Barton and Wallace (1979) reported for the first time a lower diversity in benthic invertebrate communities in Steepbank river that are incised inflows through natural bitumen deposits (inside McMurray formation), in comparison with upstream reference sites more recent studies have been reporting the toxicity of natural bitumen (Colavecchia et al., 2004; Colavecchia et al., 2006; Gerner et al., 2017; Tetreault et al., 2003a; Tetreault et al., 2003b). Those studies emphasize the need to quantify the background levels of contamination and respective toxicity at locations where a natural fluvial exposure to oil sands bitumen deposits occur, highlighting the importance of discriminating the effects of natural processes (fluvial erosion) from anthropogenic-related sources (mining-related activities) when assessing impacts of oil sands development on surface and groundwater contamination (Wrona et al., 2011).

Riverbanks erosion lead to an input of naturally-occurring bitumen constituents, containing mainly Polycyclic aromatic hydrocarbons (PAHs), metals, and naphthenic acids (NAs). When bitumen is in contact with river water it could be diluted/dissolved into the water stream, be deposited into the streambed and the fine particles of bitumen could also be transported across the rivers (Droppo et al., 2018). This would consequently lead to possible increments of these contaminants in local rivers and tributaries, which was confirmed for streambed sediments and surface waters of the Athabasca River basin (Headley et al., 2001; Kavanagh et al., 2009; Yergeau et al., 2012). Regarding the effects on biota, Tetreault et al. (2003b) have reported increasing Hepatic 7-ethoxyresorufin-O-deethylase (EROD) activity in two fish species collected in the Steepbank and Ells rivers, in areas where fish was exposed to i) naturally oil sands occurring compounds and ii) near surface mining activity, in comparison with fish from reference locations. Colavecchia et al. (2004) reported significant hatching alterations, early life stages

mortality, malformations and reduced size on *Pimephales promelas* exposed in the laboratory to sediments collected within the natural oil sands deposits. Colavecchia et al. (2006) highlighted the adverse effects of natural oil sands deposits in the early developmental stages of *Catostomus commersoni*, comparing their mortality, hatching, deformities, growth, cytochrome P-4501A (CYP1A) and EROD activity from native fish caught in the Athabasca Oil Sands area. Raine et al., (2017), found differences in parasitism and gill histology between brook stickleback (*Culaea inconstans*) collected from tributaries of the Athabasca River within the exposed oil sands area (infected by complex life history parasites) and reference locations (infected by parasites with simpler life histories).

All these studies plus evidence that elutriates from natural weathered bitumen samples are toxic to different invertebrates such as daphnids, snails and chironomids (Chapter 3 and 5) clearly indicate a fair amount of heterogeneity concerning the toxicity of different bitumen samples and emphasizes the need for considering the geological context of the area when assessing their ecological effects.

In the present study, the freshwater planarian *Dugesia tigrina* was used as a test organism with the primary objective of evaluating the lethal and sub-lethal effects of a natural load of weathered/eroded bitumen from the Athabasca oil sands region. The use of freshwater planarians in ecotoxicology, developmental biology and neuropharmacology is well documented (Buttarelli et al., 2008; Nano et al., 2002; Newmark and Alvarado, 2002; Ofoegbu et al., 2016; Pestana et al., 2007; Rodrigues et al., 2016; Saraiva et al., 2018; Sarnat and Netsky, 1985). Since most contaminants present in bitumen samples are known to induce cytotoxic and teratogenic effects, head regeneration of planarians together with behavioral parameters can provide sensitive and suitable information to evaluate their toxic effects. Therefore, laboratory assays were conducted using endpoints such as survival, locomotion, and time for head regeneration after decapitation to assess the effects of exposure to oil sands elutriates generated from two different natural bitumen samples collected in the riverine banks of the Athabasca river and Ells River.

4.3 Material and Method

4.3.1 – Sample area and collection

Two natural bitumen samples were collected in different locations: 1) near the banks of the Athabasca mainstream (ATB sample, 58°12'03.6"N 111°22'48.0"W) and 2) in the banks of the Ells river (ELLs sample, 57°16'49.0"N 111°42'17.0"W). From the samples tested in Chapter 3, ATB sample was used here since almost no effects were observed for daphnids, snails and bacteria. ELLs sample was used in the present study, representing the sample that induced higher toxicity to the organisms tested in Chapter 3. Both areas are not receiving any input from mining and industrial activities, and in both locations, the rivers flow through natural bitumen deposits. In both locals eroded/weathered material is in contact with the river water and oil sheens were visible in the interface between the river and the bank, due to the release of chemical substances from the oil sands solid material to the freshwater. The two sampling areas required different sampling techniques; in the Ells River samples were collected by digging into the interface between the banks and the river, while in the Athabasca mainstream, “bitumen balls” were present in the margins of the river and several individual portions were collected. Samples were then packaged and sent to the Department of Biology, University of Aveiro, Portugal.

4.3.2 – Elutriates production

Elutriates were generated using natural bitumen samples collected in Ells and Athabasca rivers (ELLs and ATB elutriates). The procedure was described before in Chapter 2; briefly, oil sands solid samples were mixed in a 1:2 ratio (solid: liquid), using ASTM as a liquid medium. Then, samples were shacked for 24h in a benchtop orbital shaker and afterward centrifuged in 50 ml Falcon tubes for 45 min, at 3220g. The supernatant was collected, stored at 4° C and tested within one week.

4.3.3 – Planarians culturing

Dugesia tigrina were obtained from a laboratory culture established for more than three years in the Department of Biology, at the University of Aveiro, Portugal. Planarians are cultured in ASTM medium (ASTM, 1980), which is renewed every two days, at 20 ± 1 °C, in the dark. Planarians were fed with bovine liver once a week, with media being renewed after feeding (coincident with one of the culture media changes). One week before the experiments, planarians with 10±1 mm with no signs of injuries and active when exposed to light were used for experiments since planarians exhibit a negative phototaxis behavior (i.e., they tend to move away from light sources).

4.3.4 – Ecotoxicological tests

The experimental setup scheme is presented in Figure 4.1. The test was divided into two parts: 1) mortality was reported after 48h of exposure followed by an evaluation of locomotion at day 8; 2) planarians were changed to clean media or maintained in the same exposure media to evaluate head regeneration after an extra 8-day period.

In the first part of the test, *D. tigrina* was exposed for 8 days to 6.25, 12.5, 25, 50, 75 and 100% of each ATB and ELLs elutriate, plus a control (ASTM only). Ten replicates were used per treatment with 2 planarians per replicate on glass crystallizing dishes with 20 ml of test solutions renewed every 48 hours. Mortality was checked after 48 h by observing immobile animals after exposure to light or those with the visible degenerating body. Mortality was reported as a percentage of total dead planarians (all replicates).

After 8 days, locomotion of planarians was evaluated only for 6.25, 12.5, 25 and 50% of ELLs elutriate, as for 75% and 100% high mortality was observed. For ATB the full dilution series till 100% was used. To measure *D. tigrina* locomotion, a video tracking system (ZebraBoxTM apparatus and the ZebraLab® v3 software, Viewpoint, France) was used. Locomotion of planarians was continuously monitored during a 13-min period (1 min of acclimation, 4 min in light conditions (550 lx), 4 min in dark conditions, and 4 min light conditions), according to the

procedures reported by Saraiva et al. (2018) and Rodrigues et al. (2016). Measured locomotion was obtained by using 20 planarians per treatment individually allocated to 24-multiwell plates with 1 mL respective experimental elutriates. Then, the locomotor activity of planarians was assessed by calculating the distance covered (mm) during a 12 minutes observation period (excluding the 1 min acclimation period).

After the behavioral assays, all organisms were decapitated below the auricles and exposed again for an extra 8-day period (total test duration 16 days) to 1) clean ASTM medium and 2) to new solutions of ATB (6.25, 12.5, 25, 50, 75 and 100%) and ELLs (6.25, 12.5, 25 and 50%) elutriates, both renewed every other day. Exposure to new elutriates and clean medium was carried out using 10 replicates with one planarian each in crystallizing dishes (Ø = 4.6 cm) containing each 20 mL of experimental solutions at 20 ± 1 °C, in the dark. Replicates were examined daily under a stereoscopic microscope following the regeneration process namely the time until the formation of new photoreceptors.

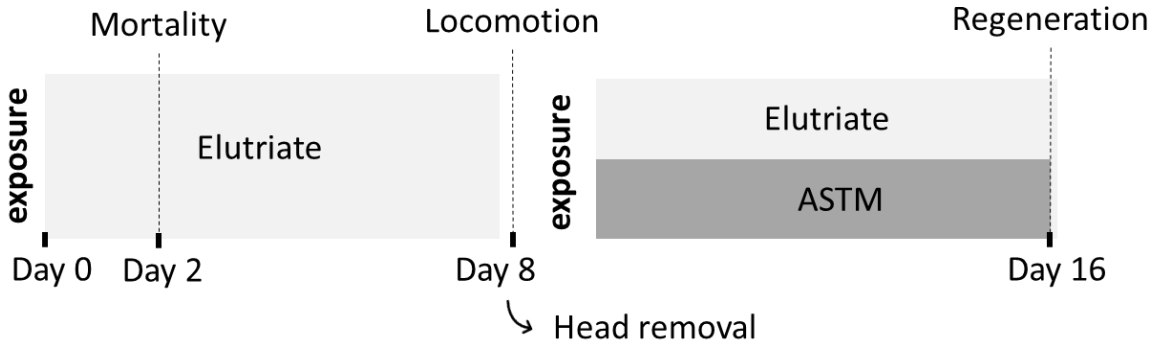

Figure 4.1 - Experimental setup scheme for the exposure of *Dugesia tigrina* to Ells and ATB elutriates. The exposure lasted a total of 16 days, where several parameters were reported in time: 2 days for mortality, 8 days for locomotion and an extra 8-day period for regeneration under elutriate or clean media (ASTM) exposure.

4.3.5 – Chemical analysis

Both solid samples and respective elutriates were analyzed regarding metal content (InnoTech Alberta, Canada), PAHs (AXYS Analytical Services Ltd, Canada) and NAs (InnoTech Alberta, Canada).

Metals were analyzed by Inductively Coupled Plasma Mass Spectrometry (ICP-MS) (Elan DRC-II with ESI SC-8XC high throughput FAST autosampler). Before analysis, digestion of samples was performed using an Ethos UP with the Maxi44 rotor (Milestone Inc). PAHs were analyzed by gas chromatography-mass spectrometry (GC-MS), through the AXYS Method MLA-02. NAs content in elutriates was analyzed using HPLC-Orbitrap-MS in water samples, previously adjusted to pH≈2, spiked with international (Dodecanoic acid-d23) and extracted by automated solid phase extraction. Compound characterization and quantification were performed using liquid chromatography coupled to Orbitrap mass spectrometer. NAs were analyzed in solid bitumen samples with a previous liquid/liquid extraction with an alkaline solution before analysis using HPLC-Orbitrap-MS.

4.3.6 – Statistical analysis

Effects of the different elutriates on planarian behavior (distance covered) and head regeneration (time to photoreceptors formation) were assessed using one-way analysis of variance (ANOVA), followed by Dunnett's post-hoc tests. Normality of data and homoscedasticity of variances were checked using Kolmogorov-Smirnov and Levene's equal variance tests, respectively. Statistical analyses were performed using the software package SigmaPlot 12.5 (Systat Software Inc.) with a significance level set at $p < 0.05$.

4.4 – Results

4.4.1 - Survival and locomotion

Elutriates from Ells river bitumen were highly toxic to *D. tigrina*, with 100% of mortality observed already at 48h of exposure to 100% of ELLs elutriate (no dilution) (Figure 4.2). Also, during this period, head reduction in planarians was observed, followed by head loss and body degeneration before death. Consequently, a clear curve of dose response with increasing mortality through the increase of oil sands ELLs elutriates was observed.

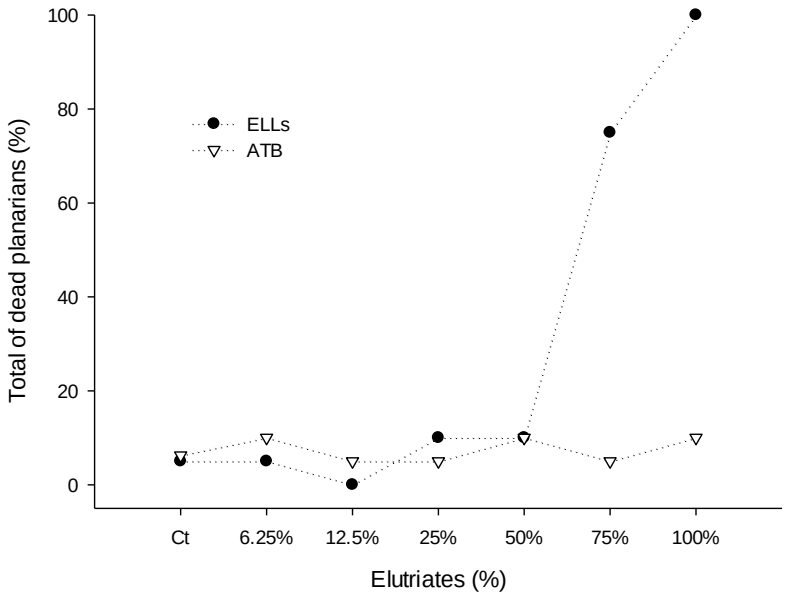

Figure 4.2 - Percentage of total dead planarians after a 48h exposure to ELLs and ATB elutriates.

Contrarily, elutriates produced using the Athabasca river bitumen did not induce mortality on planarians, with an absence of malformations or head reduction/absence during the first 48h of the test, or even during the 8-day period of the first part of the test.

Planarians' locomotion was reduced in almost 50% of the total distance covered during the 12 minutes upon exposure to 50% Ells elutriates (the highest concentration with no lethal effects) (Figure 4.3; Dunnett's test, $p<0.05$).

In contrast, exposure to ATB elutriates did not reduce the locomotion of planarians in any of the concentrations used. An increaseof 25% of the total distance covered was observed in the 100% pure elutriate compared to the control treatment (Figure 4.4; Dunnett's test, $p<0.05$).

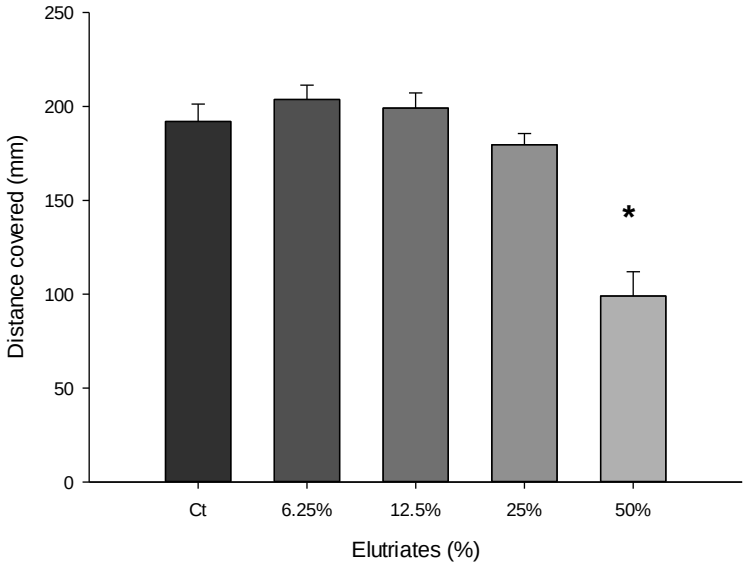

Figure 4.3 - Distance covered (mm) by *Dugesia tigrina* on a 12 minutes experiment, using the automated video tracking system, after an exposure of 8 days to ELLs elutriates. All data are presented as average ± SE, N=20. *Denotes a significant difference compared to the control, Ct (Dunnett's test, $p < 0.05$).

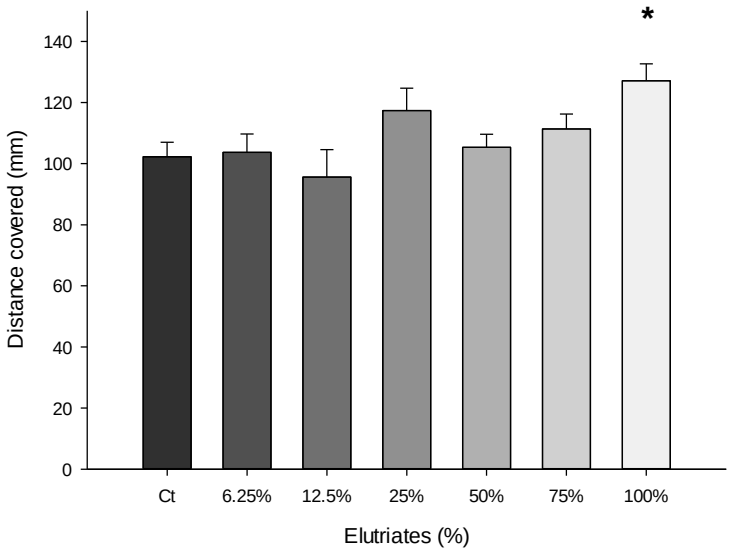

Figure 4.4 - Distance covered (mm) of *Dugesia tigrina* on a 12 minutes experiment, using the automated video tracking system, after an exposure of 8 days to ATB elutriates. All data are presented as average ± SE, N=20. * Denotes a significant difference compared to the control, Ct (Dunnett's test, $p < 0.05$).

4.4.2 - Regeneration

In the experimental set up where planarians were maintained for an extra 8-day period under the Ells elutriate exposure, a significant delay in time for photoreceptor formation was observed in comparison with organisms from the control treatment (Dunnett's test, $p<0.05$). A significant increase in the days for photoreceptor formation was reported in the three highest dilutions treatments used (12.5, 25 and 50%) (Figure 4.5). When regeneration occurred in planarians moved to clean medium, a statistical increase in the days until photoreceptor formation was observed in pre-exposed organisms to 25, and 50% of Ells elutriates (Figure 4.5; Dunnett's test, $p<0.05$).

Exposures to ATB elutriates did not induce any significant effects in the time for photoreceptors formation, nor after moved to clean media (Figure 4.6; One-way ANOVA; $p>0.05$).

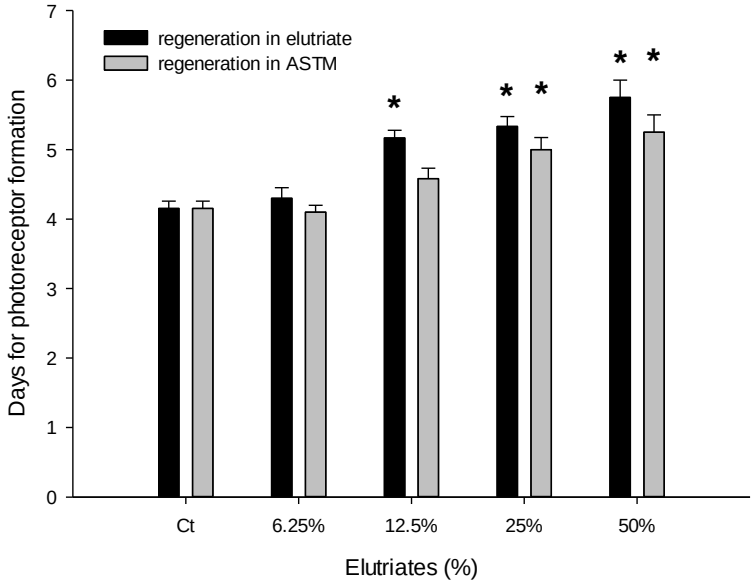

Figure 4.5 - Time for regeneration of *Dugesia tigrina* exposed to ASTM clean media and ELLs elutriates for an extra 8-day period. Data are presented as average days until photoreceptor formation ± SEM. N=20. * Denotes a significant difference compared to the control, Ct (Dunnett's test, $p < 0.05$).

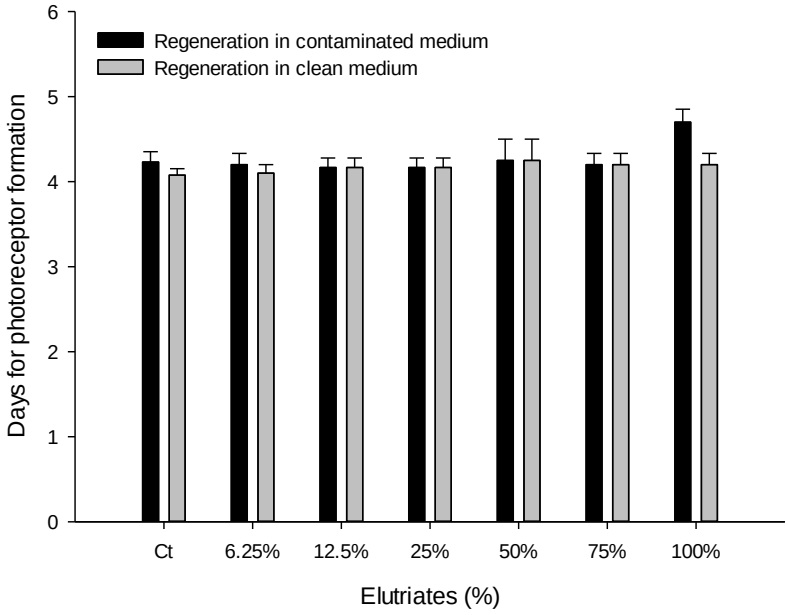

Figure 4.6 - Effects of ATB elutriates on regeneration of *Dugesia tigrina*, measured as days until photoreceptor formation. All data are presented as average ± SE. N=20.

4.4.3 - Elutriates chemical composition

Elutriates from the Athabasca River bitumen presented higher metal content (∑metals: 78003 µg/L), comparing with ELLs elutriate 64746 µg/L. The concentration of the different metals in bitumen samples and respective elutriates are presented in Table 4.1. ELLs elutriates presented higher concentrations of Aluminium, Copper, Iron, Lead and Selenium and above the maximum allowed by the Water Quality for the Protection of Aquatic Life and by the Soil Quality for the Protection of Environmental and Human Health from the Canadian Council of Ministers of the Environment (CCME). Contrarily, none of the assessed metals in ATB elutriates have concentrations higher than the maximum allowed by the CCME.

Taking into consideration the 16 priority PAHs defined by the U.S. Environmental Protection Agency, ELLs elutriates presented higher ∑PAHs content (499.35 ng/L), comparing to the ATB elutriate (35.67 ng/L). Concentrations of the different classes of PAHs are presented on Table 4.2 from natural bitumen samples collected in the banks of the Ells and Athabasca Rivers, and respective elutriates.

Also, the Ells River elutriates presented levels of Pyrene and Benzo[a]pyrene higher than the maximum allowed by CCME.

NAs presence ELLs elutriate (5630 µg/L) was higher than in the ATB elutriate (663 µg/L).

Table 4.1 - Metal content in elutriates and natural bitumen samples from ATB and ELLs rivers, Alberta, Canada, respectively. The analysis was carried out by Elan DRC-II ICPMS. Dissolved and total values were achieved for elutriates. Obtained values were compared with the maximum allowed by the Water Quality for the Protection of Aquatic Life and by the Soil Quality for the Protection of Environmental and Human Health from the Canadian Council of Ministers of the Environment (CCME). n.d.- not determined; values in bold are highlighted as above or close to the maximum allowed by CCME.

	ATB elutriate (µg/L)		**ELLs elutriate (µg/L)**		**ATB solid (µg/g)**	**ELLs solid (µg/g)**	**Maximum allowed by CCME (µg/L)**
	Total	Dissolved	Total	Dissolved	Total	Total	
Aluminium	8.5	0.85	**4040**	**2070**	7630	38400	**100**
Antimony	0.790	0.780	0.426	0.420	0.514	0.968	n.d.
Arsenic	2.05	1.94	1.74	1.09	23.5	3.02	5
Barium	48.6	46.8	60.6	41.8	131	107	n.d.
Beryllium	0.009	< 0.009	0.766	0.330	1.14	1.39	n.d.
Bismuth	0.005	0.005	0.108	0.046	0.065	0.130	n.d.
Boron	46.0	45.4	420	372	27.2	70.2	1500
Cadmium	0.019	0.019	0.061	0.019	0.309	0.091	0.09
Calcium	67500	66900	16300	14400	16000	2000	n.d.
Chloride	9950	9930	38200	32700	463	356	n.d.
Chromium	< 0.03	< 0.1	7.15	3.3	15.5	27.7	8.9
Cobalt	0.942	0.042	5.72	2.21	16.3	13.2	n.d.
Copper	1.05	0.98	**11.5**	**5.19**	12.1	11.2	**2**
Iron	72.4	2.8	**5190**	**1410**	42700	16300	**300**
Lead	0.084	0.013	**12.6**	**5.10**	9.41	11.7	**1**
Lithium	4.29	4.23	60.1	53.7	11.8	90.0	n.d.
Manganese	175	95.6	57.7	32.0	578	677	n.d.
Molybdenum	1.12	1.11	1.59	1.57	3.96	2.19	73
Nickel	1.15	1.14	14.7	7.12	47.3	36.7	25
Selenium	0.40	0.40	**1.01**	0.69	1.07	0.57	**1**
Silver	0.003	0.002	0.147	0.073	0.469	0.315	0.25
Strontium	182	180	240	238	101	74.9	n.d.
Thallium	0.0269	0.0264	0.0708	0.0501	0.235	0.238	0.8
Thorium	0.0090	0.0089	7.03	3.00	10.2	7.27	n.d.
Tin	< 0.003	< 0.003	0.164	0.159	0.466	1.10	n.d.
Titanium	0.94	0.62	86.9	85.8	2090	1800	n.d.
Uranium	0.529	0.519	1.43	0.901	2.53	1.16	15
Vanadium	2.45	2.19	14.4	7.09	74.2	66.9	n.d.
Zinc	4.3	3.73	9.9	5.20	45.5	32.6	30

Table 4.2 - PAHs content in elutriates and natural bitumen samples from ATB and ELLs rivers, analyzed by gas chromatography-mass spectrometry (GC-MS). Obtained values were compared with the maximum allowed by the Water Quality for the Protection of Aquatic Life and by the Soil Quality for the Protection of Environmental and Human Health from the Canadian Council of Ministers of the Environment (CCME). <D.L. – above the detection limited; n.d.- not determined.

	ATB elutriate (ng/L)	ELLs elutirate (ng/L)	ATB solid (ng/g)	ELLs solid (ng/g)	Maximum allowed by CCME (ng/L)
Naphthalene	22.9	23.2	113	93.9	1100
Acenaphthylene	<D.L.	<D.L.	<D.L.	<D.L.	n.d.
Acenaphthene	<D.L.	<D.L.	<D.L.	<D.L.	5800
2-Methylfluorene	<D.L.	<D.L.	<D.L.	<D.L.	n.d.
C2 Phenanthrenes/Anthracenes	<D.L.	110	546	2070	n.d.
Fluorene	1.35	2.18	<D.L.	<D.L.	300
Phenanthrene	5.08	12.6	<D.L.	<D.L.	400
Anthracene	<D.L.	<D.L.	<D.L.	<D.L.	12
C1 Phenanthrenes/Anthracenes	<D.L.	24.2	<D.L.	<D.L.	n.d.
Fluoranthene	1.85	23.4	<D.L.	166	40
Pyrene	2.06	**95.6**	<D.L.	752	**25**
Benz[a]anthracene	<D.L.	<D.L.	<D.L.	<D.L.	18
Chrysene	2.43	186	1070	1330	n.d.
Benzo[b]fluoranthene	<D.L.	46.3	417	356	n.d.
Benzo[j,k]fluoranthenes	<D.L.	6.87	<D.L.		n.d.
Benzo[e]pyrene	<D.L.	114	619	721	n.d.
Benzo[a]pyrene	<D.L.	**22.6**	227	205	**15**
Perylene	<D.L.	88.6	1190	488	n.d.
Dibenz[a,h]anthracene	<D.L.	13.9	<D.L.	95.9	n.d.
Indeno[1,2,3-cd]pyrene	<D.L.	19.1	207	101	n.d.
Benzo[ghi]perylene	<D.L.	47.6	341	275	n.d.
2-Methylnaphthalene	10.7	15.3	125	90.1	n.d.
1-Methylnaphthalene	5.18	7.66	64.7	57.3	n.d.
C1-Naphthalenes	15.9	22.9	190	90.1	n.d.
Biphenyl	11.5	9.78	51.4	43.8	n.d.
C1-Biphenyls	6.47	10.9	111	76.3	n.d.
C2-Biphenyls	23.5	22.7	<D.L.	<D.L.	n.d.
C2-Naphthalenes	22.3	24	<D.L.	<D.L.	n.d.
1,2-Dimethylnaphthalene	<D.L.	<D.L.	<D.L.	<D.L.	n.d.
2,6-Dimethylnaphthalene	<D.L.	2.71	<D.L.	<D.L.	n.d.
C3-Naphthalenes	6.52	26.4	<D.L.	<D.L.	n.d.
2,3,6-Trimethylnaphthalene	<D.L.	3.32	<D.L.	<D.L.	n.d.
2,3,5-Trimethylnaphthalene	<D.L.	<D.L.	<D.L.	<D.L.	n.d.
C4-Naphthalenes	2.67	50.7	56.2	1010	n.d.
C1-Acenaphthenes	<D.L.	<D.L.	<D.L.	<D.L.	n.d.
C1-Fluorenes	2.48	9.59	107	134	n.d.
1,7-Dimethylfluorene	<D.L.	<D.L.	<D.L.	<D.L.	n.d.
C2-Fluorenes	<D.L.	49.3	551	518	n.d.
C3-Fluorenes	11.6	283	1760	3250	n.d.
Dibenzothiophene	0.947	2.88	<D.L.	<D.L.	n.d.
C1-Dibenzothiophenes	<D.L.	4.62	<D.L.	<D.L.	n.d.
2/3-Methyldibenzothiophenes	<D.L.	<D.L.	<D.L.	512	n.d.
C2-Dibenzothiophenes	2.75	257	880	3090	n.d.
2,4-Dimethyldibenzothiophene	<D.L.	<D.L.	<D.L.	<D.L.	n.d.
C3-Dibenzothiophenes	6.94	1470	13700	13900	n.d.

	ATB elutriate (ng/L)	ELLs elutirate (ng/L)	ATB solid (ng/g)	ELLs solid (ng/g)	Maximum allowed by CCME (ng/L)
C4-Dibenzothiophenes	10.3	1440	34700	24700	n.d.
3-Methylphenanthrene	<D.L.	9.54	<D.L.	<D.L.	n.d.
2-Methylphenanthrene	<D.L.	<D.L.	<D.L.	<D.L.	n.d.
2-Methylanthracene	<D.L.	<D.L.	<D.L.	<D.L.	n.d.
9/4-Methylphenanthrene	<D.L.	14.7	<D.L.	<D.L.	n.d.
1-Methylphenanthrene	<D.L.	<D.L.	<D.L.	<D.L.	n.d.
3,6-Dimethylphenanthrene	<D.L.	11.4	<D.L.	173	n.d.
2,6-Dimethylphenanthrene	<D.L.	<D.L.	<D.L.	144	n.d.
1,7-Dimethylphenanthrene	<D.L.	8.4	<D.L.	193	n.d.
1,8-Dimethylphenanthrene	<D.L.	<D.L.	<D.L.	<D.L.	n.d.
C3-Phenanthrenes/Anthracenes	1.89	590	2590	8420	n.d.
1,2,6-Trimethylphenanthrene	<D.L.	15.4	<D.L.	<D.L.	n.d.
Retene	<D.L.	<D.L.	<D.L.	<D.L.	n.d.
C4-Phenanthrenes/Anthracenes	20.3	1510	25600	28200	n.d.
C1-Fluoranthenes/Pyrenes	7.77	896	4920	7390	n.d.
3-Methylfluoranthene/Benzo[a]fluorene	<D.L.	134	<D.L.	1310	n.d.
C2-Fluoranthenes/Pyrenes	14.8	1950	16300	19400	n.d.
C3-Fluoranthenes/Pyrenes	9.1	1600	21800	20700	n.d.
C4-Fluoranthenes/Pyrenes	4.07	1120	8730	7650	n.d.
C1-Benzo[a]anthracenes/Chrysenes	2.52	394	5530	4230	n.d.
5/6-Methylchrysene	<D.L.	48.4	523	660	n.d.
1-Methylchrysene	<D.L.	17.7	486	187	n.d.
C2-Benzo[a]anthracenes/Chrysenes	4.16	530	10100	7470	n.d.
5,9-Dimethylchrysene	<D.L.	124	2120	1600	n.d.
C3-Benzo[a]anthracenes/Chrysenes	0.918	198	6440	4950	n.d.
C4-Benzo[a]anthracenes/Chrysenes	<D.L.	60.9	835	758	n.d.
C1-Benzofluoranthenes/Benzopyrenes	<D.L.	326	3480	2970	n.d.
7-Methylbenzo[a]pyrene	<D.L.	45.9	330	307	n.d.
C2-Benzofluoranthenes/Benzopyrenes	0.995	171	2560	2100	n.d.
1,4,6,7-Tetramethylnaphthalene	<D.L.	6.67	<D.L.	193	n.d.

4.5 – Discussion

In the present study, planarians exposed in the laboratory to elutriates from natural bitumen collected in the Ells River showed reduced survival and locomotion, and a delay in head regeneration. These results together with the absence of effects observed for the elutriated produced from bitumen samples from Athabasca river clearly demonstrate that natural eroded bitumen can exert a toxic effect to aquatic biota, but these effects are site-specific given the heterogeneity of bitumen samples across oils sand area.

Elutriate extraction methodology was successfully applied, with a described methodology in Chapter 2, and successfully applied on a more complex approach

in Chapter 3 using a battery of aquatic organisms exposed to different oil sands elutriates. In this study, both elutriates differed in composition regarding metals, PAHs, and NAs, with ELLs elutriates generally containing higher levels of PAHs and NAs and some metals than ATB elutriates. Metals are known to induce cytotoxic effects on planarians, with cadmium inducing cell toxicity, decreasing mitotic activity of neoblasts, different mitotic and chromosomal abnormalities as well as mitotic delays in the planarian *Polycelis feline* (Kalafatić et al., 2004). The same species presented its neoblast mitotic activity reduced by manganese (Kopjar et al., 1997). Later, Kovačević et al., (2009) reported epithelial and muscular damages when planarians were exposed to aluminum, a metal that is highly present in the ELLs elutriates. Cell division was also known to be affected by the presence of mercury, cadmium and lead in different planarian species (Chakravarty and Srivastava, 1992; Stallwitz and Haeder, 1993; Xu and Xiu, 1993). Considering this, it would be expected that the measured metals' concentration in both elutriates would induce cytotoxicity in planarians. However, this did not occur in the ATB elutriates.

Even though PAHs can impair the life traits of aquatic organisms due to the high toxicity to aquatic invertebrates (Ikenaka et al., 2013), the effects of PAHs in planarians is poorly studied. The higher levels of PAHs found in ELLs elutriates can also contribute to the highest toxicity observed. ELLs elutriates presented 14 times the ∑PAHs content of the 16 priority PAHs defined by the U.S. Environmental Protection Agency of ATB elutriates. Best and Morita, (1991) found that Benzopyrene induced teratogenic effects, inducing lethally in asexual planarians *Dugesia dorotocephala*. Interestingly, Pyrene and Benzo[a]pyrene were detected at high concentrations in ELLs elutriates, providing one of the possible explanations for their higher toxicity.

In the best of our knowledge, no previous work has been conducted examining the toxicity mechanisms of NAs in planarians. Despite the mechanisms through which NAs exert their toxicity are still not entirely clear, seems that NAs could act as membrane disruptors or narcosis inducers (Frank et al., 2006). This may be explained by the similarity in structure of some aromatic NAs and steroid hormone

(Rowland et al., 2011) or as more recently discovered, by inducing genotoxic effects on the organisms (Lacaze et al., 2014).

Nevertheless, NAs are a naturally-occurring, aliphatic or alicyclic carboxylic acids found in petroleum reserves, known to become toxic to various organisms (Scott et al., 2008) and are also reported as one of the primary drivers of toxicity to aquatic organisms when are exposed to oil sands materials (Gerner et al., 2017).
Alongside the critical lethal effects that planarians suffered in the presence of ELLs elutriate, exposure to sublethal elutriate dilutions also affected their locomotion and regeneration. Effects on locomotion can lead to possible implications at the population level increasing the susceptibility to the attack of predators and impacting planarian feeding behavior which in turn can lead to deleterious effects in growth and reproduction (Thorp and Covich, 2009). Since planarians are also known to be efficient predators, a possible reduced feeding activity can also indirectly affect prey species populations. Since planarians move by gliding (through ciliary activity and crawling), effects on muscular activity will have consequences in the locomotion of planarians. Therefore, the findings of Kovačević et al., (2009), where aluminum-induced epithelial and muscular damages in planarians, may also provide a hint on the low locomotion activity upon exposure.

Regeneration is also an important parameter that highlights the significance of using freshwater planarians as useful indicators for water quality and pollution (Kent, 1974). Regeneration is the base for asexual reproduction of some planarians; the observed delay in head formation when in the presence of ELLs elutriates can lead to significant negative implications for population dynamics. The regeneration of blastema involves the proliferation of totipotent stem cells (neoblasts) in a process crucial to the formation of new tissues while old tissues are remodeled (Reddien and Sánchez-Alvarado, 2004; Reddien et al., 2005). This could be the mechanism how elutriates may have impaired head regeneration, by reducing the neoblast proliferation and also inducing apoptotic effects.

The same elutriates used in the present study were already used in Chapter 3, where *Daphnia magna*, *Physa acuta,* and *Vibrio fischeri* were negatively affected

by the presence of these oil sands elutriates, with more pronounced effects when organisms were exposed to elutriates from Ells river bitumen, when compared to the ATB elutriate exposure, which is in line with the results obtained in the present study. Also, different approaches reported the effects of oil sands natural bitumen on different life traits of aquatic biota, which included higher fish larvae mortality in Ells river sediment than in Steepbank river sediments (Colavecchia et al. (2004, 2006). Fish collected from both Ells and Steebank rivers also presented increased liver enzymes (Tetreault et al., 2003a; 2003b), but in this case, the ones from the Steepbank river revealed a stronger physiological effect.

4.6 – Conclusion

The present study highlighted the heterogeneity of the oil Sands area regarding natural bitumen samples toxicity, with two different sampling sites revealing different levels of bitumen toxicity in planarians. This difference in toxicity is a factor that increases the complexity in the interpretation of toxicity related to the natural effects of bitumen from oil sands.

D. tigrina revealed to be an interesting and promising organism to study effects from the exposure to contaminants present in bitumen samples, and that can be washed to the river water system. The use of sub-lethal parameters such locomotion and regeneration were crucial for that as they were detected in diluted elutriates. The findings of the present study provide new data for a more accurate risk assessment of the Oil Sands region, in order to discriminate between natural materials and mining activities related hazards.

4.7 – Acknowledgments

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Chapter 5: Contaminated sediment with natural Oil sands bitumen impaired the *Chironomus riparius* life-history under laboratory condition.

Contaminated sediment with natural Oil sands bitumen impaired the *Chironomus riparius* life-history under laboratory condition

5.1 – Abstract

Athabasca oil sands in Alberta, Canada are large deposits of bitumen (extremely heavy crude oil) representing one of the largest petroleum reserves in the world. In these oil sands and given the zero-discharge policy established by the Canadian government the fate, toxicity and remediation of waters produced by the separation process of bitumen from the sand, which is rich in hydrocarbons and other contaminants, is the focus of most environmental risk assessments. The scientific community already spotted the existence of natural contamination in Canadian Oil Sands, since rivers flow through bitumen deposits. By natural processes, bitumen or bitumen-related materials will end up in rivers potentially affecting aquatic biota. Here, using laboratory assays and the midge *Chironomus riparius* as test species, we aimed at studying the effects of different natural bitumen samples in sediments along with a simulation of bitumen washing, through testing elutriates. Chironomids were sensitive when bitumen was present in the sediment, and oil sands materials from different sites elicited different levels of toxicity. Bitumen samples collected in Ells river induced the most severe effects in all studied endpoints followed by natural bitumen samples from one Steepbank river location, Athabasca River and from the second site in Steepbank river, highlighting the heterogeneity of the area regarding of toxicity induced by natural bitumen in aquatic biota. Exposure to natural weathered bitumen elutriates did not cause any adverse effects to *C. riparius*. The present study adds toxicity data for risk assessment studies within the Athabasca river basin showing that natural bitumen reaching stream sediments can affect benthic organisms. These results can help in the prediction of the potential impacts that industrial and mining activities in the region adding data on the toxicity of Oil Sands background material.

Keywords: Chironomidae; background toxicity; erosion of river banks; natural contamination, sediment contamination

5.2 – Introduction

One of the largest reserves of petroleum in the world is found in three major deposits in Northern Alberta, Canada. In these oils sands, surface mining extracts separate the bitumen from sand to produce crude oil (Conly et al., 2007), producing large volumes of water which are consequently stored in tailing pounds due to the zero-discharge policy (Government of Canada, 2015a Government of Canada, 2015b). These waters are alkaline, rich in organic acids and acutely toxic to aquatic biota (Anderson et al., 2011; Hrudey, S.E. 1975; Morandi et al., 2015; Morandi et al., 2017; Raine et al., 2017; Raine et al., 2018). Canadian oil Sands mining activities are increasing leading to concerns on the protection and ecological status of local freshwaters such as the Athabasca River basin (Griffiths et al., 2006).

For a more accurate ecological risk assessment of oil sands mining/upgrading activities on the surrounding ecosystems, quantification of background levels of contaminants at locations that are “non-impacted” by an anthropogenic disturbance is crucial. Baseline monitoring is thus needed to establish benchmarks and allow comparison with sites that are potentially impacted by oil sands mining/upgrading activities (Wrona et al., 2011). The Athabasca River and its tributaries flow through natural bitumen deposits, with aquatic systems receiving a continuous input of natural eroded/weathered contaminants, especially increased during high flow periods due to the greatest erosive forces and when the overland flow contribution is high (Droppo et al. 2018). Therefore, erosion of the slumping areas, snow melting, and increase in river water levels are examples of natural processes that can make bitumen or bitumen-related material contaminants available in freshwaters (Barton and Wallace, 1979). Droppo et al. (2018) reported that eroded bitumen from McMurray formation is a mixture of fine-grained (silts and clays) and coated with natural hydrophobic oils. Due to its composition, poor settling and long-range transport are expected, alongside with a longitudinal increase in Polycyclic aromatic compounds (PACs) concentration from upstream

to downstream of rivers, with higher levels of PACs in ELLs river than in Steepbank river. Consequently, when bitumen enters into freshwaters, it is expected that: 1) parts of the eroded bitumen are immediately dissolved into the water; 2) the remaining natural eroded bitumen will settle on the top sediment of the river and 3) the fine-grained part is transported through the river to downstream areas.

After Barton and Wallace (1979) have reported the inherent risks of natural weathered bitumen in the oil sands zone towards freshwaters. Their work found a reduction in biodiversity in areas where waters are influenced by bitumen formations, different studies have been reporting deleterious effects in different aquatic organisms caused by increasing concentrations of contaminants (Colavecchia et al., 2004; Colavecchia et al., 2006; Gerner et al., 2017; Tetreault et al., 2003). Moreover, these studies have also shown some heterogeneity concerning different samples of bitumen collected at different sites within the oil sands area with no influence of mining activities. On an attempt to predict the potential associated impacts that industrial and mining activities could pose to the Athabasca river basin, there is thus the need to separate those impacts from the ones caused by natural occurring of contaminants, especially metals, Polycyclic aromatic hydrocarbons (PAHs) and Naphthenic acids (NAs).

Within this in mind, and to provide data on the potential background toxicity in these Canadian oil sands, this study aimed to evaluate the ecotoxicological effects of natural bitumen loads (i.e., not related to human activities) in aquatic organisms to simulate their natural appearance in rivers. For that, the model species *Chironomus riparius* whose larvae live in close association with river sediments was chosen to assess effects concerning two types of exposure: i) bitumen contaminated sediments simulating the deposition of bitumen into sediments and ii) bitumen elutriates simulating the release of contaminants from solid natural bitumen into river waters. To achieve this objective, oil sands bitumen samples collected in four different sites within the oil sands area in Alberta, Canada were used.

5.3 – Material and method

5.3.1- Study area – bitumen collection

Rivers in oil sands area flow through north and will end up in the Athabasca basin. Natural bitumen samples were collected in four different sites in three rivers: two samples collected in the Steepbank river, distancing 7 Km from each other (SP sample - 56°58'47.3"N 111°17'53.0"W and STB sample - 56°59'55.1"N 111°24'12.1"W), one in the Ells river (ELLs sample - 57°16'49.0"N 111°42'17.0"W) and one in the Athabasca River (ATB sample - 58°12'03.6"N 111°22'48.0"W). These field sites do not suffer any influence from mining activities, but in all cases, the aquatic systems flow through bitumen deposits and are continuously exposed to fresh bitumen. Also, Colavecchia et al. (2004) defined the Ells and Steepbank rivers as typical rivers with natural inputs of oil sands material. In Ells and Steepbank rivers, where SP, STB, and ELLs samples were collected, oil sheens were visible, and rivers had streambeds that resembled asphalt-like pavement (Figure 5.1).

Figure 5.1 – Margins of Steepbank river, in the Canadian Oil Sands, with asphalt like pavement in the interface bank/water, where clear oil sheens are visible with the dissolution of oil sands material into the water. Elutriates will simulate the present scenario.

Contrarily to samples SP, STB and ELLs, where the river flow erodes the slumping areas (and consequently the bitumen), the Athabasca River sample (ATB sample) was characterized by spherical shape balls since they were eroded and rolled down through the slumping areas (5-8 meters) ending up in the rivers (Figure 5.2). Here, oil sheens were also visible in the interface water/sand.

Figure 5.2 – Margins of the Athabasca River, in the Canadian Oil sands, with "bitumen balls" in the interface with the river. The physical processes will shape their form, ending in the interface bank/water with that spherical form. Dissolution of natural bitumen will create visible oil sheens in the water.

After collection, all solid samples were packed in heavy gauge, food-safe plastic bags, and shipped in coolers to the Department of Biology, University of Aveiro, Portugal where they were stored at 4° C.

5.3.2 - Test organisms

Chironomus riparius larvae used in all tests were collected from laboratory cultures established at the University of Aveiro. Cultures were maintained at room temperature of 20±1 ◦C and 16:8 h light-dark photoperiod in previously burnt inorganic fine sediment. American Society for Testing Materials (ASTM) hard water used as the medium was renewed weekly with larvae fed *ad libitum* every two days with a suspension of macerated Tetramin® (Germany).

5.3.3 - Elutriates production and bitumen incorporation in sediments

For elutriate production, the same method used in Chapter 2 was followed, already adapted by the study of Loureiro et al. (2005). Briefly, the four collected samples (SP, ATB, STB, and ELLs) were mixed in a 1:2 ratio (solid: liquid), using the ASTM media. In a benchtop orbital shaker, samples were mixed for 24h, and centrifuged in 50 ml *Falcon* tubes for 45 min, at 3220g. The supernatant was then collected stored at 4° C and used as an elutriate for testing within one week of preparation.

For the sediment contamination, fine inorganic sediment (< 1 mm) was previously burned at 500 °C for four hours and mixed with bitumen fractions corresponding to 5, 10 and 20% of the four solid natural bitumen samples; sediment was mixed with oil sands solid samples in plastic containers and then assorted to glass test vessels.

5.3.4 - Chronic 28-days partial life cycle test

Ecotoxicological assays with *C. riparius* were conducted using standardized protocols for both contaminated sediment (OECD, 2004a) and spiked water – in the form of elutriate in this case (OECD, 2004b), respectively.

In both experiments, test conditions were the same as described for culturing and organisms were fed every two days with a suspension of macerated Tetramin® (0.5 mg per organism per day). For the tests with contaminated sediment, 30g of sediment were used in each replicate (1 cm layer of sediment) with 150 mL of ASTM hard water. For the elutriates exposure, 30g of non-contaminated inorganic fine sediment (<1 mm, previously burned at 500 °C during 4 h) was used in each vessel of the test. Then, 150 mL of the test solutions were used. Dilutions of the four previously produced elutriates (6, 12.5, 25, 50, 75 and 100%) were used in each vessel of the test.

In both experiments, 10 replicates with five *C. riparius* first instar larvae (less than 48 h post-hatching) were used. Five replicates per treatment were sacrificed at the end of 10 days of exposure to check for survival and measure larvae body length. For that, larvae were placed in 70% ethanol, and the total length measured with a

stereo dissecting microscope fitted with a calibrated eyepiece micrometer. The initial size of larvae (mm) was established at the beginning of the test by measuring a pool of 30 larvae (Length $_{initial}$). To calculate the growth ratio (mm) of larvae after 10 days of the test, equation 1 was followed: (Length $_{final}$ -Length $_{initial}$)/ Length $_{initial}$. At the end of the test, the cumulative percentage of emergence, the male/female ratio, the average time to emergence of organisms and adult size (male and female) was obtained with the remaining five replicates in each treatment. For that, *C. riparius* imagoes were collected daily from emergent traps with the aid of an aspirator, counted, gender discriminated and preserved in 70% ethanol. Imagoes were later dried at 50 ◦C for 24 h and weighed in a microbalance (Mettler UMT2).

5.3.5 - Chemical analysis

Chemical analysis for metals, PAHs and NAs were carried out on the 4 samples tested (SP, ATB, STB, ELLs) and for both bitumen solid sample and respective elutriate. All samples were analyzed for metals and NAs (InnoTech Alberta, Canada) and PAHs (AXYS Analytical Services Ltd, Canada). NAs aqueous solutions were analyzed using HPLC-Orbitrap-MS in water samples, previously adjusted to pH≈2, spiked with international (Dodecanoic acid-d23) and extracted by automated solid phase extraction. Compound characterization and quantification were performed using liquid chromatography coupled to Orbitrap mass spectrometer. NAs were analyzed in solid bitumen samples with a previous liquid/liquid extraction with an alkaline solution before analysis using HPLC-Orbitrap-MS. Metals were analyzed by Inductively Coupled Plasma Mass Spectrometry (ICP-MS) (Elan DRC-II with ESI SC-8XC high throughput FAST autosampler). Before analysis, digestion of samples was performed using an Ethos UP with the Maxi44 rotor (Milestone Inc). PAHs were analyzed by gas chromatography-mass spectrometry (GC-MS), through the AXYS Method MLA-02.

5.3.6 – Statistical analysis

The effects of the four natural bitumen samples and respective elutriate on the different *C. riparius* endpoints (growth ratio, emergence rate, development time

and imagoes weight) were evaluated using a One-Way ANOVA's followed by Dunnett's test using the treatments without solid material or elutriate as control treatments. Normality and homoscedasticity of data were verified by the Shapiro-Wilk and Levene´s tests, respectively.

5.4 – Results

5.4.1 - *Chironomus riparius* responses to contaminated sediment with natural bitumen

Survival in the control treatment was higher than 95% and pH, oxygen and conductivity followed the requested in the guideline. Also, in both cases, cumulative emergence in control treatments reached 100% thus meeting the validation criteria (OECD, 2004a).

C. riparius larval growth was significantly reduced after 10 days of exposure to sediments contaminated with natural bitumen. The presence of 10% and 20% of ATB natural bitumen in sediments reduced considerably larval size in comparison with control (Dunnet´s test; $p<0.05$) (Figure 5.3). All concentrations of STB and ELLs bitumen samples induced statistical decreases in larvae growth ratio of *C. riparius* (Dunnet´s test $p<0.05$). The presence of SP bitumen was the only sample not inducing any statistical effect on *C. riparius* larvae growth.

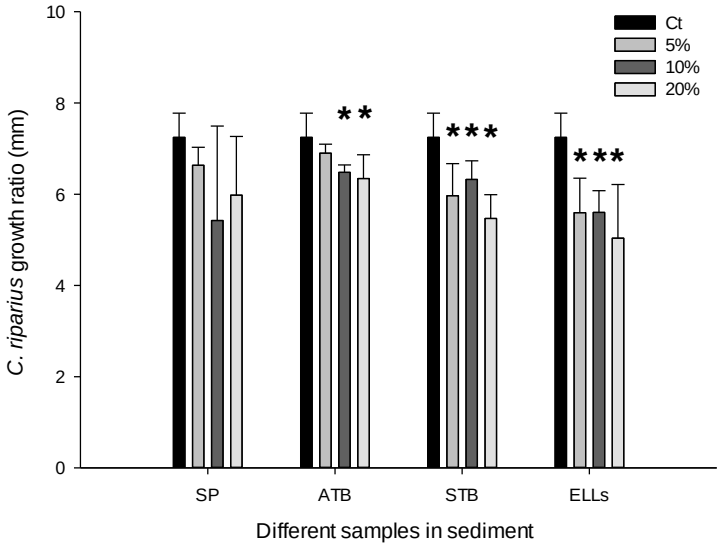

Figure 5.3 – *Chironomus riparius* larvae growth ratio over a 10-day exposure to natural bitumen from different locations in Canadian oil Sands applied at different % into the sediment. Data are presented as average ± SE; * denotes significant differences compared to the control treatment (Ct; clean sediment) (Dunnett's test, $p < 0.05$).

The average time to emerge was slightly affected by the presence of ELLs bitumen in the sediment. Males of *C. riparius* exposed to 20% of ELLs bitumen in sediment needed more time to develop and emerge in comparison to larvae from the control treatment (Dunnet´s test $p<0.05$). Other treatments did not affect the development of *C. riparius*, providing a comparable emergence as organisms under control conditions.

C. riparius cumulative percentage of emergence was also affected by the presence of natural bitumen in sediments. At the end of the 28 days of the test, statistically differences were found in all concentrations of ELLs natural bitumen samples, with a 46% reduction in cumulative emergence when midges were exposed to 20% of ELLs sample in the sediment, compared to the control (Dunnet´s test; $p<0.05$) (Figure 5.4). A reduction in *C. riparius* total emergence caused by exposure to STB bitumen in sediment was observed, with a decrease between 20 and 35% of their total emergence in the different concentrations even though no statistical differences were found. SP and ATB samples did not induce any effect in the *C. riparius* emergence rate (One-way ANOVA; p>0.0

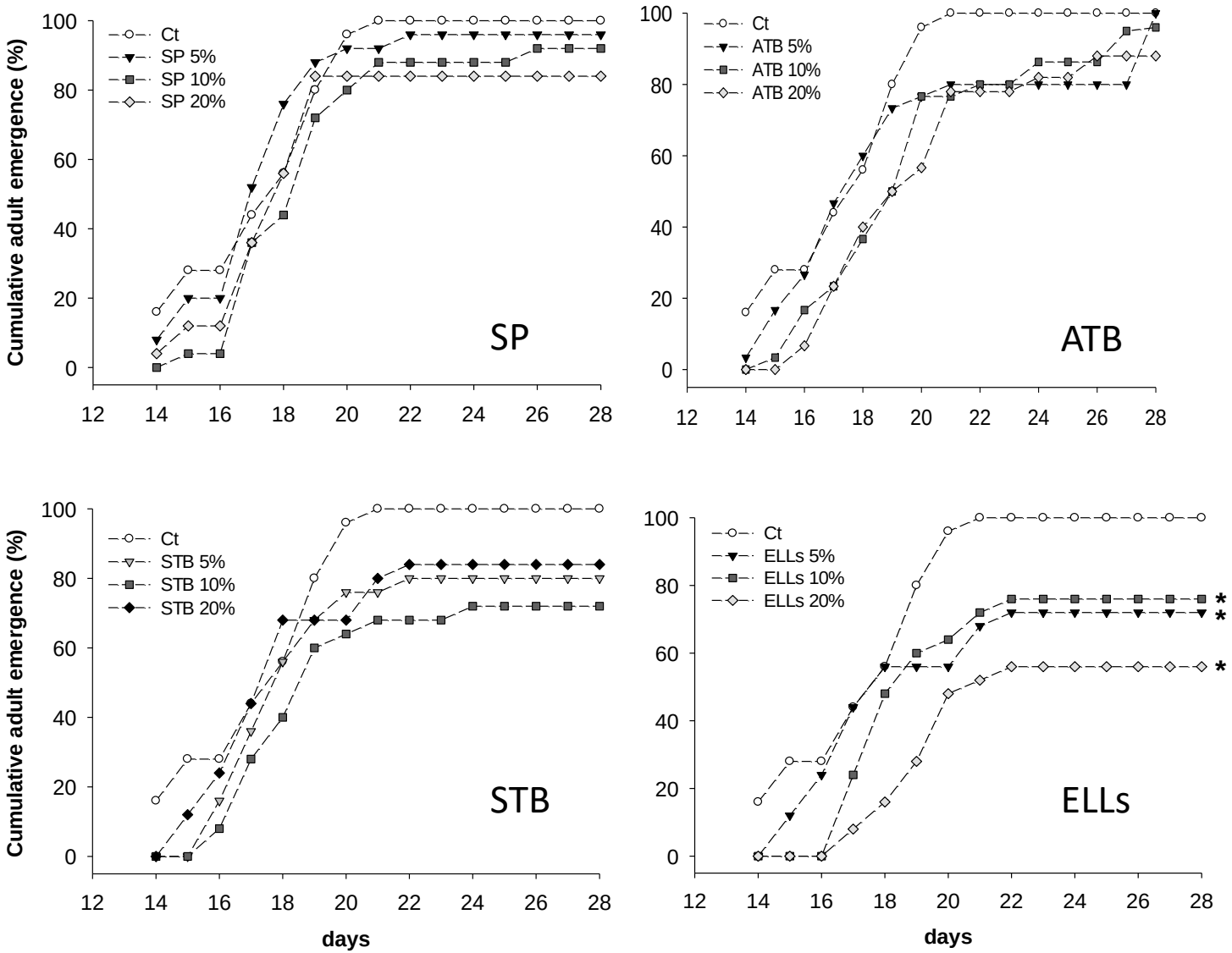
Cumulative adult emergence (%)
Ct
SP 5%
SP 10%
SP 20%
SP
ATB 5%
ATB 10%
ATB 20%
ATB
STB 5%
STB 10%
STB 20%
STB
ELLs 5%
ELLs 10%
ELLs 20%
ELLs
*
days

Figure 5.4 – Cumulative emergence (%) of *Chironomus riparius* after exposure to natural bitumen from different locations in Canadian oil Sands applied at different % into sediment. * denotes a significant difference in the total emergence of imagoes compared to the control treatment (Ct) (Dunnett's test, $p < 0.05$).

The weight of *C. riparius* imagoes was also affected by the presence of natural bitumen in the sediment. The presence of 5% and 20% of the ELLs sample in sediment reduced the weight of female imagoes (Dunnet´s test $p<0.05$) (Figure 5.5 A). Also, male imagoes exposed to 20% of ELLs solid sample showed weight reduction (Dunnet´s test $p<0.05$) (Figure 5.5 B). The other three samples, SP, ATB, and STB, did not induce significant alterations in imagoes weight (ANOVA, $p>0.05$).

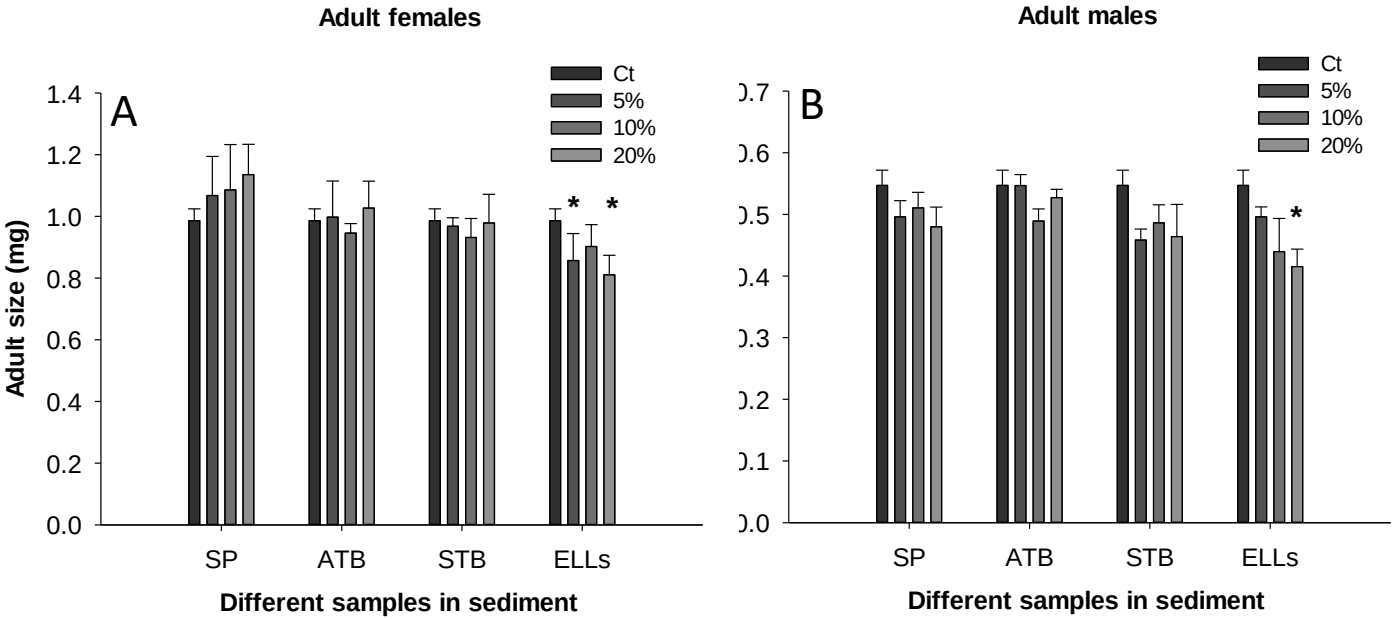

Figure 5.5 – Weight of *Chironomus riparius* adult females (A) and males (B), resulting from their larvae exposure to natural bitumen samples applied at different % in sediment, * denotes a significant difference in emergences compared to the control treatment (Ct) (Dunnett's test, $p < 0.05$).

5.4.2 – *Chironomus riparius* exposed to oil sands elutriates

For *C. riparius* assays using elutriates, the same validity criteria of the test were met. Survival in control was higher than 95% and pH, oxygen and conductivity following the standardized protocols (OECD, 2004a). Once again, cumulative emergence in control was equal to 100%.

The range of elutriates' concentration from the different natural bitumen samples did not induce any effects in any of the parameters analyzed (ANOVA p>0.05; Figure 5.6, 5.7 and 5.8).

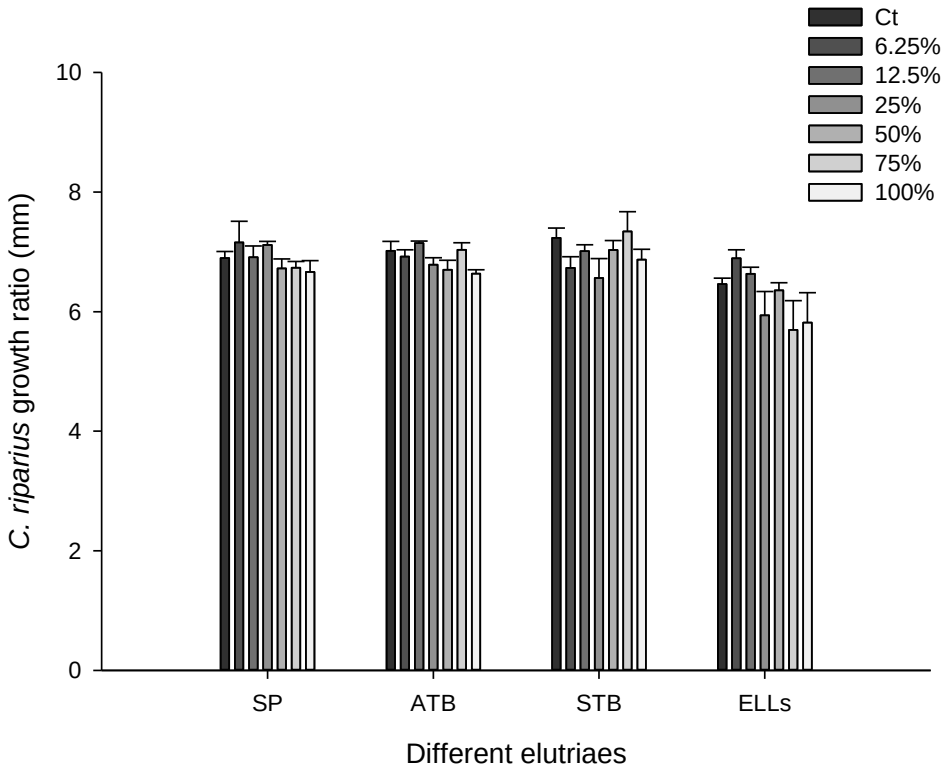

Figure 5.6 – *Chironomus riparius* larval growth ratio (mm) over a 10-day exposure to different concentrations of oil sands elutriates (mm; mean ± SE).

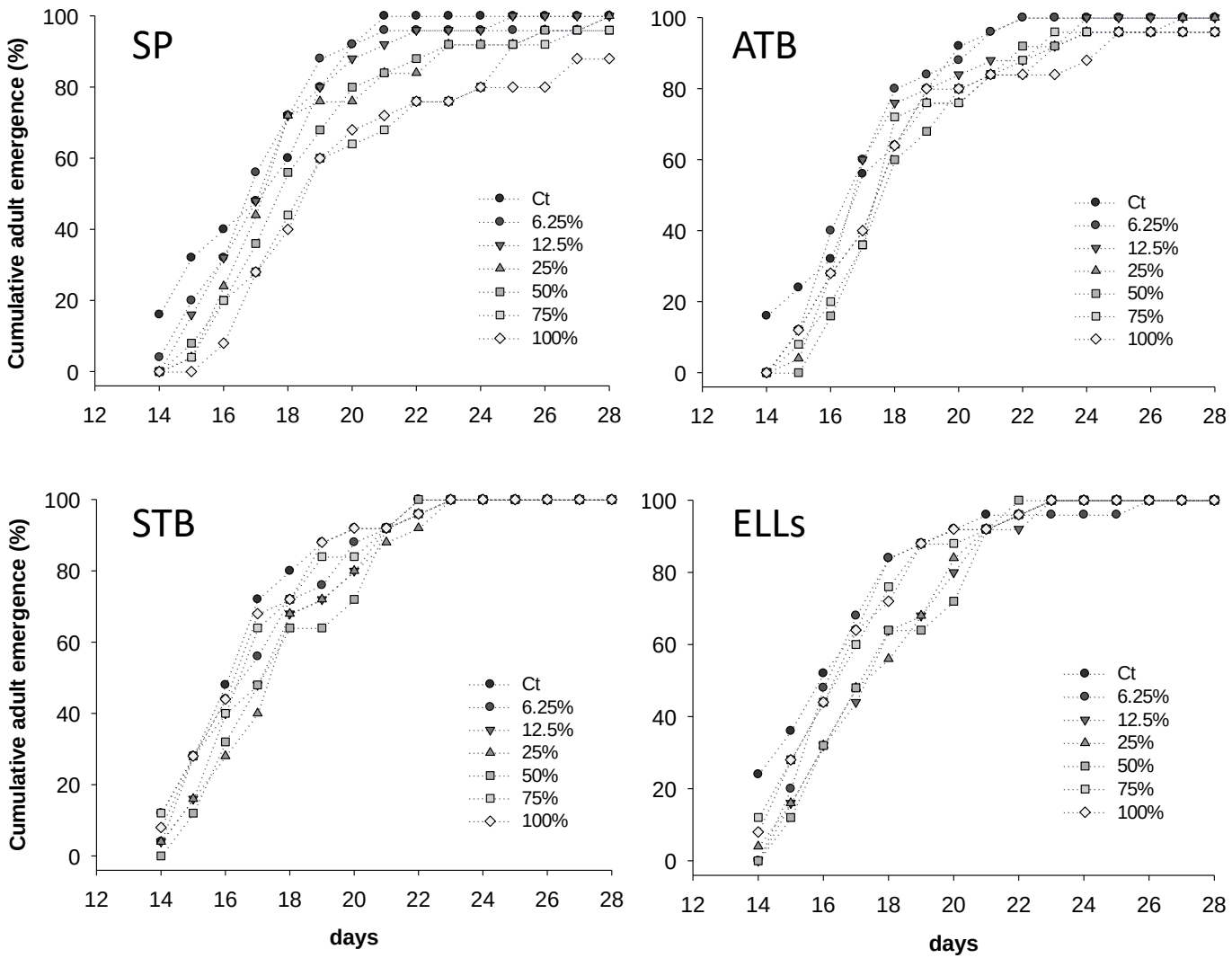

Figure 5.7 – *Chironomus riparius* cumulative emergence (%) after exposure to different concentrations of oil sands elutriates.

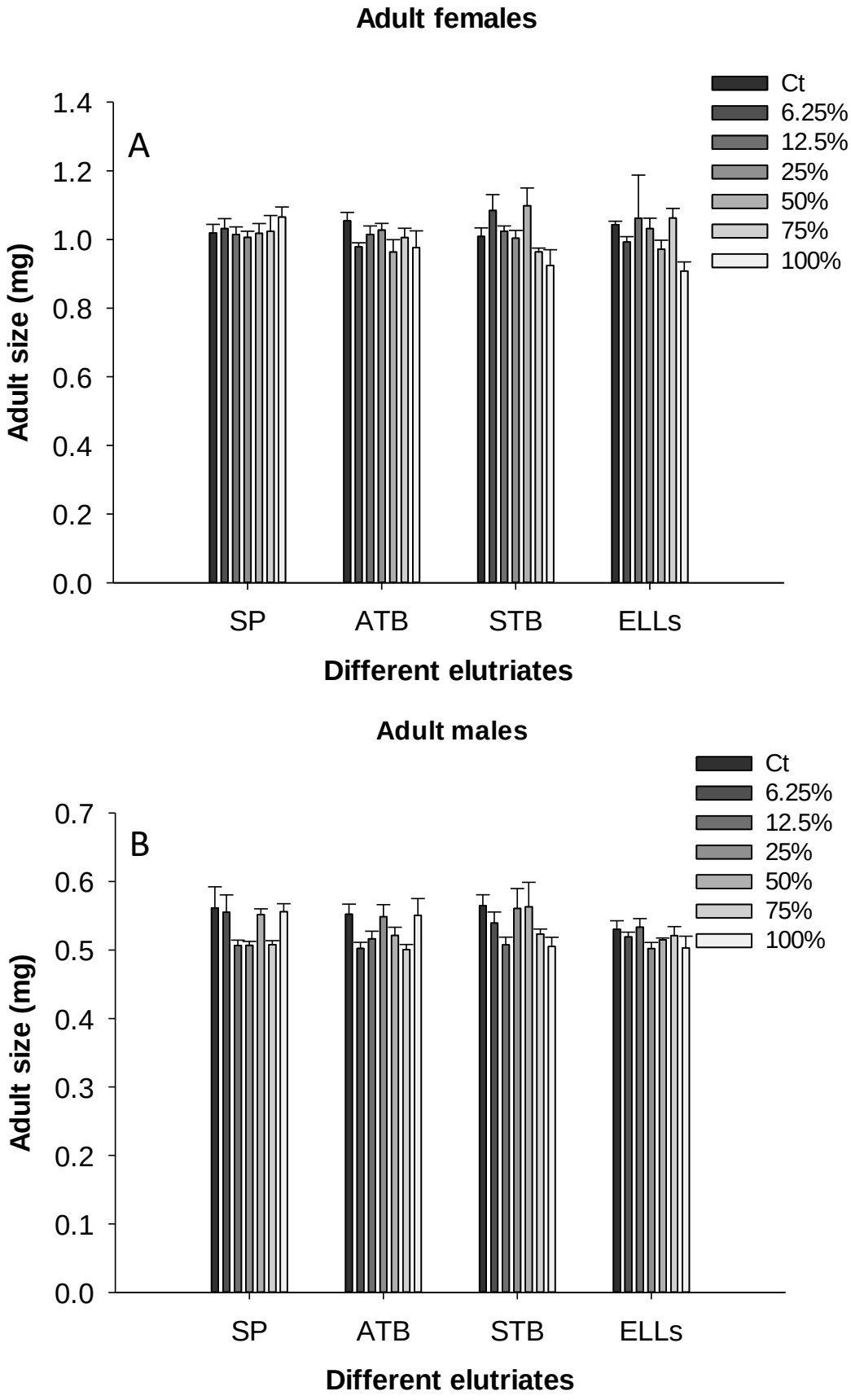

Figure 5.8 – Size of *Chironomus riparius* adult females (A) and males (B) in mg, resulting from their larvae exposure to different concentrations of oil sand elutriates.

5.4.3 - Metal, PAHs, and NA content in bitumen samples and elutriates

Both elutriates and natural bitumen solid samples were analyzed for content in metals, PAHs and NAs. Generally, solid samples were rich in aluminum, boron, cobalt, copper, lead, and selenium (Table 5.1). The SP solid bitumen sample presented the highest levels of all the assessed metals compared with ATB, STB, and ELLs solid samples. Different levels of PAHs, especially in samples STB and ELLs, were also detected, with higher levels of pyrene and Benzo[a]pyrene in ELLs solid samples (Table 5.2). The presence of Naphthenic acids in natural solid bitumen was high, with SP sample (collected in one of the banks of the Steepbank river) presenting the lowest total NAs comparing with the other three samples (Table 5.3). Also, the presence of low molecular NAs was predominant in the STB and ELLs samples, compared to ATB and SP samples (data not shown).

The chemical analysis of elutriates also reflected some heterogeneity between bitumen-related samples regarding metal contents. Aluminum, boron, cobalt, copper, lead, and selenium concentrations in elutriates exceeded the maximum allowed by the Water Quality for the Protection of Aquatic Life and by the Soil Quality for the Protection of Environmental and Human Health from the Canadian Council of Ministers of the Environment (CCME) (Table 5.1). Elutriates collected from the Ells river solid sample presented the highest levels of PAHs, followed by STB, ATB and SP samples, respectively (Table 5.2); pyrene and Benzo[a]pyrene were the PAHs with values above the maximum allowed by CCME for freshwaters. Higher NAs contents were also derived from elutriates produced by the sample collected in the banks of the ELLs river, with almost twice the total amount of NAs of the STB sample, eight times more than ATB sample and 200 times more than SP sample (Table 5.3).

Table 5.1 - Metal content of natural bitumen samples from SP, ATB, STB, and ELLs, collected from local rivers of the oil sands area in Alberta, Canada, and respective elutriates. Dissolved and total values are presented for elutriates. The right-side column presents the maximum level allowed by the Water Quality for the Protection of Aquatic Life and by the Soil Quality for the Protection of Environmental and Human Health from the Canadian Council of Ministers of the Environment (CCME). n.d.- not determined; values in bold highlight values above or close to the limit established by the CCME.

	SP elutriate (µg/L)		**ATB elutriate (µg/L)**		**STB elutriate (µg/L)**		**ELLs elutriate (µg/L)**		**SP solid (µg/g)**	**ATB solid (µg/g)**	**STB solid (µg/g)**	**ELLs solid (µg/g)**	**Maximum allowed by CCME (µg/L)**
	Total	Dissolved	Total	Dissolved	Total	Dissolved	Total	Dissolved	Total	Total	Total	Total	
Aluminium	7.8	0.75	8.5	0.85	**835**	**90.5**	**4040**	**2070**	133000	7630	10300	38400	**100**
Antimony	0.577	0.569	0.790	0.780	0.181	0.179	0.426	0.420	1.05	0.514	0.092	0.968	n.d.
Arsenic	0.386	0.381	2.05	1.94	0.896	0.611	1.74	1.09	21.7	23.5	2.24	3.02	5
Barium	27.3	26.8	48.6	46.8	26.4	24.8	60.6	41.8	552	131	28.3	107	n.d.
Beryllium	0.030	0.030	0.009	< 0.009	0.044	0.012	0.766	0.330	3.62	1.14	0.327	1.39	n.d.
Bismuth	0.023	0.023	0.005	0.005	0.007	0.007	0.108	0.046	0.485	0.065	0.025	0.13	n.d.
Boron	**1740**	**1730**	46.0	45.4	22.5	22.2	420	372	250	27.2	12.0	70.2	**1500**
Cadmium	0.082	0.081	0.019	0.019	0.010	0.010	0.061	0.019	0.247	0.309	0.025	0.091	0.09
Calcium	256000	255000	67500	66900	43500	43200	16300	14400	30900	16000	1270	2000	n.d.
Chloride	22000	21600	9950	9930	13700	13500	38200	32700	415	463	228	356	n.d.
Chromium	0.04	< 0.1	< 0.03	< 0.1	0.80	0.1	7.15	3.3	85.5	15.5	6.80	27.7	8.9
Cobalt	0.272	0.067	0.942	0.042	0.831	0.612	5.72	2.21	34.1	16.3	3.58	13.2	n.d.
Copper	**4.35**	**3.77**	1.05	0.98	1.38	1.36	**11.5**	**5.19**	70.3	12.1	2.8	11.2	**2**
Iron	15.4	1.4	72.4	2.8	**501**	**118**	**5190**	**1410**	116000	42700	6000	16300	**300**
Lead	0.080	0.054	0.084	0.013	0.665	0.186	**12.6**	**5.10**	29.4	9.41	4.71	11.7	**1**
Lithium	261	261	4.29	4.23	8.65	8.54	60.1	53.7	125	11.8	13.8	90.0	n.d.
Manganese	2.95	0.58	175	95.6	10.3	10.3	57.7	32.0	723	578	50.5	677	n.d.
Molybdenum	1.90	1.86	1.12	1.11	1.52	1.50	1.59	1.57	2.12	3.96	3.55	2.19	73
Nickel	12.4	11.7	1.15	1.14	7.33	6.67	14.7	7.12	83.1	47.3	35.3	36.7	25
Selenium	**3.73**	**3.68**	0.40	0.40	0.41	0.34	**1.01**	0.69	2.07	1.07	0.33	0.57	**1**
Silver	0.022	0.021	0.003	0.002	0.017	0.005	0.147	0.073	0.743	0.469	0.137	0.315	0.25
Strontium	5150	5060	182	180	110	108	240	238	376	101	23.8	74.9	n.d.
Thallium	0.0642	0.0634	0.0269	0.0264	0.0312	0.0308	0.0708	0.0501	0.810	0.235	0.118	0.238	0.8
Thorium	0.0493	0.0487	0.0090	0.0089	0.0741	0.0482	7.03	3.00	15.7	10.2	4.32	7.27	n.d.
Tin	0.032	0.031	< 0.003	< 0.003	0.023	0.007	0.164	0.159	2.00	0.466	0.226	1.10	n.d.
Titanium	1.23	0.80	0.94	0.62	16.4	6.95	86.9	85.8	3340	2090	799	1800	n.d.
Uranium	1.16	1.14	0.529	0.519	0.060	0.057	1.43	0.901	3.54	2.53	0.800	1.16	15
Vanadium	0.31	0.28	2.45	2.19	1.97	0.76	14.4	7.09	168	74.2	67.3	66.9	n.d.
Zinc	8.3	7.65	4.3	3.73	4.9	3.35	9.9	5.20	162	45.5	12.2	32.6	30

Table 5.2 - PAHs content (analyzed by gas chromatography mass spectrometry (GC-MS)) in natural bitumen present in the SP, ATB, STB and ELLs samples, collected from local rivers of the oil sands area in Alberta, Canada, and respective elutriates. The right-side column presents the maximum level allowed by the Water Quality for the Protection of Aquatic Life and by the Soil Quality for the Protection of Environmental and Human Health from the Canadian Council of Ministers of the Environment (CCME). <D.L. – below detection limit; n.d.- not determined.

	SP elutriate (ng/L)	ATB elutriate (ng/L)	STB elutriate (ng/L)	ELLs elutriate (ng/L)	SP solid (ng/g)	ATB solid (ng/g)	STB solid (ng/g)	ELLs solid (ng/g)	Maximum allowed by CCME (ng/L)
Naphthalene	22.7	22.9	25.1	23.2	102	113	105	93.9	1100
Acenaphthylene	<D.L.	<D.L.	<D.L.	<D.L.	<D.L.	<D.L.	<D.L.	<D.L.	n.d.
Acenaphthene	<D.L.	<D.L.	<D.L.	<D.L.	<D.L.	<D.L.	<D.L.	<D.L.	5800
2-Methylfluorene	<D.L.	<D.L.	<D.L.	<D.L.	<D.L.	<D.L.	<D.L.	<D.L.	n.d.
C2 Phenanthrenes/Anthracenes	2.15	<D.L.	9.45	110	40.3	546	2580	2070	n.d.
Fluorene	1.92	1.350	1.57	2.18	<D.L.	<D.L.	<D.L.	<D.L.	300
Phenanthrene	7.49	5.08	4.88	12.6	40	<D.L.	<D.L.	<D.L.	400
Anthracene	<D.L.	<D.L.	<D.L.	<D.L.	<D.L.	<D.L.	<D.L.	<D.L.	12
C1 Phenanthrenes/Anthracenes	2	<D.L.	<D.L.	24.2	<D.L.	<D.L.	<D.L.	<D.L.	n.d.
Fluoranthene	2.34	1.85	2.58	23.4	13.7	<D.L.	296	166	40
Pyrene	1.39	2.06	9.34	**95.6**	21	<D.L.	1720	752	**25**
Benz[a]anthracene	0.412	<D.L.	<D.L.	<D.L.	<D.L.	<D.L.	<D.L.	<D.L.	18
Chrysene	1	2.43	15.2	186	17.8	1070	3030	1330	n.d.
Benzo[b]fluoranthene	<D.L.	<D.L.	4.78	46.3	<D.L.	417	700	356	n.d.
Benzo[j,k]fluoranthenes	<D.L.	<D.L.	<D.L.	6.87	<D.L.	<D.L.	<D.L.		n.d.
Benzo[e]pyrene	<D.L.	<D.L.	8.16	114	<D.L.	619	1200	721	n.d.
Benzo[a]pyrene	<D.L.	<D.L.	<D.L.	**22.6**	<D.L.	227	411	205	**15**
Perylene	0.778	<D.L.	19.6	88.6	71.3	1190	1080	488	n.d.
Dibenz[a,h]anthracene	<D.L.	<D.L.	<D.L.	13.9	<D.L.	<D.L.	165	95.9	n.d.
Indeno[1,2,3-cd]pyrene	<D.L.	<D.L.	2.54	19.1	18.1	207	228	101	n.d.
Benzo[ghi]perylene	<D.L.	<D.L.	4.33	47.6	14	341	495	275	n.d.
2-Methylnaphthalene	10.3	10.7	9.41	15.3	90.8	125	95.9	90.1	n.d.
1-Methylnaphthalene	5.37	5.18	5.03	7.66	55.7	64.7	51.9	57.3	n.d.
C1-Naphthalenes	15.7	15.9	14.4	22.9	146	190	95.9	90.1	n.d.
Biphenyl	11	11.5	9.81	9.78	67.6	51.4	49.4	43.8	n.d.
C1-Biphenyls	9.59	6.47	8.49	10.9	86.1	111	94.3	76.3	n.d.
C2-Biphenyls	34.5	23.5	34.5	22.7	<D.L.	<D.L.	<D.L.	<D.L.	n.d.
C2-Naphthalenes	23.6	22.30	15.5	24	<D.L.	<D.L.	<D.L.	<D.L.	n.d.

	SP elutriate (ng/L)	ATB elutriate (ng/L)	STB elutriate (ng/L)	ELLs elutriate (ng/L)	SP solid (ng/g)	ATB solid (ng/g)	STB solid (ng/g)	ELLs solid (ng/g)	Maximum allowed by CCME (ng/L)
1,2-Dimethylnaphthalene	<D.L.	<D.L.	<D.L.	<D.L.	<D.L.	<D.L.	<D.L.	<D.L.	n.d.
2,6-Dimethylnaphthalene	1.87	<D.L.	<D.L.	2.71	<D.L.	<D.L.	<D.L.	<D.L.	n.d.
C3-Naphthalenes	9	6.52	18.2	26.4	<D.L.	<D.L.	<D.L.	<D.L.	n.d.
2,3,6-Trimethylnaphthalene	0.972	<D.L.	2.19	3.32	<D.L.	<D.L.	<D.L.	<D.L.	n.d.
2,3,5-Trimethylnaphthalene	<D.L.	<D.L.	<D.L.	<D.L.	<D.L.	<D.L.	<D.L.	<D.L.	n.d.
C4-Naphthalenes	1.67	2.67	23	50.7	50.3	56.2	861	1010	n.d.
C1-Acenaphthenes	<D.L.	<D.L.	<D.L.	<D.L.	<D.L.	<D.L.	<D.L.	<D.L.	n.d.
C1-Fluorenes	2.84	2.48	3.36	9.59	<D.L.	107	469	134	n.d.
1,7-Dimethylfluorene	<D.L.	<D.L.	<D.L.	<D.L.	<D.L.	<D.L.	<D.L.	<D.L.	n.d.
C2-Fluorenes	2.66	<D.L.	5.91	49.3	<D.L.	551	2360	518	n.d.
C3-Fluorenes	6.47	11.6	48.7	283	<D.L.	1760	6890	3250	n.d.
Dibenzothiophene	0.737	0.947	<D.L.	2.88	<D.L.	<D.L.	<D.L.	<D.L.	n.d.
C1-Dibenzothiophenes	<D.L.	<D.L.	<D.L.	4.62	<D.L.	<D.L.	445	<D.L.	n.d.
2/3-Methyldibenzothiophenes	<D.L.	<D.L.	<D.L.	<D.L.	<D.L.	<D.L.	445	512	n.d.
C2-Dibenzothiophenes	1.5	2.75	27.7	257	52	880	5000	3090	n.d.
2,4-Dimethyldibenzothiophene	<D.L.	<D.L.	<D.L.	<D.L.	<D.L.	<D.L.	<D.L.	<D.L.	n.d.
C3-Dibenzothiophenes	3.96	6.94	68.6	1470	118	13700	22900	13900	n.d.
C4-Dibenzothiophenes	4.9	10.3	172	1440	360	34700	45000	24700	n.d.
3-Methylphenanthrene	1.17	<D.L.	<D.L.	9.54	<D.L.	<D.L.	<D.L.	<D.L.	n.d.
2-Methylphenanthrene	0.828	<D.L.	<D.L.	<D.L.	<D.L.	<D.L.	<D.L.	<D.L.	n.d.
2-Methylanthracene	<D.L.	<D.L.	<D.L.	<D.L.	<D.L.	<D.L.	<D.L.	<D.L.	n.d.
9/4-Methylphenanthrene	<D.L.	<D.L.	<D.L.	14.7	<D.L.	<D.L.	<D.L.	<D.L.	n.d.
1-Methylphenanthrene	<D.L.	<D.L.	<D.L.	<D.L.	<D.L.	<D.L.	<D.L.	<D.L.	n.d.
3,6-Dimethylphenanthrene	0.255	<D.L.	<D.L.	11.4	<D.L.	<D.L.	310	173	n.d.
2,6-Dimethylphenanthrene	<D.L.	<D.L.	<D.L.	<D.L.	<D.L.	<D.L.	198	144	n.d.
1,7-Dimethylphenanthrene	0.356	<D.L.	<D.L.	8.4	8.54	<D.L.	256	193	n.d.
1,8-Dimethylphenanthrene	<D.L.	<D.L.	<D.L.	<D.L.	<D.L.	<D.L.	<D.L.	<D.L.	n.d.
C3-Phenanthrenes/Anthracenes	1.13	1.89	22.4	590	24.7	2590	11000	8420	n.d.
1,2,6-Trimethylphenanthrene	<D.L.	<D.L.	<D.L.	15.4		<D.L.	<D.L.	<D.L.	n.d.
Retene	<D.L.	<D.L.	<D.L.	<D.L.	21.8	<D.L.	<D.L.	<D.L.	n.d.
C4-Phenanthrenes/Anthracenes	3.57	20.3	256	1510	91.7	25600	57000	28200	n.d.
C1-Fluoranthenes/Pyrenes	3.51	7.77	86.1	896	52.8	4920	16900	7390	n.d.
3-methylfluoranthene/Benzo[a]fluorene	1.63	<D.L.	10.5	134	<D.L.	<D.L.	2680	1310	n.d.

	SP elutriate (ng/L)	ATB elutriate (ng/L)	STB elutriate (ng/L)	ELLs elutriate (ng/L)	SP solid (ng/g)	ATB solid (ng/g)	STB solid (ng/g)	ELLs solid (ng/g)	Maximum allowed by CCME (ng/L)
C2-Fluoranthenes/Pyrenes	4.15	14.8	154	1950	139	16300	37900	19400	n.d.
C3-Fluoranthenes/Pyrenes	<D.L.	9.1	154	1600	48.9	21800	42400	20700	n.d.
C4-Fluoranthenes/Pyrenes	<D.L.	4.07	76.7	1120	49.4	8730	22200	7650	n.d.
C1-Benzo[a]anthracenes/Chrysenes	0.81	2.52	35.6	394	35.3	5530	9130	4230	n.d.
5/6-Methylchrysene	<D.L.	<D.L.	2.15	48.4	<D.L.	523	1020	660	n.d.
1-Methylchrysene	<D.L.	<D.L.	2.19	17.7	9.42	486	453	187	n.d.
C2-Benzo[a]anthracenes/Chrysenes	0.742	4.16	87.3	530	68.6	10100	17900	7470	n.d.
5,9-Dimethylchrysene	<D.L.	<D.L.	22.2	124	<D.L.	2120	3680	1600	n.d.
C3-Benzo[a]anthracenes/Chrysenes	<D.L.	0.918	35	198	13.5	6440	10400	4950	n.d.
C4-Benzo[a]anthracenes/Chrysenes	<D.L.	<D.L.	11.2	60.9	<D.L.	835	2120	758	n.d.
C1-Benzofluoranthenes/Benzopyrenes	<D.L.	<D.L.	34.1	326	<D.L.	3480	5200	2970	n.d.
7-Methylbenzo[a]pyrene	<D.L.	<D.L.	<D.L.	45.9	<D.L.	330	523	307	n.d.
C2-Benzofluoranthenes/Benzopyrenes	<D.L.	0.995	19.7	171	36	2560	3710	2100	n.d.
1,4,6,7-Tetramethylnaphthalene	<D.L.	<D.L.	1.81	6.67	<D.L.	<D.L.	205	193	n.d.

Table 5.3 – Total NAs content in SP, ATB, STB and ELLs natural bitumen samples and respective elutriates, assessed by analyzed by HPLC-Orbitrap-MS.

	Total NAs (µg/L)	Total NAs (µg/g)
SP Elutriate	28.6	-
ATB Elutriate	663	-
STB Elutriate	3330	-
ELLs Elutriate	5630	-
Solid SP	-	33.4
Solid ATB	-	191
Solid STB	-	185
Solid ELLs	-	154

5.5. – Discussion

The present study reports deleterious effects induced by samples collected along river banks within the Athabasca oil sands region to *C. riparius* under controlled laboratory conditions, highlighting a potential hazard to freshwater benthic organisms. Also, different levels of toxicity for the different samples were observed showing stronger effects when using natural bitumen samples collected from the banks of the Ells river. This study adds thus essential data for risk assessment studies within the oil sands area and explicitly allowing for discrimination between natural and anthropogenic-related pollution.

From the different natural bitumen samples collected from three different rivers and mixed in the sediments, the ELLs sample was the most toxic sample inducing more severe effects in *C. riparius* such as reductions in larval growth, development, cumulative emergence and weight of imagoes after a 28 days exposure. The STB sample, collected in the banks of the Steepbank river, was the second most toxic sample to *C. riparius*, followed by ATB and SP samples. These results demonstrate that if natural weathered bitumen reaches river sediments, it could negatively affect benthic communities namely sediment dwelling invertebrates.

Surprisingly the SP natural bitumen sample presented the lowest toxicity to *C. riparius*, despite the higher levels of metals when compared to the other three solid samples. Contrarily, when looking at the PAHs and NAs content in solid samples, the pattern was different with ELLs, STB an ATB solid samples presenting higher levels of PAHs and NAs, comparing with SP solid sample, matching the higher toxicity pattern induced especially by STB and ELLs bitumen samples. It is important to refer that despite no clear differences concerning the total NAs concentration in natural bitumen samples, the higher presence of low molecular NAs in ELLs sample could also relate to the highest toxicity induced towards chironomids. Lower molecular weight NA (especially acyclic compounds) already demonstrated higher toxicity due to their capacity to more easily penetrate and disrupt cell membranes, severely affecting organisms (Jones et al., 2011).

On the other hand, our results also showed that elutriates produced from these four different bitumen samples were not toxic to chironomids, with no significant effects observed for any of the life-history endpoints analyzed. The elutriates used in the present study were derived similarly to those used in Chapter 2, 3 and 4 where adverse chronic and acute effects were observed for daphnids, snails, bacteria, and planarians. This highlights the importance of using different exposure routes in different organisms, to assess effects accurately, and relate them to exposure and organismal traits. The difference in toxicity obtained between natural bitumen mixed in the sediment and elutriates could be explained by the bioavailability of contaminants in solid samples through surface contact or even by bitumen ingestion, in contrast with a lower bioavailable fraction present in elutriates. *Chironomus* species were already used to assess the toxic effects that oil sands mining and upgrading activities pose to aquatic organisms. Studies reported deleterious effects of exposure to fresh oil sands process waters (OSPW) used in bitumen treatment on *Chironomus dilutus* (Anderson et al., 2012; Wiseman et al., 2013), or to test the effectiveness of ozonation to reduce the toxicity of OSPW (Anderson et al., 2011). This species was also exposed to untreated OSPW, and OSPW treated with different levels of metallic salt coagulants of aluminum sulfate and to the polymer polyDADMAC to infer on the remediation potential to OSPWs (Pourrezaei et al., 2011). Therefore, chironomids

have shown a good potential to evaluate these types of contaminants, already in this area of concern, but approaching issues derived from anthropogenic activities.

Even though elutriates did not cause significant effects, ELLs natural bitumen sample induced the highest toxicity towards *C. riparius* in the present study. Our results are in line with the findings of Droppo et al. (2018) where higher loads of PACs in Ells river, comparing with the Steepbank river were reported, suggesting a strong spatial variation of natural chemistry within the oil sands region. The different chemical composition and consequently toxicity between the different natural samples collected in the field attest for the heterogeneity in composition and potential toxicity of bitumen in the oil sands area. Natural bitumen collected from different tributaries/rivers' banks presented different levels of toxicity, as well as those from the same river. In the present study, samples SP and STB collected from the Steepbank river presented different toxicities to chironomids. Therefore, it is difficult to define the background toxicity of the area due to its heterogeneity.

Till now, only a few studies devoted their attention to the natural effects that bitumen induces on aquatic organisms, namely using vertebrate species and collecting sediment, water, and organisms from the field. Colavecchia et al. (2004) found hatching alterations, increased mortality and malformations, and reduced size in early life stages of *Pimephales promelas* exposed to sediments from natural oil sand deposits and OSPW. Effects of naturally contaminated sediment were also evaluated by Colavecchia et al. (2006) reporting that sediments contaminated with oil sands material impaired the development and survival of white sucker *Catostomus commersoni* eggs and larvae in laboratory conditions. Tetreault et al. (2003) also found biochemical changes in fish that were collected within the proximity areas of the mining activities when compared with fish populations residing in reference conditions.

In the present study, ecotoxicological effects of natural bitumen incorporated in sediment were depicted under laboratory conditions in test organisms from laboratory cultures. Since the aim of the present study is to evaluate the effects induced by a natural load of bitumen in natural river populations, the uncertainty caused by the use of laboratory organisms instead of native species, and the

ability of organisms to avoid or adapt in natural systems are always factors that should be taken in consideration. On the other hand, in laboratory experiments, all exposure conditions are controlled, and no extra factors are influencing results. In natural conditions, changes in temperature, water flow, predation, among several other biotic and abiotic factors can contribute to an increment of stress to organisms. Related to this, Beery et al. (2017) conducted a study to assess the local and natural adaptation of native populations of *Hyalella azteca* in two different oil sands wetlands not influenced by anthropogenic activities. Surprisingly, no evidence of local adaptation was observed, with a lower survival rate in local populations than “foreign” species. Also, in the study of Beery et al. (2017), it is highlighted that studies using laboratory species may still provide ecologically relevant results since laboratory cultures of *Hyalella azteca* had the same response to oil sands natural wetlands than native populations. Those results strengthened our findings, supporting the relevance of effects in non-native species.

The present study is, therefore, a step forward in the evaluation of the natural effects induced by a load of weathered natural bitumen into the aquatic systems, using *C. riparius* as model species. Different works already reported the existence of the inherent background toxicity in Canadian oil sands. Therefore, the present study adds more information for future risk assessment studies of the mining/upgrading activities, highlighting the need to take into consideration the natural background toxicity in this oil sands area. It is also important to highlight the heterogeneity of the area regarding toxicity, where different areas could pose different levels of effects/toxicities to the aquatic biota given the different concentrations of PAHs, NAs, and metals in different bitumen samples, even within the same river.

5.6 – Conclusion

Chironomids life history and growth were significantly affected by the presence of natural bitumen in sediments, previously collected from the banks of the Steepbank and Ells river that contained high levels of NAs, PAHs, and metals. At the same time, no adverse effects were observed when chironomids were

exposed to elutriates produced from these bitumen samples simulating the natural load into the river, and the natural washing processes occurring in rivers. The results from the present study also highlight the chemical heterogeneity of the natural bitumen samples collected within the oil sands regions and their consequently differential toxicity. The present study calls therefore for the importance of considering the natural background effects caused by the presence of bitumen within river sediments.

5.7 – Acknowledgments

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Chapter 6: Changes in macroinvertebrate communities caused by exposure to Oils sands bitumen in mesocosms.

Changes in macroinvertebrate communities caused by exposure to Oils sands bitumen in mesocosms.

6.1 – Abstract

In the Canadian oil sands, rivers and tributaries cut through bitumen deposits and are consequently exposed to an input of fresh, natural weathered/eroded bitumen. Therefore, it is crucial to predict inherent adverse effects of bitumen to aquatic biota and to distinguish those from anthropogenic perturbation, related to mining and upgrading activities. Following a series of studies using single-species tests with natural bitumen collected from different rivers in the Athabasca region of northern Alberta (Canada), a mesocosm experiment was carried out. The present study aimed at evaluating the effects of natural bitumens collected from the banks of the Ells river on a natural benthic community. After 7 days of the experiment, natural weathered bitumen mixed in sediment did not induce effects on primary production (as chlorophyll *a* contents) or leaf decomposition. However, bitumen in sediment was able to reduce the macroinvertebrates abundance, with a reduction in the percentage of EPTs (total number of organisms that belongs to Ephemeroptera, Plecoptera, and Trichoptera), specially explained by a reduction of *Ephemera* sp. and *Chironomus* sp. that feed on fine sediment particles. The present study confirms the previously observed toxicity to Chironomus, using now a higher tier approach. Losses on biodiversity were probably caused mainly by physical alterations induced by the ingestion and filtration-effects induced by suspended bitumen fine sediment and also due to the covering of the substrate, with possible implications to the macroinvertebrate community structure.

Key-words: eroded natural bitumen**;** macroinvertebrate community structure; river biodiversity; EPT; Species abundance, artificial streams

6.2 - Introduction

Athabasca oil sand deposits near Fort McMurray, Alberta, Canada, are the largest Canadian petroleum reserves, and the third largest in the world (Gerner et al., 2017), covering an area of 42000 km^2 (Conly et al., 2007). Since the Athabasca River and its tributaries are incised in the McMurray formation, they are naturally exposed to bitumen that is continuously eroded/weathered from their banks (Barton and Wallace, 1979). Despite the increasing concerns about the environmental impact that oil extraction/mining and upgrading activities could have into the surrounding aquatic ecosystems (Conly et al., 2002; Headley et al., 2001), it is paramount to predict and distinguish natural from anthropogenic effects in aquatic biota from Canadian oil sands rivers (Gerner et al., 2017). As such it is critical to define baseline information concerning the effects of these natural sources of contamination (Conly et al., 2007; Droppo et al., 2018).

Some studies have already assessed the effects induced by bitumen deposits on specific organisms' life traits, by collecting sediments from the oil sands formation and exposing fish in laboratory conditions (Colavecchia et al., 2004; Colavecchia et al., 2006; Colavecchia et al., 2007), or assessing physiological and biochemical responses of collected fish exposed to natural and mining-related contamination (Tetreault et al., 2003a; Tetreault et al., 2003b). Also, oil sands elutriates generated using natural bitumen collected in the banks of different rivers, showed to be toxic to aquatic organisms (Chapter 2, 3 and 4). Moreover, the heterogeneity and disparity in toxicity obtained from natural bitumen samples collected from different sites have also been reported for *Daphnia magna* (Cladocera), *Vibrio fisheri* (Bacteria), *Physa acuta* (snails) (Chapter 3), *Dugesia tigrina (*planaria) (Chapter 4) and *Chironomus riparius* (Diptera) (Chapter 5).

Standard ecotoxicological studies are used to evaluate the induced effects of single chemicals to single species individuals (Chapman, 2002; Santos et al., 2018), only looking at specific species effects which may not express consequences at higher levels of biological organization. Some individual based effects can be extrapolated to population effects (e.g., mortality, reproduction), but disregard any biological interaction between organisms (e.g., predation, symbiosis,

competition), that are present in biological communities in the field. (Van den Brink et al., 2005; Santos et al., 2018; Vighi and Villa, 2013). Also, laboratory assays usually use species that could be more, or less sensitive to environmental changes than representative natural communities.

The use of natural communities in mesocosms experiments tries, therefore, to fill in this gap of knowledge, aiming to achieve a more realistic approach, where specific hypotheses are tested under a more ecologically relevant scenario. While effects at higher tiers usually regard impacts already occurring at lower organizational levels (organism level), mesocosms can help assessing indirect effects such as resilience, and recovery, which are useful parameters when assessing ecological risks at community, and ecosystem levels (Van den Brink et al., 2005; Odum, 1984).

In the present study, natural bitumen collected in the banks of the Ells River was used since was previously defined as highly toxic to aquatic laboratory cultured invertebrates (Chapter 3, 4, and 5). Here, this material was used to mimic a natural load of weathered fresh bitumen and infer on the effects induced to a natural macroinvertebrate community in a mesocosm trial (higher tier approach). This natural bitumen sample was mixed in sediment deposited in the artificial streams. Structural and functional measures were assessed for seven days of exposure (leaf decomposition, primary production, and benthic macroinvertebrate community's structure). Leaf decomposition and primary production are functional parameters indicative of primary energy sources provided to lotic freshwater systems, which are considered critical and reliable indicators of ecosystems health (Abelho et al., 2016; Young et al., 2008). Biodiversity abundance and structure are crucially related to ecosystems functions (namely the previous mentioned), being therefore of significant importance to study. The present study states as the null hypothesis that natural bitumen from banks of the Ells River when deposited/mixed in sediment will keep the natural macroinvertebrates community abundance and structure, along with ecosystem functions.

6.3 – Material and method

6.3.1 - Natural bitumen sample collection

Natural bitumen samples were collected from the banks of the Ells River, Alberta, Canada (ELLs sample, 57°16'49.0"N 111°42'17.0"W) where no input from mining and industrial activities is perceived. In this specific location, the Ells River flows through natural bitumen deposits and due mainly to the erosion of river banks, the bitumen enters into the streams (Figure 6.1-A), being present on bed streams at different levels, from sand entirely covered by deposited bitumen (Figure 6.1-B) to sites where no bitumen is visible. Also, oil sheens were evident in the interface between the river and the bank due to washing of bitumen in the river (Figure 6.1-C). These observations mean that bitumen could be deposited in the sediments and also that chemicals released from bitumen disperse in the water.

The samples were collected by digging into the interface banks/river and after collection, samples were packed into a heavy gauge, food-safe plastic bags, and placed into coolers for shipping and sent to the Department of Biology, University of Aveiro, Portugal.

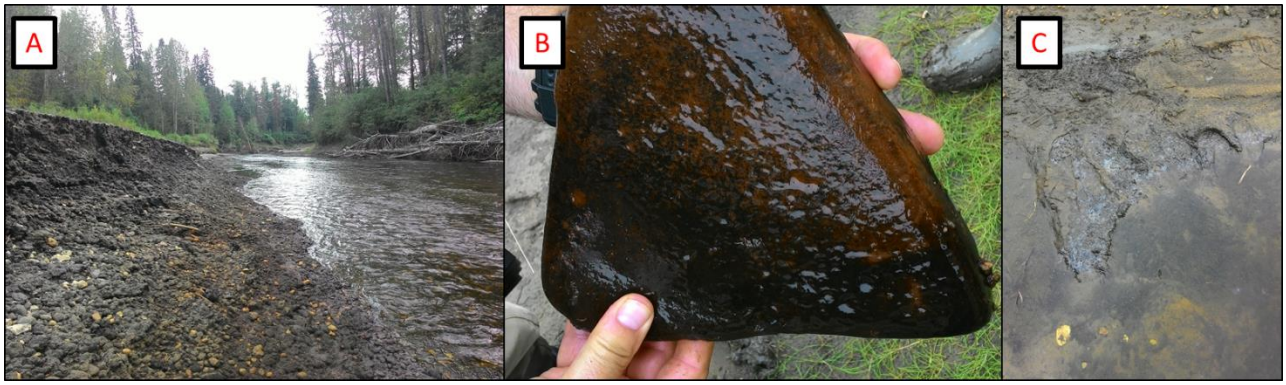

Figure 6.1 – Pictures from the Ells river in Alberta, Canada, (A) cutting into oil sands deposits, exposed to an input of natural bitumen; (B) stone collected from the bottom of the river, covered by sticky bitumen and (C) visible oil sheens in the interface between the sands and the river.

6.3.2- Macroinvertebrates and leaf collection

Alder leaves were previously collected from riparian vegetation at São Pedro de Alva, Portugal (40°16'38.8"N, 8°11'52.8"W) in the autumn of 2017. After collection, leaves were left to air dry and stored in the darkness at room temperature.

Previous to invertebrate sampling in Mau River (Sever do Vouga, Portugal), the macroinvertebrate composition of the river was determined (data not shown) using a Surber sampler to obtain four representative quantitative samples. Benthic invertebrates collected were preserved in 70% ethanol and were identified to genus level. The four samples provided information on the natural densities of that stream, and the abundance of the different genus was used to define the distribution of the different taxa among the artificial test streams.

The macroinvertebrates for inoculation in the artificial streams were then collected by kick sampling in riffle habitats in the same river (-40°44'21.2"N, 8°24'6.8"WW), a considered pristine river with no history of contamination, also presenting an excellent ecological quality status (Rodrigues et al., 2018; Vidal et al., 2014). Macroinvertebrates were transported to the laboratory in river water, were sorted by taxa and inoculated to artificial streams according to their composition in the natural river previously determined. In terms of abundance, 53.8% of the total functional feeding groups were fine sediment collectors/filters (*Hydropsyche* sp., *Ephemera* sp., *Chironomus* sp., and Oligochaeta), followed by shredders with 34.5% (*Sericostoma* sp., *Perla* sp. and *Calamoceras* sp.), grazers with 7.81% (Baetidae, Heptageniidae, and Leutridae), and the less abundant group were the predators representing a total of 3.9% (*Boyeria* sp., *Onychogomphus* sp., *Calopteryx* sp., and Athericidae). The total number of each species/family is described in Table 6.1.

Table 6.1 – Number of organisms of each species/family used at the beginning of the mesocosms experiment, per artificial stream. Macroinvertebrate composition of the river was previously determined and used in the present experiment.

Species/family	Number of organisms per river
***Hydropsyche* sp.**	9
***Ephemera* sp.**	54
***Chironomus* sp.**	100
Oligochaeta	2
***Sericostoma* sp.**	71
***Perla* sp.**	2
***Calamoceras* sp.**	33
Baetidae	14
Heptageniidae	3
Leutridae	7
***Boyeria* sp.**	2
***Onychogomphus* sp.**	2
***Calopteryx* sp.**	2
Athericidae	6
TOTAL	307

6.3.3 – Mesocosms experimental design

To test the effects of natural weathered bitumen when attached to sediment on the aquatic biota, experiments were conducted in an indoor modular mesocosms system for 7 days using 9 individual streams with 2 m length, 20 cm width, 22.5 cm depth each (Figure 6.2-A and 6.2-B). Three independent treatments were tested, each one with 3 replicates (streams): 10 and 20% of ELLs natural bitumen sample mixed in sediment and a negative control treatment (streams with clean sediment). Each stream contained approximately 280 L of Artificial Pond Water (APW) enriched with phosphate and nitrate to simulate the natural composition of Mau River. For each stream, 7 Kg of inorganic fine sediment (<1 mm, previously burned at 500 °C during 4 h), 3 leaf packs (10 mm mesh size) containing approximately 1 g of *Alnus glutinosa* leaves and 5 unglazed ceramic tiles (20 cm^2) were also used in each stream (Figure 6.2-C). Leaf packs were previously conditioned for 15 days using Mau River water in the laboratory at 15º C, while ceramic tiles were left in Mau River for two weeks before the experiment to allow

biofilm colonization. Artificial stream flow was maintained at a constant rate of 4 L/min.

At day 0 of the experiment, all artificial streams contained APW enriched medium, sediment (contaminated and non-contaminated with natural bitumen), leaf packs and ceramic tiles colonized with biofilm. Macroinvertebrates were sorted and distributed per stream randomly and according to the abundances described in section 6.3.2. Water physic-chemical parameters were measured every other day during the 7 days of exposure.

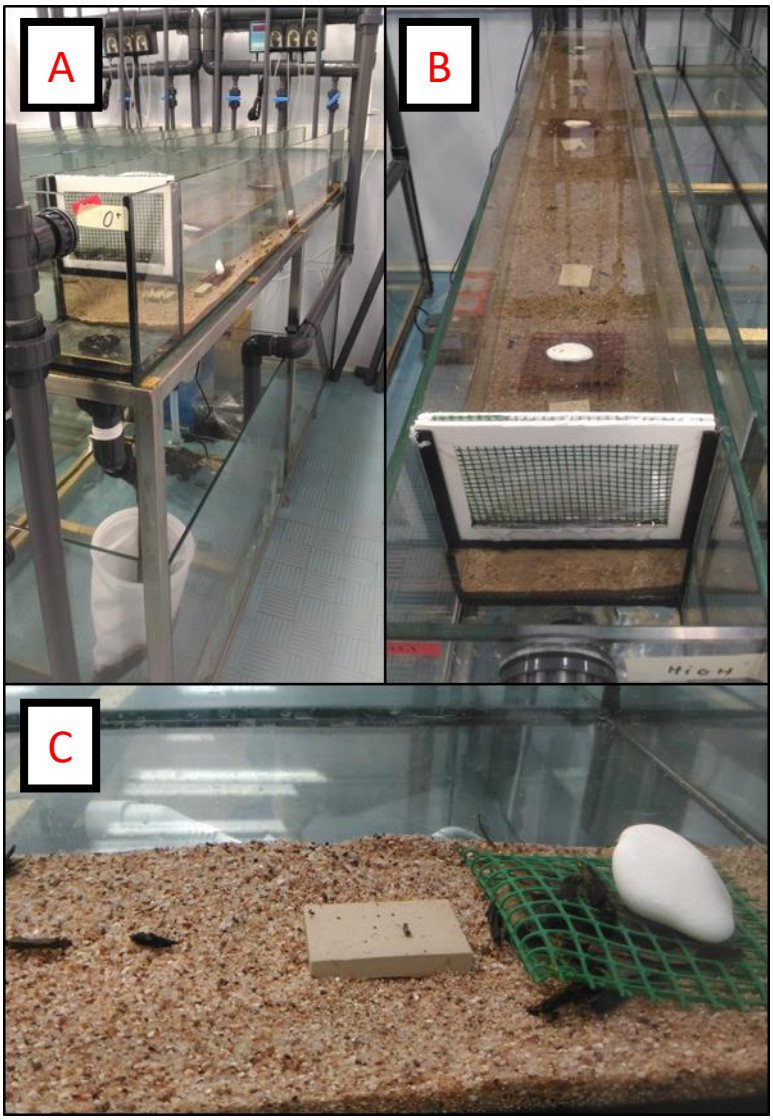

Figure 6.2 – Mesocosms facility at the Department of Biology, University of Aveiro, Portugal. (A) and (B) perspectives from an artificial stream; (C) leaf packs and unglazed ceramic tiles on the top sediment.

At the end of the test:

1) Leaf packs were carefully removed rinsed and cleaned with a soft brush (organism attached were collected). Leaves were then dried at 50°C during 48h and weighted to check the leaves decomposition through the weight loss of leaves.
2) Ceramic tiles were scrubbed with a soft brush and rinsed in water. The extract was then filtered with GF/C filters (1.2 µm). Filters were stored in falcon tubes protected from light at -20°C until analysis. Chlorophyll *a* analysis was carried out using 90% acetone solution followed by centrifugation (3000 rpm / 10 minutes / 4°C) and Chlorophyll *a* content measured spectrophotometrically, following Jeffrey and Humphrey (1975) to measure primary production in the different treatments.
3) Macroinvertebrate community was hand collected using soft tip tweezers and by sediment sieving. The total amount of organisms was then added to the ones found attached to leaf packs. All organisms (divided by replicate stream were then preserved in 70% of ethanol and identified under a stereomicroscope (MS5, Leica Microsystems, Houston, USA). The community structure was assessed by calculating the species richness (number of different macroinvertebrate families collected), total macroinvertebrates abundance and percentage of EPTs (Ephemeroptera, Plecoptera, and Trichoptera) after 7 days in comparison compared to the initial EPT. Also, the abundance of each one of the functional feeding groups (fine sediment collectors/filters, shredders, grazers, and predators) was also reported.

6.3.4 – Chemical analysis

ELLs Natural bitumen sample was analyzed for metals and Naphthenic Acids (NAs) (InnoTech Alberta, Canada) and polycyclic aromatic hydrocarbons (PAHs) (AXYS Analytical Services Ltd, Canada). NAs aqueous solutions were analyzed using HPLC-Orbitrap-MS in water samples, previously adjusted to pH≈2, spiked

with international (Dodecanoic acid-d23) and extracted by automated solid phase extraction. Compound characterization and quantification were performed using liquid chromatography coupled to Orbitrap mass spectrometer. NAs were analyzed in solid bitumen samples with a previous liquid/liquid extraction with an alkaline solution before analysis using HPLC-Orbitrap-MS. Metals were analyzed by Inductively Coupled Plasma Mass Spectrometry (ICP-MS) (Elan DRC-II with ESI SC-8XC high throughput FAST autosampler). Before analysis, digestion of samples was performed using an Ethos UP with the Maxi44 rotor (Milestone Inc). PAHs were analyzed by gas chromatography-mass spectrometry (GC-MS), through the AXYS Method MLA-021.

6.3.5 – Statistical Analysis

All data were checked for normality and homoscedasticity using the Shapiro-Wilk test and the Levene's equal variance test, respectively. Leaf weight loss after 7 days of exposure, primary production, species richness, total macroinvertebrates abundance and percentage of EPTs (compared to the initial EPTs organisms) and the abundance of each functional feeding groups in bitumen treatments were compared with control treatment through a one-way ANOVA followed by Dunnett's *post-hoc* tests. Analysis were performed using SigmaPlot 12.5 software.

Differences in benthic communities induced by the presence of different levels of bitumen in sediments were evaluated using permutational multivariate analysis of variance (PERMANOVA), conducted on a Bray-Curtis dissimilarity matrix. The dissimilarity between communities exposed to the different treatments was visualized by a non-metric multidimensional scaling ordination (nMDS), using a Bray-Curtis dissimilarity (Clarke and Ainsworth, 1993). Similarity percentages analysis (SIMPER) was used to determine the contribution of individual taxa to the average Bray-Curtis dissimilarity between treatments and exposure period. All the multivariate analysis were performed using R 3.4.4 software, vegan package (Oksasen et al., 2018).

6.4 – Results

After 7 days of exposure, primary production was not affected by the presence of natural bitumen in the sediment, with no significant differences observed on chlorophyll *a* concentration between control and the different bitumen treatments (ANOVA, p=0.105) (Figure 6.3-A). Similarly, no significant difference in the leaf mass loss was observed between bitumen treatments and controls (ANOVA, p= 0.625) (Figure 6.3-B).

Concerning macroinvertebrates' community, after the 7 days of exposure, species richness was not affected by the presence of riverine natural bitumen (ANOVA, p= 0.206) (Figure 6.4-A). However, total macroinvertebrates abundance was decreased by the presence of natural bitumen in sediment, for both 10% and 20% of bitumen in sediment, comparing to the control abundance (ANOVA, p< 0.05) (Figure 6.4-B). Also, the percentage of EPTs abundance (Ephemeroptera, Plecoptera, and Trichoptera) in both treatments with bitumen was significantly lower than in control treatments (ANOVA, p< 0.05) (Figure 6.4-C).

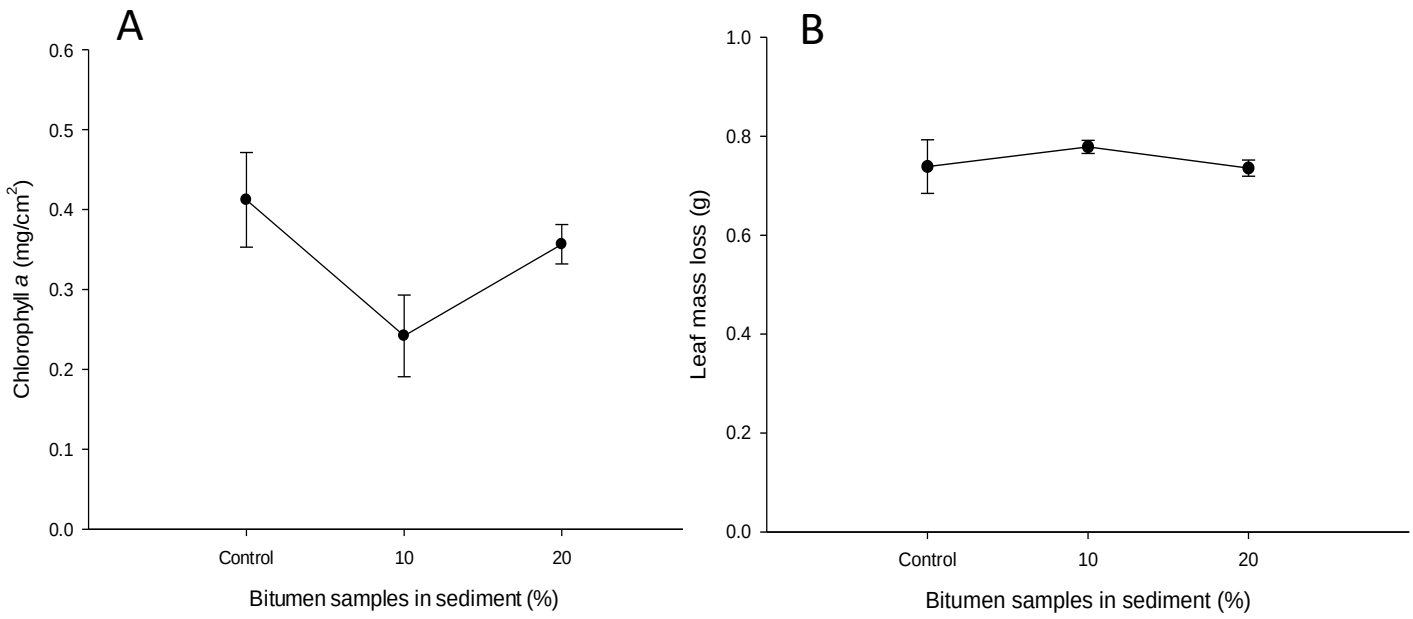

Figure 6.3 – Chlorophyll *a* (A) and leaf weight loss (B) in artificial streams with clean sediment (control) and two percentages of natural bitumen mixed in sediment (10% and 20%). All data are presented as mean ± SE.

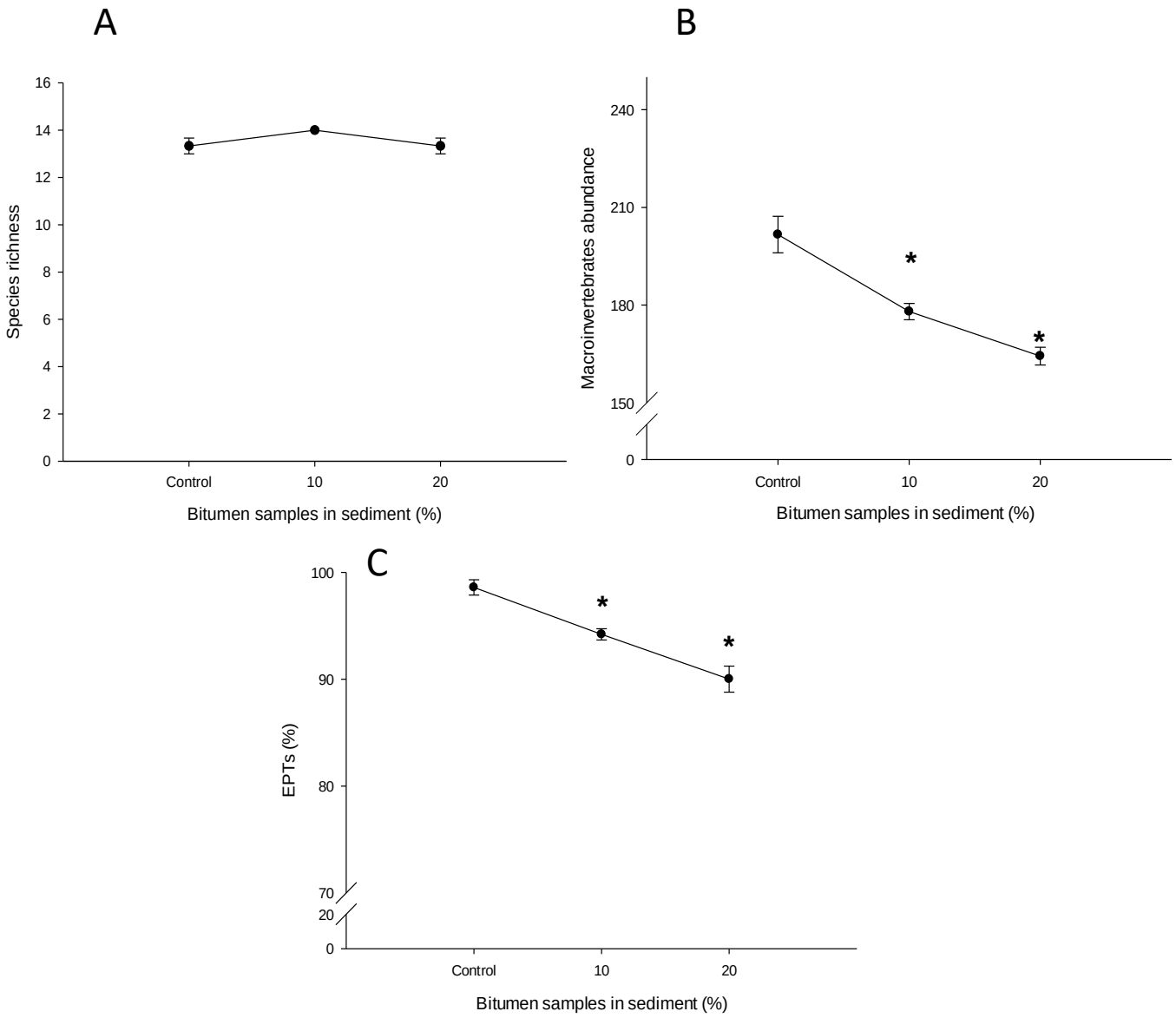

Figure 6.4 – Species richness (A), macroinvertebrates abundance (B) and EPT (Ephemeroptera, Plecoptera, and Trichoptera) in artificial streams with clean sediment (control) and two natural bitumen treatments (10 and 20% of bitumen). EPT is expressed as % of the initial community. * Denotes a significant difference compared to the control, Control (Dunnett's test, $p < 0.05$).

The abundance of shredders, grazers, and predators in bitumen treatments did not statistically differ from control (ANOVA, $p > 0.05$). In turn, the abundance of fine sediment collectors/filters was drastically reduced in both treatments compared to control (ANOVA, $p < 0.05$).

PERMANOVA (p< 0.05), identified differences in the community structure when comparing control and the 20% bitumen treatment (PERMANOVA, p=0.001) (Figure 6.5). According to the SIMPER analysis, 68% of those differences were mainly due to the decrease in the number of *Chironomus* sp. and *Ephemeroptera* sp..

During exposure, mainly when the highest natural bitumen concentration was present in sediment, fine particles of bitumen were visible in the interface between sediment and water, forming a greyish fine sediment layer, observed in Figure 6.6-A and 6.6-B.

Natural bitumen sample was analyzed regarding its composition, and the values for the different assessed metals are presented in Table 6.1. Different classes of PAHs were also assessed, and their concentrations are presented in Table 6.2. The sample collected from the banks of the Ells river had a concentration of 154 (µg/g) of total NAs.

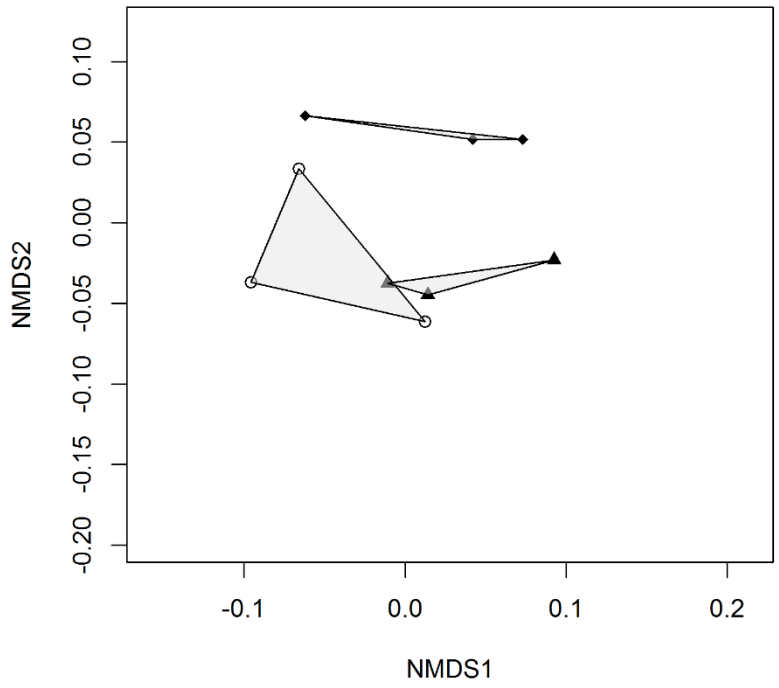

Figure 6.5 - Two-dimensions NMDS plots of the macroinvertebrate communities based on the Bray-Curtis dissimilarity matrix. Circles represent control communities, triangles represent communities exposed to low percentage of bitumen (10%) and diamonds represent communities exposed to high percentage of bitumen (20%).

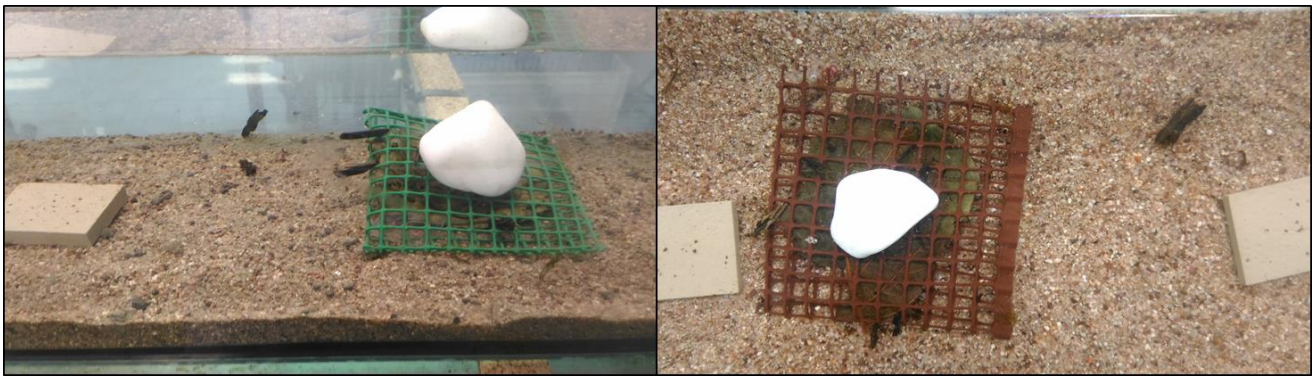

Figure 6.6 – Detailed view of A) artificial stream with 20% of bitumen in the sediment, presenting a grey layer of bitumen fine sediment, comparing with B) with clean sediment without bitumen.

Table 6.2 - Metal content in natural bitumen sample from ELLs river, Alberta, Canada, respectively. The analysis was carried out by Elan DRC-II ICPMS.

	ELLs bitumen (µg/g)
	Total
Aluminum	38400
Antimony	0.968
Arsenic	3.02
Barium	107
Beryllium	1.39
Bismuth	0.130
Boron	70.2
Cadmium	0.091
Calcium	2000
Chloride	356
Chromium	27.7
Cobalt	13.2
Copper	11.2
Iron	16300
Lead	11.7
Lithium	90.0
Manganese	677
Molybdenum	2.19
Nickel	36.7
Selenium	0.57
Silver	0.315
Strontium	74.9
Thallium	0.238
Thorium	7.27
Tin	1.10
Titanium	1800
Uranium	1.16
Vanadium	66.9
Zinc	32.6

Table 6.3 - PAHs content in natural bitumen sample from ELLs river, analyzed by gas chromatography-mass spectrometry (GC-MS). <D.L. – above the detection limited.

	ELLs bitumen (ng/g)
Naphthalene	93.9
Acenaphthylene	<D.L.
Acenaphthene	<D.L.
2-Methylfluorene	<D.L.
C2 Phenanthrenes/Anthracenes	2070
Fluorene	<D.L.
Phenanthrene	<D.L.
Anthracene	<D.L.
C1 Phenanthrenes/Anthracenes	<D.L.
Fluoranthene	166
Pyrene	752
Benz[a]anthracene	<D.L.
Chrysene	1330
Benzo[b]fluoranthene	356
Benzo[j,k]fluoranthenes	<D.L.
Benzo[e]pyrene	721
Benzo[a]pyrene	205
Perylene	488
Dibenz[a,h]anthracene	95.9
Indeno[1,2,3-cd]pyrene	101
Benzo[ghi]perylene	275
2-Methylnaphthalene	90.1
1-Methylnaphthalene	57.3
C1-Naphthalenes	90.1
Biphenyl	43.8
C1-Biphenyls	76.3
C2-Biphenyls	<D.L.
C2-Naphthalenes	<D.L.
1,2-Dimethylnaphthalene	<D.L.
2,6-Dimethylnaphthalene	<D.L.
C3-Naphthalenes	<D.L.
2,3,6-Trimethylnaphthalene	<D.L.
2,3,5-Trimethylnaphthalene	<D.L.
C4-Naphthalenes	1010
C1-Acenaphthenes	<D.L.
C1-Fluorenes	134
1,7-Dimethylfluorene	<D.L.
C2-Fluorenes	518
C3-Fluorenes	3250
Dibenzothiophene	<D.L.
C1-Dibenzothiophenes	<D.L.
2/3-Methyldibenzothiophenes	512
C2-Dibenzothiophenes	3090
2,4-Dimethyldibenzothiophene	<D.L.
C3-Dibenzothiophenes	13900
C4-Dibenzothiophenes	24700

	ELLs bitumen (ng/g)
3-Methylphenanthrene	<D.L.
2-Methylphenanthrene	<D.L.
2-Methylanthracene	<D.L.
9/4-Methylphenanthrene	<D.L.
1-Methylphenanthrene	<D.L.
3,6-Dimethylphenanthrene	173
2,6-Dimethylphenanthrene	144
1,7-Dimethylphenanthrene	193
1,8-Dimethylphenanthrene	<D.L.
C3-Phenanthrenes/Anthracenes	8420
1,2,6-Trimethylphenanthrene	<D.L.
Retene	<D.L.
C4-Phenanthrenes/Anthracenes	28200
C1-Fluoranthenes/Pyrenes	7390
3-Methylfluoranthene/Benzo[a]fluorene	1310
C2-Fluoranthenes/Pyrenes	19400
C3-Fluoranthenes/Pyrenes	20700
C4-Fluoranthenes/Pyrenes	7650
C1-Benzo[a]anthracenes/Chrysenes	4230
5/6-Methylchrysene	660
1-Methylchrysene	187
C2-Benzo[a]anthracenes/Chrysenes	7470
5,9-Dimethylchrysene	1600
C3-Benzo[a]anthracenes/Chrysenes	4950
C4-Benzo[a]anthracenes/Chrysenes	758
C1-Benzofluoranthenes/Benzopyrenes	2970
7-Methylbenzo[a]pyrene	307
C2-Benzofluoranthenes/Benzopyrenes	2100
1,4,6,7-Tetramethylnaphthalene	193

6.5 – Discussion

The adverse effects previously reported in the other Chapters of this thesis were validated by the observed effects at a higher tier level, on the macroinvertebrates community structure, when Ells river bitumen was applied to sediment. Natural bitumen collected in the banks of the Ells River was chosen among other bitumen natural samples given its toxicity towards *C. riparius* (Chapter 5), with effects including reductions in larvae growth, reduction in the cumulative emergence and imagoes weight. Also, in Chapter 2, 3 and 4 of the present thesis, when ecotoxicological assays with bitumen elutriates were conducted, deleterious effects on daphnids, snails, planarians, and bacteria were observed.

The erosion of bitumen from river banks and its entrance into aquatic systems is a topic of concern in these Athabasca oil sands. Droppo et al., (2018) proved that polycyclic aromatic compounds (PACs) associated with suspended sediment transported in the Athabasca River and local tributaries are mainly from natural sources, resulting from the erosion of the river banks. Consequently, incorporation of natural eroded bitumen into sediments is expected to occur due to physical and biological (e.g., bioturbation) processes that will eventually lead to some negative impacts in these systems (Yergeau et al., 2013). Having this in mind, ecotoxicological information regarding this scenarios is crucial to perceive risks associated with natural contaminants and to discriminate them from anthropogenic pressure.

In the present study, an excellent compromise between the ecosystems complexity and the highly artificial settings of laboratory experiments was carried out in mesocosms experiments using a natural macroinvertebrate community along with functional bioassays. Primary production, which is a fundamental process within freshwater ecosystems (Peters et al., 2013), was not affected by bitumen exposure. Besides the influence of nutrients and availability of light, primary production is dependent on the environmental media (in this case the presence of a stressor) and the density and feeding behavior of grazers that feed on the producers (Abelho et al., 2016; Mckie and Malmqvist, 2009). In the present study, biofilm was exposed to bitumen washing from the sediment, which may not have been enough to induce adverse effects. Also, grazers were not affected and therefore their pressure was similar between treatments.

Similarly, leaf litter degradation was not affected, which is in accordance with the no effects observed on shredders abundance, the group majorly responsible for this decomposition related function. This may be a question of (low) chemical availability or (short) time of exposure, considering the percentages of bitumen used, or the 7-day exposure period. Our results, given the no effects observed for these two functional measures also suggest the absence of significant sub-lethal effects within grazers and shredders (i.e., toxic anorexia).

On the other hand, this 7 day period was enough to induce changes in the EPT abundance, which is considered a highly sensitive endpoint in freshwater quality monitoring trials. As it is known, small physical or chemical changes induced by contamination of streams may lead to a reduction in the abundance of EPTs (Compin and Céréghino, 2003; Hynes, 1959; Wallace et al., 1996). Besides the decrease in abundance of *Ephemera* sp. (within the EPT), the effects on the community structure were also driven by the reduction in the abundance of *Chironomus* sp.. This reduction occurred mainly in the treatment with the highest bitumen content where the fine suspended material (fine sediment) was visible in the water column during the 7 days of exposure (Figure 6-A). Effects were then observed in organisms that ingest fine sediment (e.g., *Chironomus* sp.) and on filter feeders (e.g., *Ephemera* sp.), by affecting their gills and filaments with small bitumen particles, with consequent effects on their normal physiological functions. Since these organisms were the dominant in the collected natural community (Table 6.1), their abundance reduction led to a statistical effect on the exposed community to bitumen treatments. Effects on *Chironomus* sp. could be primarily attributed to the ingestion and direct contact with small bitumen particles incorporated in the sediment that proved to be very toxic to the aquatic biota due to the presence of bitumen and related contaminants (metals, NAs and PAHs) (Chapter III; Gerner et al., 2017). Since *Ephemera* sp. presented the highest gill area from the tested organisms, the adherence of particles to the gill surface may result from mucus secretion as a response to physical irritation, which could impair their life traits (Lemly, 1982).

Although we have not tested the benthic macroinvertebrate communities of the Ells Rriver with the respective natural conditions where are included different climate regimes, this study is a step further to evaluate how natural contamination in Oils Sands rivers affect natural benthic macroinvertebrate communities and ecosystem functioning. Also, our results are in line with the findings previously reported in the literature regarding the way natural bitumen affected natural biota. Barton and Wallace (1979) compared the performance of macroinvertebrate communities in two areas of the Steepbank river: one that cuts through the oil sands deposits, and an upstream part of the river with no influence of natural

bitumen. A reduction in the abundance of organisms was primarily attributed to the physical alteration of the substrate and the presence of particles of oil sands in suspension that physically affected Plecoptera and Trichoptera by direct contact and by ingestion, which is precisely what occurred in the present study.

In the study of Lemly (1982), indirect effects of streambeds sedimentation pinpointed as a disruptor of feeding habits of invertebrates, by inducing general habitat destruction, especially for filter feeding taxa. Also, the same study stated that decomposition of compounds associated with materials in the bed load might depress pH and eliminate acid-sensitive species of Plecoptera and Ephemeroptera. Later, Rabení et al. (2005) reported that different feeding groups were sensitive to deposited sediment, with a significant decrease in all the feeding groups' densities and taxa richness (apart from shredders) with increasing deposited sediment. Larsen et al. (2011) confirmed that even low to moderate amounts of fine sediment on streambed could alter the richness, abundance, community composition, trait diversity and trait composition of benthic invertebrates. All these studies revealed that physical alterations of the substrate could impair the life traits of natural communities, especially the filter communities.

In summary, the present study highlights the possible effects induced by natural exposure to bitumen in the oil sands area, demonstrated that natural communities might be affected by the presence of natural eroded/weathered bitumen in sediment, even at low percentages. Effects were mainly due the presence of this fine bitumen particles that reduced the survival of filter feeders and collectors through ingestion or adsorption to gills. The absence of effects in functional parameters were also a result to highlight, with no effects on primary production and leaf consumption during the mesocosms experiment. Despite the possible limitations of laboratory and mesocosms experiments, our results validated laboratory single species tests and are validated by field studies conducted in the local rivers located in these oil sands area.

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Chapter 7: General discussion and conclusion

7 - General discussion and conclusions

This thesis is a step forward on the understanding of the ecotoxicological effects of natural eroded bitumen to aquatic biota. The ecotoxicological data obtained in the present study, in combination with already published studies related to the effects of bitumen deposits in aquatic biota (Barton and Wallace, 1979; Colavecchia et al., 2004; Colavecchia et al., 2006; Colavecchia et al., 2007; Tetreault et al., 2003a; Tetreault et al., 2003b), adds valuable data for future risk assessment studies regarding the ecological effects of natural contamination in Oil sands area.

It is known that the increased levels of contaminants (such as PAHs, NAs, and metals) in local rivers is mainly due to the direct contact between bituminous riverbanks and rivers (Kelly et al., 2010; Kelly et al., 2009), but to the best of our knowledge, it is not reported the ecotoxicological effects of bitumen into aquatic invertebrates.

The following highlights were derived from the present study:

- **Contaminants from natural bitumen may be bioavailable to freshwater organisms living in the water column**

The methodology used in the present thesis to obtain elutriates from bitumen samples showed that contaminants, at least partially, passed from the solid phase to an aqueous phase, corroborated by the chemical analyses and the observed toxicity of elutriates to the tested organisms. Elutriates generated from solid samples were the more suitable option found to perform a more realistic possible liquid extract from bitumen samples. Since this work aimed to understand the direct effects of bitumen input into streams, elutriates provided reliable information about the dissolution of bitumen into freshwaters. An interesting observation is reported in Chapter 2, where 3 extraction cycles simulated the continuous washing/weathering of bitumen into rivers. Interestingly, the 3rd cycle of extraction provided the most toxic elutriate to daphnids when compared to the 1st and 2nd

cycle of extraction. This result is of the most importance since bitumen will face the normal washing processes in rivers, and the high toxicity found with the increased washing time could indicate that the initial toxicity of bitumen could be underestimated when compared with the toxicity after releasing another type of contaminants.

- **Heterogeneity in toxicity was found through the different bitumen samples and respective elutriates, even when samples were collected in the same river**

One of the significant findings of the present study is related to the heterogeneity of toxicity and presence of contaminants regarding the different samples collected in the banks of local rivers. In Chapter 2, three samples were collected distancing 5 meters of each other. It was reported that one of the samples could be highlighted as “the most toxic”, with a substantial effect on the reproductive capacity of daphnids. In Chapter 3, this heterogeneity in toxicity was confirmed. Daphnids, snails, and bacteria were strongly affected by elutriates generated from the bitumen sample collected in the banks of the Ells river (ELLs sample), compared with samples collected in two sites on the Steepbank river (SP and STB samples) and one in the Athabasca River (ATB sample). In Chapter 4, *Dugesia tigrina* was only exposed to ATB and ELLs elutriates and planarians were acutely affected by ELLs elutriates with high mortality, a decrease in locomotion and regeneration capacity. In Chapter 5, *Chironomus riparius* were used as test species evaluating the effects of contaminated sediment vs contaminated liquid medium (using elutriates). Again, the pattern of toxicity was the same, with reduced larval size, increased time to emergence, and decrease in the total emergence of chironomids when solid bitumen material collected in the banks of the Ells River was present.

- **Bitumen was toxic to the exposed organisms in both solid form (as collected in the banks of rivers) and when liquid extracts were performed (in the form of elutriates). Chironomids were only affected by bitumen particles in sediment indicating different routes of exposure of contaminants is the key factor in the toxicity mediated effects.**

When bitumen enters in freshwaters, it is expected that parts of the eroded bitumen are immediately dissolved into the water. The remaining natural eroded bitumen will settle on the top sediment of the river and eventually the fine-grained portion is transported through the river to downstream areas (Droppo et al., 2018). In the present thesis, both elutriates and solid bitumen in sediments induced toxicity to the exposed organisms. In Chapter 5, the effects of elutriates and bitumen exposure were assessed, using *Chironomus riparius* as a model species. Contrary to the results obtained in chapter 2, 3 and 4, *C. riparius* were not affected when exposed through oil sands elutriates. However, a strong effect was observed when *C. riparius* was exposed to Ells bitumen sample in sediment, impairing their larval growth, emergence and imagoes size. This result was later validated in chapter 6, where macroinvertebrate community structure was affected mainly by the reduction in the abundance of *Chironomus* sp. and *Ephemera* sp., when exposed to the same bitumen material on a mesocosms experiment.

On an integrated analysis, our results demonstrated that toxicity will change from organism to organism, depending essentially from the main route of exposure. Since *C. riparius* is mainly exposed to bitumen through surface contact or even by bitumen ingestion, it is expected an increased toxicity (with consequent effects) when bitumen is attached to sediment, comparing with an exposure to elutriates.

The results suggested that bioavailability of contaminants is the key factor that induces the toxic effects to the organisms, that is directly connected to the route of exposure of each organism.

- **Considering the two types of exposure assessed in the present study, *Daphnia magna* was the most sensitive species to elutriates' exposure and *Chirnomus* sp. and *Ephemera* sp. to bitumen in sediment.**

Daphnids were the most sensitive organisms when in the presence of oil sands elutriates, followed by the bacteria *Vibrio fischeri* and the snail *Physa acuta*, by comparing the EC_{50}s and LC_{50}s. Lari et al. (2017) demonstrated that chronic exposure to OSPW affected growth and fitness of *D. magna* that probably resulted from a reduction in energy intake inducing a depletion in their energy reserves. The same study reported that OSPW reduced the number and the size of their offspring and it is suggested that effects on the reproductive performance of *D. magna* are mediated by the toxicity of its chemical components. Since no studies reported the effects of natural bitumen on daphnids, the study of the effects of OSPW (originated from the separation of bitumen from sands) is the closest that we can compare regarding the effects of oil sands on daphnids and the similarity of the constituent chemicals.

Regarding the effects of solid bitumen samples, the macroinvertebrate community was affected mainly by the reduction in abundance of *Chironomus* sp. and *Ephemera* sp., after 7 days on a mesocosm experiment with natural communities and bitumen material in sediment. Effects on *Chironomus* sp. could be primarily attributed to the ingestion and direct contact with small bitumen particles incorporated in the sediment, which is in accordance with the findings reported in chapter 5 (Chapter 5; Gerner et al., 2017). It is also hypothesised that effects of oil sands suspended particles to *Ephemera* sp. could be related with the attachment of Oil sands material on gills and filaments of the filter feeders, impairing their normal physiological functions and reducing their abundance when bitumen was in sediment.

- **Ecotoxicological results are in accordance with chemical analysis results, with severe effects on exposures when bitumen samples had higher concentrations of PAHs and NAs – SP and ATB were the less toxic and STB and ELLs the most toxic samples.**

These strong effects on the aquatic organisms are in accordance with the results from the chemical analysis. As mentioned in the thesis, the presence of metals, PAHs, and NAs could mediate the toxicity in aquatic organisms exposed to bitumen samples (or elutriates). The higher content in PAHs and NAs apparently was the main factor that led to the higher toxicity observed in the ELLs elutriates. With the ecotoxicological and chemical data, it is clear that SP and ATB were the lest toxic samples and STB and especially ELLs samples were the most toxic samples to the studied organisms. This pattern of toxicity follows the increase of NAs content in the 4 elutriates as following: SP<ATB<STB<ELLs, with the ELLs elutriate having 200, 8 and 1.7 times more NAs content than SP, ATB, and STB elutriates, respectively. NAs were previously reported in the literature as the most contributing contaminants to the toxicity observed in the aquatic biota, using OSPWs as test medium (Morandi et al. 2015).

- **The importance of discriminating between natural and anthropogenic sources of contamination, and future studies for improving an accurate risk assessment in canadian Oil Sands.**

The present study reinforces the need to perform more studies related to the effects of natural bitumen input into Oil sands regional rivers. Only a joint approach where chemical analysis, information about changes in biodiversity in local rivers, and the ecotoxicological data can provide a reliable and complete analysis of the real effects that natural sources of contamination can pose to regional aquatic systems. The present study adds valuable information to the scientific community, concluding that natural bitumen samples will induce negative effects on key aquatic organisms. On the other hand, factors such as high seasonal variability of

flow and dilution capacity of bitumen in rivers may change these effects and should be considered in the future.

Taking into consideration all the mentioned before, it is possible to redefine the background toxicity in different rivers in Canadian oil sands. The heterogeneity in toxicity is one of the main problems regarding this redefinition of background toxicity, due to the difficulties in transposing the effects of bitumen from different areas of the same river. Chemical, ecological and ecotoxicological data should be provided in several areas of rivers on a more holistic perspective regarding this problem.

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