

In-situ Caged Wood Frog (*Rana sylvatica*) Survival and
Development in Wetlands Formed from Oil Sands Process-
Affected Materials (OSPM)

**A Thesis Submitted to the College of
Graduate Studies and Research
in Partial Fulfillment of the Requirements
for the Degree of Master of Science
in the Toxicology Graduate Program
University of Saskatchewan
Saskatoon, Saskatchewan
Canada**

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PREFACE

This thesis has been organized as a series of manuscripts that will be submitted for publication in scientific journals. Some repetition of introductory and methodological material was unavoidable.

ABSTRACT

Currently there are three companies producing bitumen from the Athabasca Oil Sands Region located near Fort McMurray, Alberta, Canada. Extraction of bitumen produces solid (sand) and liquid (water with suspended fine particles) tailings material, called oil sands process-affected materials (OSPM). These waste materials are stored on site due to a “zero discharge” policy and must be reclaimed when operations end. The OSPM is known to contain naphthenic acids (NAs) and polycyclic aromatic hydrocarbons (PAHs) and has high pH and salinity. A possible method of reclamation is the “wet landscape” approach, which involves using OSPM to form wetlands that would mimic natural wetland ecological functioning. This study investigated the effects of wetlands formed with OSPM on wood frog larvae (*Rana sylvatica*), using endpoints including survival, growth, time to metamorphosis, hormonal status, and detoxification enzyme induction [ethoxyresorufin-*o*-dealkylase (EROD) activity].

In-situ caging studies were completed in 2006 and 2007. Four wetlands were studied in 2006 and 14 wetlands were studied in 2007. The 2006 season saw a host of problems that were resolved for the 2007 season. In 2006, tadpole survival did not differ among reference wetlands and old OSPM-affected wetlands but there was 100% mortality of tadpoles in the young OSPM-affected sites that contain the highest concentrations of toxic components. Results were similar in 2007, with tadpoles raised in young OSPM-affected wetlands having 41.5%, 62.6%, and 54.7% higher tadpole mortality than old OSPM-affected, young reference, and old reference wetlands, respectively. In 2007, tadpoles from young OSPM-affected sites had delayed metamorphosis (12 days longer than tadpoles from old reference wetlands and 18 days longer than tadpoles in old OSPM-affected wetlands). The thyroid hormone ratios of tadpoles in young OSPM-affected wetlands were between 25% and 42% lower than tadpoles in all other wetlands groups. The EROD activity of tadpoles in young OSPM-affected wetlands was an average 223% higher than those in old OSPM-affected wetlands, showing us that tadpoles were responding to higher levels of contaminants in young OSPM-affected wetlands. Size differences were only noted in 2007, most likely not as a result of exposure to OSPM, but due to differences in population density. The results of this study lead us to believe that toxicity due to OSPM decreases as wetlands get older and OSPM-affected wetlands could support native amphibian populations if they are

allowed to mature. Since we considered wetlands to be old if they were seven years or older and the fact that old-OSPM wetlands showed effects on tadpoles similar to those of reference wetlands and showed much less toxicity than young OSPM-containing wetlands, we believe wetlands that are at least seven years old would sustain amphibian life.

ACKNOWLEDGEMENTS

First, I would like to thank my supervisor, Dr. Judit Smits, for her guidance and encouragement. I am also greatly appreciative of my other committee members Drs. Mark Wickstrom and Barry Blakley, who were always willing to share their expertise. As well I would like to thank my external examiner Dr. Peter Flood for his time and knowledge. Funding for this research was generously provided by the Natural Science and Engineering Research Council of Canada as well as our industry partners, Syncrude Canada Ltd., Suncor Energy Inc., Albian Sands Energy Inc., Canadian Natural Resources Ltd., Imperial Oil Ltd., Petro-Canada and Total E and P Ltd.

This research could not have been complete without the help of many people in the field and in the lab, as well as the support of students in the Toxicology Centre. Field work was made much easier by field assistants Hussein Keshwani and Keegan Hicks. Other researchers from the Universities of Alberta, Windsor and Guelph, also working with the Carbon Dynamics, Food Web Structure, and Reclamation Strategies in Athabasca oil sands wetlands (CFRAW) project, made months of living and working in Fort McMurray a blast. Technical support from members of our partner companies was also greatly appreciated. Most notably, Wayne Tedder and Christine Daly of Suncor Energy Inc. and Clara Qualizza, Terry Van Meer, Chris Beierling, Nadia Loubiri, and Lori Cyprien from Syncrude Canada Ltd. Sukhbir Nain, Mandy Olsgard, Niti Gupta, and Brenda Trask, must all be credited for their assistance in the lab.

I would also like to thank Lynnette Dagenais who always had time to help me, as well as my friends and family who were of constant support.

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LIST OF ABBREVIATIONS

7-ER	7-ethoxyresorufin
AOS	Athabasca Oil Sands
AEUB	Alberta Energy and Utility Board
ANOVA	Analysis of Variance
CT	Consolidated tailings
d	Days
EIA	Enzyme immunoassay
EOR	Enhanced oil recovery
EROD	7-ethoxyresorufin- <i>o</i> -dealkylase
HPT	Hypothalamic-pituitary-thyroid axis
KS	Kolmogorov-Smirnov test
MFT	Mature fine tailings
NADPH	Reduced nicotinamide adenine dinucleotide phosphate
NAs	Naphthenic acids
NaOH	Sodium hydroxide
NW	Natural Wetland
OSPM	Oil sand process-affected materials
PCBs	Polychlorinated biphenyls
<i>R. sylvatica</i>	<i>Rana sylvatica</i>
SSC	Sweet synthetic crude
SWSS	South West Sands Storage area
T3	Triiodothyronine
T4	Thyroxine
THs	Thyroid hormones
TP	Test pond

CHAPTER 1: LITERATURE REVIEW

1.1 Oil Sands History and Background

Bituminous sands, more commonly known as oil sands, are heavy oil (bitumen) packed between sand particles that are associated with silt, clay, and water (FTFC, 1995). Oil sands deposits are found in many areas of the world including Venezuela and the United States, with some of the largest being located in western Canada (Rogers, 2003).

Oil sands deposits in Canada are found in the provinces of Alberta and Saskatchewan. In Alberta there are three main deposits that cover an area of approximately 146 280 km² (AEUB, 2006). These deposits are the Peace River, Cold Lake, and Athabasca Oil Sands (AOS) deposits (Figure 1.1). The Athabasca deposit is the largest, covering approximately 102 760 km², and is the only deposit in which conventional surface mining methods are used (AEUB, 2006; Gentes et al., 2006).

The origin of oil sands is a disputed issue (Speight, 1999). The most widely accepted theory as stated by Squires (2005) is called the remote origin by Speight (1999) and states that bitumen was formed by oil that migrated into a sand deposit. At some point after migration, pressure from overburden was removed allowing lighter fractions of oil to escape, leaving heavier portions behind that we call bitumen.

Oil sands have been known to exist for a long period of time. However, it was not until the past century that exploitation of these resources was attempted on an industrial scale. Large scale production of oil by extraction of bitumen from the AOS did not progress beyond pilot projects until the 1960s and 1970s (Scott and Fedorak, 2004). Currently, there are three major companies (Syncrude Canada Ltd., Suncor Energy Inc., and Albian Sands Energy Inc.) mining oil sands using surface mining techniques, with many other surface mining operations in the pre-production phase. Due to recent increases in production, approximately 25 percent of Canada's oil production comes from the oil sands and this amount is expected to increase as demand for oil increases and more companies begin mining (Leung et al., 2003). An increase in production will be accompanied by a large increase in the production of tailings materials and disturbed land. Currently, there is a large amount of research being conducted into reclamation options and their effects on the environment.

1.1.2 Mining

Surface mining operations in the Athabasca oil sands deposit are completed using typical mining methods. Vegetation is stripped and other overlying materials (overburden), such as soil and rock, are removed. Once exposed, raw oil sands are dug up and hauled away using large stripping shovels and heavy-haul trucks (Squires, 2005).

Surface mining techniques are only feasible for bitumen deposits that exist under less than 80 metres of overlying materials (AEUB, 2006). Any oil sands covered by more than 80 metres overburden must be recovered by other, more economically and technologically feasible methods. These include different forms of in-situ oil recovery, such as steam stimulation, in-situ combustion, and other forms of enhanced oil recovery (EOR) that increase the oil's ability to flow (Speight, 2007). After EOR techniques have been employed oil can be pumped to the surface in a fashion similar to conventional oil deposits. Currently less than ten percent of Canada's oil sand deposits are suitable for surface mining (Speight, 1999).



Figure 1.1 Map showing Alberta's major oil sand deposits (modified from Source: Einstein, 2006)

1.1.3 Extraction and Upgrading

Oil bound in the form of oil sand must be extracted from the associated solid particles. The extraction method used today is an adaptation of the Hot Water Extraction Process (Figure 1.2) developed by Dr. Karl Clark (Clark and Pasternack, 1932; FTFC, 1995). Once raw oil sands are mined they are sent to the extraction plant as a slurry of sand and water. In the extraction plant this slurry is mixed with sodium hydroxide (NaOH) and hot water (Clemente and Fedorak, 2005). Tailings water has a pH in the range between 8 and 9 (Rogers, 2003). The increase in pH resulting from addition of NaOH causes naturally occurring carboxylic acids (naphthenic acids) to become soluble in water. Once naphthenic acids are dissolved, they exhibit surfactant-like properties that aid in the separation of oil from the sand (Schramm et al., 2000). This resulting slurry is then screened to remove materials too large for the upgrading process and sent on to the primary separation vessels, where components of oil sands are segregated based on density. Heavy components such as sand move to the bottom, while bitumen floats to the top as froth and is removed and sent for upgrading (Schramm et al., 2000). The left over materials include sand and water with suspended fine solids.

Once extracted, bitumen is sent for upgrading. The final product of upgrading is called sweet synthetic crude (SSC). The SSC oil has a shorter carbon chain, lower molecular weight, and is less viscous than bitumen. Further upgrading and processing results in the formation of other petroleum products such as gasoline and kerosene. A more in-depth review of upgrading and refining process can be found in Speight (1999 and 2007).

1.1.5 Production of Tailings Materials

Surface mining for oil sands creates a major environmental disturbance by removing large amounts of overburden and oil sand [approximately two of oil sand must be extracted to produce one barrel of crude oil (Mikula et al., 1996)], as well as using large amounts of water in the extraction process. The extraction process also produces large amounts of tailings waste. Approximately four cubic meters of fluid tailings are generated for each cubic metre of oil sand

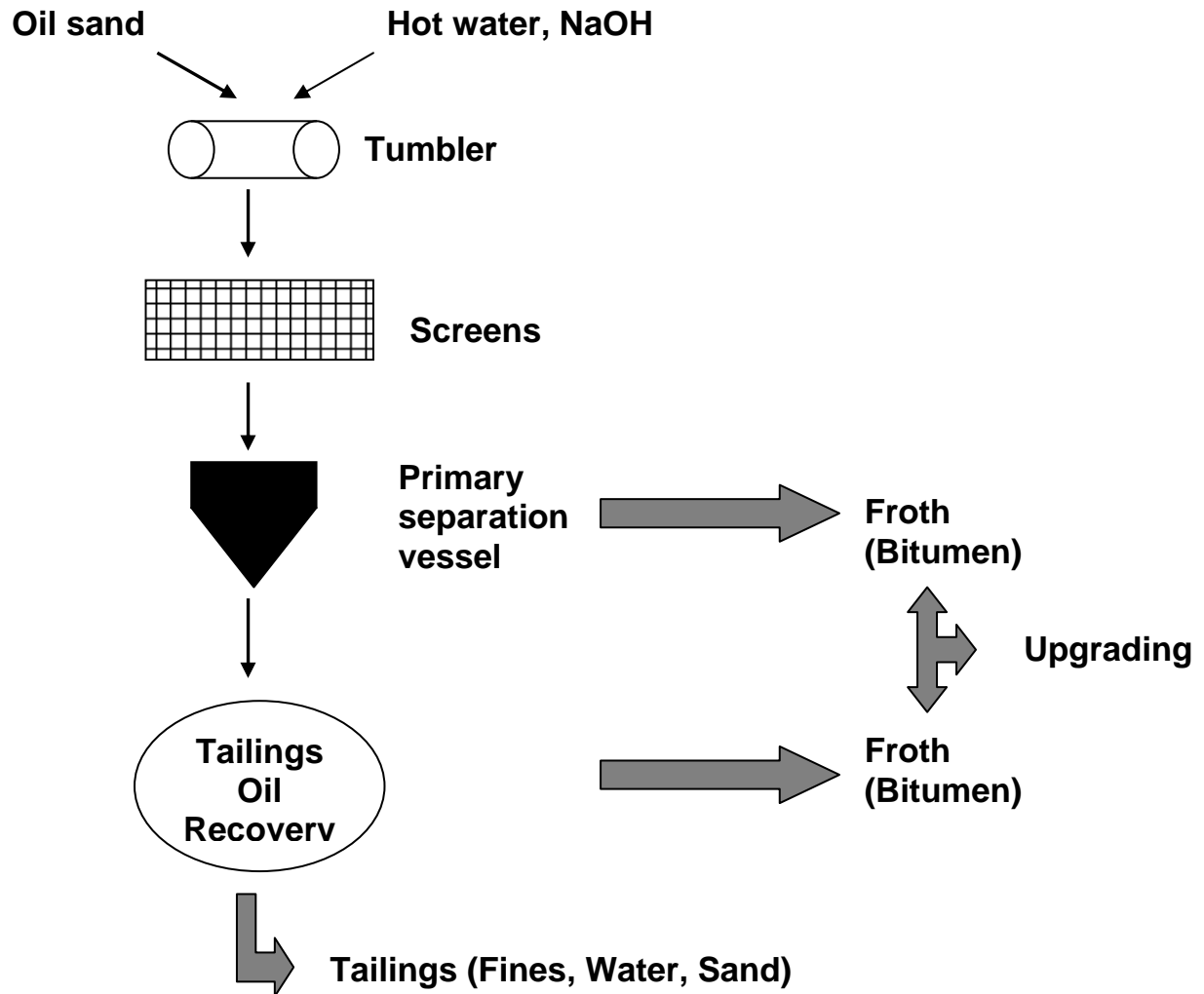


Figure 1.2 Diagram outlining the Hot Water Extraction Process (Clark and Pasternak, 1932) that is used for separating bitumen from raw oil sand.

that is extracted (Madill et al., 2001). Oil sands tailings are comprised of sand, silt, clays, water, and other dissolved materials. Also associated with the tailings is unrecoverable bitumen, which accounts for approximately two percent (FTFC, 1995) of the final tailings volume (Holowenko et al., 2002). The solid and liquid portions of the tailings waste can collectively be referred to as oil sand process-affected materials (OSPM). Provincial legislation does not allow oil sands companies to discharge any of the tailings waste into the surrounding environment, so all of the OSPM is stored on-site in large ponds (tailings ponds). Here heavier solids, mainly sand, settle out quickly and form a beach in tailings ponds leaving silt and clay (fines) suspended in water. Liquid tailings consist of process-affected water and fine tails. After further settling and compaction of fine particles in water, the resulting material is called mature fine tailings (MFT). The MFT are approximately 30% solids and 70% process-affected water (Mikula et al., 1996).

Another tailings product is called consolidated tailings (CT). The fine tails portion of MFT can take thousands of years to completely settle out if not treated (Mikula et al., 1996). One method of treating MFT is by the addition of coagulants, such as gypsum, which can greatly increase the speed of settlement of suspended fine particles. The coagulated final product is CT, which can help speed reclamation by using the MFT to produce a stable material that can be used in reclamation (Chalaturnyk et al., 2002). Although a large portion (> 70%) of tailings water is recycled back into the process (Quagraine et al., 2005), liquid tailings materials are accumulating at 100 000 m³ / day (Madill et al. 2001). It is predicted that more than one billion cubic metres of tailings will eventually have to be reclaimed (Leung et al., 2001).

As well as the sheer volume of tailings there is concern due to proven toxicity of many tailings constituents to living organisms. Increased salinity, polycyclic aromatic hydrocarbons in the form of unrecovered bitumen, and concentrated naphthenic acids are all sources of toxicity in liquid tailings material (Leung et al., 2001). Tailings have been shown to adversely affect fish (Nero et al., 2006a,b; Peters et al., 2007; Siwik et al., 2000), amphibians (Pollet and Bendell-Young, 2000), birds (Gentes et al., 2006; Gurney et al., 2005; Smits et al., 2000), mammals (Rogers, et al., 2002), and plants (Renault, 2005).

1.1.6 Reclamation of Mines and Tailings Materials

After mine closure, mining companies are required by law to return the mine lease areas to a state similar to the pre-production state. This means that the area must be able to support a functioning ecosystem, one that can sustain populations of native flora and fauna expected to inhabit the area naturally (Madill et al., 2001).

One method of reclamation proposed is called the “wet landscape approach”. This involves the formation of different types of wetlands (ponds, lakes) from tailings materials such as MFT and CT (Gulley and MacKinnon, 1993; Madill et al., 2001). To form these wetlands, mined out pits, which would not allow OSPM to escape, would be lined with tailings (CT and MFT) and filled with process-affected water and/or clean water. In time, it is hoped these created wetlands will support a natural wetland ecosystem (Siwik et al., 2000). However, since tailings materials have been shown to cause toxic effects to wildlife (Gentes et al., 2006) these wetlands have to be proven to become less toxic as they mature and be able to support organisms expected to inhabit the area. This study used a native amphibian, the wood frog (*Rana sylvatica*), as a bio-indicator to increase knowledge of the success of detoxification and the ability of wetlands formed with OSPM to sustain populations of indigenous amphibians.

1.2 Bioindicators

The use of bioindicators as a method for assessing environmental impacts caused by human activities has been increasing greatly in recent years (Venturino et al., 2003). A bio-indicator, as described in Olsgard (2007), is a living species that can act as an “early warning” of potential toxicity or damage to an ecosystem, as a result of exposure to contaminants. A bioindicator should have an important function in its ecosystem, be widely distributed, and have measurable biological responses that are reproducible when exposed to pollutants. Amphibians can be considered to be good bio-indicators because they are sensitive to contaminant exposure, are representative of aquatic life (Cooke, 1981; Gupta et al., 2007), and have been well studied in relation to environmental problems. Also, amphibian metamorphosis is a highly visible biological change which can be altered by environmental conditions and exposure to pollutants.

They reflect both terrestrial (adults) and aquatic (larvae) habitats, and they are also integral to food webs, playing important roles as both a prey and a predator species. For these reasons, *R. sylvatica* (Pollet and Bendell-Young, 2000) and other amphibians (Fort et al., 1999; Harris et al., 1998) have been used as indicators of environmental health in various ecosystems.

1.2.1 Amphibian use in Toxicology

The use of amphibians in toxicology has increased in recent years. This has been a result of the acknowledgement that amphibians are sensitive to environmental contaminants, are representative of aquatic life spending their larval (tadpole) life stage in the aquatic environment, and may show readily visible signs, such as deformations, as a result of exposure to contaminants (Cooke, 1981; Storrs and Kiesecker, 2004).

Amphibians are thought to be sensitive to man-made contaminants for a number of reasons. First, their skin is thinner and therefore more permeable to contaminants than other forms of wildlife (Storrs and Kiesecker, 2004). Also, frogs have an area of skin on their abdomen that is more absorptive of water (Wassersug, 1997) and therefore likely to absorb toxicants more readily. Secondly, amphibians have a complicated life cycle that is under highly coordinated hormonal control (Shi, 2000; Fort et al., 2007), which can be used as an indicator of exposure to contaminants. Further, their eggs, larvae, and adult stages may differ in response to exposure to the same toxicants such as pesticides (Cooke, 1972; Harris et al. 2000). As a result, amphibians have been used as test animals in environmental toxicology studies pertaining to many different anthropogenic contaminants, such as polychlorinated biphenyls (PCBs) (Gutleb et al., 2000), petrochemicals (Huang et al., 2007), and pesticides (Freeman and Rayburn, 2005; Fort et al., 2004; Diana et al., 2000).

1.2.2 Amphibian Caging Studies

Amphibian *in-situ* caging studies involve raising amphibians or their larvae in an enclosure or cage. Cages are usually made of a wooden or plastic frame and some type of mesh screen and are located in an area (usually a wetland) to be investigated (Harris et al., 2001).

Recently, amphibian caging studies have been identified as a valuable method for environmental studies in toxicology (Fort et al., 1999; Chinathamby et al., 2006) and ecology (Eaton et al., 2004; Harris et al., 2001). Caging studies allow researchers to control some variables associated with field studies, such as predation and difficulties in obtaining adequate numbers of study organisms. At the same time, caging studies permit researchers to keep environmental parameters such as temperature, photoperiod, and water chemistry identical to those encountered in regular field studies (Harris et al., 2001). In this respect, in-situ caging studies would fall somewhere in between laboratory and field based studies. The trade-off for having a higher density of animals in a restricted area and the need for food supplementation is off-set by the benefit of animals being able to experience naturally encountered conditions.

1.2.3 Wood Frog (*Rana sylvatica*) Life History

The wood frog is native to the northeastern United States and most of Canada (including the AOS), including Alaska and the Northwest Territories (Figure 1.3). Wood frogs are a species of “true frog”, which have the general characteristics of webbed toes on their hind limbs and visible dorso-lateral ridges on their trunk. Wood frogs usually have a dark mask around the eyes. Their color is generally brownish but may range from light brown to green to nearly black (Government of Alberta, 2002).

Wood frogs are generally found in moist, shaded areas such as marshes and damp woodlands. They are quite mobile in search of food items, such as insects and other small invertebrates. The ability of the wood frog to reside as far north as Alaska is due to their ability to endure freezing. Wood frogs endure freezing by increasing the concentration of glucose in cells, which acts as a cryoprotectant (Hemmings and Storey, 1996). Also, the presence of specialized blood proteins, called ice nucleating proteins, control ice formation in body fluids (Storey and Storey, 1992). These proteins also allow them to be active and begin reproductive activities as early as March when ice may still be present on wetlands, which is earlier in the year than any other anurans in the area.

Wood frogs mate between early April and mid-June. The large, round, jelly-like egg masses laid by the females can contain several thousand eggs. Wood frogs congregate at breeding areas and many egg masses are usually found together. Eggs hatch in approximately three weeks into the larval (tadpole) stage. The tadpoles metamorphose into frogs in 6 to 12 weeks depending on water temperature (Government of Alberta, 2002; Northern Prairie Wildlife Research Center, 2006).

1.2.4 Amphibian Metamorphosis

Anurans, such as *Rana sylvatica*, have a dual life stage after hatching from eggs. Metamorphosis is a period of rapid changes and development from the free swimming, larval (tadpole) form to the mostly terrestrial, tetrapod, juvenile frog stage. During metamorphosis, almost every body system undergoes drastic changes (Degitz et al., 2005). New structures are also formed, such as limbs. A detailed review of amphibian metamorphosis is published by Shi (2000).

Metamorphosis has three phases. First, premetamorphosis occurs without the influence of thyroid hormones (THs). It is distinguished by formation of hind limbs. Secondly, prometamorphosis is a period of time when THs start to increase in concentration. Thirdly, the metamorphic climax is defined by the emergence of forelimbs, resorption of the tail, and a large spike in the amount of thyroid hormone concentrations. Extensive changes in internal organs, such as remodeling of the gastro-intestinal tract and a change from ammonia excretion to urea excretion within the liver, also take place during the metamorphic climax (Shi, 2000). In the Gosner (1960) format for staging amphibians, the metamorphic climax starts at stage 42. Metamorphosis has been extensively studied and is known to be under control of thyroid hormones and the hypothalamic-pituitary-thyroid axis (HPT) (Galton, 1988, 1992; Shi, 2000; Tata, 2006; Fort et al., 2007). After metamorphic climax, the tails of tadpoles are fully resorbed and the organism left is a juvenile frog.

1.3 Biomarkers of Exposure to Contaminants

A biomarker, as defined by Shugart et al. (1992), is a biochemical or cellular change that occurs as a result of exposure to a xenobiotic, which is any foreign chemical naturally occurring or anthropogenic (Klaassen, 2001) that can be measured in a living sample or biological system. In this manner, biomarkers are similar to bio-indicator species in that they can act as an early warning system, allowing researchers to detect changes in a test organism or population of organisms before visible changes, such as direct physical damage or reduced survival, occur. Common biomarkers that can be measured are hormones, detoxification enzymes, enzyme activities, and immunological changes. Smits et al. (1996, 2002) have used immune function changes to monitor responses of mink (*Mustela vison*) to effluent from a Kraft pulp mill and American kestrels (*Falco sparverius*) to PCBs. Ethoxyresorufin dealkylase (EROD) activity, an enzymatic assay that is used to detect exposure to contaminants, has been used by Gauthier et al. (2004) in the amphibian *Xenopus laevis* in relation to river contamination. Also in amphibians, analysis of different parts of the thyroid system have been used as biomarkers of exposure to cadmium (Sharma and Patino, 2008), PCBs (Gutleb et al., 2000) and estrogenic compounds (Hogan et al., 2006).

1.3.1 Thyroid hormones (T3 and T4)

Thyroid hormones (THs) have widespread functions in the body. In amphibians there are two main THs, thyroxine (T4) and the more biologically active triiodothyronine (T3). They control basal metabolic rates and growth rates in many species (Brent, 1994; Helbing et al., 2006). T4 is the main product secreted by the thyroid glands. However, for thyroid hormones to be active, T4 must be converted to T3. This is done mainly in peripheral tissues, most notably the liver, but can also be done in the thyroid gland, by 5-deiodinase (Fort et al., 2007). Another deiodinase enzyme 5-deiodinase can convert T3 and T4 to reverse T3 (rT3) and T2 respectively. To cause a change in physiology, THs must bind to thyroid hormone receptors (TRs) located in the nucleus of cells. By binding to TRs, thyroid hormones alter gene expression and ultimately cause the intended change. Since rT3 and T2 have low affinity for thyroid hormone receptors they effectively inhibit or inactivate thyroid hormone (Shi, 2000).

In amphibians such as *R. sylvatica*, thyroid hormones control the highly specialized process of metamorphosis (Brown and Cai, 2007; Denver, 1997; Galton, 1988; Shi et al., 1996; Tata, 2006). Without thyroid hormones, metamorphosis will not progress, resulting in abnormally large tadpoles that never complete the change to frogs (Shi, 2000). Fort (2007) describes six areas of control of thyroid hormone and their function. Each of these areas could be possible targets for toxicants and could lead to alterations in thyroid hormone production and function, ultimately leading to alterations in metamorphosis. In-depth reviews of thyroid hormone and amphibian metamorphosis are given by both Shi (2000) and Fort (2007).

Due to the important functions of THs in many species, many studies have investigated the effects of chemicals that can potentially interfere with thyroid hormone, effectively using them as a biomarker. Oil sands process-affected material caused an increase in thyroid hormone in tree swallows (*Tachycineta bicolor*), while PCBs suppressed THs in American kestrels (*Falco sparverius*) (Smits et al., 2002). In amphibians, cadmium altered thyroid gland activity (Sharma and Patino, 2008).

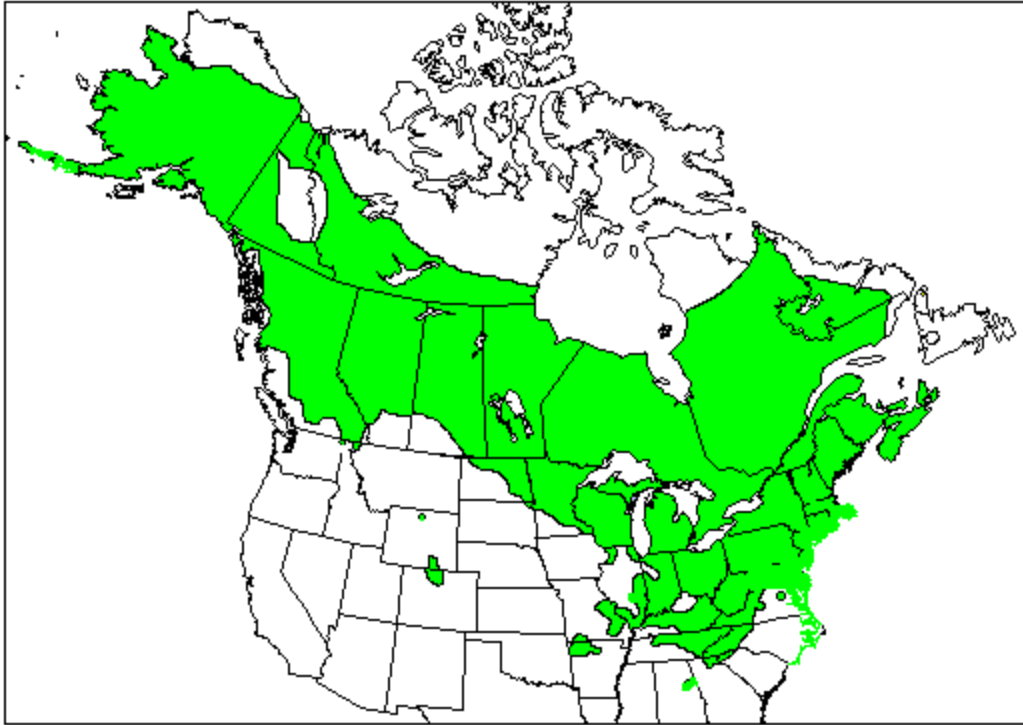


Figure 1.3 Map of geographical range of *Rana sylvatica* (modified from Northern Prairie Wildlife Research Center, 2006).

CHAPTER 2: RESEARCH GOALS, OBJECTIVES AND HYPOTHESES

2.1 Overall Goal

The main purpose of this research is to determine how wetlands formed from oil sands process-affected materials (OSPM) may affect the survival and development of *Rana sylvatica* larvae, which are expected to inhabit reclaimed wetlands after mine closure and reclamation. Furthermore, this study will determine how the effects of OSPM created wetlands on *R. sylvatica* larvae may change as the wetlands mature by comparing wetlands formed with OSPM that are of different ages.

2.2 Objectives

- 1) To raise *Rana sylvatica* larvae (tadpoles) from early pre-metamorphic stages until metamorphic climax in cages placed in wetlands of different ages that either contain OSPM or do not contain OSPM.
- 2) To measure physiological variables such as length, weight, and energy metabolism through triglyceride concentrations, as well as survival, which may show measurable changes as a result of the chemical differences in the wetlands.
- 3) To examine effects on development and metamorphosis related to differences in wetlands through endocrinological endpoints, specifically thyroid hormone (T3 and T4) status.

4) To determine liver ethoxyresorufin-*o*-dealkylase (EROD) induction. This reflects biotransformation efforts of tadpoles that are exposed to different levels of contaminants through placement in OSPM and reference wetlands.

2.3 Hypotheses

Null Hypothesis 1- There will be no difference in *Rana sylvatica* larval survival, growth and development, thyroid hormone concentrations, EROD enzyme activity, and whole body triglyceride concentrations as a result of being raised in OSPM-affected wetlands compared with reference wetlands.

Null Hypothesis 2- *Rana sylvatica* larval survival, growth and development, thyroid hormone concentrations, EROD enzyme activity, and whole body triglyceride concentration, will not be affected by the maturity (age) of the wetlands.

CHAPTER 3: TADPOLE SURVIVAL, GROWTH AND DEVELOPMENT

3.1 Introduction

The demand for oil is increasing, while reserves of oil are decreasing. As a result, production of oil from unconventional oil deposits, such as Alberta's oil sands, is increasing rapidly. There are currently three companies using surface mining to excavate oil sand, from which bitumen (heavy crude oil) is then extracted. Oil sands production accounts for approximately 25% of Canada's total crude oil production and this percentage is expected to increase as more companies begin production (Leung et al., 2003).

Crude oil that is extracted from oil sand is dense and highly viscous. In the ground bitumen is associated with sand, fine clay particles, and water (Speight, 2007). Surface mining of oil sands involves clearing land, digging out raw oil impregnated sand with excavation equipment, and transporting the oil sand with heavy haul trucks to the extraction facilities (ECT, 1995). Bitumen is separated from the associated materials by a hot water floatation process (Clark and Pasternak, 1932). This produces large quantities of solid (sand) and liquid (process-water containing suspended fine particles) wastes, called tailings or oil sands process-affected materials (OSPM), which are stored on mine sites in large dyked tailings ponds. Quagraine et al. (2005) states that by the year 2025 as much as one billion cubic metres of OSPM may be stored in tailings ponds. Government legislation requires the mine sites to be returned to a state similar to that of the area before production began (Madill et al., 2001). One strategy adopted by the oil sands industry to achieve this reclamation, the "wet landscape approach", involves the use of

stored tailing material to form lakes, ponds, and other types of “constructed wetlands” expected to cover 20 to 40% of the final landscape (Bendell-Young et al., 2000; Gentes et al., 2006; Siwik et al., 2000).

Since these constructed wetlands are meant to form a working ecosystem over time, they must be able to support communities of wildlife that would naturally inhabit the region. This may be problematic since OSPM and some of its constituents can be toxic to many organisms such as plants (Renault et al., 1998), amphibian larvae (Pollet and Bendell-Young, 2000), mammals (Rogers et al., 2002), fish (Nero et al., 2006a; van den Heuvel et al., 2000), aquatic invertebrates (Leung et al., 2003), and bird species (Gentes et al., 2006; Smits et al., 2000). It is thought that increased concentrations of salts, polycyclic aromatic hydrocarbons, and naphthenic acids (NAs) are the main cause of the toxicity to wildlife (Gentes et al., 2007; Nero et al., 2006a, b). However, OSPM toxicity is expected to diminish over time (Leung et al., 2003). Little is known about the effects of OSPM on amphibians, and chronic, low grade toxicity may interfere with the formation of a stable ecosystem that can support amphibian life.

Amphibians that are native to the northern boreal regions, such as the wood frog (*Rana sylvatica*) are expected to live in and around wetlands formed with OSPM. The larvae (tadpoles) of the wood frog are entirely aquatic until they complete metamorphosis. Amphibians and their larvae have been used as model subjects in many types of toxicological and ecological studies including investigations of the effects of pesticides (Cooke, 1972, 1981; Materna et al., 1995), industrial wastes (Huang et al., 2007; Snodgrass et al., 2004), and oil sands tailings water (Pollet and Bendell-Young, 2000). Recently, the use of confining amphibians in a wetland of interest (*in-situ* caging) has been recognized as a practical method for toxicology studies. Caging reduces variables associated with field collection of wild specimens (predation, stress/unreliability of capture, variations in diet, and unknown confounding elements), while still exposing amphibians to realistic environmental conditions such as light, temperature, and water chemistry (Harris et al., 2001). For these reasons *R. sylvatica* larvae are excellent subjects for an in-situ toxicological study of wetlands formed with OSPM. This study is intended to provide information on the viability of using wetlands containing OSPM, as part of a reclamation strategy that could be implemented by oil sands companies in northern Alberta.

During the course of this study, survival, growth, and developmental (morphometric) endpoints of indigenous *R. sylvatica* larvae were assessed to determine the ecological sustainability of the wetlands, and whether this sustainability held true for wetlands of different ages. Triglyceride concentration in the larvae was also measured to reveal possible alterations in energy storage or use by amphibians exposed to OSPM, which may reduce their ability to flourish in such ecosystems. This study was part of a multi-university collaborative project called Carbon Dynamics, Food Web Structure, and Reclamation Strategies in Athabasca Oil Sands Wetlands (CFRAW).

3.2 Materials and Methods

3.2.1 Experimental Design

3.2.1.1 Year One Experimental Design

In year one, four wetland sites were chosen for the study (Figure 3.1). Two sites contained OSPM and two did not, the latter being considered reference sites. Of these four wetlands, one OSPM and one reference site were experimental trenches on the Suncor mine lease (Figure 3.2), each consisting of three similar trenches. The three OSPM-impacted trenches were filled with water from Natural Wetland, a wetland formed from tailings water (OSPM) seepage on the Suncor lease; the three reference trenches were filled with water from Weir 1, a commonly used source of ‘clean’ water unaffected by mining and extraction activities, on the Suncor lease site. The two other sites were single, distinct bodies of water. One was a reference wetland off Tower Road, 20 km from the oil sands leases near the city of Fort McMurray, Alberta. The other was called 4m CT, an OSPM-affected created wetland on the Suncor lease site. Three enclosures were placed in each wetland or trench and forty young tadpoles were placed in each mesocosm. Tadpoles were collected from a wetland, Bill’s Lake, which did not contain OSPM-containing but was located on the Syncrude Mildred Lake lease site. Tadpoles were fed boiled lettuce *ad libitum*. Uneaten lettuce was removed from the enclosures and replaced with new lettuce every second day.

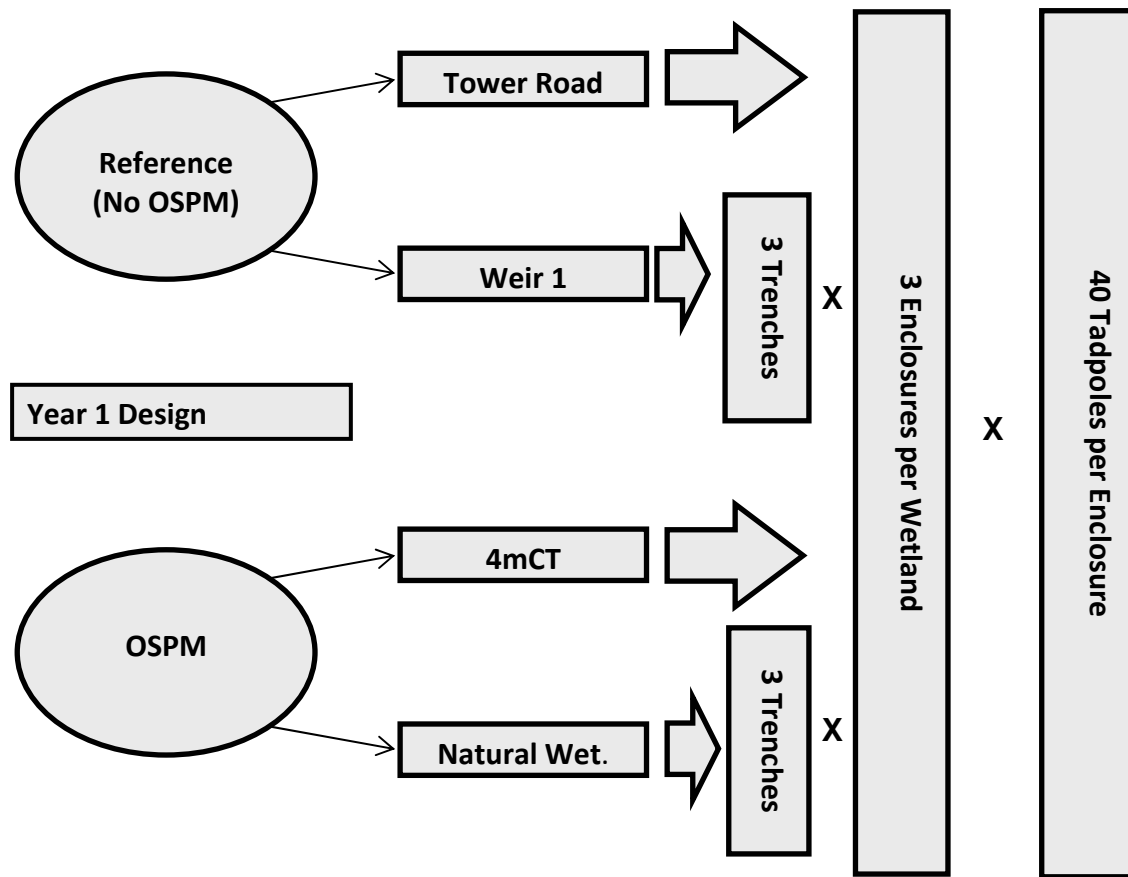


Figure 3.1 Year 1 (2006) experimental design diagram Note: Abbreviations are as follows: OSPM – oil sands process-affected material; 4m CT – Four Metre Consolidated Tailings Wetland; Wet. - Wetland



Figure 3.2 Picture of the experimental trenches on Suncor Energy Inc.'s mine lease.

3.2.1.2 Year Two Experimental Design

The year two (2007) design (Figure 3.3) was greatly expanded to encompass 14 different sites, seven reference sites (no OSPM) and seven OSPM impacted sites. All cages were placed directly in the wetland to be studied, with no use of experimental trenches. Also, all wetlands were categorized by age. Wetlands were considered “young” if they were seven years old or less, and “old” if they were eight years or older. In year two I also increased the number of enclosures per wetland from three to four. Also, the number of tadpoles placed in each mesocosm was increased to 50 individuals. Tadpoles were collected from Bill’s Lake. Tadpoles were fed boiled lettuce *ad libitum*. Uneaten lettuce was removed from the enclosures and replaced with new lettuce every second day.

3.2.1.3 Wetland Selection and Classification

Wetlands of the four classes (young OSPM, old OSPM, young reference, and old reference) were available in different numbers since they were selected on the basis of relevance to a multi-university collaborative project, availability, and security. Firstly, wetlands were selected because of their inclusion and relevance to a multi-disciplinary ecological study of wetlands formed with OSPM (CFRAW). A suite of wetlands was chosen for CFRAW and these wetlands were used in several research projects as part of CFRAW. Secondly, large numbers of OSPM wetlands were available, while relatively few young reference sites were available. Reference wetlands were wetlands containing no OSPM, but could be man-made or naturally occurring. Finally, only wetlands located on a mine site were chosen to prevent unwanted human disturbances. These were three limiting factors causing unequal numbers of wetlands among the four wetland classes.

Enclosures from each wetland were used to provide tadpoles for all analyses, unless problems were encountered that made samples unavailable or unreliable. Some reasons for not including certain enclosures in analyses were physical damage to enclosures allowing escape of tadpoles, large changes in water levels (evaporation/draining) altering metamorphosis or survival, and death of tadpoles resulting in inadequate numbers of tadpoles for all analyses.

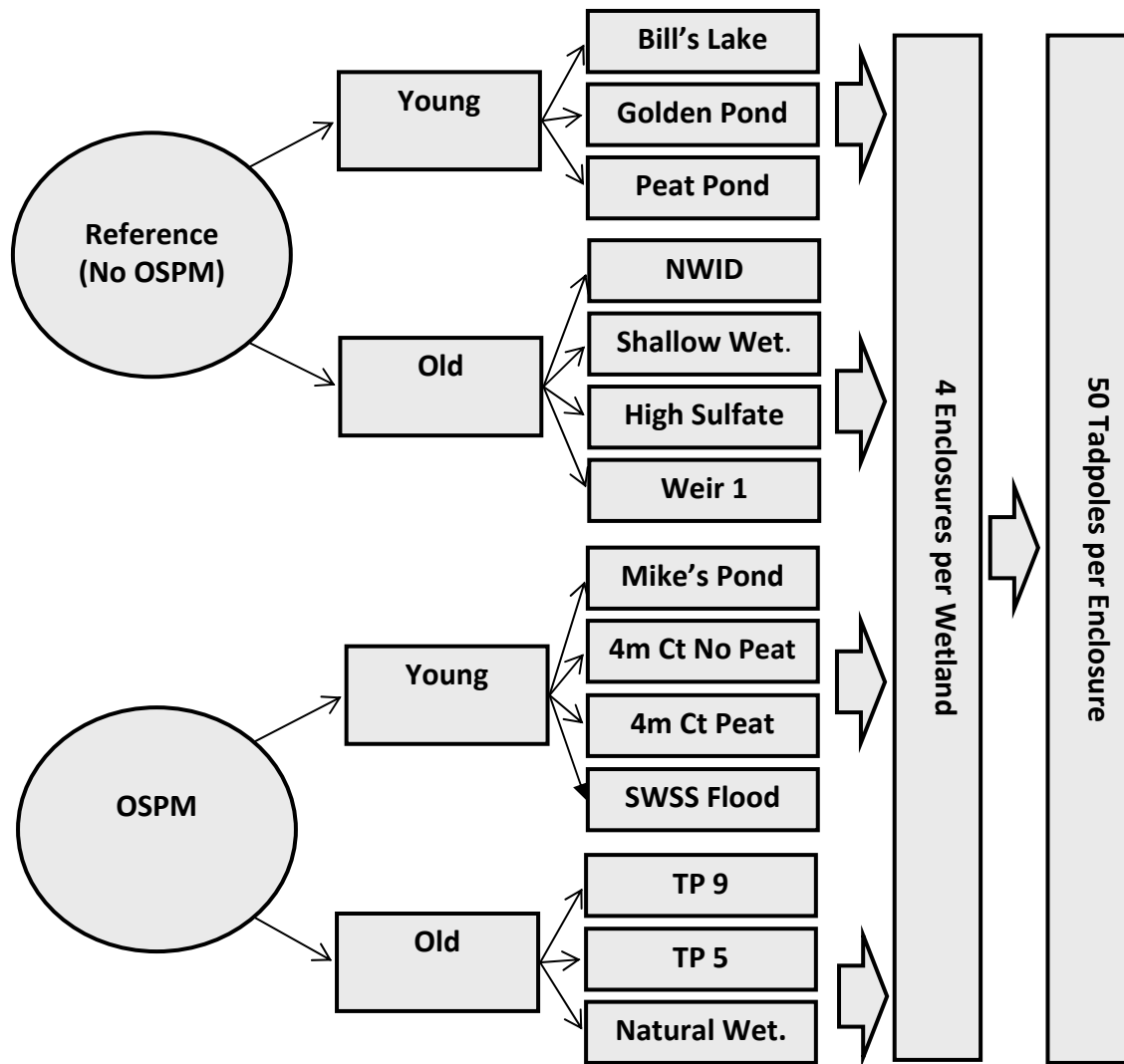


Figure 3.3 Year 2 (2007) experimental design diagram Note: Abbreviations are as follows: OSPM – oil sands process-affected materials; 4m CT – Four Metre Consolidated Tailings Wetland; Wet. – Wetland; NWID – North West Interceptor Ditch Wetland; SWSS – South West Sand Storage Complex; TP 9 – Test Pond 9; TP 5 – Test Pond 5

Complete lists of all enclosures and analyses they were used in are provided in Tables 3.1 and 3.2.

3.2.3 Enclosure Design

Enclosure design was adopted and modified from several sources (Harris et al., 2001; Materna et al., 1995). The design used in this study (Figure 3.4) consisted of a rectangular wooden frame measuring 76 cm x 44.5 cm x 44.5 cm. A wooden framed lid, secured in place with removable nails, also measured 76 cm x 44.5 cm. The sides and bottom of the rectangular frame and the associated lid were covered with 1600 μm fiberglass mesh. Wooden stakes were also attached to the frame allowing the enclosures to be secured in the wetlands. Black landscaping cloth was attached to one side of the lid and draped over one side of the enclosure, acting to shade the developing larvae. When placed in wetlands, approximately three quarters of the enclosures' total height was submerged. The initial density of tadpoles in each mesocosm was approximately 0.44 tadpoles per litre.

3.2.4 Study Duration

Tadpoles were added to the enclosures at early pre-metamorphic stages (approximately stages 24 to 26) and were kept in the enclosure until they reached metamorphic climax (stage 42). I called this period the "time to metamorphosis". The method I used for determining the stage of development was that of Gosner (1960). The study was ended on an enclosure-by-enclosure basis at the time when greater than 75 percent of the remaining tadpoles reached metamorphic climax.

3.2.5 Tadpole Survival

Year 1 Tadpole survival was the percentage of tadpoles alive at the end of the study period out of the original 40 individuals.

Year 2 Tadpole survival was measured and reported as the percentage of tadpoles surviving out of the original 50 individuals, after 52 days. Enclosures were checked every second day and dead or dying tadpoles were removed.

Table 3.1 Wetland descriptions, including area (m²).

Wetland	OSPM Status	Age	Area (m²)	Other Information
Bill's Lake	Reference	Old	5821	Located at Syncrude Canada's South Hills
Peat Pond	Reference	Young	6624	Located at Syncrude Canada's South Hills
Golden Pond	Reference	Young	5677	Located at Syncrude Canada's South Hills
Mike's Pond	OSPM	Young	16920	Located on Syncrude Canada's mine lease
Test Pond 5	OSPM	Old	675	Located on Syncrude Canada's mine lease
Test Pond 9	OSPM	Old	3732	Located on Syncrude Canada's mine lease
West Interceptor Ditch Wetland	Reference	Old	3155	Located on Syncrude Canada's mine lease
South West Sands Storage (Flood Wetland)	OSPM	Young	11954	Located on Syncrude Canada's mine lease
Test Pond 14 (Shallow Wetland)	Reference	Old	35000	Located on Syncrude Canada's mine lease
Natural Wetland	OSPM	Old	12227	Located on Suncor Energy Inc.'s mine lease
High Sulfate Wetland	Reference	Old	2394	Located adjacent to Suncor Energy Inc.'s mine lease
Weir 1	Reference	Old	56419	Gravel pit filled with water on Suncor Energy Inc.'s mine lease
4 Metre CT - No Peat Zone	OSPM	Young	4006	Located on Suncor Energy Inc.'s mine lease
4 Metre CT - Peat Zone	OSPM	Young	4006	Located on Suncor Energy Inc.'s mine lease

Table 3.2 List of wetlands and enclosures (four possible in each wetland) studied in year two describing the length of time until the end of study in each enclosure and explanations of why an enclosure was not included in statistical analysis for some or all endpoints. All enclosures were used for all analyses unless otherwise stated.

Wetland	Enclosure	Days To End of Study	End Points Analyzed
Bill's Lake	1	62	All Bill's Lake enclosures were used in all analysis with the exception of #1 and #2 in the triglyceride assay and #1 in the EROD assay
	2	62	
	3	62	
	4	62	
Peat Pond	1	58	Used in all analyses
	2	58	
	3	58	
	4	58	
Golden Pond	1	58	Used in all analyses
	2	58	
	3	58	
	4	58	
Mike's Pond	1	66	Used in all analyses
	2	68	Used in all analyses
	3	68	Not used in triglyceride analysis - too little extract
	4	N/A	Not used in any analyses except survival due to death of all tadpoles before metamorphosis
Test Pond 5	1	52	All Test Pond 5 enclosures used in all analyses except #1 and #2 were not used in triglyceride assay - not enough extract after thyroid hormone assay
	2	52	
	3	52	
	4	52	
Test Pond 9	1	55	Used in all analyses
	2	56	
	3	56	
	4	56	
West Interceptor Ditch Wetland (WID)	1	60	Used in all analyses
	2	60	
	3	60	
	4	60	
South West Sands Storage (Flood Wetland)	1	N/A	All Flood Wetland enclosures were used in survival analyses only – due to death of all tadpoles before metamorphosis
	2	N/A	
	3	N/A	
	4	N/A	
Test Pond 14 (Shallow Wetland)	1	60	Used in all analyses
	2	60	Used in all analyses
	3	60	Used in all analyses
	4	N/A	Not used in any analyses - enclosure damaged
Natural Wetland	1	N/A	No Natural Wetland enclosures were used in any analyses except survival - death of all tadpoles
	2	N/A	
	3	N/A	

	4	N/A	
High Sulfate Wetland	1	57	Used in all analyses
	2	57	
	3	56	
	4	57	
Weir 1	1	N/A	Not used in any analyses - enclosure damage
	2	58	Used in all analyses
	3	58	Used in all analyses
	4	58	Used in all analyses
4 Metre CT - No Peat Zone	1	75	Used in all analyses
	2	75	Used in all analyses
	3	75	Used in all analyses
	4	N/A	Not used in any analyses except survival-death of tadpoles
4 Metre CT - Peat Zone	1	75	Used in all analyses
	2	N/A	Not used in any analyses except survival-death of tadpoles
	3	75	Used in all analyses
	4	N/A	Not used in any analyses - enclosure damaged

Table 3.3 Water chemistry (averages over duration of study)

Wetland	pH	Conductivity ($\mu\text{s}/\text{cm}$)	Naphthenic Acids (mg/L)	Dissolved Oxygen (mg/L)	NH ₄ (mg/L)
Bills Lake	7.38	531.7	0.68	10.10	0.26
Peat Pond	7.90	1058	0.62	7.50	0.34
Golden Pond	8.54	1154	1.68	8.00	0.15
Mike's Pond	8.58	4047.5	22.76	9.83	0.68
Test Pond 5	8.75	2224	10.23	10.15	0.67
Test Pond 9	8.96	1946.5	20.30	9.530	0.16
Test Pond 14 ^a	8.09	373	0.73	7.23	0.43
WID ^b	7.99	582.5	0.87	8.40	0.58
SWSS ^c	8.08	4130	40.80	N/A	N/A
Natural Wet. ^d	8.55	1463.3	53.00	10.60	0.66
HS ^e	8.55	3380	7.0	8.16	0.45
4m CT peat ^f	8.38	2695	35.65	7.10	BDL
4mCT no peat ^f	8.09	2770	30.90	7.30	0.24

^aAlso called Shallow Wetland

^bWID – West Interceptor Ditch Wetland

^cFlood wetland on South West Sands Storage area

^dNatural Wetland

^eHigh Sulfate Wetland

^fTwo different areas of the same wetland – one area peat amended, one area no peat amendment

3.2.6 Tadpole Length, Weight, and Average Daily Length and Weight Gain

Both tadpole weight and length were measured immediately following euthanasia at the termination of the field study. Length (snout to tail) was measured with a Westward electronic caliper (Acklands-Grainger, Fort McMurray, AB, Canada). Body weight was measured with a Sartorius TE313S scale (max 310 g, d = 0.001) (Sartorius AG, Goettingen, Germany) after drying excess moisture from the tadpole by gentle blotting with tissue paper and placing the tadpole on a piece of laboratory film.

Average daily length and weight gain were determined by dividing the tadpole's length and weight by the number of days the tadpole was caged (time to metamorphosis).

3.2.7 Whole-Body Triglyceride and Thyroid Hormone Extraction

The method for extraction used was developed from Brasfield et al. (2004). Whole tadpoles and livers removed from selected tadpoles were frozen in liquid nitrogen (Praxair, Saskatoon, SK, Canada) immediately following collection and measurement of body weight and body length. A total of five tadpoles were sampled from each enclosure. Once returned to the Toxicology Centre in Saskatoon, SK, samples were transferred to a -80 °C freezer until the time of sample preparation and analysis.

The same whole body extract from the tadpole was used for both triglyceride and thyroid hormone quantification. During sample homogenization and extraction, all materials (scissors, forceps, glass tubes, etc.) and buffers were placed on ice. Homogenization buffer consisting of 1mM 6-propyl-2-thiouracil (Sigma Aldrich, Oakville, ON, Canada) in 95% ethanol was made prior to extraction and stored at -20 °C in a glass bottle. The first step in both extraction processes was to remove the tadpoles from cryovials and place them in plastic weigh boats (VWR International, Mississauga, ON, Canada). A volume of homogenization buffer equal to that of the tadpole was then added. Tadpoles were finely minced using scissors and transferred along with the buffer to a 16 mm x 100 mm glass culture tube (VWR International, Mississauga, ON, Canada). Another volume of homogenization buffer equal to the volume of the tadpole was then added to the tadpole homogenate in the glass tube.

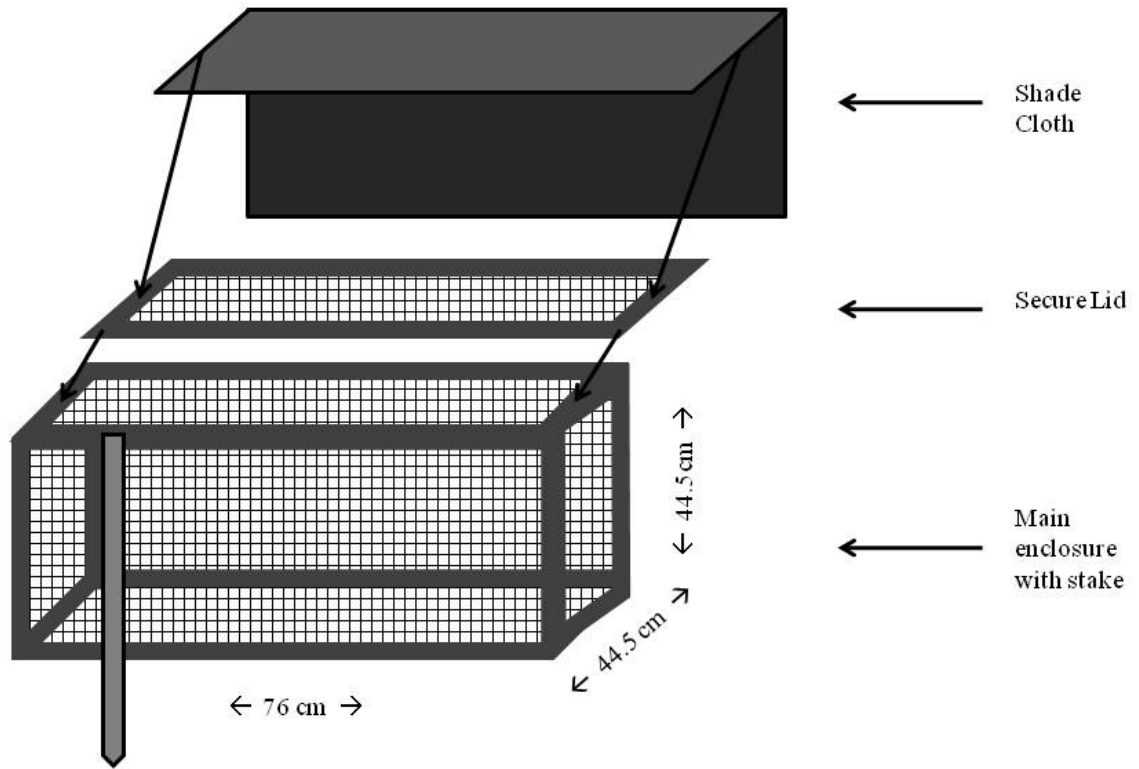


Figure 3.4 Enclosure design showing landscaping cloth used as shade cloth, enclosure with lid covered with 1600 μm mesh screen and a stake for securing the enclosure to the bottom of the wetland.

Further homogenization was completed using a Tissue Tearor (BioSpec Products, Inc., Bartlesville, OK, USA) in three bursts of ten seconds each. Next, samples were vortexed vigorously for one minute, then stored on ice. Each sample was then centrifuged at 2900 rpm at 4 °C for ten minutes in an Eppendorf 5810 R centrifuge and swinging bucket rotor [model A-4-62 (Eppendorf Canada, Mississauga, ON, Canada)]. Supernatant was carefully removed and transferred to a clean glass tube. Another volume equal to twice the volume of the tadpole was then added to the remaining pellet, which was then re-suspended by vortexing for one minute. The re-suspended homogenate was then centrifuged again, as described above. The resulting supernatant, which contained ethanol used in the extraction, thyroid hormones, and triglycerides was again carefully poured off and combined with the previous supernatant. This sample was then evaporated under a stream of nitrogen in a water bath at 50 °C so that the final volume was equal to that of the initial tadpole. Once the desired volume was reached, the extract was divided into as many 150 µl aliquots as possible and stored at –80 °C until assays were completed.

3.2.8 Whole-body Triglyceride Quantification

Quantification of total body triglycerides was completed using clinical kit reagents from Sigma Aldrich (Oakville, ON, Canada). The method was adopted from McGowan et al. (1983). For this assay, aliquots of the tadpoles previously homogenized and extracted with ethanol, were removed from the –80 °C freezer and stored on ice until sufficiently thawed. Samples were vortexed vigorously, and then centrifuged at 2500 rpm for five minutes at 4 °C to ensure samples were free of un-dissolved matter. For quantification, pure glycerol standard (Sigma #G7793-5ml) was diluted with 95% ethanol to concentrations ranging from 0.0391 mg/ml to 2.5 mg/ml to form a standard curve. First, 180 µl of reagent A (glycerol kinase; Sigma #F6428-40ml) and 10 µl of all standards and samples were added in duplicate to a clear, flat-bottom, Nunc 96-well plate (VWR International, Mississauga, ON, Canada). The plate was then incubated at 37 °C for five minutes in the same microplate spectrophotometer (Molecular Devices, Sunnyvale, CA, USA) that was used for reading the plate. After shaking, 45 µl of reagent B (lipase; Sigma #T2449-10ml) was added to all wells. The plate was then shaken again for 20 minutes at 37 °C, after which the plate was read at 540 nm. The final concentration of triglycerides in each well was calculated by comparing the reading from each sample to the glycerol standard curve.

3.2.9 Statistics

In year two (2007), each wetland was not compared to each of the other wetlands separately. Based on the knowledge that as OSPM-affected wetlands get older there is a reduction in toxicity, due to bioremediation, I grouped wetlands according to their age (either young or old) and OSPM status (OSPM or no OSPM). I assumed that wetlands of the same group (young OSPM-affected, old OSPM-affected, young reference, old reference) have similar characteristics, such as concentration of NAs. This method of grouping allowed me to complete the two-way ANOVA described below to test the hypothesis that there will be an interaction between age and OSPM status of wetlands. I predicted that older OSPM-affected wetlands would be similar to reference wetlands and much less toxic than young OSPM-affected wetlands.

Statistics were carried out using SPSS statistical software package (Version 16.0.1., SPSS Inc., Chicago, IL, USA). The level of significance was $p < 0.05$. All data were tested for normality and equality of variances assumptions using the Shapiro-Wilks test and Levene's test, respectively. In year one, the experimental design was planned for a two-way ANOVA. However, due to loss of a reference site, a one-way ANOVA was conducted to determine if there were differences in survival (percentage of initial numbers), body weight, body length, and concentration of triglycerides (whole-body) among the three remaining experimental sites. If assumptions of normality or homogeneity of variances were violated, data presented as percentages were arcsin square root transformed, and regular, numeric data were \log_{10} transformed. If assumptions were met after transformation, an ANOVA was performed on the transformed data. If assumptions could not be met the non-parametric Kruskal-Wallis test was performed. In year two, if assumptions were met, a two-way ANOVA was completed on the same variables as in year one. Age of wetlands (young or old) and OSPM status (OSPM or no OSPM) of the wetlands were the two factors in the two-way ANOVA. If assumptions of normality and homogeneity of variances were violated the same method for transforming data as in year one was taken. After transformation, if assumptions were met a two-way ANOVA was performed. If assumptions were not met, the Scheirer-Ray-Hare extension of the Kruskal-Wallis test [a non-parametric variation of a two-way ANOVA test (Sokal and Rohlf, 2003)], was performed as in Rickwood et al. (2008).

3.3 Results

3.3.1 Year One (2006)

3.3.1.1 Problems

Year one of field research presented many unexpected problems that were beyond control of the researcher. Some data were lost or were not used due to the introduction of variables not expected to be encountered normally in wetlands. First, the off-site reference wetland near Fort McMurray was tampered with by unknown person(s); all enclosures had been emptied of their tadpoles and piled on shore. This site was completely abandoned just short of one month into the study and no data were obtained. Secondly, water level in some of the experimental trenches on Suncor's lease fluctuated and two were inadvertently, completely drained for approximately 12 hours. Most of the fluctuations in water level were due to seepage out of the trenches as well as high rates of evaporation. This problem was remedied later in the year once a system of tanks and pipes were set up, allowing researchers to add water to individual trenches when needed. At the end of this field season it was necessary to exclude data from the two trenches (both containing process-affected water from Natural Wetland) that had been drained and were subsequently refilled. Due to the loss of two of the three OSPM-affected trenches, as well as my offsite reference, most of the data used for publication will come from the year two part of the study, which was much more successful.

Given the lack of security at off-site wetlands and the poor control of water levels at the experimental trenches, which resulted in poor quality data, changes were made to the experimental design in year two. First, all wetlands in the study were located on secure oil sands lease sites, preventing interference from outside persons. Second, the experimental trenches on the Suncor site were abandoned in favour of traditional wetlands that were either natural or constructed since fluctuations in water levels would be less likely. The second field season was much more successful because of these adjustments. However, the Natural Wetland site had to be excluded from all analyses because water levels in the wetland decreased significantly over the summer, resulting in extremely low water levels in enclosures, which would likely have had concentrated levels of toxic components (Daly, 2007). Also, one enclosure from the Weir 1 site

was lost due to damage in a storm. Nevertheless, sample collection was quite good and resulted in sufficient samples from almost all study sites.

3.3.1.2 Survival

There were differences in survival between reference and OSPM-containing sites in year one (Kruskal-Wallis, $p=0.027$). The young OSPM site, 4m CT, showed greatly decreased survival (100% mortality within 2 weeks of the beginning of the study) when compared to the reference site and more mature (older) wetland containing OSPM (Natural Wetland) (Figure 3.5).

3.3.1.3 Tadpole Length, Weight, and Average Daily Length and Weight Gain

Tadpoles from wetlands containing OSPM had similar body weights [(ANOVA, $p = 0.371$) (Figure 3.6)] and body lengths [(ANOVA, $p = 0.266$) (Figure 3.7)] to tadpoles raised in wetlands containing no OSPM. Daily length gain [(ANOVA, $p = 0.432$) (Figure 3.8)] and daily weight gain [(ANOVA, $p = 0.625$) (Figure 3.9)] of tadpoles were also not significantly different between wetlands.

3.3.1.4 Whole-body Triglyceride Concentrations

In the first study year triglyceride concentrations in tadpoles were not determined due to lack of specimens for analysis.

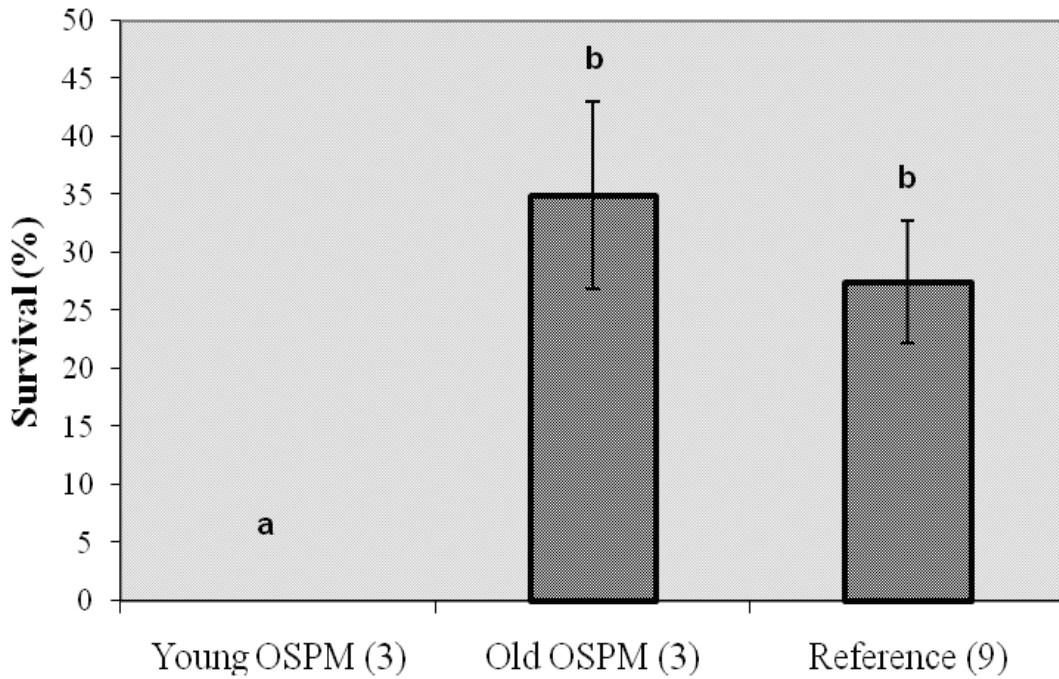


Figure 3.5 Year one mean survival ($\% \pm$ S.E.) of *Rana sylvatica* tadpoles raised in a reference wetland, an old oil sands process-affected material (OSPM) containing wetland, and a young OSPM-affected wetland. The number of enclosures per type of wetland is shown in parentheses (n), differences were considered significant when $p < 0.05$. A significant difference was detected (Kruskal-Wallis, $p = 0.027$). A tukey post-hoc test determined young OSPM wetlands were different from all others (different letters above bars indicate a significant difference, $p < 0.05$).

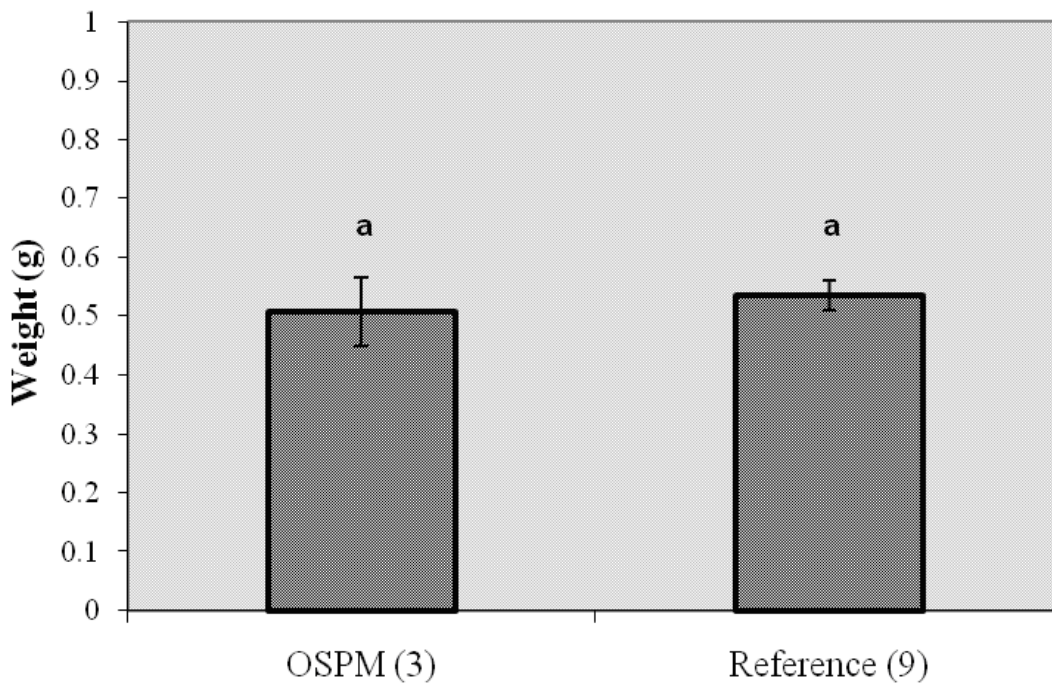


Figure 3.6 Year one mean body weight ($g \pm$ S.E.) of *Rana sylvatica* tadpoles raised in a reference wetland and an old oil sands process-affected material (OSPM) containing wetland. The number of enclosures per type of wetland is shown in parentheses (n), differences were considered significant when $p < 0.05$. No significant difference were detected (ANOVA, $p = 0.371$). Different letters above bars indicate a significant difference, $p < 0.05$.

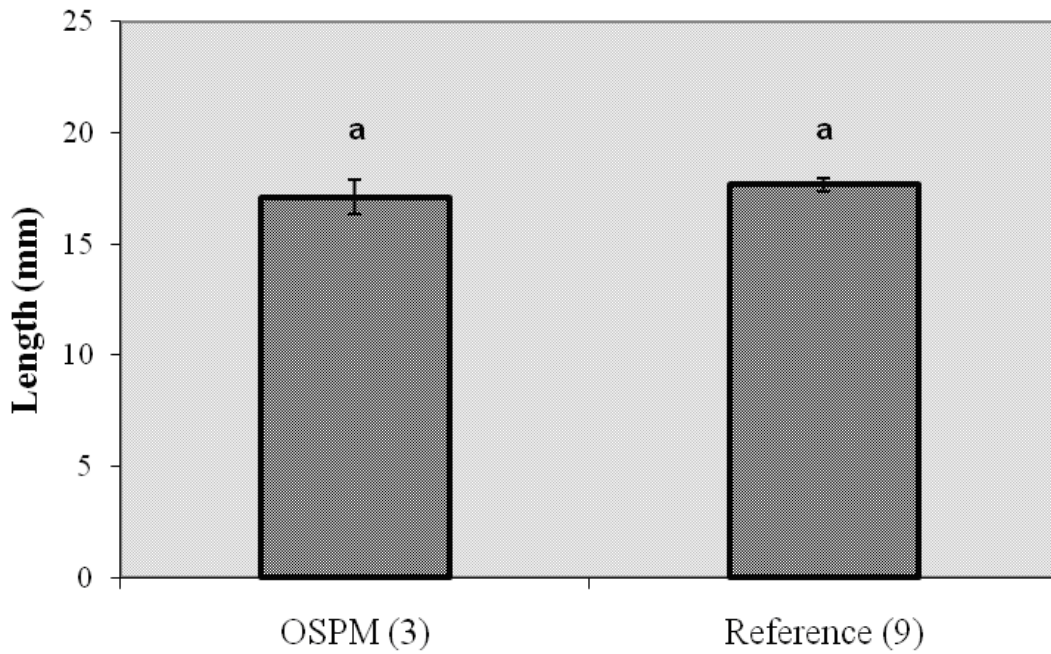


Figure 3.7 Year one mean body length (mm \pm S.E.) of *Rana sylvatica* tadpoles raised in a reference wetland and an old oil sands process-affected material (OSPM) containing wetland. The number of enclosures per type of wetland is shown in parentheses (n), differences were considered significant when $p < 0.05$. No significant differences were detected (ANOVA, $p = 0.266$). Different letters above bars indicate a significant difference, $p < 0.05$. Different letters above bars indicate a significant difference, $p < 0.05$.

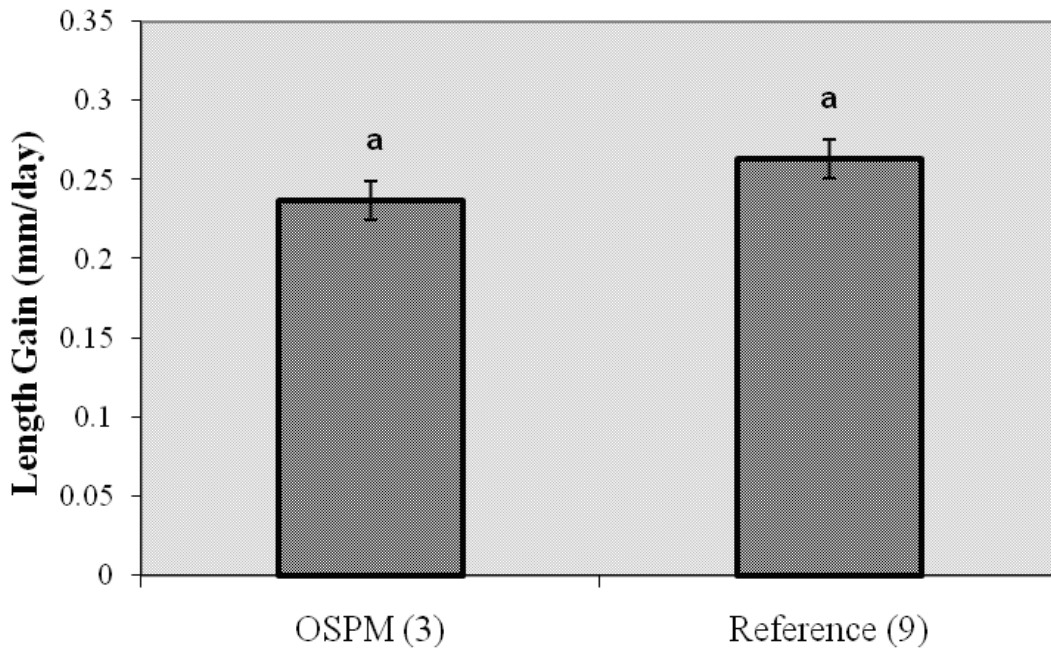


Figure 3.8 Year one mean daily length (mm/day \pm S.E.) gain of *Rana sylvatica* tadpoles raised in a reference wetland and an old oil sands process-affected material (OSPM) containing wetland. The number of enclosures per type of wetland is shown in parentheses (n), differences were considered significant when $p < 0.05$. No significant differences were detected (ANOVA, $p = 0.432$). Different letters above bars indicate a significant difference, $p < 0.05$.

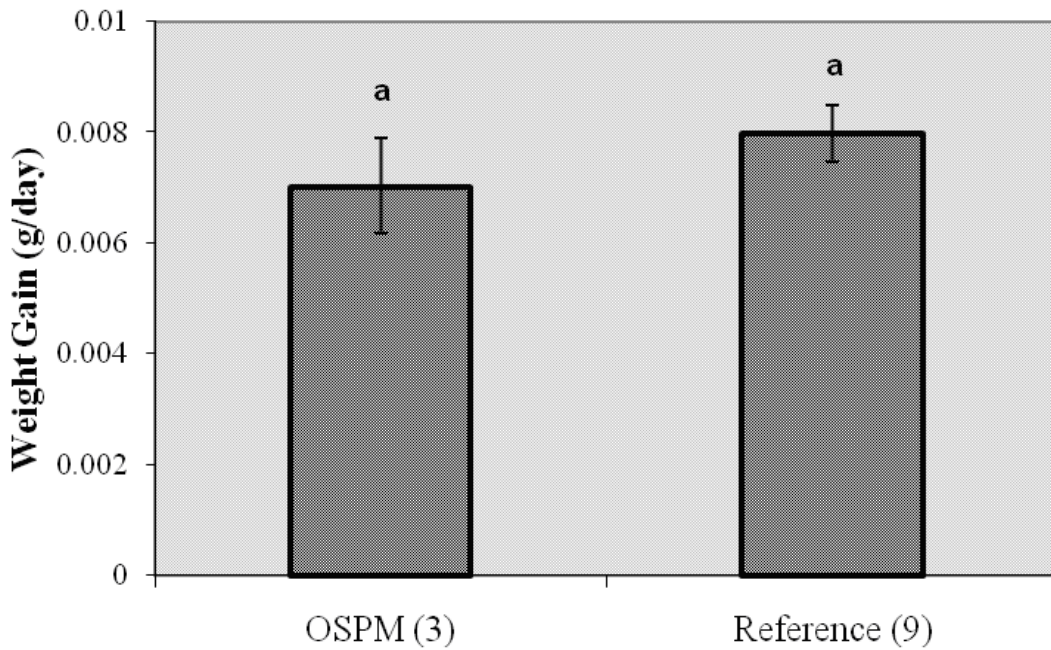


Figure 3.9 Year one mean daily weight gain (g/day \pm S.E.) of *Rana sylvatica* tadpoles raised in a reference wetland and an old oil sand process-affected material (OSPM) containing wetland. The number of enclosures per type of wetland is shown in parentheses (n), differences were considered significant when $p < 0.05$. No significant differences were detected (ANOVA, $p = 0.625$). Different letters above bars indicate a significant difference, $p < 0.05$.

3.3.2 Year Two (2007)

3.3.2.1 Survival

Survival was much lower in young wetlands containing OSPM than all other classes of wetlands. Old OSPM-containing wetlands showed survival similar to that of both young and old reference wetlands. There was a significant interaction effect between the factors of age and OSPM status. Treatment effects of age and OSPM status were also found to be significant [(two-way ANOVA, interaction $p < 0.001$, age $p = 0.004$, OSPM $p < 0.001$) (Figure 3.10)].

3.3.2.2 Tadpole Length, Weight, and Average Daily Length and Weight Gain

Tadpoles that were raised in young OSPM-containing wetlands were heavier than tadpoles raised in the other classes of wetlands. For body weight of tadpoles, there was a significant interaction effect between the wetland's age and OSPM status but not treatment effects of age or OSPM status alone [(two-way ANOVA, interaction $p = 0.016$, age $p = 0.441$, OSPM status $p = 0.293$) (Figure 3.11)]. For body length, a significant interaction between treatment effects was detected, but not for treatment effects alone [(two-way ANOVA, interaction $p < 0.001$, age $p = 0.920$, OSPM status $p = 0.890$) (Figure 3.12)]. Weight gain per day was not different among tadpoles in any wetland type [(two-way ANOVA, interaction $p = 0.226$, age $p = 0.721$, OSPM status $p = 0.620$) (Figure 3.13)]. For daily length gain, no significant difference due to an interaction between treatment effects or OSPM status was found, but a significant difference due to age was detected (two-way ANOVA, interaction $p = 0.733$, age $p < 0.001$, OSPM status $p = 0.137$) (Figure 3.14)].

3.3.2.3 Whole-body Triglyceride Concentrations

There was a significant difference in triglyceride concentrations of tadpoles raised in different wetland types due to an interaction between the factors of age and OSPM status, but not treatment effects alone [(two-way ANOVA, interaction $p = 0.041$, age $p = 0.739$, OSPM status $p = 0.711$) (Figure 3.15)]. Tadpoles raised in old reference wetlands had the highest concentrations

of triglycerides followed by young OSPM-containing wetlands. Young reference sites and old OSPM sites were similar to each other and had the lowest concentrations of triglycerides.

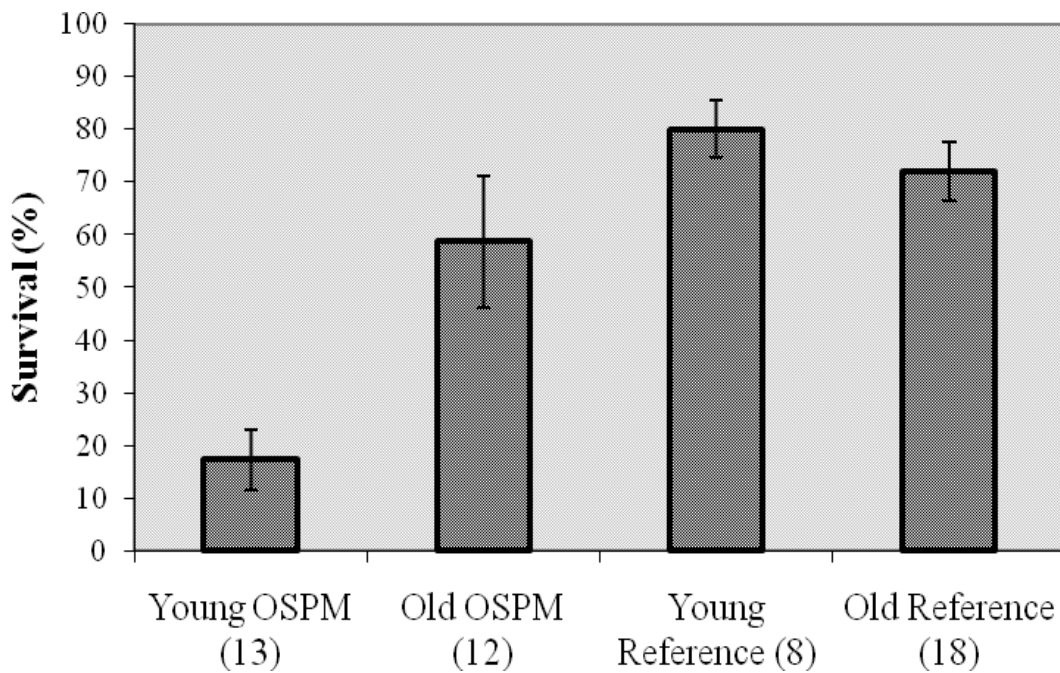


Figure 3.10 Year two mean survival ($\% \pm$ S.E.) per mesocosm of *Rana sylvatica* tadpoles raised in different wetlands grouped by the factors of age (young or old) and oil sand process-affected material (OSPM) status (OSPM containing or reference). The number of enclosures per type of wetland is shown in parentheses (n), differences were considered significant when $p < 0.05$. A significant difference due to a treatment interaction, as well as age and OSPM status effects alone was detected (two-way ANOVA, interaction $p < 0.001$, age $p = 0.004$, OSPM status $p < 0.001$).

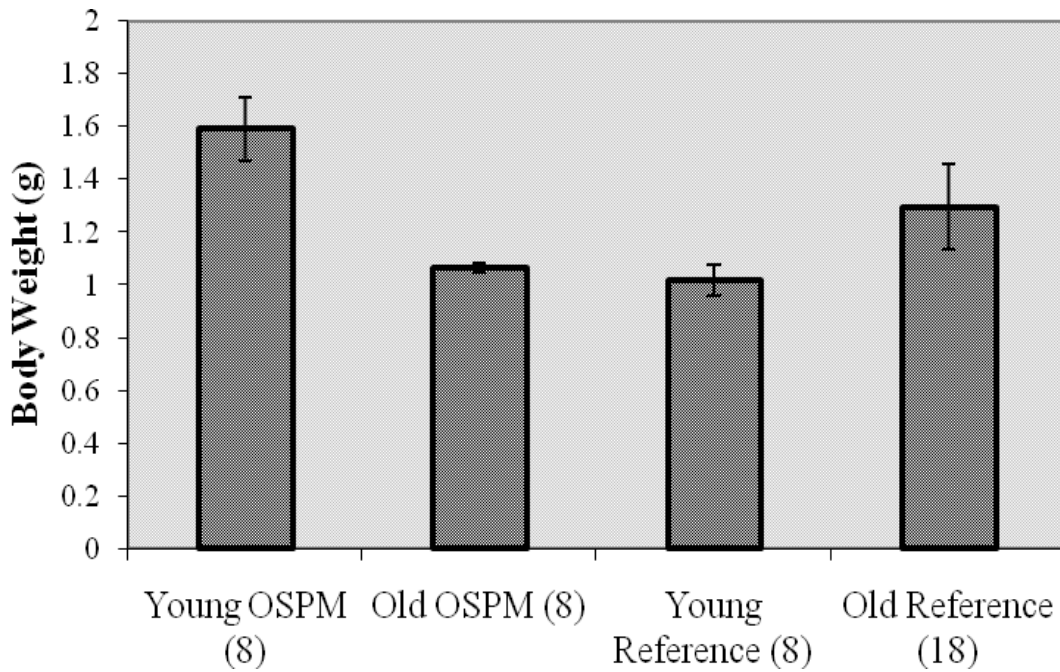


Figure 3.11 Year two mean body weight ($g \pm S.E.$) of *Rana sylvatica* tadpoles raised in different wetlands grouped by the factors of age (young or old) and oil sand process-affected material (OSPM) status (OSPM containing or reference). The number of enclosures per type of wetland is shown in parentheses (n), differences were considered significant when $p < 0.05$. A significant difference due to an interaction between treatment effects was detected, but not treatment effects alone (two-way ANOVA, interaction $p = 0.016$, age $p = 0.441$, OSPM status $p = 0.293$).

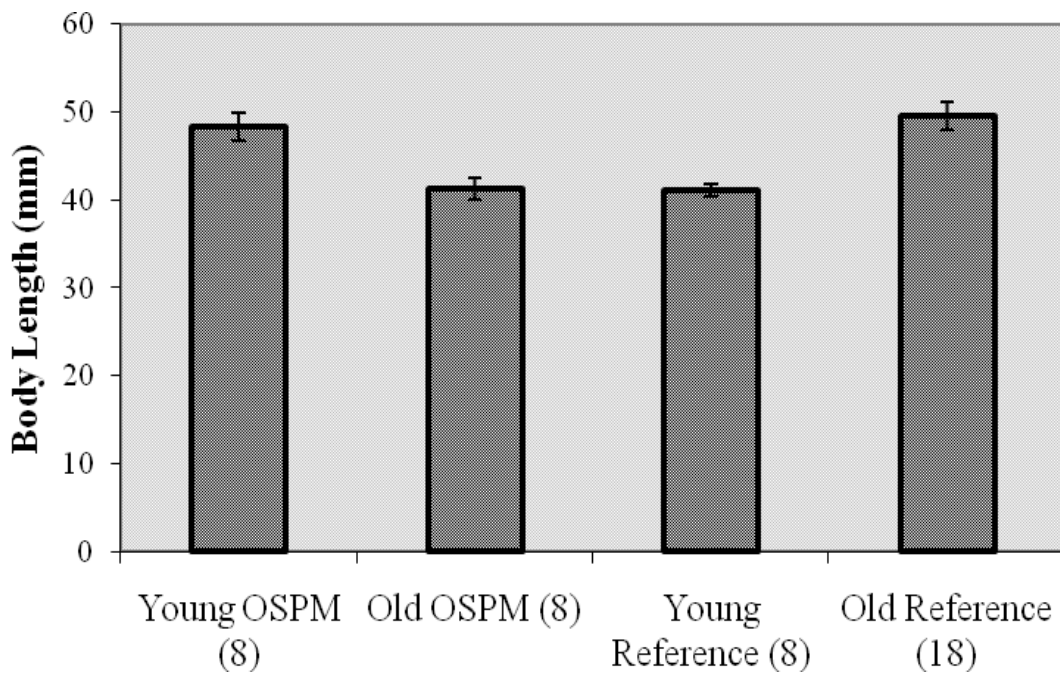


Figure 3.12 Year two mean body length (mm \pm S.E.) of *Rana sylvatica* tadpoles raised in different wetlands grouped by the factors of age (young or old) and oil sand process-affected material (OSPM) status (OSPM containing or reference). The number of enclosures per type of wetland is shown in parentheses (n), differences were considered significant when $p < 0.05$. A significant difference due to an interaction between treatment effects was detected, but not for treatment effects alone (two-way ANOVA, interaction $p < 0.001$, age $p = 0.920$, OSPM status $p = 0.890$).

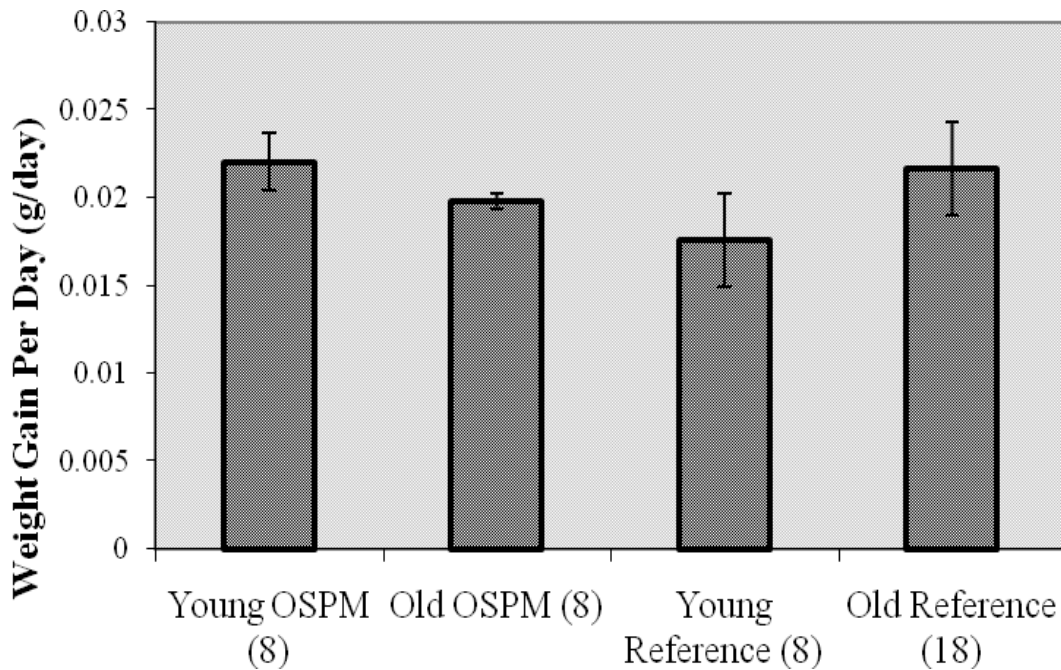


Figure 3.13 Year two mean weight gain per day (g/day \pm S.E.) of *Rana sylvatica* tadpoles raised in different wetlands grouped by the factors of age (young or old) and oil sand process-affected material (OSPM) status (OSPM containing or reference). The number of enclosures per type of wetland is shown in parentheses (n), differences were considered significant when $p < 0.05$. No significant difference due to an interaction between treatment effects or treatment effects alone was detected (two-way ANOVA, interaction $p = 0.226$, age $p = 0.721$, OSPM status $p = 0.620$).

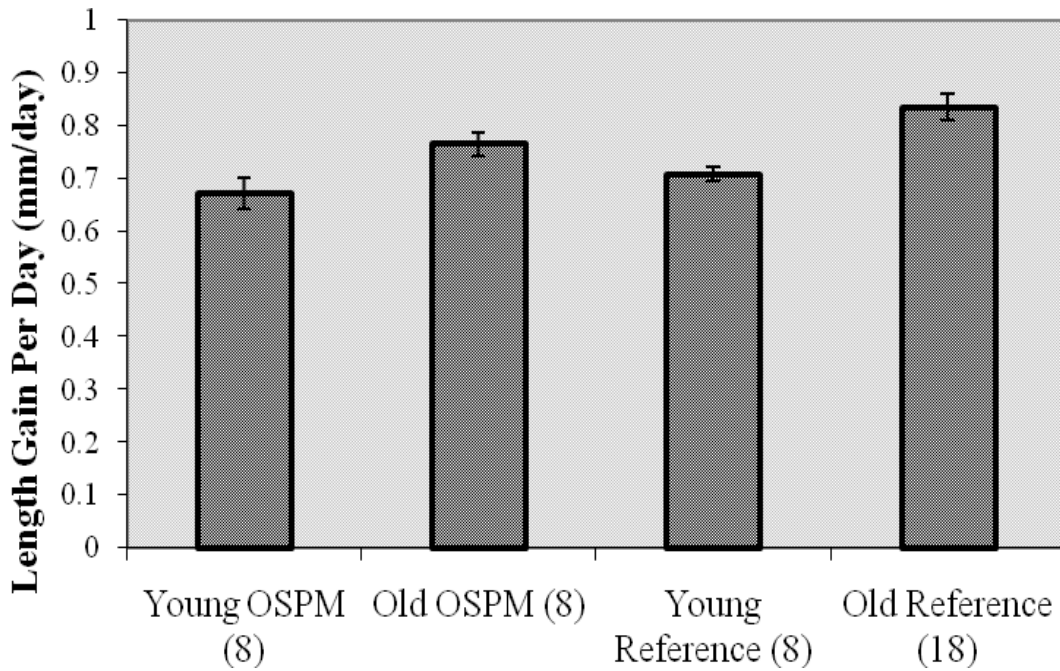


Figure 3.14 Year two length gain per day (mm/day \pm S.E.) of *Rana sylvatica* tadpoles raised in different wetlands grouped by the factors of age (young or old) and oil sand process-affected material (OSPM) status (OSPM containing or reference). The number of enclosures per type of wetland is shown in parentheses (n), differences were considered significant when $p < 0.05$. No significant difference due to an interaction between treatment effects or OSPM status was found, but a significant difference due to age was detected (two-way ANOVA, interaction $p = 0.733$, age $p < 0.001$, OSPM status $p = 0.137$).

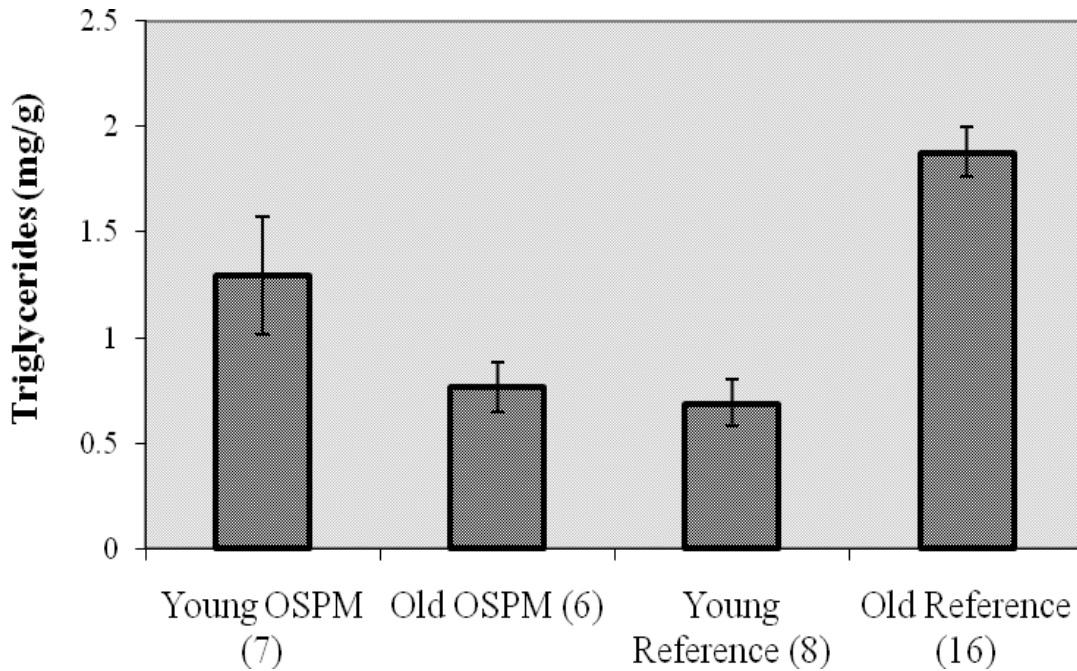


Figure 3.15 Year two mean whole-body triglyceride concentrations (mg/g \pm S.E.) of *Rana sylvatica* tadpoles raised in different wetlands grouped by the factors of age (young or old) and oil sand process-affected material (OSPM) status (OSPM containing or reference). The number of enclosures per type of wetland is shown in parentheses (n), differences were considered significant when $p < 0.05$. A significant difference due to an interaction between treatment effects was detected, but not treatment effects alone (two-way ANOVA, interaction $p = 0.041$, age $p = 0.739$, OSPM status $p = 0.711$).

3.4 Discussion

3.4.1 *Rana sylvatica* Survival

Rana sylvatica tadpoles raised in the 4m CT wetland, which is formed from young OSPM and receives input of fresh tailings water during the spring and summer, showed 100% mortality in the 2006 field season. In comparison, survival was not significantly different among wetlands containing no OSPM (Weir 1) and Natural Wetland, which contained older OSPM. Similarly in 2007, the lowest survival of tadpoles occurred in wetlands that were contained young OSPM. These results support those of Pollet and Bendell-Young (2000), who found tadpoles exposed to OSPM had low survival. The results from both years showed similar survival between old OSPM and young and old reference wetlands, which was much higher than the young OSPM sites in all cases. These results strongly suggest that toxicity from OSPM is diminishing over time. However, it is not known which components of the OSPM are causing the toxic effects. Many previous studies (Holowenko et al., 2001; Rogers et al., 2002; Quagraine et al., 2005) have shown that a large part of the toxicity from OSPM (mainly tailings water) is a result of naphthenic acids (NAs). NAs are a complex mixture of naturally occurring carboxylic acids that are of several different molecular weights, chain lengths, and may have several ring structures. Other possible sources of OSPM toxicity are from high levels of salinity (Renault et al., 1998; Nero et al., 2006), which has been shown to be toxic to amphibians (Rios-Lopez, 2008; Christy and Dickman, 2002). Polycyclic aromatic hydrocarbons (PAHs) may also contribute to more acute toxicity. Studies have shown that natural aging of OSPM-affected wetlands can reduce toxicity (Quagraine et al. 2005, references there in). Research has shown that microbes can degrade PAHs (Madill et al. 2001) and NAs (Herman et al., 1994; Lai et al., 1996), which is thought to be an important factor in the reduction of toxicity of OSPM over time (Madill et al. 2001). Toor et al. (2008) have shown that NAs degrade over time, with the majority of the NAs degraded are of lower molecular weight and have the lowest number of rings, which causes a reduction in acute toxicity of tailings water. However, some chronic toxicity remains that is thought to be due to remaining NAs with high molecular weight and more ring structures. The results of this study will be of considerable interest to oil sands companies since young tailings material is toxic to *R. sylvatica* larvae. This means that amphibians are unlikely to survive in

wetlands constructed from young OSPM, and those wetlands should somehow be made inaccessible to amphibians to avoid potentially severe detrimental effects on populations of native amphibians in the area. On the encouraging side, this work presents solid evidence, through low mortality rates among tadpoles caged both in more mature OSPM and reference wetlands, that amphibian populations may be able to form part of sustainable communities in wetlands containing more mature OSPM.

3.4.2 Tadpole Growth

In both years of this study, measures of growth (length, weight, and their change over time) were analyzed. Tadpole growth is a very important endpoint since it has been shown that survival of juvenile frogs is positively related to size at metamorphosis (Morey and Reznick, 2001). In 2006, growth of tadpoles in only one OSPM site could be compared with that of one reference site due to problems described in section 3.3.1.1., and growth was similar between sites. Any real differences from the ‘treatment’ effect of different maturity wetlands would have been obscured because of the 100% mortality of tadpoles raised in young OSPM-affected wetlands. Rather than acute and severe toxicity, the expectation was to see decreased growth due to the higher exposure of all tailings related contaminants.

In 2007, body size among tadpoles was higher in those raised in young OSPM-containing wetlands. This was an unexpected result because normally organisms dealing with toxic insult are expected to be smaller, expending more energy towards detoxification efforts, rather than growth (Berven, 1990; Morey and Reznick, 2001). I do not believe this is directly related to exposure to OSPM. Rather, the increased tadpole size is most likely due to lower density of tadpoles as a result of significantly higher mortality of tadpoles in these enclosures. This result is supported by Eaton et al. (2005), who found that increased densities of tadpoles resulted in smaller size at the time of metamorphosis. Many other researchers have also discussed similar correlations (Berven, 1990; Morey and Reznick, 2001). Food limitation is the mechanism that is most often used to explain density dependent effects on growth and survival (Kupferberg, 1997). However, we provided a steady, unlimited, diet of boiled lettuce to the tadpoles. Therefore, food limitation was not likely, but energy expenditure in acquiring food may have been different for

tadpoles in cages with high density versus cages with low tadpole density. Another possible reason for increased size in tadpoles raised in young OSPM-containing wetlands is that these tadpoles were significantly older at the time of metamorphic climax, meaning they had more time to grow. Metamorphosis and related hormonal endpoints will be further discussed in chapter four of this thesis. The observation that tadpoles cannot complete metamorphosis yet continue to grow has been reported several times (Allen, 1929; Shi, 2000). Because of amphibians' abilities to keep growing without completing metamorphosis (Boone, 2005) it was decided to analyze growth rate in terms of weight and length gain per day, which gives a more accurate representation of growth because it takes into consideration the difference in growth periods. Tadpole weight gain per day in 2007 was not different among wetland classes. Length gain per day was different but only due to effects of age of wetland, not OSPM status. This result was not readily explainable, but may be due to the high plasticity of tadpoles causing variation within populations as well as amongst different populations (Morey and Reznick, 2001). The results of daily growth suggest that tadpole growth is not being affected directly by contamination and more likely to be changing due to differing growth periods and/or lower tadpole densities in enclosures from young OSPM-containing wetlands.

3.4.3 Whole Tadpole Triglyceride Concentrations

Tadpole whole-body triglyceride concentrations were only measured in the second year (2007) of this study. Triglycerides are a main form of energy storage in many types of animals, and are a common biomarker used in ecotoxicology studies of many species (Owen et al., 2005), especially in fish (Adams, 1999; Bennett and Janz, 2007). Our goal was to use total body triglyceride concentration as a possible indicator of overall condition and energy storage or consumption due to exposure to OSPM. We found that triglyceride concentrations were different among wetlands; however, concentrations did not show an expected pattern with tadpoles exposed to the wetlands with highest contaminant levels having the lowest triglyceride concentrations. Tadpoles from young OSPM-containing wetlands had triglyceride concentrations higher than all other wetland classes except for old reference sites, which had the highest concentrations. This is best explained by the lower tadpole densities in wetlands that caused the highest mortality, similar to the growth effects, which may have resulted in less energy

expenditure due to less competition with other tadpoles. In young OSPM-containing wetlands tadpole survival was 41.5% lower than in old OSPM-containing wetlands, 62.6% lower than in young reference wetlands, and 54.7% lower than in old reference sites. This means that young OSPM-containing wetlands had 41.5%, 62.6%, and 54.7% lower tadpole densities than old OSPM-affected, young reference, and old reference wetlands, respectively. Another possible explanation for these unexpected results is the delay to metamorphosis in tadpoles raised in young OSPM wetlands, which could have allowed them more time to accumulate more stores of triglycerides. It has been postulated that greater stores of lipids would be advantageous to amphibians under starvations conditions. This could possibly allow for greater survival of amphibians that are larger, which generally have larger lipid stores (Scott et al., 2007).

3.5 Conclusions

Although morphometric endpoints in tadpoles did not show expected differences, less mature (young) OSPM does not look to be a good option for the formation of wetlands. Survival of tadpoles was generally poor in wetlands that were young and contained OSPM. In the longer term this may decrease the chances of sustainable amphibian populations from forming in wetlands constructed on oil sands mine sites. However, if the OSPM wetlands were allowed to mature for a sufficient period of time, which this study suggests is at least seven years, toxicity may decrease to a level comparable with reference wetlands as shown by the survival rates of tadpoles in wetlands formed with more mature OSPM. Wetlands formed from older OSPM may therefore be able to support native amphibian populations. It was beyond the range of this study to determine what caused the reduction in OSPM toxicity over time, but as discussed earlier it is known that some known toxic constituents of OSPM, such as NAs and PAHs, can be degraded by microbes, which would reduce toxicity of OSPM-containing wetlands. For a more complete picture of the ability of wetlands formed with OSPM to support amphibian populations, reproductive assays should be completed in a field type study. As well, juvenile and adult stage frogs should be investigated.

CHAPTER 4: TADPOLE ENDOCRINE FUNCTION, METAMORPHOSIS, AND HEPATIC ENZYME ACTIVITY

4.1 Introduction

In the past few decades, amphibians have been increasingly studied as indicators of environmental degradation and contamination from various sources. Pesticides (Cooke, 1972; Diana et al., 2000; Fort et al., 2004), estrogenic compounds (Hogan et al., 2006), and contaminants from the petrochemical chemical industry (Huang et al., 2007; Pollet and Bendell-Young, 2000) have all presented causes for concern for which amphibians have been used as an indicator of toxicity. The oil sands of northern Alberta are another place where amphibians could be useful as an environmental indicator species. Currently, the oil sands industry is rapidly expanding and both solid and liquid tailings materials, called oil sand process-affected materials (OSPM), are accumulating in very large quantities (Madill et al. 2001; Quagraine et al., 2005). Government regulation requires liquid tailings to be stored in large dyked tailings ponds until reclamation can take place (Crowe et al., 2001). The “wet landscape” approach to reclamation of OSPM, which involves creating wetlands from fluid tailings in mined-out pits, is one method for the reclamation of OSPM (Madill et al. 2001). To be viable, wetlands constructed with OSPM must be able to support a functioning ecosystem. However, OSPM has proven toxic to many life forms (Clemente and Fedorak, 2005; Franklin et al., 2002; Leung et al., 2003; Rogers et al., 2002) and it is unknown if formation of functioning ecosystems is possible. Toxicity from OSPM appears to decrease over time (Lai et al., 1996); therefore, older wetlands formed with OSPM are expected to be more ecologically viable.

As an important part of native ecosystem function in the Athabasca Oil Sands Region (Figure 1.1) amphibians must be able to live sustainably in oil sands constructed wetlands if they

are to be considered ecologically viable. Amphibians are sensitive to environmental contamination (Cooke, 1981; Gupta et al., 2007) and have a unique transition between two distinct life stages (metamorphosis) that can be used as an important bioindicator. These factors make them ideal indicator species for use in testing the ecological viability of constructed wetlands. Metamorphosis must be completed if amphibians such as *Rana sylvatica* are to reproduce successfully. Amphibian metamorphosis has been extensively studied and is hormonally regulated, with thyroid hormone (TH) playing the most important role (Galton, 1992; Shi, 2000; Buchholz et al., 2007; Tata, 2006; Fort et al., 2007). Thyroid hormones are present in two forms, with triiodothyronine (T3) having the greatest biological activity. The other hormone, thyroxine (T4) (Denver, 1998), has to be converted to T3 to function actively (Shi, 2000). Thyroxine produced by the thyroid gland is converted to T3 by deiodinase enzymes in the thyroid and peripheral tissues such as the liver (Denver, 1998; Huang et al., 2001; Cai and Brown, 2004). As a result, both T3 and T4 concentrations need to be measured to understand thyroid status during amphibian development (Fort et al., 2007) and can be used as markers of exposure to contaminants. Contaminant interference with thyroid hormone production or its action on target tissues may have negative effects on metamorphosis, which would preclude establishment of healthy *R. sylvatica* populations in oil sands constructed wetlands.

In this study, amphibian metamorphosis and thyroid hormone status are used as indicators of the ecological sustainability of wetlands formed with OSPM. 7-ethoxyresorufin-o-dealkylase (EROD) activity was also measured as a biomarker of exposure to potential toxicants found in wetlands formed with OSPM. The EROD activity is a well established biomarker, since EROD activity increases with increasing contaminant exposure (Whyte et al., 2000). The EROD assay has been previously used in birds and mammals exposed to contaminants from the oil sands region (Gentes, 2006). Sensitivity of amphibians to contaminants, their unique development, and the fact they are indigenous to the oil sands region were my reasons for choosing *R. sylvatica* larvae as a test organism for the investigation of toxicity of wetlands formed with OSPM.

4.2 Materials and Methods

4.2.1 Experimental Design

See section 3.2.1

4.2.2 Time To Metamorphosis

Time to metamorphosis was defined as the number of days it took tadpoles to reach metamorphic climax [Gosner stage 42 (Gosner, 1960)], with day zero being the day early pre-metamorphic tadpoles were placed in the enclosures. The study was started on May 9, 2006 and May 11, 2007.

4.2.3 Thyroid Hormones

4.2.3.1 Whole-Body Thyroid Hormone Extraction

See section 3.2.6 for details, since triglyceride and thyroid hormone extractions follow the same technique, initially.

4.2.3.2 Thyroid Hormone Quantification

Thyroid hormones were quantified in an extract that was produced from single whole tadpoles. Commercially available competitive enzyme immunoassay kits were used for quantifying the hormones. Individual kits specific for Triiodothyronine (T3) and Thyroxine (T4) were used. In year one, kits were obtained from MP Biomedicals, Orangeburg, NY, USA (T3 – 07BC-1005 and T4 07BC-1007). In year two, to be consistent with another researcher conducting parallel research, very similar kits were acquired from BioQuant Inc., San Diego, CA, USA (T3 - BQ043T and T4 -BQ044T).

4.2.4 Liver Somatic Index (LSI)

The wet weights of tadpole livers were measured at the time of necropsy. The liver somatic index (LSI) of each tadpole was then calculated by the following formula:

$$\text{LSI} = \text{Liver weight} / (\text{Body weight} - \text{Liver weight}) \quad (4.1)$$

The LSI gives a measure of each frog's liver size in relation to its body weight.

4.2.5 Hepatic Detoxification Enzyme Activity

4.2.5.1 Liver Microsome Production

Microsome production in this study used an adaptation of the methods described in Papp et al. (2005). Tadpole livers were removed and snap frozen in liquid nitrogen within five minutes of euthanasia, by placing tadpoles in a 500 mg/L solution of MS222 (ethyl-*m*-aminobenzoate methanesulfonate salt, MP Biomedicals, Solon, OH, USA) dissolved in water. The livers of five wood frog larvae from each enclosure were pooled and homogenized. If five livers were not available due to poor survival, as few as three livers were pooled. If fewer than five livers were used, this was adjusted in calculating the final the amount of resorufin formed per mg of protein per minute. All chemicals for preparation of buffers were acquired from Sigma Aldrich, (Oakville, ON, Canada). During the entire process, tools, buffers, and samples were kept cool by working on crushed ice.

While still frozen, pooled livers forming each sample were removed from cryovials and placed in 2ml glass homogenization tubes (Wheaton Science Products, Millville, NJ, USA). One milliliter of HEPES (4-2-hydroxyethyl) -1-piperazineethanesulfonic acid) homogenization buffer (0.02 M HEPES: 0.15 M KCL; pH 7.5) was then pipetted into the glass tube and the sample homogenized by 20 – 30 up and down strokes of a hand homogenizer. The resulting homogenate was poured into a 36ml centrifuge tube (VWR International, Mississauga, ON, Canada) and the glass homogenization tube was then rinsed with homogenization buffer until all particles were removed. The rinsate was also added to the centrifuge tube and the final volume was made to 35 ml with homogenization buffer. Six samples were then centrifuged (Sorvall WX90, Thermo Scientific, Waltham, MA, USA) for 20 minutes at 10 000 g at a temperature of 4 °C. The centrifuge rotor was pre-cooled in a refrigerator prior to use. After centrifugation, the supernatant was carefully poured into a clean centrifuge tube, preventing the transfer of fat, which formed a layer on the surface. Fat was removed before pouring by quickly inserting and removing a plastic micropipette tip, to which the fat adhered. The volume in the new tubes was again made to 35 ml with homogenization buffer. These samples were centrifuged for one hour at 100 000 g at 4 °C. After this final centrifugation, supernatants were carefully poured off and the inside of the tubes

were dried using tissue paper, being careful not to touch the pellet at the bottom. The resulting microsome pellets were then resuspended in 600 μ l of buffer [0.05 M tris (2-amino-2-hydroxymethyl-1, 3-propanediol); 1mM EDTA (ethylenediamine tetraacetic acid); 20% v/v glycerol]] and stored as 300 μ l aliquots in cryovials at - 80°C until further analysis.

4.2.5.2 Ethoxyresorufin -o-Deethylase Activity (EROD)

The EROD activity was measured using an adaptation of the method published by Papp et al. (2005) and Gentes et al. (2006). Enzyme activity was quantified by measuring the production of the fluorescent compound, resorufin (Sigma Aldrich, Oakville, ON, Canada). Since EROD was the only enzyme being measured, the only required substrate was 7-ethoxy resorufin (7-ER) (Sigma Aldrich, Oakville, ON, Canada), which was prepared in methanol at a concentration of 207 mM and stored at -20 °C. At the time of the reaction the 207 mM 7-ER was diluted to 11.7 mM with phosphate buffer (sodium phosphate 0.05 M, pH 8.0). The reaction was carried out in a 96-well, flat bottom, microtitre plate. All samples were run in triplicate, except in cases of insufficient sample when duplicates were run, with a specific blank used for all cases. The specific blank contained all reagents except for microsomes and was used to separate fluorescence produced by resorufin from that of other reagents. The final volume in each well was 180 μ l, which consisted of the following (added in the order mentioned): for sample wells - 40 μ l phosphate buffer, 80 μ l microsomes (see section 4.2.2.2), and 30 μ l 7-ER working solution. This mixture was then incubated for ten minutes at room temperature. After incubation, 30 μ l of NADPH (2 mg/ml in phosphate buffer) was added to start the enzymatic reaction. The reaction was run for 40 minutes then stopped by adding 60 μ l of acetonitrile, which contained 600 μ g/ml fluorescamine used for protein quantification. The fluorescence of the resorufin product was then measured by excitation at 530 nm and emission at 590 nm using a microplate fluorometer (Dynex Technologies, Chantilly, VA, USA).

Resorufin formation was quantified by subtracting the specific blank's fluorescence from the mean of the triplicate sample. This value was then compared to a standard curve generated from stock resorufin salt (Sigma Aldrich, Oakville, ON, Canada) solution. The EROD enzyme activity was reported as the amount of resorufin formed per milligram of protein per minute.

4.2.5.4 Total Protein Quantification

Protein content of each sample was quantified using a fluorescamine method adapted from Kennedy and Jones (1994) and Olsgard (2007). In this method, total protein in each sample is determined at the same time in the same well as production of resorufin by the enzyme ethoxy resorufin-O-deethylase (CYP450-1A). A bovine serum albumin (BSA) standard curve was developed to quantify the concentration of protein in each sample well. This assay was carried out in a black, flat bottomed, 96-well microplates that was read using a microtitre plate fluorometer (Dynex Technologies, Chantilly, VA, USA).

To stop the enzyme reaction by precipitating the enzymes, 60 μ l of a 0.6 mg/ml solution of fluorescamine in acetonitrile was added to each well. The addition of the fluorescamine (Sigma Aldrich, Oakville, ON, Canada) allowed for the measurement of total protein simultaneously with the enzyme activity. Immediately after stopping the enzyme reaction and measuring the EROD activity (see section 4.2.2.3), fluorescence of each sample was read at 390 nm excitation and 460 nm emission on the previously mentioned microplate fluorometer. The reading of fluorescence was then compared with the BSA standard curve generated earlier and presented as total amount of protein per ml of microsomes.

4.2.6 Statistics

In year two (2007), each wetland was not compared to each of the other wetlands separately. Based on the knowledge that as OSPM-containing wetlands get older there is a reduction in toxicity, due to bioremediation, I grouped wetlands according to their age (either young or old) and OSPM status (OSPM or no OSPM). I assume that wetlands of the same group (young OSPM-containing, old OSPM-containing, young reference, old reference) have similar characteristics, such as concentration of NAs. This method of grouping allowed me to complete the two-way ANOVA described below to test the hypothesis that there will be an interaction between age and OSPM status of wetlands. We predict that older OSPM-containing wetlands will be similar to reference wetlands and much less toxic than young OSPM-containing wetlands.

Statistics were carried out using SPSS statistical software package (Version 16.0.1, SPSS Inc., Chicago, IL, USA). Differences were considered to be significant when $p \leq 0.05$. All data were tested for normality and equality of variances assumptions using the Shapiro-Wilks test and Levene's test, respectively. In year one, the experimental design was planned for a two-way ANOVA. However, due to loss of a reference site, a one-way ANOVA was completed to determine if there were differences in tadpole time to metamorphosis, thyroid hormone status, liver weight and LSI, and EROD activity, among the three remaining experimental sites. If assumptions of normality or homogeneity of variances were violated, data presented as percentages were arcsin square root transformed, and regular, numeric data were \log_{10} transformed. If assumptions were met after transformation, an ANOVA was performed on the transformed data. If assumptions could not be met the non-parametric Kruskal-Wallis test was performed. In year two, if assumptions were met, a two-way ANOVA was completed on the same variables as in year one. Age of wetlands (young or old) and OSPM status (OSPM or no OSPM) of the wetlands were the two factors in the two-way ANOVA. If assumptions of normality and homogeneity of variances were violated the same method for transforming data as in year one was taken. After transformation, if assumptions were met a two-way ANOVA was performed. If assumptions were not met, the Scheirer-Ray-Hare extension of the Kruskal-Wallis [a non-parametric variation of a two-way ANOVA test (Sokal and Rohlf, 2003)], was performed as is in Rickwood et al. (2008).

4.3 Results

4.3.1 Year One (2006)

4.3.1.1 Time to Metamorphosis

Tadpoles raised in a young OSPM-containing site were not included in this analysis because they all died early in the study. Data from two of three trenches (six of nine enclosures) were not included in the analysis because water levels were reduced, which can cause metamorphosis to occur earlier than it would normally (Shi, 2000). This effect was observed in the two trenches with reduced water levels, with metamorphosis of tadpoles being completed at a much smaller than normal size soon after reduction of water levels. The tadpoles in remaining

trenches that were included in the analysis showed no difference in time to metamorphosis in year one (ANOVA, $p = 0.275$) ; (Figure 4.1).

4.3.1.2 Whole-body Thyroid Hormone Concentration

Only triiodothyronine (T3) was quantified in the entire bodies of tadpoles because sample size was insufficient. Whole-body T3 concentrations in *R. sylvatica* tadpole raised in the OSPM-containing wetland were not significantly different from those raised in the reference wetland (ANOVA, $p = 0.310$); (Figure 4.2).

4.3.1.3 Resorufin Production

The production of resorufin (EROD assay) was not complete due to lack of liver tissue.

4.3.2 Year Two (2007)

4.3.2.1 Time to Metamorphosis

Tadpoles raised in young OSPM-containing sites took a significantly longer time to complete metamorphosis than tadpoles in any of the other classes of wetlands. A significant difference due to an interaction between treatment effects, as well as the treatment effect of age alone was detected, but not due to OSPM status alone (two-way ANOVA, interaction $p < 0.001$, age $p < 0.001$, OSPM status $p = 0.566$); (Figure 4.3).

4.3.2.2 Whole-body Thyroid Hormone Concentration

Similar to year one, whole-body T3 concentrations in tadpoles did not differ among wetlands. No significant difference due to an interaction between treatment effects or treatment effects alone was detected (two-way ANOVA, interaction $p = 0.571$, age $p = 0.133$, OSPM status $p = 0.474$); (Figure 4.4). However, in year two, tadpole whole-body concentrations of T4 did differ among wetlands. A significant difference due to an interaction between treatment effects was detected, but not treatment effects alone (two-way ANOVA, interaction $p = 0.011$, age $p = 0.782$, OSPM status $p = 0.281$); (Figure 4.5). Tadpoles raised in young OSPM-containing wetlands had the highest concentration of T4. The lowest concentration of T4 was found in tadpoles raised in young reference sites. Also for thyroid hormone ratio (T3:T4), a significant

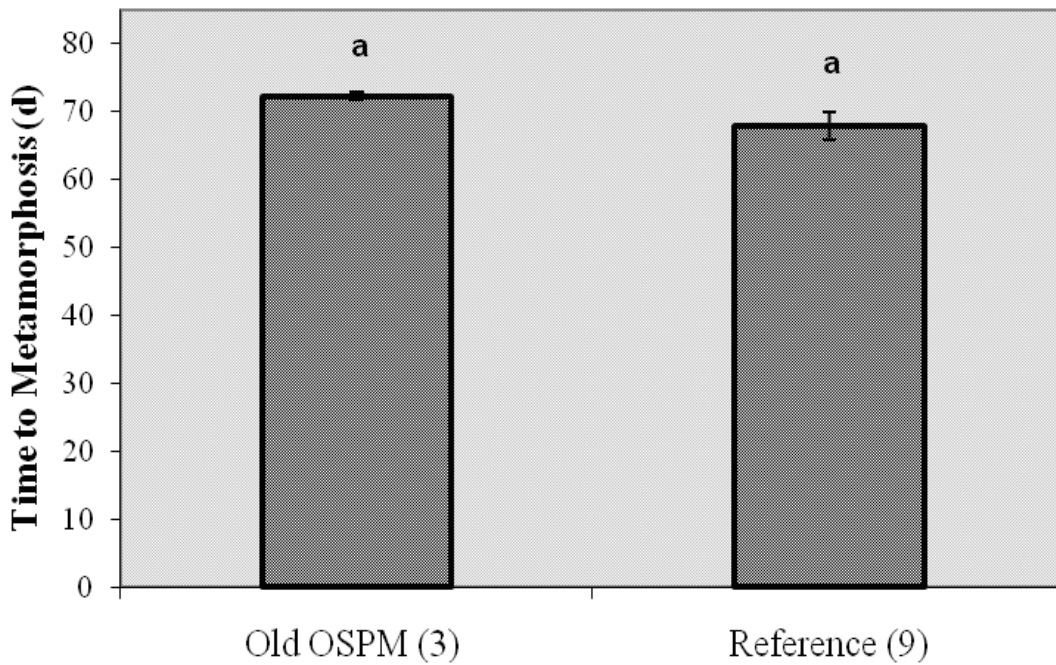


Figure 4.1 Year one mean time until metamorphosis ($d \pm S.E.$) of *Rana sylvatica* tadpoles raised in a reference wetland and an old oil sands process-affected material (OSPM)-containing wetland. The number of enclosures per type of wetland is shown in parentheses (n), differences were considered significant when $p < 0.05$. No significant differences were detected (ANOVA, $p = 0.275$).

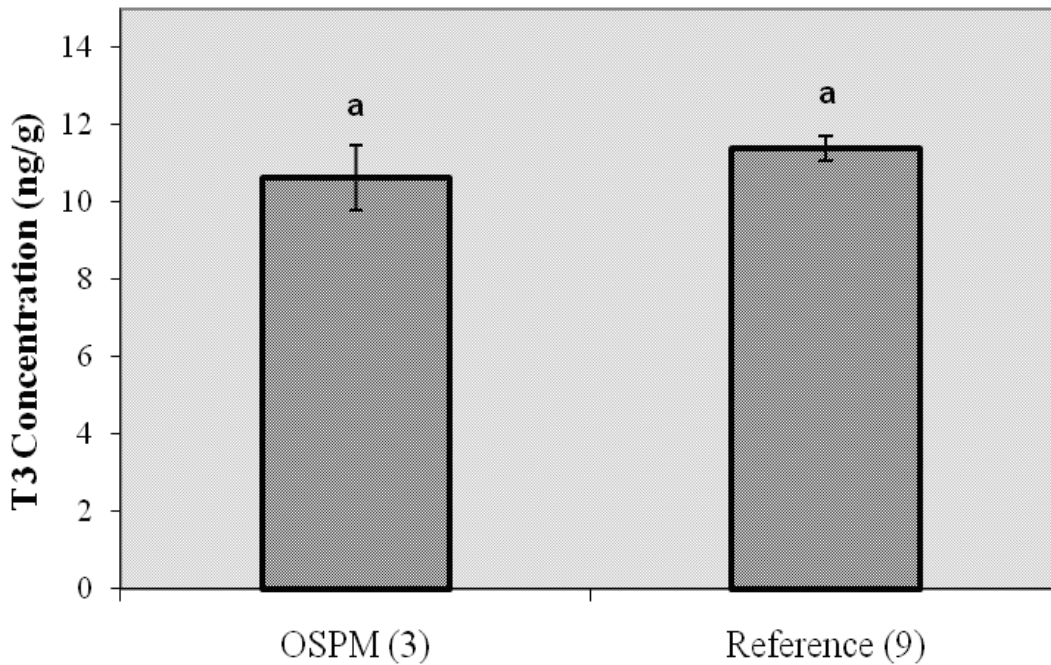


Figure 4.2 Year one mean whole-body triiodothyronine (T3) concentration (ng/g \pm S.E.) of *Rana sylvatica* tadpoles raised in a reference wetland and an old oil sands process-affected material (OSPM)-containing wetland. The number of enclosures per type of wetland is shown in parentheses (n), differences were considered significant when $p < 0.05$. No significant differences were detected (ANOVA, $p = 0.310$).

difference due to an interaction between treatment effects was detected, but not treatment effects alone (two-way ANOVA, interaction $p = 0.006$, age $p = 0.314$, OSPM status $p = 0.064$); (Figure 4.6).

4.3.2.3 Liver Weight and Liver Somatic Index (LSI)

As in year one, there was no difference in the weight of the livers of tadpoles that were raised in different classes of wetlands. No significant differences due to an interaction between treatment effects or treatment effects alone were detected (two-way ANOVA, interaction $p = 0.794$, age $p = 0.592$, OSPM status $p = 0.233$); (Figure 4.7). For LSI, a significant difference due to an interaction between treatment effects, as well as the treatment effect of OSPM status alone, was detected, but not due to age alone (two-way ANOVA, interaction $p < 0.001$, age $p = 0.448$, OSPM status $p < 0.001$); (Figure 4.8).

4.3.2.4 Resorufin Production (EROD activity)

The production of resorufin, and therefore EROD activity, was much higher in young OSPM-containing wetlands when compared to all other classes of wetlands. A significant difference due to an interaction between treatment effects was detected, but not treatment effects alone (two-way ANOVA, interaction $p = 0.002$, age $p = 0.126$, OSPM status $p = 0.437$); (Figure 4.9).

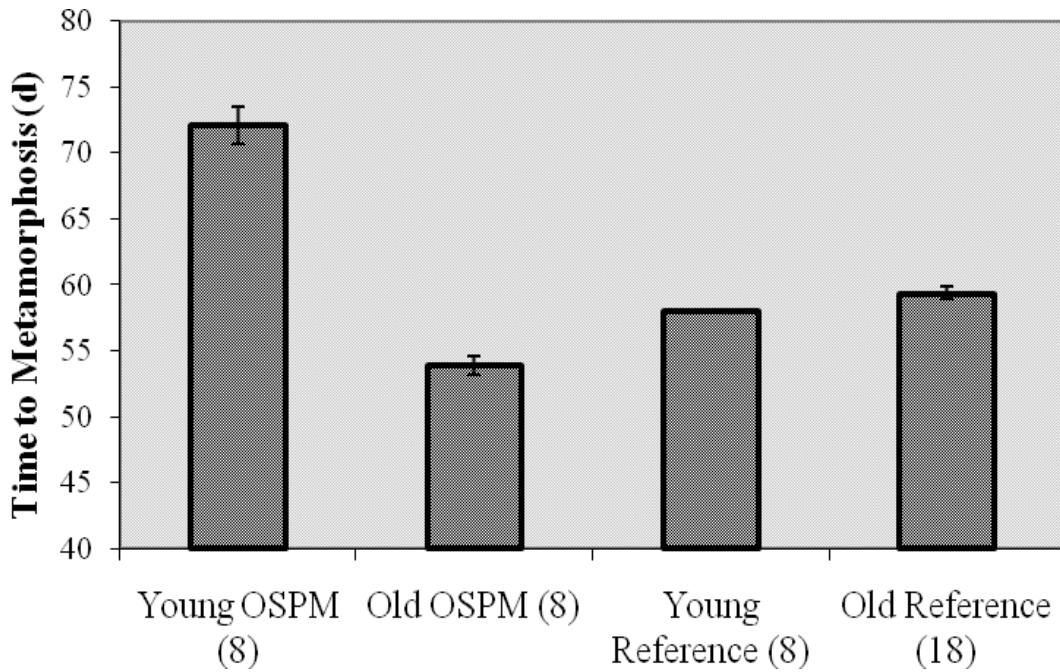


Figure 4.3 Year two mean time to metamorphosis ($d \pm S.E.$) of *Rana sylvatica* tadpoles raised in different wetlands grouped by the factors of age (young or old) and oil sand process-affected material (OSPM) status (OSPM containing or reference). The number of enclosures per type of wetland is shown in parentheses (n), differences were considered significant when $p < 0.05$. A significant difference due to an interaction between treatment effects, as well as the treatment effect of age alone was detected, but not due to OSPM status alone (two-way ANOVA, interaction $p < 0.001$, age $p < 0.001$, OSPM status $p = 0.566$).

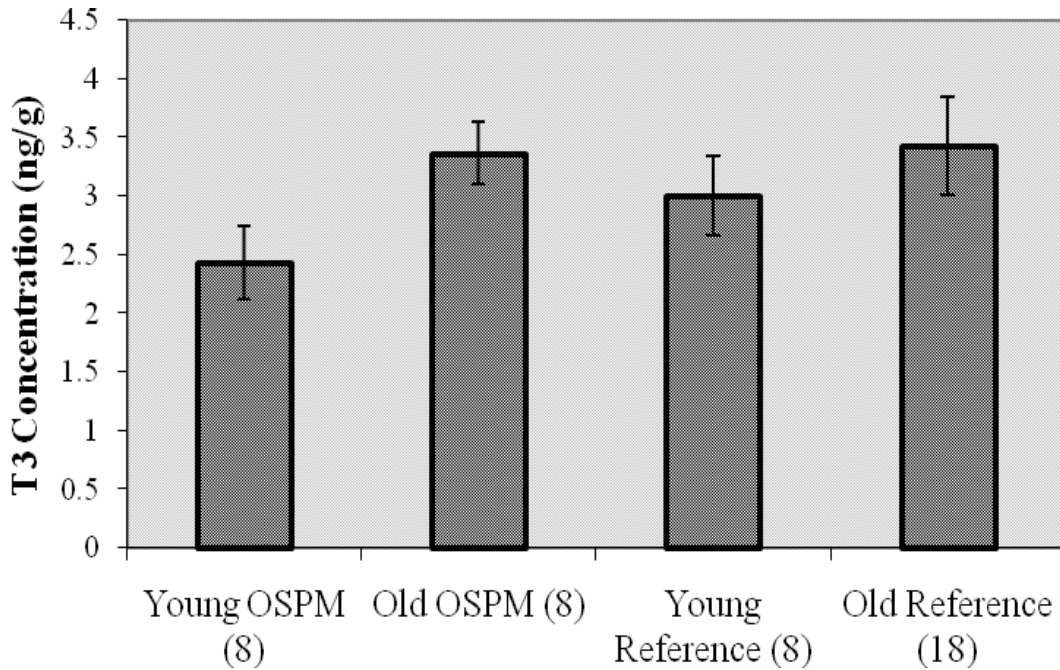


Figure 4.4 Year two mean whole-body triiodothyronine (T3) (ng/g \pm S.E.) concentration of *Rana sylvatica* tadpoles raised in different wetlands grouped by the factors of age (young or old) and oil sand process-affected material (OSPM) status (OSPM containing or reference). The number of enclosures per type of wetland is shown in parentheses (n), differences were considered significant when $p < 0.05$. No significant difference due to an interaction between treatment effects or treatment effects alone was detected (two-way ANOVA, interaction $p = 0.571$, age $p = 0.133$, OSPM status $p = 0.474$).

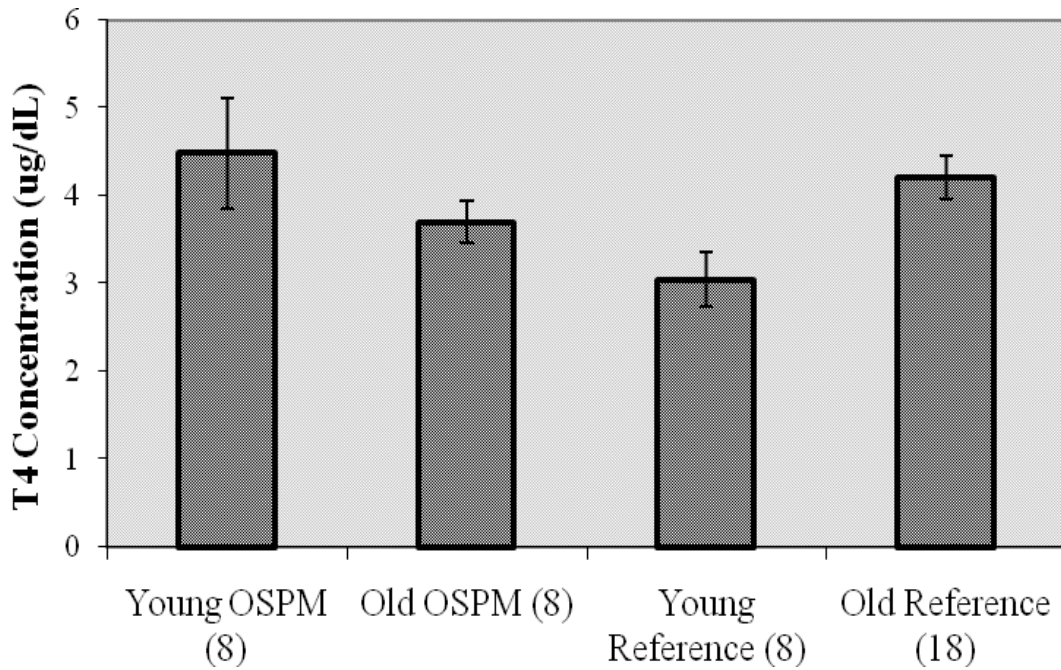


Figure 4.5 Year two mean whole-body thyroxine (T4) concentration (ug/dL \pm S.E.) of *Rana sylvatica* tadpoles raised in different wetlands grouped by the factors of age (young or old) and oil sand process-affected material (OSPM) status (OSPM containing or reference). The number of enclosures per type of wetland is shown in parentheses (n), differences were considered significant when $p < 0.05$. A significant difference due to an interaction between treatment effects was detected, but not treatment effects alone (two-way ANOVA, interaction $p = 0.011$, age $p = 0.782$, OSPM status $p = 0.281$).

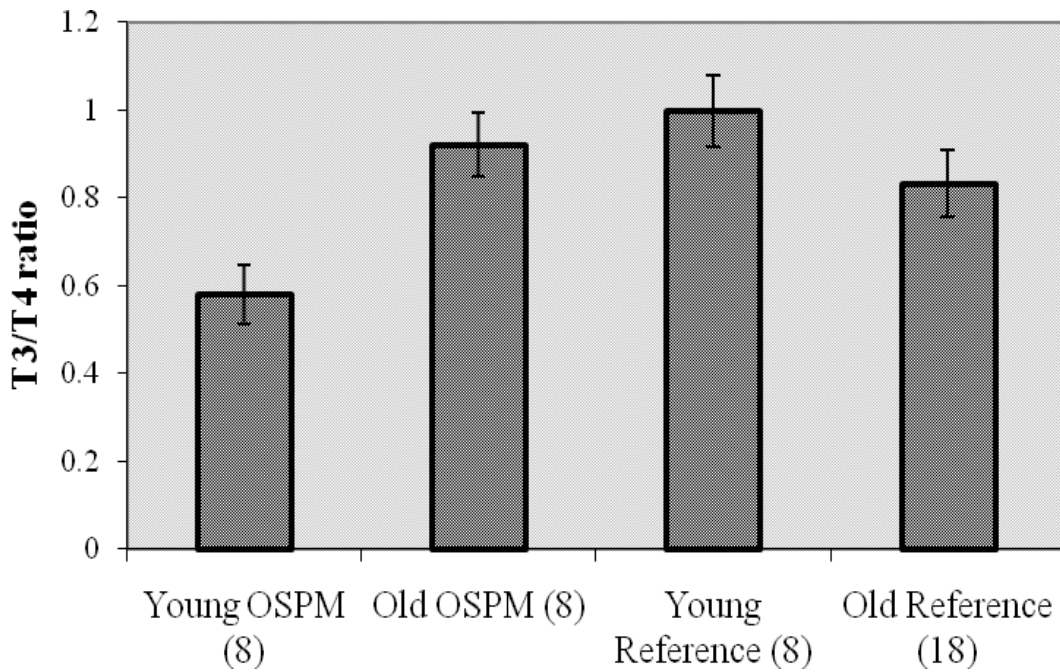


Figure 4.6 Year two mean thyroid hormone ratio ($T3/T4 \pm S.E.$) of *Rana sylvatica* tadpoles raised in different wetlands grouped by the factors of age (young or old) and oil sand process-affected material (OSPM) status (OSPM containing or reference). The number of enclosures per type of wetland is shown in parentheses (n), differences were considered significant when $p < 0.05$. A significant difference due to an interaction between treatment effects was detected, but not treatment effects alone (two-way ANOVA, interaction $p = 0.006$, age $p = 0.314$, OSPM status $p = 0.064$).

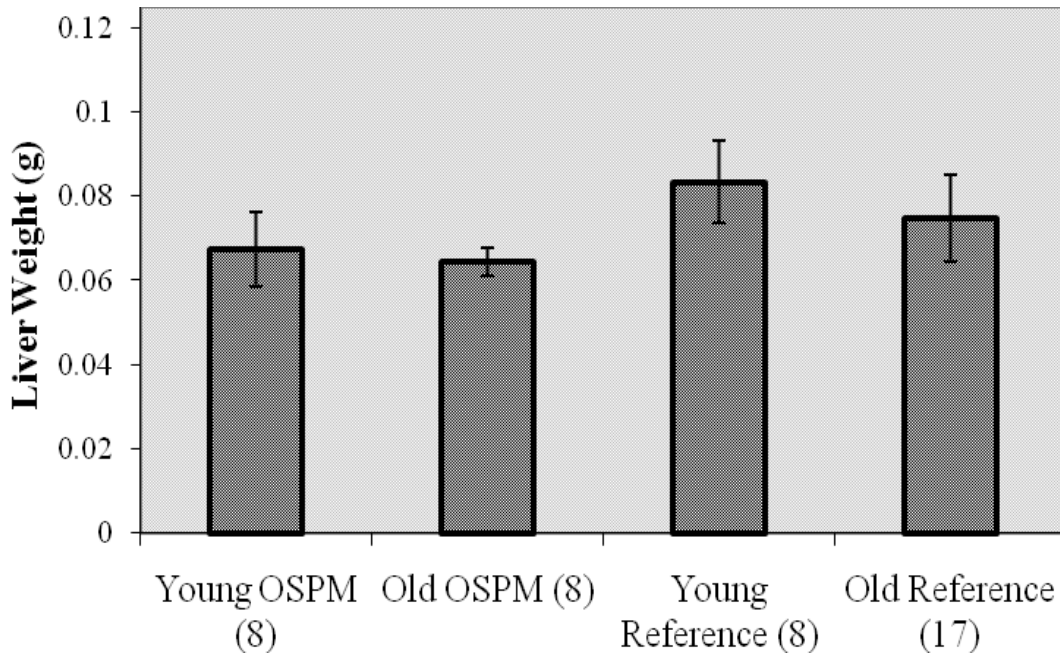


Figure 4.7 Year two mean liver weight ($g \pm S.E.$) of *Rana sylvatica* tadpoles raised in different wetlands grouped by the factors of age (young or old) and oil sand process-affected material (OSPM) status (OSPM containing or reference). The number of enclosures per type of wetland is shown in parentheses (n), differences were considered significant when $p < 0.05$. No significant difference due to an interaction between treatment effects or treatment effects alone were detected (two-way ANOVA, interaction $p = 0.794$, age $p = 0.592$, OSPM status $p = 0.233$).

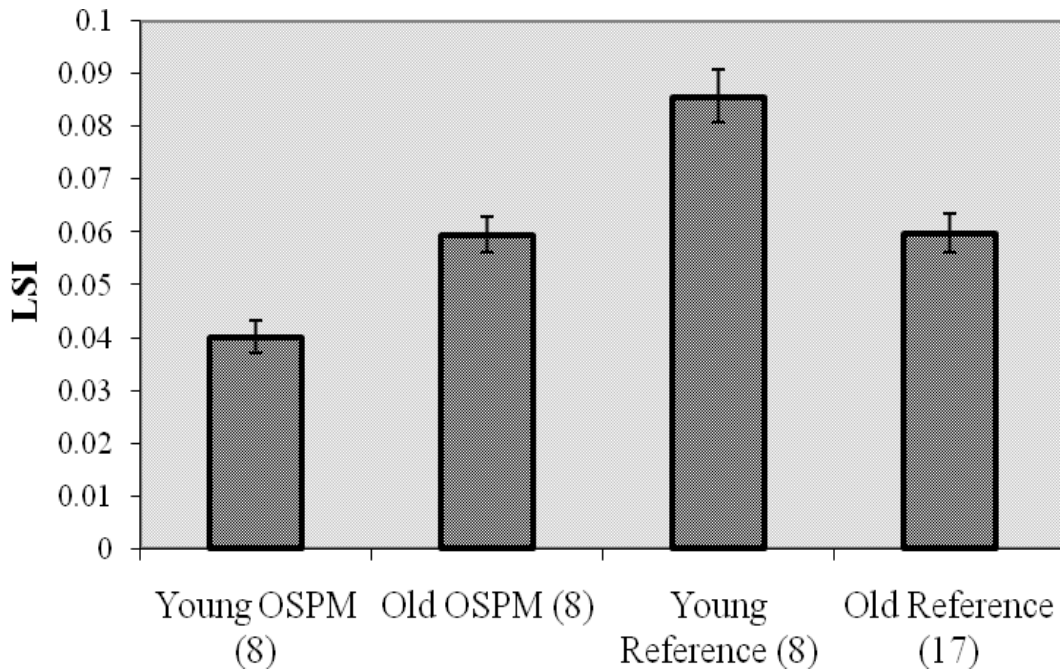


Figure 4.8 Year two mean liver somatic index (LSI) ($LSI \pm S.E.$) of *Rana sylvatica* tadpoles raised in different wetlands grouped by the factors of age (young or old) and oil sand process-affected material (OSPM) status (OSPM containing or reference). The number of enclosures per type of wetland is shown in parentheses (n), differences were considered significant when $p < 0.05$. A significant difference due to an interaction between treatment effects, as well as the treatment effect of OSPM status alone was detected, but not due to age alone (two-way ANOVA, interaction $p < 0.001$, age $p = 0.448$, OSPM status $p < 0.001$).

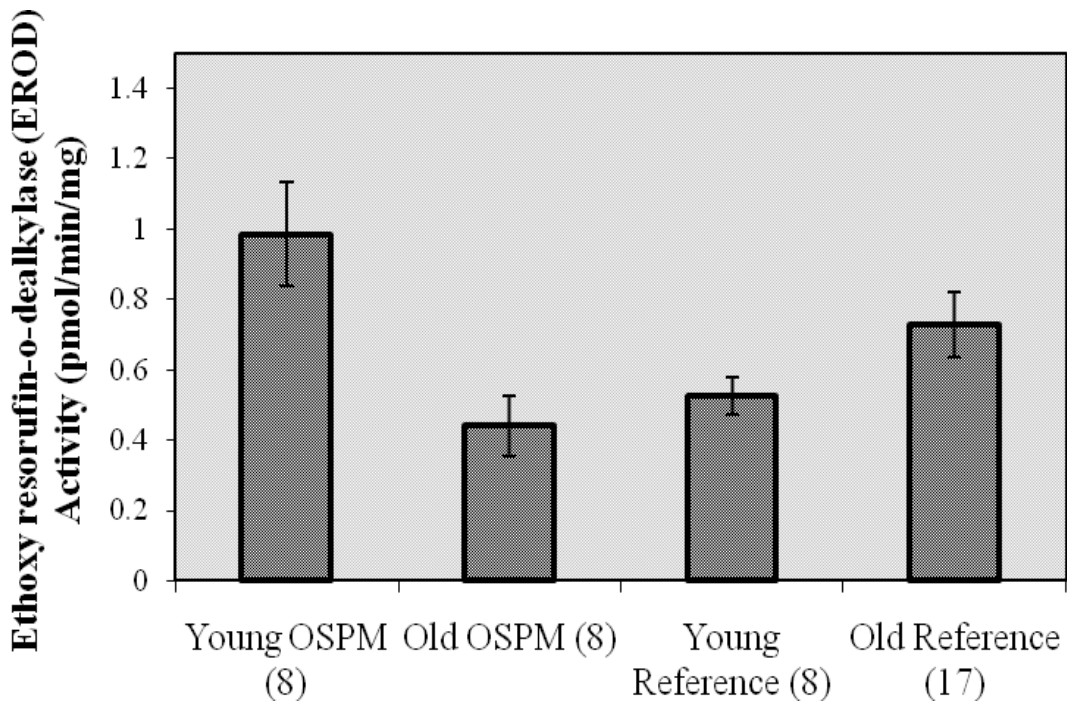


Figure 4.9 Year two mean ethoxyresorufin-o-dealkylase (EROD) activity (pmol/min/mg \pm S.E) in *Rana sylvatica* tadpoles raised in different wetlands grouped by the factors of age (young or old) and oil sand process-affected material (OSPM) status (OSPM containing or reference). The number of enclosures per type of wetland is shown in parentheses (n), differences were considered significant when $p < 0.05$. A significant difference due to an interaction between treatment effects was detected, but not treatment effects alone (two-way ANOVA, interaction $p = 0.002$, age $p = 0.126$, OSPM status $p = 0.437$).

4.4 Discussion

4.4.1 Time to Metamorphosis

In year two the results of this study were similar to those of Pollet and Bendell-Young (2000). Tadpoles (*Bufo boreas* in the case of Pollet and Bendell-Young) raised in reference wetlands completed metamorphosis faster than those raised in wetlands containing by OSPM. However, the present study classified wetlands by age (young or old) and OSPM status (OSPM-containing or reference). Only tadpoles raised in young, assumed to be more toxic, OSPM-containing wetlands showed a delay in metamorphosis. This is consistent with the idea that as OSPM-affected wetlands age, toxicity of the wetland is reduced due to natural biochemical processes such as biodegradation (Lai et al., 1996) and photolysis of contaminants such as naphthenic acids and PAHs. This delay indicates that increased exposure to contaminants in young OSPM-containing wetlands causes a physiological stress in tadpoles that delays metamorphosis. Due to the complex mixture of contaminants it is not possible to identify any specific contaminant as the cause of the delayed metamorphosis.

Late metamorphosis negatively affects amphibians. Tadpoles completing metamorphosis later have decreased survival when they reach the young adult stage (Berven, 1990; Morey and Reznick, 2001). As well, tadpoles that complete metamorphosis earlier have the advantage of being larger once they reached the juvenile frog stage and are able to reproduce earlier (Berven, 1990; Pollet and Bendell-Young, 2000).

For amphibians such as *R. sylvatica* to be part of a functioning ecosystem, they must reproduce successfully, which entails completion of metamorphosis and survival until adulthood. This is compromised in *R. sylvatica* from young OSPM-containing wetlands, which ultimately would lead to poor establishment of local amphibian populations.

4.4.2 Thyroid Hormone Status

Thyroid hormones (THs) have many functions in many different species such as regulation of growth, development, and metabolism (Brent, 1994). Thyroid hormones are also

the main governing factor in amphibian metamorphosis (Shi, 2000; Fort et al., 2007). These compounds have complex functions and affect multiple events during metamorphosis such as tail resorption and forelimb development. Without THs, metamorphosis can become arrested. Interestingly, this arrested metamorphosis can be completed if THs are provided at a later time (Rot-Nikcevic and Wassersug, 2004). The current study found no differences in T3 concentrations. T4 concentrations were different among young OSPM wetlands, which had higher concentrations than young reference wetlands. Overall this difference was very small and difficult to explain. However, the ratio of T3:T4, which gives a measure of the rate of deiodination of T4 to T3 (Picard-Aitken et al., 2007), was the lowest in tadpoles raised in young OSPM-containing wetlands. These alterations in thyroid status of *R. sylvatica* I believe to be responsible for the delayed/arrested metamorphosis in tadpoles living in young OSPM-containing wetlands, because of the strong link between THs and metamorphosis I have discussed. As well, alterations in thyroid status may also have other, unmeasured effects on amphibian metabolism, since THs control basal metabolic rate along with other metabolic processes.

4.4.3 Hepatic Detoxification Enzyme [7-ethoxyresorufin dealkylase (EROD)] Activity

The activity of EROD is a commonly used biomarker of exposure to a variety of industrial contaminants (Havelkova et al., 2007). The activity of the cytochrome P450 1A (CYP 450) enzyme family is linked to the enzyme 7-ethoxyresorufin-o-dealkylase (EROD). Exposure of test organisms to contaminants often results in an increased EROD enzyme activity. Rogers (2003) showed that naphthenic acids, which are considered to be one of the major toxic components of OSPM, may induce the EROD enzyme. Also, OSPM has been shown to contain PAHs (Madill et al. 2001) that are known to induce CYP 450 enzymes and EROD activity (van der Oost et al., 2003). Therefore, a difference in EROD activity of tadpoles among wetlands may indicate increased exposure of our test organisms to contaminants, which would affect their physiology and ultimately their ability to function in a particular wetland.

This study found the highest EROD activity in tadpoles caged in young OSPM-containing wetlands. This activity was significantly higher than old OSPM-containing sites as

well as young reference sites. These results are consistent with higher levels of contaminants in the young OSPM wetlands. These findings also agree with water chemistry data of current naphthenic acid concentrations (Appendix 2) plus those previously reported, as well as higher PAH concentrations (Clemente and Fedorak, 2005; Gentes, 2006; Madill et al. 2001). These results are similar to those of Gauthier et al. (2004), who found that amphibians exposed to contaminated water exhibited increased EROD activity when compared to control sites. Our overall EROD activity results were lower than those found by Gauthier [11.7 pmol/min/mg protein (control) versus 77.5 pmol/min/mg protein (exposure)]. This may be due to the difference in species and their size but it may also be a result of sample storage. There are conflicting reports noted in a review published by Whyte et al. (2000) that state that sample EROD activity can be stable for up to 24 months, while others have stated that even at -80 °C a reduction in activity by 50% can occur after seven days. Livers in this study were stored at -80 °C for a period of approximately 4 months.

When tadpoles caged in OSPM-containing wetlands were compared to those in old reference sites their EROD activity was higher, but it was not statistically different. This result may be explained by the variation caused by exposure to different environmental factors or differences in physiological factors among tadpoles (Goksoyr and Forlin, 1992; Havelkova et al., 2007) and not an increased amount of contaminants in the reference wetland. Another possible explanation may be that PAHs, which are known EROD inducers, occur naturally in the soil throughout the oil sand region (FTFC, 1995). This could have exposed tadpoles in all wetlands to PAHs of varying concentrations.

4.5 Conclusions

During the 2007 field study, metamorphosis was severely delayed, thyroid status was altered, and EROD enzymes were induced in tadpoles raised in young constructed wetlands formed with OSPM. Tadpoles caged in older OSPM-containing wetlands were similar to both young and old reference wetlands in terms of time to completion of metamorphosis, thyroid status, and EROD activity. These findings were in agreement with the findings of chapter 3, which found decreased survival in young OSPM-containing wetlands but not in old OSPM-

affected wetlands. These results suggest that as wetlands age they become less toxic, most likely due to a reduction in contaminant levels. This study shows that metamorphosis of tadpoles is delayed or completely arrested with exposure to OSPM contaminants. This experiment also indicates the wet landscape approach to reclamation has the potential to support populations of native amphibians, but only if the wetlands are sufficiently aged (7 years or more).

CHAPTER 5: GENERAL DISCUSSION

Currently, there are three oil sands companies producing bitumen by surface mining methods from deposits near Fort McMurray, Alberta, Canada. The number of companies involved in the mining of oil sands is increasing rapidly and so is the output of oil. This increase in the oil production also increases the amount of problematic by-products, including tailings materials. A major concern with the large amount of tailings is their toxicity. Adverse biological effects of solid and liquid tailings are believed to be associated with increased pH, increased salinity, PAHs from unrecovered bitumen, and naphthenic acids. Naphthenic acids are considered to be the greatest source of toxicity to wildlife. A proposed method for reclamation of liquid oil sands tailings material is the wet landscape approach in which 20 to 40% of the final reclamation area will be comprised of wetlands. These may include small, shallow wetlands and large lakes formed in mined-out pits. Before such a method can be implemented, researchers are trying to determine whether or not wetlands created with oil sands process-affected material (OSPM) can, with time, support a viable ecosystem. During early research into the use of OSPM in wetland construction, a number of wetlands were built to serve as real-world working models for use in experiments. This study examined the ability of these created wetlands to support a viable native ecosystem by using *Rana sylvatica* larvae as species that is representative of other aquatic life and will show biological responses to toxicants (bioindicator).

Bioindicators have been used extensively in studies on oil sands impact and reclamation research. To date, organisms from different trophic levels have been investigated, notably plants (Crowe et al., 2001), phytoplankton (Leung et al., 2003), birds (Gentes et al., 2007; Gurney et al., 2005), and fish (Nero et al., 2006). Amphibians, which represent a native, upper trophic level, aquatic and terrestrial vertebrate, are expected to inhabit reclaimed oil sands mining and tailings storage areas after reclamation. Amphibians are a unique and valuable study organism because

they live a fully aquatic existence during their larval stage, but live a mostly terrestrial life as adults. The larval stage was chosen for this study due to its suitability for representing aquatic life (Cooke, 1981). The main goal of the third chapter of this thesis was to investigate if amphibians that are native to the oil sands region could survive and grow in wetlands formed with OSPM. The fourth chapter investigated the metamorphosis and thyroid hormone status of *Rana sylvatica* larvae (Degitz et al., 2005; Opitz et al., 2005; Zhang et al., 2006; Shi, 2000). The relationship between metamorphosis and thyroid hormone status has been previously used as an indicator of exposure to environmental toxicants (Fort et al., 2007; Zhang et al., 2006). Due to the well-studied process of metamorphosis and its dependence on thyroid hormone, this work, along with several others, have examined metamorphosis in amphibians for detection of endocrine and thyroid disrupting compounds (Degitz et al., 2005; Opitz et al., 2005; OECD, 2008). As well, the hepatic detoxification enzyme (CYP450 1A) activity in *Rana sylvatica* was measured as a biomarker of exposure to contaminants.

The first field season (2006) had several problems that interfered with obtaining sufficient samples for all intended measurements, assays, and statistical analyses. Only three of nine enclosures in the older OSPM-containing wetland group yielded useful samples. As well, the only data obtained from enclosures in the young OSPM-containing wetland was survival. These problems combined with the death of all tadpoles in the young OSPM-containing site reduced the number of tadpoles available for measurements and additional analysis. In spite of the limited numbers of animals available for the laboratory assays, survival data did show interesting results that supported the hypothesis of this study that the youngest OSPM-containing wetlands, which have the highest levels of toxic components, would show the highest mortality and other detrimental effects when compared to older OSPM-containing wetlands. The highest mortality was among tadpoles raised in the young OSPM-containing wetland (4m CT). In 2006, 100% of the tadpoles in this wetland died within two weeks of the start of the study. This was presumably due to high concentrations of PAHs, naphthenic acids, high salinity, or a combination of possible contaminants (Table 3.3). There was no difference in survival between the other two wetlands (one old OSPM site and one reference). This also supported the hypothesis that as wetlands containing OSPM age, they become less toxic. Similar results were obtained in 2007. The highest mortality occurred in all of the youngest OSPM-containing sites.

In 2007, the wetlands were grouped by their age and OSPM status. 4m CT again showed very high mortality but it was not 100%. It should be noted that in young OSPM-containing wetlands tadpoles either died quite quickly or survived and grew very large, as this was an unexpected result. The tadpoles that did survive seemed less susceptible or better able to deal with contaminants. Overall, these data imply that young OSPM-containing wetlands are not ideal and cannot be considered adequately reclaimed until higher viability of tadpoles can be assured, especially since this species is a logical and relevant sentinel of ecological sustainability. On the other hand, the 2007 data suggest that if OSPM-containing wetlands are allowed to age (> 7 years) the mortality rates of *R. sylvatica* are similar to those in reference wetlands. The findings of this study support those of Gentes (2006), who found that it may take 10 years for wetlands formed with OSPM to become suitable for bird populations.

In 2007, surviving tadpoles in some of the young OSPM sites were significantly larger than those in all other wetlands in 2007. This is not likely directly linked to exposure to OSPM, but rather is a result of lengthened growth period caused either by a longer time to metamorphosis, or reduced competition from very low animal densities within enclosures caused by the high mortality in young OSPM-containing wetlands. Since food was provided in each of the enclosures it is unlikely that food quality or composition caused size related differences. It has been shown on many occasions that tadpoles can keep growing even if they do not complete metamorphosis (Allen, 1929). Lengthened growth period is a possible cause of increased tadpole size in young OSPM wetlands since more time and energy could be allocated to growth, rather than to metamorphosis. A second possible cause is less social competition and crowding in enclosures in young OSPM-containing wetlands, since the high mortality rate left fewer tadpoles in these enclosures. Tadpole size has been correlated with tadpole density in wetlands (Berven, 1990; Eaton et al., 2005).

Metamorphosis is a period of rapid development and change in all amphibian tissues (Degitz et al., 2005). The time it takes amphibians to complete metamorphosis is a commonly used endpoint in amphibian ecotoxicology because it is a readily visible change that is altered by exposure to chemicals and other disturbances in habitat. Decreasing or lengthening the time to metamorphosis can adversely affect tadpole and juvenile frog survival (Berven, 1990). The problems of low water levels and evaporation in some wetlands during the first season of this

study caused apparent premature metamorphosis at a very small body size in the old OSPM and reference wetlands. Tadpoles in OSPM-containing wetlands were not included in the analysis due to 100% mortality before metamorphosis was complete. In the remaining wetlands no differences in time to metamorphosis were noted. In contrast, during year two of this study there was a significant increase in the time to metamorphosis in all exposure groups. Tadpoles caged in young OSPM wetlands experienced a lengthened or complete arresting of metamorphosis. Metamorphosis of tadpoles in old OSPM wetlands, and both young and old reference wetlands, was completed much sooner (> 20 days) than those of young OSPM-containing wetlands. These data provide more evidence that young OSPM wetlands are toxic to amphibians but this toxicity appears to decrease as the OSPM wetlands age.

In larval amphibians, such as *Rana sylvatica* tadpoles, thyroid hormones (THs) control metamorphosis (Shi, 2000; Fort et al., 2007; Tata, 2006). The thyroid hormone system has also been shown to be a possible target for certain pollutants, which could alter thyroid hormone function and thus metamorphosis (Brown et al., 2004; Degitz et al., 2005; Helbing et al., 2006; Opitz et al., 2005). Therefore, THs in developing amphibians is a logical endpoint to measure. Although significant differences in metamorphosis were found between treatment types, there were no differences in triiodothyronine (T3) in either year. In year 2 there was a difference in T4 concentrations, but only between tadpoles in young OSPM and young reference wetlands. This result is not readily explainable and is not consistent with the delayed metamorphosis in tadpoles from young OSPM wetlands. A difference in the ratio of T3: T4 was noted between tadpoles from young OSPM and young reference wetlands and may signify a change in the deiodination of T4 to T3 (Picard-Aitken et al., 2007). This combined with the fact that the slowest metamorphosis was found in the same wetland groups suggests that the delays noted in time to metamorphosis are caused by interference with THs. These results suggest that thyroid hormones in wood frogs need further study to shed light on possible mechanisms triggering alterations in timing to metamorphosis. A detailed evaluation of the thyroid system including the thyroid glands and deiodinase enzymes that produce the T3, the biologically active form of the hormone, plus thyroid hormone receptors should also be studied to give a more complete picture of the mechanisms involved in the endocrine disruption that was evident in exposed animals (Fort, 2007).

Lipids such as triglycerides are the main form of energy storage in many species and have been extensively measured in fish (Adams, 1999) and mammals. Environmental stresses, such as exposure to contaminants, have been shown to alter stores of lipids in fish (Adams, 1999). Studies have shown that amphibians and fish with lower energy stores have a poorer chance at survival and successful overwintering (Biro et al., 2004; Scott et al., 2007). This study found that tadpoles from old reference wetlands, the ideal reference sites, had the greatest stores of triglycerides followed by young OSPM wetlands, the most challenging sites regarding toxic challenge. Curiously, the lowest stores were found in young reference sites and old OSPM sites. The low energy stores in tadpoles on these latter sites may be explained by the quicker metamorphosis, which likely means energy is being used for metamorphosis and not available for storage. Metamorphosis could also lead to altered energy stores because it is a time of high energy demand and tadpoles do not eat during this transition period (Scott et al., 2007). Another explanation could be that the high densities of tadpoles in the young reference site and old OSPM site were the cause of lower lipid stores. However, the low survival of tadpoles in young OSPM wetlands probably overwhelmed more subtle effects caused by exposure to contaminants and the surviving tadpoles would be those least sensitive to toxicants. As well, the delayed metamorphosis and continuous feeding of tadpoles in young OSPM wetlands would have allowed more time for accumulation of energy stores. Previous studies have stated that exposure of fish to contaminants such as metals (Levesque et al., 2002) could deplete energy stores. A reduction in triglycerides was also seen in crabs as a result of exposure to elevated ammonia levels (Hong et al., 2007), so it may be inferred that OSPM may cause alterations in energy stores however, the data from this study did not support this. Differences in triglyceride concentrations seemed not to be caused by tadpoles spending more energy detoxifying contaminants, which is known to happen; but instead energy stores seem to be altered by indirect effects such as low densities (high mortality) or lengthened time to metamorphosis caused by exposure to OSPM. If energy stores in *R. sylvatica* were lowered due to exposure to OSPM, they may have compromised fitness and survival. This study could not conclude this because, like other assays, effects may have been masked due to very low survival in the young OSPM-containing wetlands.

The activity of the enzyme 7-ethoxyresorufin dealkylase has been used extensively as a biomarker of exposure to environmental pollutants including oil sands related contaminants. Previous research in birds (Gentes, 2006; Smits et al., 2000) and in mammals (Rogers, 2003) has shown that exposure to tailings and naphthenic acids can stimulate EROD activity. This study found that EROD activity was highest in young OSPM wetlands as predicted. This shows that amphibians exposed to young OSPM wetlands, which have higher levels of naphthenic acids and PAHs, have to expend more resources detoxifying contaminants than amphibians raised in older, presumably 'cleaner' reclaimed wetlands. This leaves fewer metabolic resources for growth, development, energy storage, and other bodily functions. On another note, other studies such as those quoted in Rogers (2003) have reported very large increases in EROD activity with exposure to some known EROD inducers. This could mean that toxicants found in OSPM are not great inducers of EROD activity, which could be an explanation for the low EROD activities. More likely, any larger induction of EROD by exposure to OSPM were masked by the large scale mortality of tadpoles in young OSPM wetlands, which we hypothesized, would cause the most EROD induction because they contain the highest level of potential EROD inducing contaminants. Another possible reason for not seeing larger differences in EROD activity among tadpoles in young OSPM wetlands and other wetlands is the fact that bitumen is found naturally in soils throughout the oil sands region. Bitumen would be a source of EROD inducing PAHs that would likely be present at all sites. More investigation into the effects of OSPM and known toxic components such as naphthenic acids on EROD induction should be conducted as it may prove to be a useful biomarker in oil sands research.

CONCLUSIONS

Over the duration of this study many biological endpoints related to growth, development, and future fitness of population stability were used as tools to assess the health of amphibians, which are expected to inhabit reclaimed oil sands mining areas. Comparisons were made between reference wetlands and OSPM-containing wetlands of different ages (4 total classes). This research has shown that young OSPM-containing wetlands will not support sustainable amphibian life due to acute toxicity to tadpoles, while old OSPM-containing wetlands can support amphibian survival similar to reference wetlands. Tadpoles raised in young OSPM-containing wetlands had delayed metamorphosis, increased liver EROD activity, and

altered thyroid status (lower T3:T4 ratio). The larger growth in tadpoles from young OSPM-containing wetlands and triglyceride differences were likely due to differences in tadpole density due to lower survival caused by contaminant exposure in young OSPM-containing wetlands. From this research it is concluded that the use of the wet landscape approach to reclamation will be able to support populations of indigenous amphibians such as *Rana sylvatica*, but only after wetlands have matured, so biotic and abiotic processes can reduce the compounds responsible for acute toxicity. This study confirms that toxicity of OSPM is decreasing over time. However, more work needs to be completed to further the knowledge of how well amphibians will perform in wetlands formed with old OSPM, as well as more investigation into how reclaimed wetlands will perform ecologically. If implementation of this reclamation method does occur on a large scale, an ecological monitoring program should be established for amphibians because amphibians are integral to a functioning ecosystem in both aquatic and terrestrial environments. They could also continue to serve as an important monitoring tool for ongoing reclamation efforts.

Currently, the only toxicological studies on amphibians are based on larval amphibians up to the completion of metamorphosis. Further in-situ studies on amphibians must be completed to gain a better understanding of how different life stages of amphibians such as *Rana sylvatica* will perform in reclaimed areas on the oil sands. Included should be health and reproductive studies on adult amphibians and the hatchability of their eggs. Clearly these two phases are critical to population stability (Berven, 1990). Harris et al. (2000) have shown that amphibians at various stages of development are differentially sensitive to toxicants. Also, more species of amphibians must be investigated since the previous study by Pollet and Bendell-Young (2000) has shown differences in biological response of different amphibian species (*R. sylvatica* and *B. boreas*) to OSPM. Similarly, other researchers have noted strong species differences among amphibians exposed to contaminants in the environment. Harris et al. (2000) noted differences in the sensitivities of the frog *R. pipiens* and *B. americanus* to pesticides, while Snodgrass et al. (2004) found that *R. clamitans* showed more toxic responses to coal combustion wastes than *R. sylvatica*. Although it cannot completely replace a well-controlled laboratory experiment for reducing external variation or a field study for dealing with the most realistic environmental

variables (Harris et al., 2001), this study is another example of how caging tadpoles can be a useful technique for studying environmental contamination.

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