EFFECTS OF URANIUM MINING AND MILLING EFFLUENTS ON JUVENILE FISH BIOENERGETICS, GROWTH AND OVERWINTER SURVIVAL

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ABSTRACT

To assess potential impacts of effluents from Key Lake and McClean Lake uranium operations on freshwater systems, morphometric (weight, length, condition factor) and biochemical (total body lipids and triglycerides, liver triglycerides, muscle protein, muscle RNA/DNA ratio) measures of growth and bioenergetics were determined in young-of-the-year (YOY) fishes collected in fall and spring. It was predicted that fishes exposed to mining and milling effluents would be in poorer condition relative to fishes from reference sites and that fishes would be depleted in lipids and triglycerides in the spring compared to the previous fall following a northern winter. Various total body lipid and triglyceride measurement methods were initially compared and validated.

Lakes receiving effluent at Key Lake (in operation > 20 years) were higher in metals, ions and ammonia compared to exposure sites at McClean Lake (in operation < 10 years). At Key Lake, there were site and season differences in total body lipids and triglycerides in YOY northern pike (*Esox lucius*) and burbot (*Lota lota*), with fishes being fattier at exposure sites compared to fishes at the reference site, and fish being fattier in spring relative to fall. A local prey item, spottail shiners (*Notropis hudsonius*), from an exposure lake were higher in triglycerides compared to shiners from a reference site, suggesting an indirect effect of uranium operation effluent on pike and burbot bioenergetics via food web enrichment. At McClean Lake, there were site and season increases in lipids and triglycerides in burbot from the exposure site, however there were no site differences in any morphometric or biochemical endpoint for northern pike. Slimy sculpin (*Cottus cognatus*) were the only species with lower triglyceride content in the spring following winter.

Overall, biochemical measures of growth (muscle protein, muscle RNA/DNA ratio) did not vary with effluent exposure at either uranium operation. Lipids and triglycerides were useful biochemical endpoints that frequently detected site and season differences in fish condition that were not noted with morphometric measures. Site and season differences in fish lipids and triglycerides at sites receiving mining and milling effluents revealed an impact of the uranium operations on indigenous YOY fish condition.

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LIST OF ABBREVIATIONS

CCME Canadian Council of Ministers of the Environment

CV coefficient of variation

DNA deoxyribonucleic acid

GSI gonado-somatic index

HSI hepato-somatic index

PCA principal component analysis

RNA ribonucleic acid

SSWQO Saskatchewan Surface Water Quality Objectives

S/V TEMS Sink/Vulture Treated Effluent Management System

YOY young-of-the-year

PREFACE

This thesis has been organized as a series of manuscripts for publication in scientific journals. Thus, there is some repetition of introductions, materials and methods and figures throughout. As well, abstracts for each data chapter are included.

Chapter 2 was submitted to Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology on April 6th 2006, Chapter 3 was submitted to the Canadian Journal of Fisheries and Aquatic Sciences on March 26th 2006 and Chapter 4 will be submitted to Freshwater Biology within the following months.

CHAPTER 1
1.0 GENERAL INTRODUCTION

1.1 Overwintering stress in juvenile fish

Maintenance of a healthy fish population is primarily influenced by recruitment of new individuals into the population. Recruitment of new individuals into a population is closely linked to survival of juvenile fish beyond their first year. For fishes in temperate and northern environments, the first winter represents a major challenge to young-of-the-year (YOY) survival and future recruitment into the population (Hurst and Conover 1998). Fish may die in the winter from cold torpor, lack of oxygen in ice-covered lakes, or long periods without feeding (Matthews 1998). During the winter, it is generally believed that fish remain relatively motionless and without feeding at the bottom of lakes and streams (Sayer and Davenport 1996, Pratt and Fox 2002, Bauer and Schlott 2004). Cool water temperatures of approximately 6°C result in lower metabolic demands for fish, while at temperatures near 0°C, fish will generally become torpid (Matthews 1998). Reduction in light during winter due to photoperiod as well as ice cover decreases productivity of lakes and impedes visual predators. Overwinter survival represents a major challenge to juvenile fishes at northern latitudes where winter's low light and low temperatures comprise a large proportion of the year.

Osmoregulatory failure may also contribute to winter mortality in fish. At cold temperatures (< 4°C), changes in membrane permeability can alter ion transport in fish (Morris and Bull 1968). This could result in a loss of essential ions such as Na^+ and eventually lead to death (Morris and Bull 1968, Johnson and Evans 1996). Smaller fish

may be more prone to osmoregulatory failure than larger fish since smaller individuals have a larger gill area, on a per gram basis, than larger individuals (Johnson and Evans 1996). Since smaller fish are generally also younger fish, osmoregulatory failure during low winter water temperatures will have a proportionally higher impact on YOY fish compared to older age classes.

During the first summer of life, it is important for YOY fish to allocate energy to growth as well as energy storage for overwinter survival (Adams 1999, Sogard and Olla 2000; Figure 1.1).

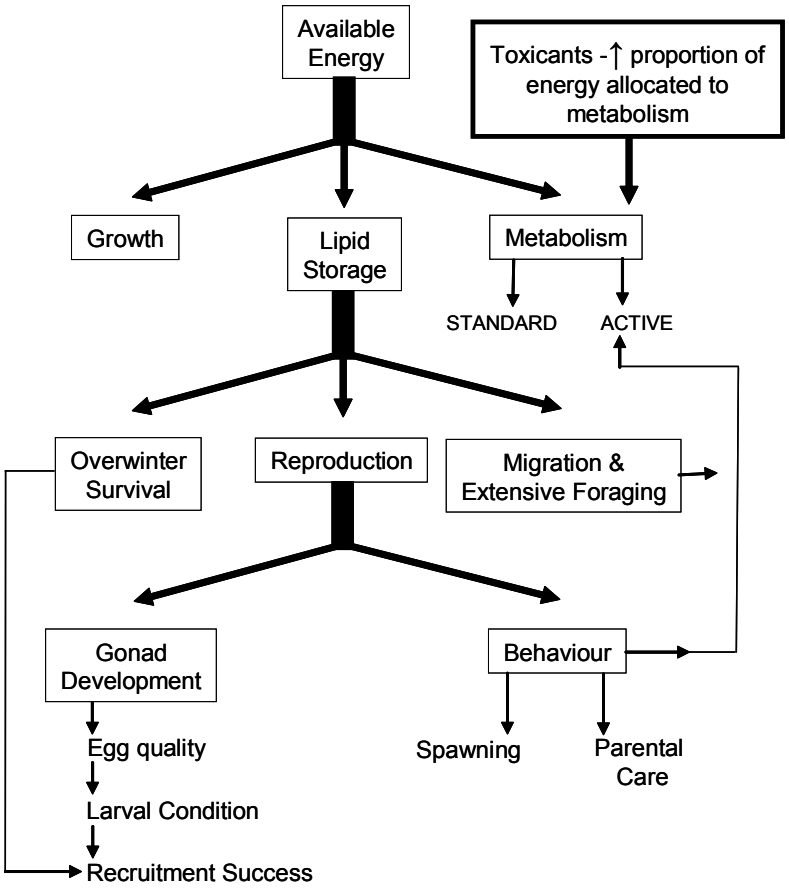

Figure 1.1 General allocation strategy of available (assimilated) energy into the main functional processes of growth, metabolism, lipid storage and reproduction for a typical temperate or north-temperate fish species (modified from Adams 1999).

Body size has an important influence on an animal's energetic requirements, its potential for resource exploitation and its susceptibility to natural enemies (Werner and Gilliam 1984). Smaller fish have a relatively higher metabolic rate than larger individuals (Love 1980) and, therefore, have an increased demand for stored energy over the winter compared to larger, older individuals (Cunjak 1988). Since smaller fish have a higher mass-specific metabolic rate and lower energy density than larger fish, size-dependent overwinter mortality may occur (Peters 1983, Post and Parkinson 2001, McCollum *et al.* 2003). Numerous laboratory and field studies with various fish species have established that smaller YOY fish indeed suffer higher mortality over the winter than larger individuals (Oliver *et al.* 1979, Toneys and Coble 1980, Post and Evans 1989, Johnson and Evans 1991, Griffiths and Kirkwood 1995, Foy and Paul 1999, Gotceitas *et al.* 1999, Kristiansen *et al.* 2000, Sogard and Olla 2000, Grant and Tonn 2002, Biro *et al.* 2004). In addition, size-dependent predation can regulate winter mortality, as smaller fish are more vulnerable to gape-limited predators (Werner and Gilliam 1984, Kristiansen *et al.* 2000). In summary, young-of-the-year fish are therefore under evolutionary pressure to attain a sufficient size in order to avoid predation, attain resources, decrease relative metabolism and ultimately, survive the first winter of their lives.

Lipids are a primary source of energy and are important for both fitness and survival of fish (Adams 1999). Winter poses a significant stressor for fishes as fasting, cold temperatures and reduced activity are generally accompanied with decreasing lipid levels (Toneys and Coble 1980, Cunjak 1988, Lemly 1996, Berg and Bremset 1998, Sogard and Olla 2000, Post and Parkinson 2001). During winter, age-0 fish rely on stored energy and some energy intake to meet metabolic demands (Johnson and Evans

1991). Therefore, it is critical for fish to attain sufficient lipid stores before the relative inactivity of winter. Fish that obtain fat reserves before winter have a higher probability of winter survival (Thompson *et al.* 1991, Lemly 1996, Post and Parkinson 2001). Decreased lipid levels can lead to starvation as well as compromised osmoregulation (Lemly 1996, Adams 1999). Certainly, YOY fish are at the greatest risk of experiencing overwinter mortality as lipid depletion leads to starvation.

In summary, given the high potential for winter mortality, survival during the first winter for fish can determine future year-class strength for a population. Young-of-the-year fish experience a tradeoff between energy allocation to growth versus energy allocation to storage (Adams 1999, Post and Parkinson 2001, Figure 1.1). Small differences in body size can have a large effect on survival through various factors such as increased relative metabolism, osmoregulatory failure, increased risk of predation or decreased ability to exploit resources. Sufficient lipid stores prior to winter are also important for fish survival and eventual recruitment. Clearly, overwinter survival represents a major challenge to YOY fishes, particularly at northern latitudes where winters are long and cold.

1.2 Combination of stressors: winter and environmental contamination

The combination of winter and an environmental stressor may significantly reduce the overwinter survival of young-of-the-year fishes, thereby reducing their recruitment into the population. The term "winter stress syndrome" was coined by Lemly (1993) to describe a condition of metabolic distress in fish associated with the combination of a metabolic stressor and winter conditions. The three conditions noted by Lemly (1996) for winter stress syndrome to occur are: (1) the presence of a metabolic

stressor, (2) cold water temperatures and (3) fish respond to cold with reduced activity and foraging. Lemly (1996) notes that a variety of chemical and biological stressors can potentially induce winter stress syndrome. More specifically, the potential that a given stressor will lead to winter stress syndrome depends on its propensity to increase metabolism (Figure 1.1). Metabolic stressors can include such variables as exposure to inorganic or organic toxicants, parasites, altered pH or high suspended sediment. Obviously, the presence of multiple metabolic stressors will increase the probability of the syndrome developing. However, the concept of winter stress syndrome has never been tested in the field, as Lemly's theory was generated in a laboratory setting under simulated winter conditions.

Contaminants may act directly or indirectly on fish condition. A contaminant could indirectly be altering the operative environment in an aquatic system such as temperature, predation risk, resource availability or cover (Congdon *et al.* 2001). The release of industrial effluent may substantially increase water flow within a stream ecosystem. This could result in removal of organic debris, algae, macrophytes, invertebrates, spawning gravel, eggs, and possibly crowding fish into refugia or stranding fish in temporary pools (Matthews 1998). Indirect, food web mediated effects of metal contamination have been reported to impact yellow perch (*Perca flavescens*) bioenergetics as metal contaminated lakes created gaps in prey size structure which resulted in slow growing perch populations (Iles and Rasmussen 2005).

Contaminants can act directly on fish development and physiology and affect such things as activity level, metabolic rate or energy assimilation (Congdon *et al.* 2001). If metabolic costs are increased in response to a toxicant, production processes such as

growth and lipid storage could be reduced (Calow 1991, Figure 1.1). Presence of an environmental stressor can increase standard metabolism in a variety of ways. Metabolism could be increased as a result of problems with ion regulation, damage to gills or increased activity of detoxification mechanisms, thereby decreasing the proportion of energy available for allocation to lipid storage and growth (Figure 1.1). For instance, metabolic stress associated with winter conditions increased the toxicity of selenium to juvenile blue gill (*Lepomis macrochirus*) (Lemly 1993). Common carp (*Cyprinus carpio*) responded to various concentrations of sublethal copper exposure with increased metabolic demands and reduced feeding (De Boeck *et al.* 1997). In addition, contaminant exposure can adversely affect fish behaviours involving sensation, perception, cognition, co-ordination and motor function (Atchison *et al.* 1987, Døving 1991, Scott and Sloman 2004). Avoidance behaviour has been observed in lake whitefish (*Coregonus clupeaformis*) exposed to copper, lead and zinc (Scherer *et al.* 1998). Exposure to cadmium inhibited performance of predator avoidance behaviours in juvenile rainbow trout (*Oncorhynchus mykiss*) through accumulation in the olfactory system (Scott *et al.* 2003). Organisms can respond to changes in their environment at all levels of biological organization, from the molecular to the population levels. Unfortunately, energetically expensive changes in physiology or behaviour resulting from contaminant exposure will reduce the allocation of assimilated energy towards energy storage and growth, which are critical for winter survival and recruitment (Figure 1.1).

Environmental stressors can alter both the quality and quantity of lipids in fish (Adams 1999), which directly influences the ability of juvenile fishes to survive the potentially energy-depleting winter months. Alternatively, decreased lipids can result in

increased susceptibility of fish to environmental stressors (Adams 1999). Higher rates of lipid metabolism and lower lipid, specifically triglyceride levels, in fish experiencing chronic metal exposure in the fall relative to fish inhabiting uncontaminated lakes has been reported in a previous study (Levesque *et al.* 2002) and death from lipid exhaustion has been noted to occur with a variety of fish species (Oliver *et al.* 1979, Adams *et al.* 1985, Henderson *et al.* 1988, Hurst and Conover 1998, Finstad *et al.* 2004). Since winter is a physiologically stressful time for fish, the addition of a contaminant has the potential to significantly decrease overwinter survival.

1.3 Measurement of growth and condition in fishes

1.3.1 Morphometric endpoints

Traditional morphometric measures used in fish biology such as weight, length and condition factor are easy to measure, but provide rather crude estimates of overall condition in fish. Weight can be used as a measure of energy storage, while length can be used to measure growth. The generally accepted assumption is that a heavier weight for a given length corresponds to better condition. Fulton's condition factor is a simple formula and is one of many used to evaluate the weight-length relationship (reviewed by Bolger and Connolly 1989). Fulton's condition factor ($weight/length^3*100$) converts a two-dimensional weight-length relationship into a single statistic that provides a simple indication of the well being of a fish.

Organ weights, such as livers or gonads in adults, can be related to body weight in a hepato-somatic (HSI) or gonado-somatic (GSI) index, respectively (organ weight/total weight*100). Liver weight is generally a measure of energy storage and gonad weight in sexually mature individuals is a commonly used measure of reproductive status. The

hepato-somatic index is strongly related to growth rate and condition factor, and can be an indicator of energy content in juvenile Atlantic cod (*Gadus morhua*) (Lambert and Dutil 1997, Couture *et al.* 1998). In general, variation in the HSI reflects mobilization or accumulation of lipid in the liver. In addition, the HSI is commonly used as a biomarker of contaminant exposure (Goede and Barton 1990, Facey *et al.* 2005). Since the liver is very important for detoxification, exposure to contaminants can lead to hypertrophy (an increase in size) or hyperplasia (an increase in the number of cells) or both (Goede and Barton 1990, Hinton and Lauren 1990). Therefore, determining morphometric endpoints in fishes will provide information on growth, energy storage, reproductive potential and response to toxicants.

1.3.2 Biochemical endpoints

Before changes in weight, length and condition factor occur, changes in biochemical composition should become apparent (De Boeck *et al.* 1997). Biochemical measures of condition have been proposed to provide innovative and sensitive techniques to evaluate fish condition, since sub-organismal processes determine whether an individual attains sufficient lipids and establishes appropriate energy allocation (Adams 1999, Congdon *et al.* 2001, Post and Parkinson 2001). The four major classes of biological macromolecules are lipids, proteins, carbohydrates and nucleic acids (Voet *et al*. 1999), although in fishes, lipids along with proteins are the major constituents, while carbohydrates are proportionally lower compared to other vertebrates (Tocher 2003).

1.3.2.1 Muscle RNA/DNA ratio

Muscle ribonucleic acid (RNA) to deoxyribonucleic acid (DNA) ratio provides an estimate of short-term (hours to days) growth rate (Bulow 1970, Clemmesen 1988).

While DNA per cell remains relatively constant, the amount of RNA will vary with physiological status, requirements for protein synthesis and growth (Bulow 1970, Raae *et al.* 1988, Buckley *et al.* 1999). There are different forms of RNA which have different roles in development, feeding and growth: ribosomal RNA (rRNA), transfer RNA (tRNA) and messenger RNA (mRNA). However, since rRNA represents the majority of total RNA, changes in total RNA and RNA/DNA ratio primarily reflect changes in rRNA (Buckley *et al*. 1999). Therefore, the ratio of RNA to DNA provides an index of protein-synthesizing potential per cell (McLaughlin *et al.* 1995) or a cell's metabolic intensity (Clemmesen 1988). Since temperature and food availability are two main environmental factors affecting growth, changes in either of these parameters in a study site will impact RNA/DNA ratio in fish. Indeed, numerous studies have found that fish fed high rations of food have higher RNA/DNA ratios than food deprived fish (Clemmesen 1988, Raae *et al.* 1988, Steinhart and Eckmann 1992, Weber *et al.* 2003). The RNA/DNA ratio can also be used as a bioindicator of contaminant stress, as growth rates can change with contaminant exposure. Kearns and Atchison (1979) found that yellow perch RNA/DNA ratio was negatively correlated with metal levels. Using a fluorometric dye-binding assay (Clemmesen 1988), the concentration of both RNA and DNA can be determined in fish muscle, providing an RNA/DNA ratio and a measure of an individual's short-term growth rate prior to collection.

1.3.2.2 Muscle protein concentration

Total protein content provides a measure of longer-term growth (days to weeks) and to a lesser extent, energy storage. Protein synthesis in an active tissue such as caudal muscle reflects recent growth (Couture *et al.* 1998) and therefore nutritional status of the

individual fish. Tissue concentrations of protein, like RNA/DNA ratio, also tend to be higher in fed fish than in food deprived individuals (Smith 1981, Steinhart and Eckmann 1992, McLaughlin *et al.* 1995, Weber *et al.* 2003). Proteins can be exploited as an energy source, although they tend to be utilized only under conditions of severe stress (Benton *et al.* 1994). McLaughlin *et al.* (1995) found that muscle protein concentration is a good indicator of nutritional status in recently emerged brook trout (*Salvelinus fontinalis*), as fed fish had higher muscle protein values compared to food deprived individuals.

1.3.2.3 Lipids

Total body lipids provide an estimate of lipid stores in individual fish (Bligh and Dyer 1959, Weber *et al.* 2003). Most stored lipid is of exogenous origin, since only a small fraction is synthesized in the liver (Voet *et al*. 1999). Lipids can be defined as compounds soluble in organic solvents and usually contain either a fatty acid esterified to alcohol groups in the case of glycerides or a fatty acid esterified to amino groups, as found in sphingolipids (Tocher 2003). In fishes, lipids can be divided into two general groups: the polar lipids composed primarily of phospholipids and the neutral or non-polar lipids, composed principally of triacylglycerols, which are more commonly known as triglycerides (Henderson and Tocher 1987, Tocher 2003)

Total body lipids are typically determined using a solvent extraction method, such as the Bligh and Dyer (1959) chloroform-methanol extraction. Here, a tissue sample is homogenized and combined with chloroform and methanol in a precise ratio. Following separation, lipophilic substances in the tissue sample will have migrated to the chloroform fraction. Lipid weight can then be determined gravimetrically by simply

evaporating the chloroform and measuring the remaining lipid in the sample (Figure 1.2). An alternative method of total body lipid gravimetric determination is the sulphophosphovanillin method (Figure 1.2), where unsaturated fatty acids are measured in a lipid solvent extract (Knight *et al.* 1972, Weber *et al.* 2003).

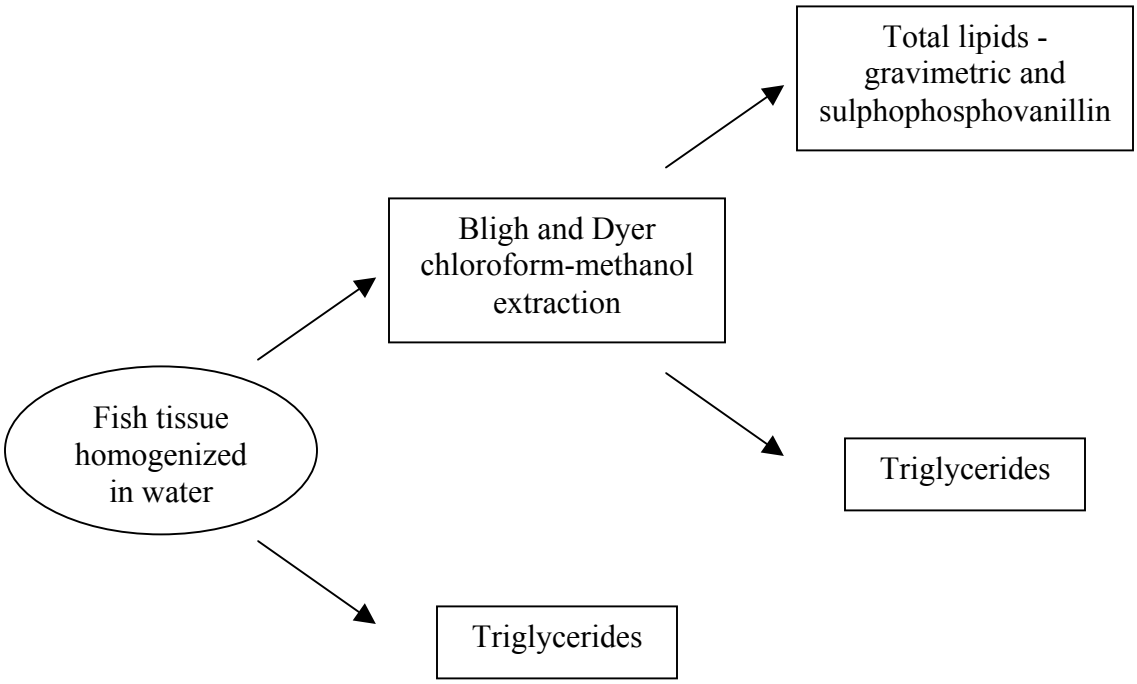

Figure 1.2 Preparations for different total body lipid and triglyceride measurements methods.

1.3.2.4 Triglycerides

Depending on the fish species, triglycerides are stored in varying amounts in muscle, liver, subdermal tissues and mesenteries (Adams 1999). In general, triacylglycerols are the predominant lipid class in the diet of freshwater fishes (Tocher 2003). During feeding, excess dietary fatty acids are exported from the liver in the form

of lipoproteins and stored in the form of triglyceride in specific lipid storage areas (Tocher 2003). The primary site for long-term energy storage is in mesenteric adipose tissue, although some fish species store significant amounts of fat within white muscle and between skin and muscle (Henderson and Tocher 1987). Triglycerides are stored as a long-term source of energy to be used when the energy requirements of the fish exceed the energy available from the diet (Sheridan 1988, Tocher 2003). Lipids are mobilized by lipolytic enzymes which hydrolyze stored triglycerides and release free fatty acids (Sheridan 1988). Subsequent β-oxidation of fatty acids provides energy for the fish in the form of ATP (Sargent *et al.* 1989). Measurement of total body lipids will include both polar lipids such as phospholipids in addition to non-polar lipids, mainly triglycerides. However, measurement of total body triglycerides provides a more ecologically and physiologically relevant endpoint than total lipids, since triglycerides are the predominant form of stored energy in fish (Henderson and Tocher 1987, Sheridan 1988, Sargent *et al.* 1989, Jobling *et al.* 1998).

Moderate amounts of lipid can be stored as triglycerides in the liver, although this is generally for short-term storage, compared to adipose tissue storage (Tocher 2003). However, in some fishes, such as the gadoids and some flatfish, the liver can be the primary lipid storage site (Sargent *et al.* 1989). Therefore, measuring liver triglycerides in fish can provide an estimate of short-term or long-term energy storage, depending on the species. Lipids in the liver are readily available as an energy source, since a high concentration of lipolytic enzymes are located in this organ.

1.3.2.5 Lipid and triglyceride measurement methods

Currently, the majority of research on fish lipids employs a non-polar solvent extraction method, for example the Bligh and Dyer (1959) chloroform-methanol lipid extraction method. These techniques are affected by problems related to the lipid extraction process, such as solvent evaporation or lipid adhering to the tissue layer during separation (Smedes and Thomasen 1996, Dickey *et al.* 2002). Previous studies have found that the efficiency of the Bligh and Dyer solvent extraction and subsequent lipid measurement is limited by the size of fish; the lipid extraction process experiences decreased performance when fish weigh < 2 g (Honeycutt *et al.* 1995, Weber *et al.* 2003). In addition, the Bligh and Dyer method is time consuming and may expose workers to toxic solvents, specifically chloroform. Therefore, there exists a need to develop a reliable, rapid and safe method to measure lipids in fish that does not require the potentially unreliable, time consuming and toxic Bligh and Dyer chloroform-methanol lipid extraction method. In addition, considering that triglycerides are the primary energy storage form in fish, it is of interest to determine triglyceride content in fish in order to determine a more ecologically and physiologically relevant estimate of fish condition than lipids.

1.4 Uranium operations in Saskatchewan

Canada is the world's leading producer and exporter of uranium, and mining facilities operating in northern Saskatchewan are the most productive, producing approximately 30% of the world's uranium supply (OECD Nuclear Energy Agency 1999, Klaverkamp *et al.* 2002). Treated mining and milling effluent is released into local aquatic ecosystems and certain metals and salts have been shown to be elevated above

background levels in water, sediment and fishes (Pyle *et al.* 2001, Klaverkamp *et al.* 2002). Although there has been substantial research effort examining the fate and effects of radionuclides within aquatic systems surrounding uranium mining operations, surprisingly little research has been conducted on potential exposure and effects of metals and salts present in mining and milling effluent on fish health. Thus, there exists a need to better understand potential long-term impacts of metals and salts released from uranium mining and milling effluent on local fauna, such as fishes.

1.4.1 Key Lake operation

Key Lake uranium operation (57°13' N, 105°38' W) is located in north-central Saskatchewan, approximately 600 km north of Saskatoon in the boreal forest (Figure 1.3).

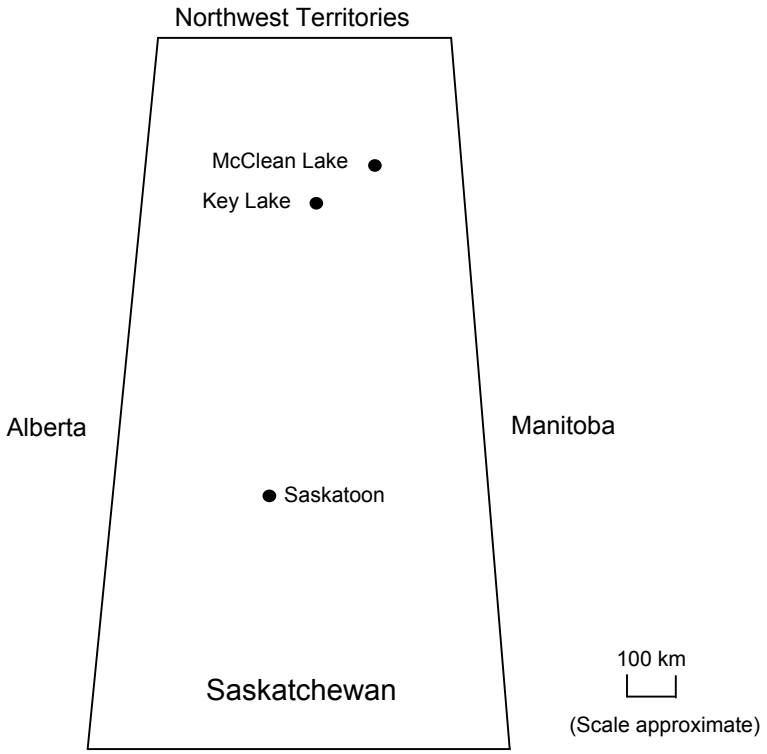

Figure 1.3 Map of Saskatchewan, Canada showing approximate locations of Key Lake and McClean Lake uranium operations.

The operation is primarily owned and operated by Cameco Corporation. The operation commenced in 1982, with open pit mining of the Gaertner ore body until 1987, followed by open pit mining of the Deilmann ore body from 1986 to 1997. In 2000, Key Lake mill began processing McArthur River ore combined with Key Lake mineralized waste. From 1994 to 1999, the Key Lake Operation was licensed to produce 4.4 million kg of uranium per year; this annual production limit was increased to 7.4 million kg of uranium in November 1999 (Klaverkamp *et al.* 2002). There are two effluent streams at Key Lake uranium operation: mine dewatering effluent is released into the McDonald Creek drainage and milling effluent is released into the David Creek drainage (Golder 2003). Both effluent types are known to contain elevated metal concentrations; mining effluent is characterized as having high nickel (Ni) content while the milling effluent contains elevated molybdenum (Mo) (Golder 2003).

During the process of uranium extraction in the mill, large volumes of waste water are produced in the mill bulk neutralization water treatment plant. Treated mill effluent is released into the David Creek drainage which consists of the following lakes joined by creeks: Wolf Lake, Fox Lake, Unknown Lake and Delta Lake (Figure 1.4). Milling effluent is released at a rate of approximately 6,000 m^3/d to the environment. When the effluent enters the Yak Creek drainage at Wolf Lake it comprises approximately 48% of Yak Creek flow. Yak Creek flows into David Creek approximately 2 km from the point of effluent release; at this point, treated effluent is about 23% of the David Creek flow. Overall, concentrations of nutrients, major ions and several metals are higher in these lakes compared to an upstream reference lake in the same watershed, David Lake (Table 1.1, Figure 1.4).

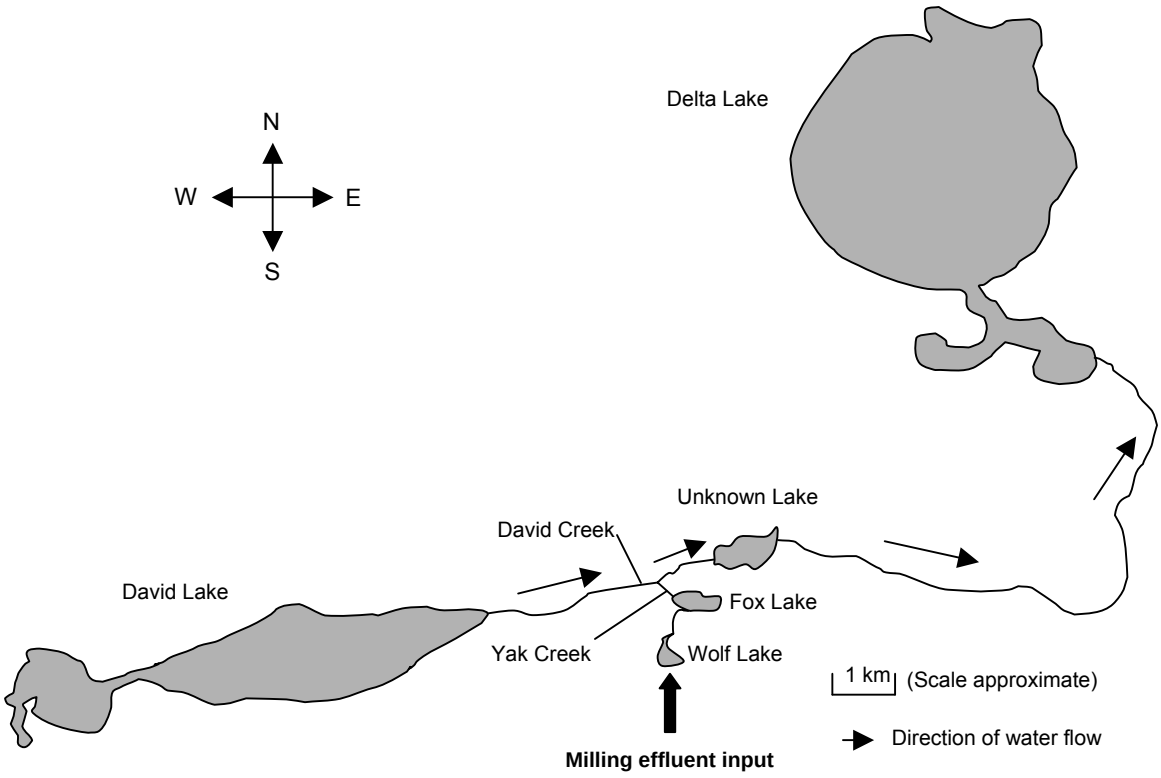

Figure 1.4 Map of David Creek drainage containing treated uranium milling effluent at Key Lake uranium operation, Saskatchewan, Canada (57°13' N, 105°38’ W). The direction of water flow is shown with arrows and lakes are shown in grey.

Table 1.1 Water quality data from 2004 for lakes at Key Lake uranium operation, Saskatchewan, Canada. Table modified from Golder (2005). Values in bold exceed either the Saskatchewan Surface Water Quality Objectives (SSWQO) or the Canadian Council of Ministers of the Environment (CCME) guidelines. Refer to Figure 1.4 for locations of sites. Values preceded by the < symbol represent the detection limit for the specific analyte.

Analyte	Units	SSWQO	CCME	Exposure Lakes		Reference Lake
				Unknown	**Delta**	**David**
Conductivity	µS/cm			725	635	29
pH		6.5 to 8.5	6.5 to 9	**5.0**	6.6	6.4
Total dissolved solids	mg/L			524	360	24
Total Hardness	mg/L			317	221	4
Ammonia (as N)	mg/L	1.9 to 2.0[(a)]	10 to >100[(a)]	**2.1**	0.19	0.03
Sulphate	mg/L			330	220	0.6
Radium-226	Bq/L	0.11	0.6	0.020	<0.005	<0.005
Arsenic	µg/L	50	5	3.8	0.9	0.1
Cadmium	µg/L	0.001	0.017	<0.5	<0.5	<0.5
Iron	mg/L	1	0.3	0.24	0.12	0.28
Lead	mg/L	0.02	0.001 to 0.007[‡]	<0.0001	<0.0001	<0.0001
Mercury	µg/L	0.1	0.026	<0.05	<0.05	<0.05
Molybdenum*	mg/L	0.7	0.073	**0.108**	**0.126**	<0.0001
Nickel	mg/L	0.025 to 0.1[‡]	0.025 to 0.150[‡]	0.0066	0.0022	<0.0001
Selenium	mg/L	0.01	0.001	**0.003**	0.001	0.0001
Silver	mg/L	0.01	0.0001	<0.0001	<0.0001	<0.0001
Strontium	mg/L			0.16	0.12	0.01
Uranium	µg/L	11 to 218[‡]		0.4	<0.1	<0.1
Zinc	mg/L	0.05	0.03	<0.005	<0.005	<0.005

[(a)] Guideline is temperature and pH dependent. Total ammonia ($NH_3 + NH_4$) values are shown for the range in pH and temperature observed in waterbodies during fall 2004

[‡] Guideline is hardness dependent, increasing with hardness

* Canadian Nuclear Safety Commission threshold limit

Certain measured variables have been reported to exceed either Saskatchewan Surface Water Quality Objectives (SSWQO) or the Canadian Council of Ministers of the Environment (CCME) guidelines in Unknown and Delta Lakes (Golder 2005; Table 1.1). Pyle *et al*. (2001) studied toxicity of uranium milling effluent by placing larval fathead minnows (*Pimephales promelas*) in Fox and Unknown Lakes; these fish had significantly higher mortality than those from the reference lake, David Lake. Although a variety of metals (As, Cd, Fe, Mo, Mn, Ni, Se, U, W) were found in increased concentrations in the exposure lakes, the authors concluded that selenium may have been responsible for the increased mortality based on existing toxicity data. Klaverkamp *et al*. (2002) found that white sucker (*Catostomus commersoni*) downstream of uranium milling effluent in Fox Lake had higher metal (Mo, As, Se, Ni, Cu) content in hepatic and/or renal tissues compared to reference fish. Clearly, there is need to determine possible effects of Key Lake operation milling effluent on local fishes.

1.4.2 McClean Lake operation

McClean Lake uranium operation (58°23'N, 103°48'W) is approximately 700 km north of Saskatoon and 100 km north-east of Key Lake Operation (Figure 1.3). The operation is within the Wheeler Upland Landscape area of the Athabasca Plain Ecoregion of north-eastern Saskatchewan and is primarily owned by AREVA, formerly COGEMA Resources Inc. Uranium mining began in 1995 and milling commenced in 1999. The waste water management system at McClean is somewhat different from the aforementioned Key Lake operation. The Sink/Vulture Treated Effluent Management System (S/V TEMS) is the single, common facility for the controlled release of all waste water generated at the McClean Lake operation (COGEMA Resources Inc. 2003,

McClean Lake Sue E Project Description 2003). Three waste water streams enter the S/V TEMS: the Jeb water treatment plant, the Jeb dewatering well system and the Sue water treatment plant. From 1995 to 1999, the Jeb water treatment plant consisted of Jeb pit mine water and surface runoff from developed areas on site. However, in 1999 uranium milling commenced at McClean and waste water from this process was also treated in the Jeb water treatment plant (COGEMA Resources Inc. 2003). The Jeb dewatering well system consists of 36 wells surrounding the perimeter of the Jeb tailings management facility and acts to remove incoming groundwater prior to contact with tailings porewater (COGEMA Resources Inc. 2003). If intercepted groundwater requires treatment, it enters the Jeb water treatment plant. However, if the dewatering well system intercepts groundwater that meets SSWQO, it is directly discharged into the S/V TEMS by merging with the Jeb water treatment plant discharge pipeline (COGEMA Resources Inc. 2003). The Sue pit began mining in 1999 and the Sue water treatment plant treats water from various sources at the Sue site. Both Jeb and Sue water treatment plants remove dissolved metals and suspended solids through a combination of precipitation, polishing, filtration, and the use of settling and sedimentation ponds. Water within holding ponds is analyzed on site and if quality requirements are met, the pond is subsequently discharged to the S/V TEMS. Alternatively, if a pond's contents do not meet required water quality levels, the entire pond may be recycled through the water treatment plant or possibly discharged to the tailings management facility (COGEMA Resources Inc. 2003).

The waste water management plan required the conversion of Sink Lake into a reservoir (Sink Reservoir) with flow control structures above and below Vulture Lake

(Figure 1.5). The waste water discharge rate from Sink Reservoir to Vulture Lake is manually regulated and water release from the reservoir occurs via a buried pipeline. An additional buried discharge pipeline at Vulture Lake outlet measures water flow and acts as an outflow diffuser to increase mixing in the downstream environment, the east basin of McClean Lake (McClean Lake Sue E Project Description 2003). There is little mixing of the effluent within McClean Lake - water samples from the west basin are close to reference and therefore, only the east basin is considered an effluent exposed area. Collins Creek carries effluent from the east basin of McClean Lake downstream approximately 15 km to Kewen Lake (Figure 1.5). The operational objectives of the S/V TEMS are summarized by the following two criteria: 1) the S/V TEMS discharge shall not exceed that amount which would cause Collins Creek flow to exceed 4.52 m^3/s and 2) the treated effluent portion of the S/V TEMS discharge shall not exceed one part treated effluent in five parts natural Collins Creek flow (COGEMA Resources Inc. 2003). Water quality in lakes and creeks receiving McClean mining and milling effluent are elevated in certain metals (Table 1.2), notably molybdenum, arsenic, nickel and uranium. Receiving water at both Key Lake and McClean Lake uranium operations are characterized by elevated conductivity, hardness, total dissolved solids and sulphates.

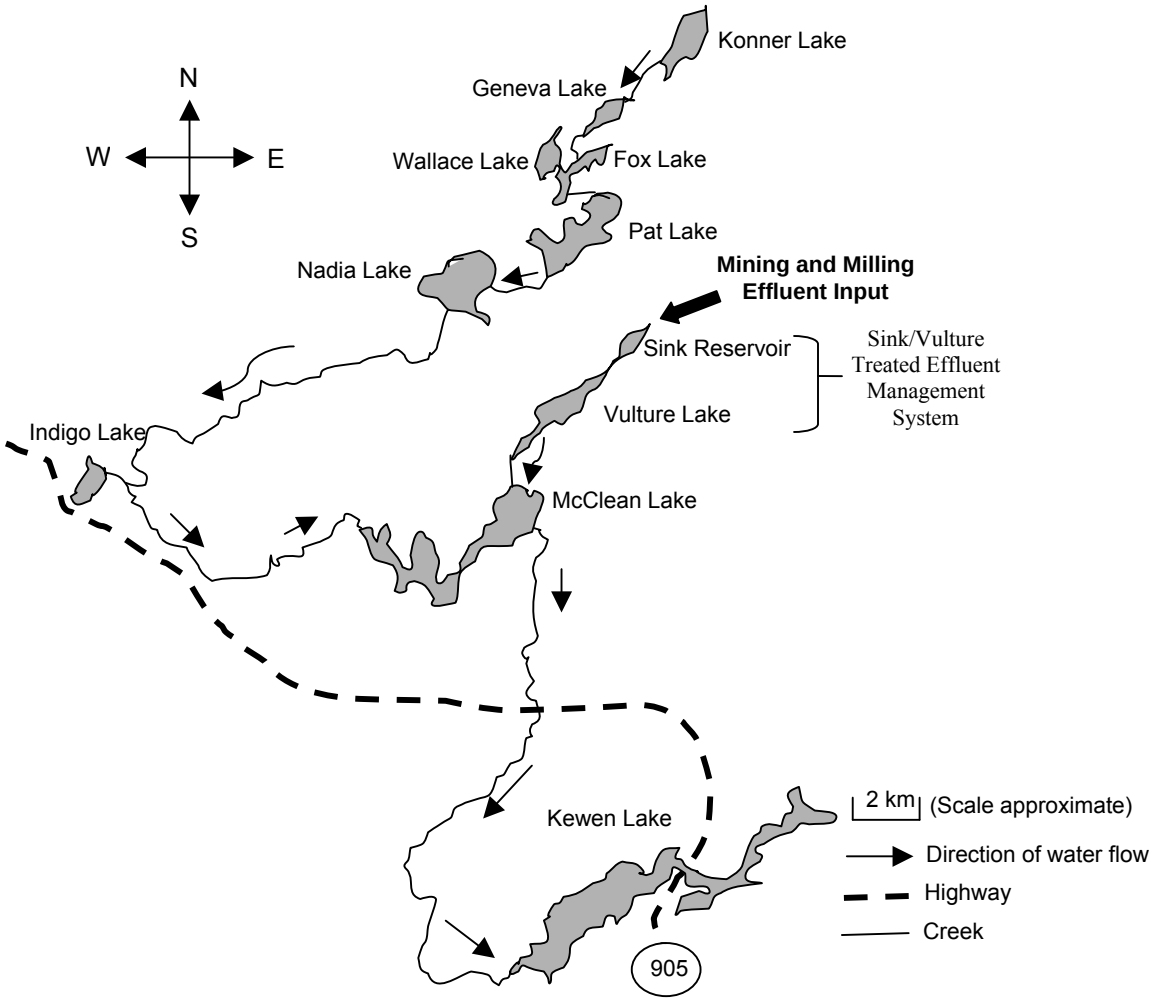

Figure 1.5 Map of the local watershed at McClean Lake uranium operation (58°23'N, 103°48'W), where lakes and creeks are receiving treated uranium mining and milling effluent. The direction of water flow in creeks is shown with arrows and lakes are shown in grey. A section of Saskatchewan highway 905 is shown with a dashed line.

Table 1.2 Water quality data for lakes at McClean Lake uranium operation, Saskatchewan, Canada. Results are mean values of monthly samples from May 2004 to November 2005 (AREVA Resources Inc. 2006). Refer to Figure 1.5 for location of lakes. Values in bold exceed either the Saskatchewan Surface Water Quality Objectives (SSWQO) or the Canadian Council of Ministers of the Environment (CCME) guidelines. Values preceded by the < symbol represent the detection limit for the specific analyte.

Analyte	Units	SSWQO	CCME	Exposure Lakes		Reference Lake
				Vulture	**Kewen**	**Mallen**
Conductivity	µS/cm			803.7	69.1	23.0
pH		6.5 to 8.5	6.5 to 9	6.8	6.6	6.6
Total dissolved solids	mg/L			556.4	51.1	24.3
Total Hardness	mg/L			321.7	21.7	6.9
Ammonia (as N)	mg/L	1.9 to 2.0	10 to >100	0.75	0.054	0.055
Sulphate	mg/L			337.1	16.7	0.69
Radium-226	Bq/L	0.11	0.6	0.0079	0.0059	0.0059
Arsenic	µg/L	50	5	2.2	0.23	0.14
Cadmium	µg/L	0.001	0.017	<0.0001	<0.0001	<0.0001
Copper	mg/L	0.01	0.002 to 0.004‡	<0.001	<0.001	<0.001
Iron	mg/L	1	0.3	0.087	0.23	0.15
Lead	mg/L	0.02	0.001 to 0.007‡	<0.002	<0.002	<0.002
Mercury	µg/L	0.1	0.026	<0.05	<0.05	<0.05
Molybdenum*	mg/L	0.7	0.073	**0.286**	0.012	0.001
Nickel	mg/L	0.025 to 0.1‡	0.025 to 0.150‡	0.013	<0.001	<0.001
Selenium	mg/L	0.01	0.001	0.00066	0.00013	0.00018
Uranium	µg/L	11 to 218‡		0.96	<0.1	<0.1
Vanadium	mg/L			<0.001	<0.001	<0.001
Zinc	mg/L	0.05	0.03	<0.005	<0.005	<0.005

(a) Guideline is temperature and pH dependent

‡ Guideline is hardness dependent, increasing with hardness

* Canadian Nuclear Safety Commission threshold limit

1.5 Indigenous fishes in northern Saskatchewan

Wild fisheries comprise about one quarter of the animal protein consumed by humans and an increasing fraction of this resource is being degraded by pollutants resulting in contaminated flesh and toxic stress to populations and the ecosystems that support them (Abramovitz 1996). The vast boreal forest is circumpolar in extent, occupying a belt as wide as 1000 km in certain regions of both North America and Eurasia (Larsen 1980). Lakes cover approximately 10% of the total boreal area (Schindler 1998) and include some of the most valuable fisheries in the world. A variety of environmental stressors are poised to disrupt thousands of years of evolution for fishes living in freshwater systems of the boreal forest. Species introductions, climate change, acidification, increased ultraviolet light exposure due to ozone thinning, aerial transport and deposition of contaminants, mining, forestry, eutrophication and man-made changes in water courses can all affect fish assemblages in the boreal forest. Although the boreal region is a very important system, it is typically overlooked by many global environmental change programs (Schindler 1998). Considering the suite of anthropogenic impacts poised to damage the boreal system, the boreal landscape may be one of the global ecoregions that will change the most in the next few decades (Schindler 1998). Since freshwaters are already scarce in many regions of the world and they are the key element in maintaining non-marine biota, including humans, they should be studied in order to maintain optimal water quality and quantity.

Aquatic systems in the boreal forest tend to be characterized by nutrient limitation (phosphorous, nitrogen), long, cold winters and relatively low species diversity (Mackay 1989). Northern lakes, rivers and streams are simple in both ecological structure and

function. For instance, there are generally fewer species at all trophic levels in northern systems like the boreal zone compared to ecosystems closer to the equator (Mackay 1989). Due to this simplicity, small changes in boreal aquatic food webs will quickly cascade and impact all other biota in the system. In Saskatchewan, common indigenous freshwater fish species include northern pike (*Esox lucius* Linnaeus), burbot (*Lota lota* Linnaeus), slimy sculpin (*Cottus cognatus* Richardson) and spottail shiners (*Notropis hudsonius* Clinton).

Northern pike have a circumpolar distribution in the northern hemisphere and are widely distributed in Canada, including Saskatchewan (Scott and Crossman 1973). Adults are sexually mature at age 3-6 for females and age 2-5 for males; spawning occurs in the spring when water temperatures reach 4.4-11.1°C. Young-of-the-year and juvenile pike are carnivorous, feeding opportunistically on benthic invertebrates and small fishes; they are a pelagic species with an active hunting strategy. Pike are a long-lived species with a life expectancy ranging from 10-26 years, depending on location, as southern populations are faster growing than arctic populations (Scott and Crossman 1973). This important sport fish is locally known as jackfish or pike.

Burbot is the only freshwater species of the cod family (Gadidae) in Canada (Scott and Crossman 1973). This fish is widely distributed across Canada and is found in waters throughout Saskatchewan. Burbot usually attain sexual maturity between 3-4 years of age and are a unique species as they spawn in late winter, under the ice. Burbot are voracious predators and hunt at night. Young-of-the-year fish and yearling fish under 50 cm in length consume invertebrates in the benthic area these fish inhabit. Burbot locate prey via olfaction and by touching the lake bottom with their pelvic fins and

barbels (Hinkens and Cochran 1988). These fish do not actively swim all day, but rather hide under rocks and forage within a small area at night by making rapid, close-range attacks on prey. Young-of-the-year burbot are known to remain within a small home range in the littoral zone of lakes (Hofmann and Fischer 2001). The maximum age for burbot in Canada is between 10 and 15 years. Burbot are colloquially known as maria, freshwater cod, ling or loche in Saskatchewan. Although the species is not a highly prized sport fish, it is commonly caught while ice-fishing (Scott and Crossman 1973).

Slimy sculpin are a small-bodied fish species (averaging 7.6 cm in total length) and are the subject of increased interest as a sentinel species in environmental monitoring (Gibbons *et al.* 1998, Galloway *et al.* 2003). The species is found throughout most of Canada, including Saskatchewan, in a variety of habitats including the deep water of lakes, rocky streams and lake bottoms (Scott and Crossman 1973). These fish feed on benthic invertebrates and can be consumed by larger predaceous fishes, including northern pike and burbot. Hill and Grossman (1987) found that slimy sculpin maintain a relatively small home range in streams, with average moves between captures ranging from 12 to 20 meters. Spawning occurs in the spring (April - June). Eggs are attached to rocks and males guard the fertilized eggs during the 28 day incubation period (van Vliet 1964). Whether a fish reaches sexual maturity at age 1 or one year later is dependent on the individual's growth rate (van Vliet 1964). Life expectancy varies, van Vliet (1964) found that the oldest slimy sculpin near La Ronge, Saskatchewan was 6 years old, although the majority of fish were less than 4 years.

Spottail shiners are an important small bodied forage fish that are both widespread and abundant in Saskatchewan. This species spawns in the spring and early summer.

They feed on plankton and aquatic invertebrates and are, in turn, consumed by almost all predaceous fishes. Spottail shiners attain a maximum age of 4 or 5 years and reach sexual maturity at age 1 or 2 (Scott and Crossman 1973).

1.6 Hypotheses and research objectives

Due to the susceptibility of YOY fishes to overwinter mortality in the presence of a potential metabolic stressor, I determined morphometric and biochemical endpoints in three indigenous fish species. In order to assess overwinter mortality, sampling was conducted in late fall and early spring, immediately before and after winter. Sampling was conducted at reference and exposure sites at the two operations described above (Key Lake and McClean Lake uranium operations), in order to assess if there were differences or similarities in fish response at the different operations.

Since physiological responses to contaminant exposure require energy, I hypothesized that fish exposed to uranium mining and milling effluent would have decreased energy storage and growth measured with traditional morphometric (weight, length, condition factor, hepato-somatic index, gonado-somatic index) and innovative biochemical (total body lipids, total body triglycerides, liver triglycerides, muscle protein, muscle RNA/DNA ratio) endpoints in both the fall and spring compared to fish from reference lakes. I also hypothesized that seasonal differences in energetic endpoints would occur, with a decrease in total body lipids, total body triglycerides and liver triglycerides in the spring compared to the fall, due to the physiological stress associated with winter.

For method validation, results from all fishes from all seasons at both mines were combined in order to compare 1) the Bligh and Dyer chloroform-methanol extracted lipid

(gravimetric and sulphophosphovanillin) measurements, 2) chloroform-methanol extracted gravimetric lipid and triglyceride measurements and 3) triglyceride determinations in solvent extracted fish tissue and whole body, unextracted fish homogenate (Figure 1.2). Results from comparisons in lipid and triglyceride measurement methods may help in subsequent data analysis for studies at the two uranium operations and future research projects using these endpoints.

Specific objectives of this study were to:

(i) Determine if uranium operation effluents have an impact on various morphometric and biochemical endpoints in juvenile indigenous fish.

(ii) Investigate potential seasonal differences in energetic biochemical endpoints in juvenile indigenous fish at both reference and uranium mining and milling effluent exposure sites.

(iii) Compare species differences in morphometric and biochemical endpoints at each uranium operation.

(iv) Compare differences in morphometric and biochemical responses for each species between two uranium operations.

(v) Validate the use of measuring triglycerides in a crude, non-solvent extracted homogenate as a rapid and reliable alternative to a chloroform-methanol extraction and subsequent lipid or triglyceride measurement.

The chapters in this thesis address these objectives as follows:

(1) Evaluate, validate and optimize lipid and triglyceride measurement methods (Chapter 2),

(2) Determine condition of fishes at Key Lake uranium operation by measuring morphometric and biochemical endpoints in YOY fishes (Chapter 3),

(3) Determine condition of fishes at McClean Lake uranium operation by measuring morphometric and biochemical endpoints in YOY fishes (Chapter 4),

(4) Compare and contrast results between the two uranium operations and summarize overall findings and recommendations (Chapter 5).

1.7 Environmental and scientific relevance

The integrative focus of this study is unique in that sensitive measures of health will be determined at the sub-organismal level and extrapolated to estimate impacts on individual survival and population structure. The combination of biochemical indices of condition and traditional morphometric measures can provide a record of recent growth and an estimate of future growth potential and survival for individual fishes. Mortality or compromised condition of young-of-the-year fishes could result in decreased recruitment and consequently affect year-class strength, overall community structure and ultimately, community level ecological interactions (Lemly 1996). This will allow the validation and comparison of the utility of different endpoints in different species in order to advise future investigators of the performance of the morphometric and biochemical endpoints in these northern aquatic systems. New uranium operations are continually being explored and initiated in Saskatchewan. Since this industry is likely to continue in Saskatchewan for the foreseeable future, the data generated in this study will be valuable to future researchers for decades to come.

In addition to the toxicological information generated, data from fish collected at reference lakes will provide new biological information on bioenergetics and growth of

juvenile indigenous fish species in boreal ecosystems. Knowledge of fish life history and dynamics is important for ecological monitoring as well as fisheries management issues. With the current and potential future anthropogenic insults on the boreal ecosystem, this baseline information on fish bioenergetics and growth will help to estimate impacts in boreal freshwater systems.

CHAPTER 2
2.0 COMPARISON OF TOTAL LIPID AND TRIGLYCERIDE MEASUREMENT METHODS IN FOUR FISH SPECIES

2.1 Abstract

Lipids are important variables in fish bioenergetics and are frequently determined using chloroform-methanol extraction methods. Triglycerides are the major energy storage form in fishes and therefore are a more ecologically and physiologically relevant measure of bioenergetics than total lipids. Total body lipids (gravimetric and sulphophosphovanillin methods) and total body triglycerides (chloroform-methanol extracted and unextracted, whole body fractions) were measured in four fish species. Total body lipids (gravimetric) were consistently higher than total body triglycerides when measured in the same solvent extracted fraction, although both measures followed similar trends. In an effort to eliminate the need for solvent extraction, the performance of the triglyceride assay was compared in both the solvent extracted fraction and a whole body, unextracted homogenate for each fish. Interestingly, the chloroform-methanol extracted triglyceride values were consistently lower than triglycerides measured in the unextracted whole body homogenate. In addition, this comparison of triglycerides measurements revealed limitations for the solvent extraction and subsequent triglyceride determination in lean fish. Thus, in addition to being simple and rapid, determination of triglycerides in a whole, unextracted fish homogenate may be a superior alternative to chloroform-methanol based methods of lipid extraction and subsequent triglyceride measurement.

2.2 Introduction

Lipids have various roles in fish nutrition and health and can be used to estimate important endpoints such as overwinter survival, energy storage, growth, reproduction and response to environmental stress (Adams 1999). Therefore, determining lipid content in wild biota, including fishes, is an ecologically important variable to consider when estimating animal condition, nutritional status and overall health. Fishes require minimum lipid levels to maintain a healthy condition, grow, reproduce and ensure the continued survival of a local population (Adams 1999). Therefore, a rapid and reliable method for measuring lipid in fish tissue is a vital component for scientific investigations related to fish ecology.

The chloroform-methanol extraction of lipids has been used in fisheries research for the past half century (Folch *et al*. 1957, Bligh and Dyer 1959). Both the Folch *et al*. (1957) and Bligh and Dyer (1959) methods require precise ratios of chloroform to methanol to water. Folch *et al*. (1957) uses a saline solution and the Bligh and Dyer (1959) method was developed for use with fish tissues, which have a high proportion of water. Lipids extracted with solvents can then be measured gravimetrically (i.e. weight). With this type of measurement method, most lipophilic substances in a fish sample will be included in the determination of lipid weight. The sulphophosphovanillin method (Knight *et al*. 1972, Weber *et al*. 2003) for lipid determination provides increased specificity and sensitivity compared to the gravimetric determination, since the assay measures the amount of unsaturated bonds which are largely due to fatty acids in a solvent extract. The gravimetric and sulphophosphovanillin methods are reliable, but both are limited by problems related to the solvent extraction technique, such as

contamination, evaporation or sample variability (Dickey *et al*. 2002). A further limitation of the chloroform-methanol extraction method is a severe decrease in extraction efficiency reported in small fish (Honeycutt *et al*. 1995, Weber *et al*. 2003). Finally, the chloroform-methanol extractions are time consuming and involve the use of toxic solvents, particularly chloroform. Since workplace exposure to toxic solvents should be minimized, a rapid and safer method of measuring lipid content in fish tissues would be a valuable assessment tool in fish research.

In ecological research, lipid content has been used to estimate fish condition (Berg and Bremset 1998, Hurst and Conover 2001, Post and Parkinson 2001, Jonsson and Jonsson 2003, Eckmann 2004, Kiessling *et al.* 2004). From an ecological perspective, total body lipids may be a poor estimate of fish condition and bioenergetics, since a solvent extracted lipid fraction will contain structural lipids (i.e. cellular membranes) in addition to energetic lipids. Triacylglycerols (triglycerides) are the primary energy storage form in fishes (Sheridan 1988, Jobling *et al.* 1998, Adams 1999). Body triglycerides are emerging as an ecologically and physiologically relevant measure of energy storage and fitness in individual fishes (Lochmann *et al.* 1995, Jobling *et al.* 1998, MacFarlane and Norton 2002, Heintz *et al.* 2004). Triglycerides have previously been measured in the solvent extracted fraction using chromatography techniques (Lochmann *et al.* 1995, Jobling *et al.* 1998, Heintz *et al.* 2004) or enzymatic assays (Benton *et al.* 1994, Weber *et al.* 2003). Measuring fish tissue triglycerides is ecologically relevant; however the current triglyceride measurement methods still employ the initial labour intensive solvent extraction process.

Therefore, further validation of measurement methods for triglycerides in fishes is required, since triglycerides are the main energy storage form in fishes and existing chloroform-methanol lipid extraction techniques are tedious and increase workplace exposure to solvents. Although various chloroform-methanol lipid extraction methods are available, only the Bligh and Dyer (1959) method was conducted here, since it is commonly used in fisheries research. The objectives of this study were to compare 1) the Bligh and Dyer chloroform-methanol extracted lipid (gravimetric and sulphophosphovanillin) measurements, 2) Bligh and Dyer extracted gravimetric lipid and triglyceride measurements and 3) triglyceride determinations in solvent extracted fish tissue and whole body, unextracted fish homogenate.

2.3 Materials and Methods

Fishes were collected using a backpack electrofishing unit from two different locations in northern Saskatchewan, Canada (57°13'N, 105°38'W and 58°23'N, 103°48'W). Fishes were collected during four sampling periods: October (2003), June, September (2004) and May (2005), overdosed with 3-aminobenzoic acid (Sigma-Aldrich Canada Ltd., Oakville, ON, Canada) and immediately stored on dry ice until transport to a -80ºC freezer at the Toxicology Centre, University of Saskatchewan. In the laboratory, fishes were dissected. Stomach contents, a piece of liver and tail muscle were removed; the remaining carcass was weighed and finely minced with scissors in ×2 volume of nanopure water. The carcass was homogenized for 3 × 30 seconds using a Tissue Tearor (BioSpec Products Inc., Bartlesville, OK, U.S.A.). Aliquots of this homogenate from each fish were used to measure all lipid endpoints as outlined in Figure 2.1. Specifically, a 2-4 ml aliquot of this homogenate was used for chloroform-methanol lipid extraction

(Bligh and Dyer 1959). The chloroform-methanol lipid extraction was conducted on all fishes using a final ratio of 1.8:2:2 for water to chloroform to methanol as per Bligh and Dyer (1959), with the following modifications: chloroform used for extractions contained 50 mg/L butylated hydroxytoluene (Sigma-Aldrich Canada Ltd., Oakville, ON, Canada) to reduce oxidation of fatty acids (Weber *et al*. 2003), samples were centrifuged instead of filtered and samples were dried at room temperature under a gentle stream of nitrogen. Lipid weight was determined in each fish (mg lipid per gram fish carcass) gravimetrically; triplicate aliquots of the chloroform fraction were evaporated in pre-weighed glass vials under a nitrogen atmosphere to prevent lipid oxidation.

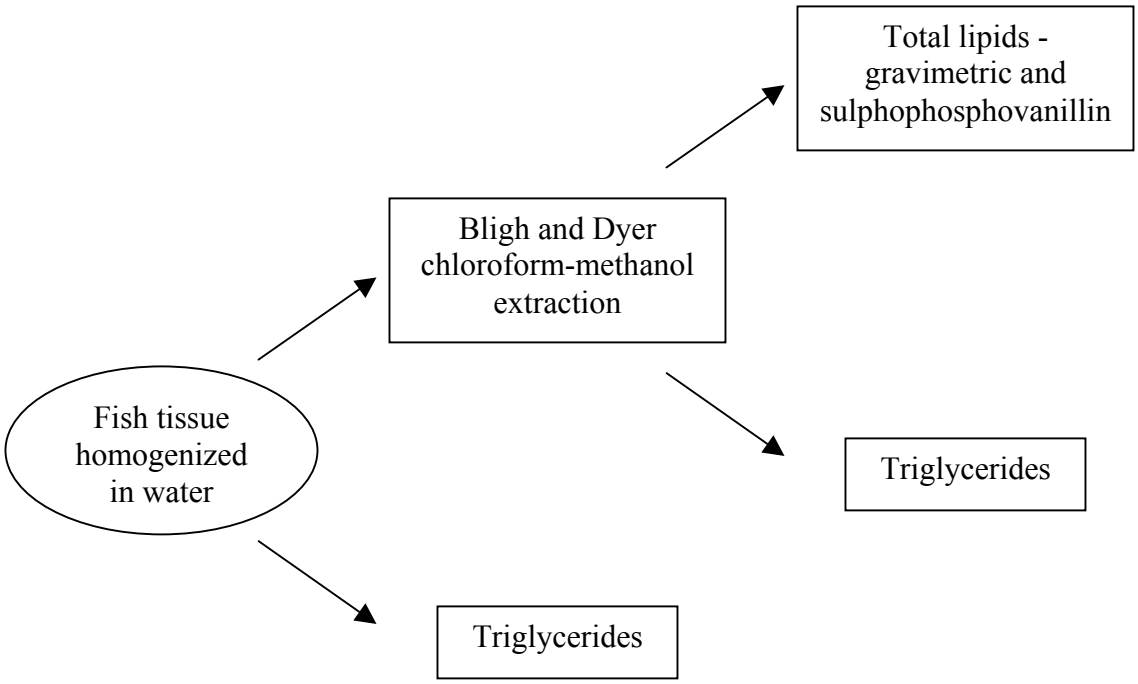

Figure 2.1 Preparations for different total body lipid and triglyceride measurements methods.

The remaining solvent extract was used to measure both total body lipids (sulphophosphovanillin assay) and total body triglycerides. A modification of the oxidation-based assay from Knight *et al*. (1972) was used (Weber *et al*. 2003) to determine total body lipids. For each species and sampling time (fall or spring), a portion of chloroform extract from individual fishes were pooled and measured gravimetrically in six replicates to determine average lipid concentration for a species in a particular season. Standards were then calculated based on the pooled species lipid content and standards and samples were developed based on the Knight *et al.* (1972) protocol and plated in duplicate 200 µl aliquots on a 96-well microplate.

Whole body triglycerides were determined in the solvent extract using a modified clinical serum assay (Sigma-Aldrich Canada Ltd., Oakville, ON, Canada) based on the McGowan *et al*. (1983) method (Weber *et al*. 2003). Solvent extracted samples were evaporated under a nitrogen stream, reconstituted in isopropanol and plated in duplicate on a 96-well microplate. Briefly, the enzymatic triglyceride assay first produces free glycerol from acyl glycerides with lipase. Glycerol content can then be determined in samples and glycerol standards spectrophotometrically after addition of glycerol kinase, since the enzymatic assay is coupled with a colourimetric reaction.

Whole body triglycerides were also determined in the unextracted fraction of the same fish carcass homogenized in ×2 volume of nanopure water. An aliquot of fish homogenate was added to an equal volume of 0.2M sodium citrate. The sample was re-homogenized on ice, placed in a heating block at 100ºC for 5 minutes and immediately returned to ice. The assay for unextracted triglycerides was the same as described above for the extracted triglycerides with one procedural addition: all samples were centrifuged

for five minutes at 500 *g* prior to assaying in order to remove insoluble materials, such as connective tissue, from the homogenate.

Intra-assay variability was calculated by determining the coefficient of variation (%CV = standard deviation/mean*100) for six replicates of a pooled sample measured in the same assay. Inter-assay variability was determined for all experimental endpoints by calculating the %CV among six replicates of the same pooled sample performed in two separate assays.

For statistical analyses, Spearman rank order correlations were used since data were not normally distributed. The correlation coefficient (r) was given at $p < 0.05$ for all correlations. Best fit linear relationships were determined using linear regression. The Mann-Whitney rank sum test was used to determine if the means of the two endpoints being correlated were significantly different. All results were reported as mean ± standard error of the mean (SEM).

2.4 Results

A total of 301 fishes were collected and analyzed: 131 northern pike (*Esox lucius* Linnaeus), 100 burbot (*Lota lota* Linnaeus), 36 slimy sculpin (*Cottus cognatus* Richardson) and 34 spottail shiners (*Notropis hudsonius* Clinton). All northern pike and burbot were aged and determined to be less than two years. The small bodied species, sculpin and shiners, were not aged and likely contained a combination of adults and juveniles.

The two methods for determining total body lipids, gravimetric determination and sulphophosphovanillin, produced similar results when values from all species were combined ($r = 0.92$, $p < 0.001$; $y = 1.01x - 1.75$, where y = total body lipids -

sulphophosphovanillin and x = total body lipids - gravimetric; Figure 2.2). Overall, the mean gravimetric lipid value (15.8 ± 0.5 mg/g) was significantly ($p < 0.001$) higher than the sulphophosphovanillin result (14.2 ± 0.6 mg/g). Species differences in total body lipid content were noted based on gravimetric lipids: sculpin (22.0 ± 1.6 mg/g) > burbot (20.7 ± 1.1 mg/g) > northern pike (12.5 ± 0.4 mg/g) > spottail shiners (7.3 ± 0.6 mg/g).

Total body lipids (gravimetric) correlated positively with solvent extracted total body triglycerides ($r = 0.80$, $p < 0.001$; y = 0.69x – 5.25, where y = extracted triglycerides and x = total body lipids) for all fish species combined. Total body lipids (15.8 ± 0.5 mg/g) were significantly greater ($p < 0.001$) than the solvent extracted triglycerides (5.7 ± 0.4 mg/g). Positive correlations between total body lipids and triglycerides were also significant ($p < 0.001$) for each species (Figure 2.3). Correlations between total body lipids and triglycerides were lower for northern pike and shiners ($r = 0.77$, $r = 0.66$, respectively) compared to burbot and sculpin ($r = 0.95$, $r = 0.91$, respectively). Slopes of linear relationships between total lipids and triglycerides were below one for all species: northern pike (0.67), burbot (0.79), slimy sculpin (0.62) and spottail shiners (0.48). Triglyceride content was also determined as a percent of total body lipids (solvent extracted triglyceride/gravimetric lipid*100%): northern pike (17.3 ± 1.4%), burbot (31.8 ± 2.2%), slimy sculpin (43.4 ± 3.0%) and spottail shiners (39.5 ± 3.3%).

Bligh and Dyer chloroform-methanol extracted total body triglycerides were significantly correlated with unextracted total body triglycerides for all species combined ($r = 0.91$, $p < 0.001$; y = 1.13x + 3.01, where y = unextracted triglycerides and x = extracted triglycerides). The mean unextracted triglyceride values were consistently and

significantly ($p < 0.001$) higher (9.4 ± 0.5 mg/g) than the mean solvent extracted triglyceride values (5.7 ± 0.4 mg/g). Separate correlations between the two triglyceride measurement methods for each species were similar (Figure 2.4): northern pike ($r = 0.90$, $p < 0.001$; y = 1.20x + 1.80), burbot ($r = 0.97$, $p < 0.001$; y = 1.05x + 1.43) sculpin ($r = 0.92$, $p < 0.001$; y = 1.58x + 1.57) and shiners ($r = 0.86$, $p < 0.001$; y = 2.86x + 5.16) where y = unextracted triglycerides and x = extracted triglycerides for all species correlations.

Differences between the extracted and unextracted triglyceride values were noted for all species, as were species differences in triglyceride concentrations (Table 2.1).

Table 2.1 Total body triglycerides (mg triglyceride per gram fish tissue) for northern pike (*Esox lucius*), burbot (*Lota lota*), slimy sculpin (*Cottus cognatus*) and spottail shiners (*Notropis hudsonius*) determined using two different methods: chloroform-methanol extraction (solvent extraction method) and non-solvent extraction (unextracted method). Data shown are mean ± standard error of the mean. Mann-Whitney rank sum test used to compare mean triglyceride values for a species determined using the two different methods.

	Total body triglycerides (mg/g)	
	Solvent extraction method	Unextracted method
Northern pike	2.8 ± 0.3	5.1 ± 0.4***
Burbot	8.7 ± 0.9	10.5 ± 1.0*
Slimy sculpin	10.3 ± 1.1	17.9 ± 2.0*
Spottail shiners	3.0 ± 0.4	13.7 ± 1.5***

* Significantly different triglyceride values for a species (*$p < 0.05$, ***$p < 0.001$)

Both intra- and inter-assay coefficients of variation were below 10% for all assays. Intra-assay variability was 3.7%, 1.6%, 2.8% and 3.0% for the gravimetric,

sulphophosphovanillin, solvent extracted triglyceride and unextracted triglyceride assays, respectively. Inter-assay variability was 5.6%, 3.8%, 3.0% and 4.7% for the gravimetric, sulphophosphovanillin, solvent extracted triglyceride and unextracted triglyceride assays, respectively.

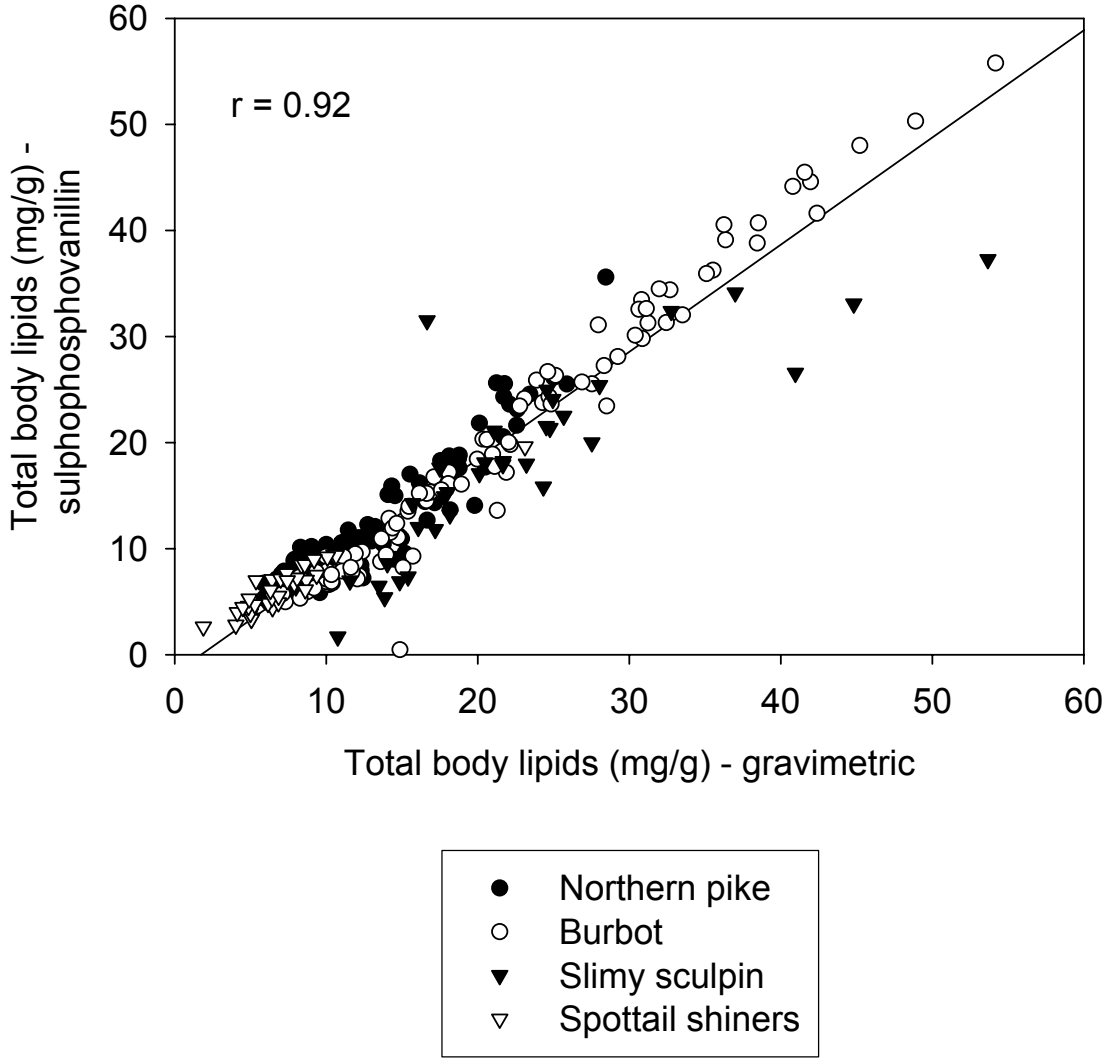

Figure 2.2 Comparison of total body lipids (mg lipid per g fish carcass) determined using gravimetric and sulphophosphovanillin methods following Bligh and Dyer chloroform-methanol extraction in four fish species: northern pike (n = 131), burbot (n = 100), slimy sculpin (n = 36) and spottail shiners (n = 34).

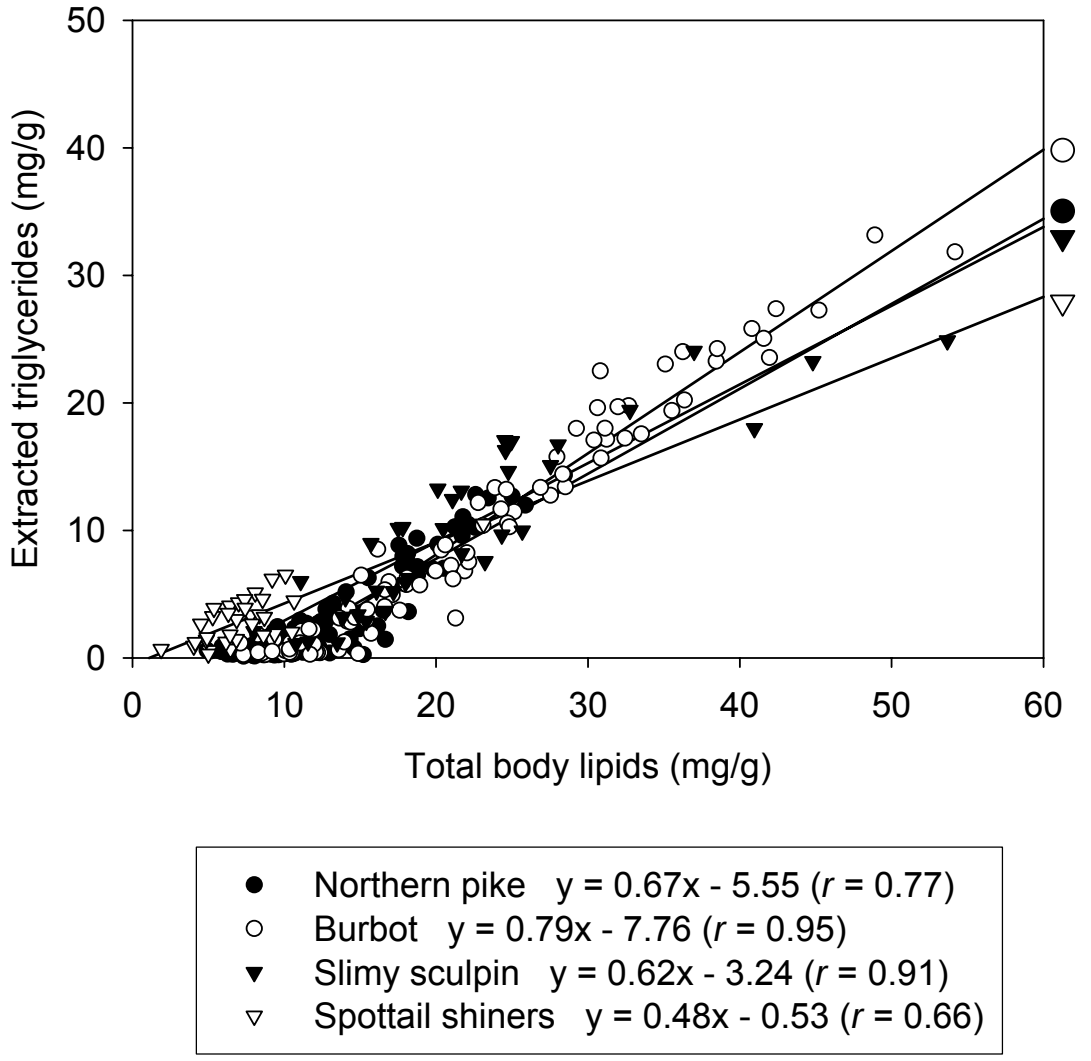

Figure 2.3 Comparison of total body triglycerides (mg triglyceride per g fish carcass) from a chloroform-methanol extracted fraction and total body lipids (mg lipid per g fish carcass) from gravimetric measurement. Results are shown separately for northern pike (n = 131), burbot (n = 100), slimy sculpin (n = 36) and spottail shiner (n = 34) with correlation coefficients (r) and best fit linear relationships.

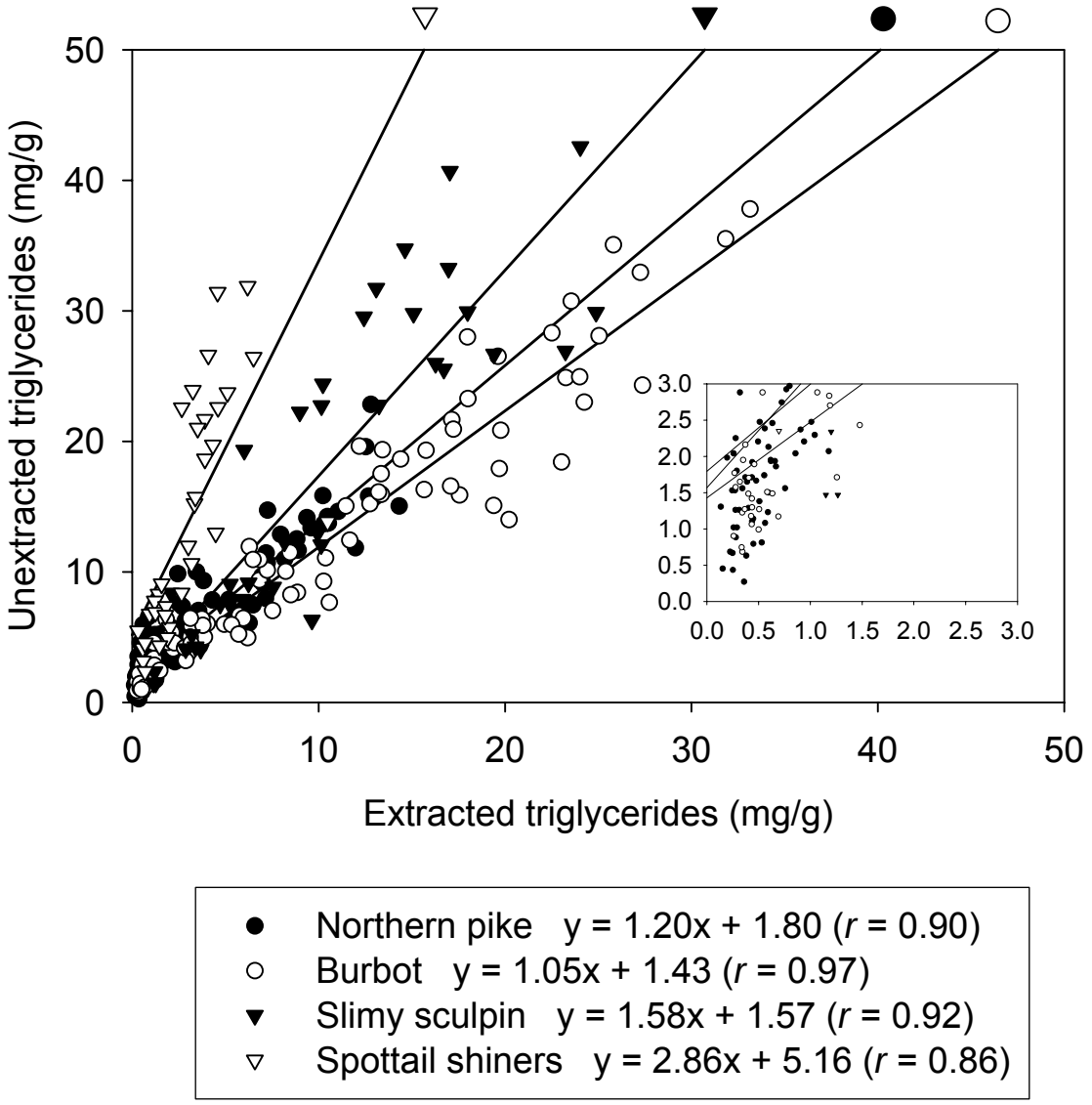

Figure 2.4 Comparison of two techniques for determining fish triglyceride concentrations (mg triglyceride per g fish carcass): total body triglycerides measured in the Bligh and Dyer chloroform-methanol extracted fraction and in the whole body, unextracted fraction. Results are shown separately for northern pike (n = 131), burbot (n = 100), slimy sculpin (n = 36) and spottail shiner (n = 34) with correlation coefficients (r) and best fit linear relationships. The inset shows an expanded view of the relationship at low (< 3 mg/g) triglyceride concentrations.

2.5 Discussion

Chloroform-methanol extraction using the Bligh and Dyer (1959) technique results in the recovery of lipid-soluble compounds including structural (membrane) lipids, triglycerides and other lipophilic molecules. Although a close correlation with a slope approaching unity was noted, total lipids determined in the present study using the gravimetric approach were consistently higher than total lipids determined with the sulphophosphovanillin assay. This is likely a result of the presence of non-fatty acid materials with saturated carbon-carbon bonds in the lipid extract being measured using the gravimetric method. Weber *et al*. (2003) found similar results and suggested that the gravimetric approach may overestimate lipid content since mucolipids, proteolipids and glycolipids are extracted and would consequently contribute to lipid mass. Since the sulphophosphovanillin assay specifically measures carbon-carbon double bonds and such bonds would arise almost exclusively from unsaturated fatty acids in the solvent extract, this assay theoretically provides a more accurate estimate of total body lipids than the gravimetric measurement. Despite this, the slope of the linear relationship between these two lipid measurements was very close to unity. Therefore, both gravimetric and sulphophosphovanillin methods provide similar estimates of total lipid content and both methods appear to be reliable based on low intra- and inter-assay variability. However, this must be interpreted with caution because both of these methods require solvent extraction, any limitations with the extraction process itself will similarly affect both methods.

Triglyceride concentrations were consistently lower than total body lipid estimates. This was expected since triglycerides are only a portion of total lipids. The

correlation between total body lipids and triglycerides among all species was the weakest comparison in this study. This could be explained by species differences in the proportion of total lipids that are triglycerides. Alternatively, differences in the types and proportions of other lipophilic substances (e.g. phospholipids) that are extracted with solvents may also vary between species. Examining species differences between total lipid and solvent extracted triglyceride measurements, it is clear that fishes with higher lipid content (burbot and slimy sculpins) have a higher correlation coefficient for the two lipid endpoints. Both northern pike and spottail shiner had more variable correlations between total lipids and extracted triglycerides, although the trends responsible for this variability were different. For the spottail shiners, a high percent of lipids were identified as triglycerides whereas for the northern pike, a lower percent of lipids were triglycerides. Overall, these results suggest significant species differences in triglyceride content when expressed on a total body lipid basis. Therefore, total body lipids estimated with the Bligh and Dyer method may be a poorer estimate of more ecologically relevant energy storage lipids, triglycerides. Fisheries research aimed at investigating fish bioenergetics, condition or nutritional status should consider measuring stored energy directly as triglycerides.

Due to the specificity of the glycerol kinase and lipase used in the triglyceride assay, triglycerides could be measured in both the unextracted fish homogenate and the solvent extracted lipid fraction. The triglyceride assay was very reliable as evidenced by low inter- and intra-assay variability for both the unextracted and the solvent extracted preparations. Overall, the unextracted and extracted total body triglyceride concentrations correlated very closely. However, it was evident that in lean fish (< 3 mg

triglyceride per g fish tissue), the solvent extracted triglyceride estimates were consistently much lower than the unextracted triglycerides. Previous studies have noted a limitation of lipid extraction methods for small fish (Honeycutt *et al*. 1995, Weber *et al*. 2003). These results suggest that the chloroform-methanol method of lipid extraction may also underestimate lipid, and therefore triglyceride, content measured in the solvent extract in lean fishes. Since fish lipid content will vary with age, species or season, it is important to employ a lipid measurement method that will work optimally and consistently for fishes of various sizes and lipid content.

Unextracted triglyceride values were consistently higher, although closely correlated with, the extracted triglyceride values. This suggests some type of systematic error. There may be sample loss during the chloroform-methanol extraction, either through loss of a portion of the lipid sample during removal of the methanol layer, or lipid remaining in the discarded tissue layer. Absorption of the chloroform fraction to tissue prevents full recovery of solvent extracted lipids and can be responsible for incomplete lipid extraction (Smedes and Thomasen 1996). Fish tissues were not re-extracted in this study, which may have increased the total lipid and triglyceride yields. However, previous preliminary experiments in this laboratory produced no measurable lipid following a second solvent extraction in a fatty fish species (farmed rainbow trout, unpublished). Other studies have endeavoured to eliminate the initial Bligh and Dyer chloroform-methanol lipid extraction prior to lipid measurement and replace it with a direct measure of lipid constituents. Dickey *et al*. (2002) determined fatty acid content in striped bass (*Morone saxatilis* Walbaum) tissue and diet using both the Bligh and Dyer method followed by a transesterification step and a direct transesterification on

unextracted samples. Increased recovery of fatty acids using the direct transesterification method was hypothesized to result from improved disruption of the matrix, since the presence of an acid environment and elevated temperatures could liberate fatty acids (Dickey *et al*. 2002). Perhaps the heating step in sodium citrate buffer employed in the current study served to free triglycerides from the tissue matrix, which may explain the higher triglyceride values obtained from the unextracted preparation.

The results of the current study and a previous study (Weber *et al*. 2003) suggest that measurement of triglycerides in the unextracted fraction may be more reliable than the results from the Bligh and Dyer extracted triglycerides, particularly for fish with low lipid levels. Weber *et al*. (2003) performed spike recovery experiments using monoolien in unextracted fish tissue and reported high recovery. This suggests that the specificity of the enzyme based triglyceride assay is not impaired in a crude homogenate and therefore, the cause of the apparent discrepancy in triglyceride values is likely an underestimation of triglyceride in the chloroform-methanol extracted fraction. Furthermore, the inability of the Bligh and Dyer lipid extraction method to perform reliably in small or lean fishes suggests that solvent extracts underestimate both total lipids and triglycerides. However, despite differences in the absolute values of triglyceride content there was a strong positive linear relationship between triglyceride determinations in solvent extracted and unextracted preparations. Although triglycerides were measured in whole fishes in this study, the results would likely be similar if specific tissues were examined, such as gonads, liver or muscle.

Using total lipid and triglyceride levels to aid in understanding energy flow through food webs and energy allocation strategies for fishes remains an important

endpoint in fisheries research. However, results of the present study indicate that the popular Bligh and Dyer chloroform-methanol extraction method is unreliable when individual fish have low lipid levels. More importantly, instead of using total lipid content to estimate fish condition, it may be more ecologically and physiologically relevant to examine triglyceride content in fishes. Previous studies have used chromatography to estimate triglyceride content in solvent extracted fish tissue (Lochmann *et al*. 1995, Jobling *et al*. 1998, Heintz *et al*. 2004). Unfortunately, these methods still involve initial solvent extraction of lipids. Although other chloroform-methanol lipid extraction methods (i.e. Folch *et al*. 1957) were not investigated here, all existing solvent extraction methods are tedious and involve worker exposure to chloroform. Therefore, a viable and possibly superior alternative to determining total body lipids in a chloroform-methanol extraction is to measure triglycerides in a whole body homogenate.

CHAPTER 3
3.0 BIOENERGETICS AND GROWTH OF YOUNG-OF-THE-YEAR NORTHERN PIKE (*ESOX LUCIUS*) AND BURBOT (*LOTA LOTA*) EXPOSED TO METAL MINING EFFLUENT

3.1 Abstract

Exposure to metal mining effluent may reduce the ability of young-of-the-year fishes to accumulate sufficient growth and energy reserves to survive the overwinter period in a Canadian boreal forest watershed. Northern pike (*Esox lucius*) and burbot (*Lota lota*) were collected just prior to ice-on (age-0) and immediately following ice-off (age-1) from two lakes receiving effluent and one reference lake. Unexpectedly, total body lipid, total body triglyceride and liver triglyceride levels were greater in effluent-exposed pike and burbot in both fall and spring. Despite this, there were no lake or season differences in growth indices of pike, including length, weight, muscle RNA/DNA ratio or muscle protein levels. Total body triglycerides were also higher in exposed spottail shiners (*Notropis hudsonius*), a major prey item of juvenile pike. In addition, total lipids and triglycerides in burbot were greater in spring compared to fall, while no seasonal differences were observed in pike, suggesting that young-of-the-year burbot continued to feed during winter. Principal component analysis indicated that total lipids, triglycerides, weight and length followed similar trends in both species. Findings suggest direct and indirect effects of metal mining effluent on lipid dynamics of juvenile fishes.

3.2 Introduction

At higher latitudes fish are subject to seasonally short periods of growth potential followed by a long over-wintering period. This seasonal environment puts constraints on the ability of young-of-the-year (YOY) fish to acquire sufficient growth and energy reserves to survive winter (Cunjak 1988, Berg and Bremset 1998, Post and Parkinson 2001). A major source of winter mortality in underyearling fish is depletion of lipid reserves (Sogard and Olla 2000, Pratt and Fox 2002, Biro *et al.* 2004). Triglycerides (triacylglycerols) are the major energy storage form in fish and have important ecophysiological relevance as indicators of growth potential and survival (Lochmann *et al.* 1995, Jobling *et al.* 1998, MacFarlane and Norton 2002, Heintz *et al.* 2004).

Environmental stressors can alter both the quality and quantity of energetic lipids in fish (Adams 1999), which directly influences the ability of YOY fishes to survive the overwinter period. In addition, metabolic costs may be increased during exposure to toxicants such as metals (McGeer *et al.* 2000, Sherwood *et al.* 2000, Levesque *et al.* 2002, Rajotte and Couture 2002, Couture and Rajotte 2003), thus decreasing production processes such as growth and lipid storage (Calow 1991, Lemly 1996, Adams 1999, Congdon *et al.* 2001). Lemly (1993) used the term "winter stress syndrome" to describe the significant overwinter lipid depletion and reduced survival that can occur in fish exposed to toxicants. Fish with reduced activity and foraging during cold winter temperatures may develop winter stress syndrome if a metabolic stressor is present (Lemly 1993, Lemly 1996). Thus, in wild fish populations inhabiting north temperate aquatic systems that are impacted by metal mining activities, recruitment of individuals into the population could be impaired (Calow 1991, Lemly 1996). However, to my

knowledge the winter stress syndrome hypothesis has not been investigated in field research involving indigenous coldwater fish species.

To test this hypothesis and assess possible impacts of metal mining effluent on juvenile fish condition and overwinter survival potential, I determined traditional morphometric measures of growth (weight, length, condition factor) and biochemical measures of energy storage (total body lipids, total body triglycerides, liver triglycerides) and growth (muscle protein, muscle RNA/DNA ratio) in juvenile fishes sampled immediately before and after the overwinter period. Young-of-the-year northern pike (*Esox lucius*) and burbot (*Lota lota*) were collected from two lakes receiving effluent from a uranium milling operation and a reference lake in northern Saskatchewan, Canada. I predicted there would be decreased growth and lipid stores in juvenile fish exposed to effluent in both fall and spring compared to fish collected from the reference lake. I also predicted decreased lipid and triglyceride content in fish collected in spring from all sites compared to fish collected in the fall, due to energy utilization during the overwinter period.

3.3 Materials and Methods

3.3.1 Site description and fish collection

Fishes were collected from two lakes downstream of uranium milling effluent discharge: Unknown Lake (high exposure), located approximately 2 km from effluent release and Delta Lake (low exposure), located approximately 10 km from effluent release (Figure 3.1). The reference lake, David Lake, is upstream of all milling activities and is located in the same watershed as the two exposure lakes (Figure 3.1). The study site is located at Key Lake uranium operation in north-central Saskatchewan, Canada

(57°13' N, 105°38’ W). At this latitude long, cold winters are typical and lakes are usually covered with ice for > 200 days. Treated mill effluent is discharged at a rate of approximately 6000 m^3/d into the David Creek drainage (Figure 3.1).

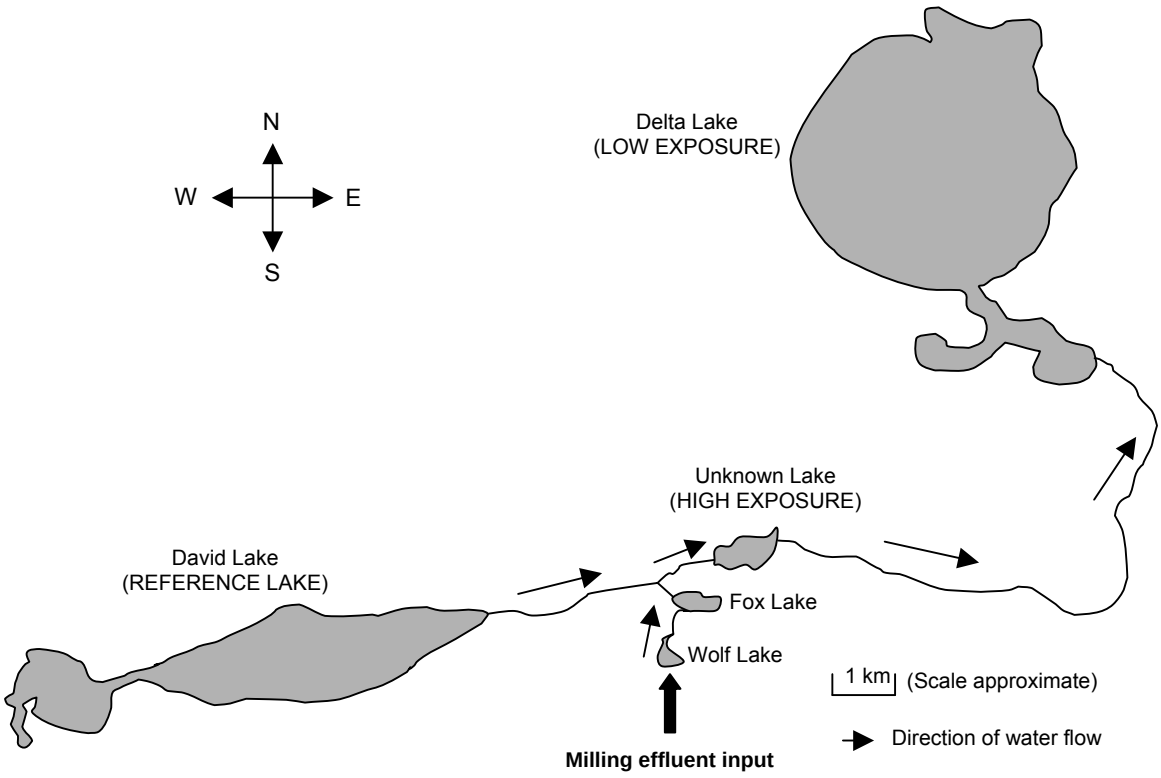

Figure 3.1 Map of David Creek drainage containing treated uranium milling effluent at Key Lake uranium operation, Saskatchewan, Canada (57°13' N, 105°38’ W). The direction of water flow is shown with arrows and lakes are shown in grey. Fishes were collected at the reference lake and the low and high exposure lakes in fall 2004 and spring 2005.

Juvenile northern pike and burbot were collected in early October 2003 and early June 2004 using boat and backpack electrofishing units. No burbot were collected from the reference lake in fall 2003. Spottail shiners (*Notropis hudsonius*) were collected in June 2004 at the low exposure and reference lakes. Fishes were captured, held for less than 1.5 hours and over-anesthetized with MS-222 (3-aminobenzoic acid). Total lengths (to the nearest 0.01 cm) and wet weights (to the nearest 0.01 g) were recorded for all fish and ageing structures (cleithra and scales) were removed from pike. Fish were immediately stored on dry ice until transport back to a -80°C freezer at the University of Saskatchewan.

Water temperature, dissolved oxygen, conductivity and pH were determined on site for reference, low and high exposure lakes in the fall and spring. Measures were made using a handheld YSI meter (YSI Environmental Incorporated, Yellow Springs, OH, U.S.A.) at a depth of 1 meter.

3.3.2 Laboratory analyses

3.3.2.1 Fish dissection

Tails and a portion of caudal muscle were cut from each fish and immediately returned to the -80°C freezer for subsequent RNA/DNA and protein determinations. Each carcass was dissected as follows. A small piece (< 270 mg) of liver was removed for triglyceride measurement and otoliths were removed from burbot. Stomach contents were removed, weighed and identified as either invertebrate or vertebrate (fish) prey items. Weight of the stomach contents was subtracted from the initial fish weight and the resulting value was used for subsequent calculations involving weight.

3.3.2.2 Total body lipids

The remaining carcass was weighed, added to ×2 volume of nanopure water, finely minced with scissors, homogenized (3 × 30 seconds) using a Tissue Tearor (BioSpec Products Inc., Bartlesville, OK, U.S.A.) and a 4 ml aliquot of the resulting homogenate was used for Bligh and Dyer solvent lipid extraction (Bligh and Dyer 1959). The chloroform-methanol lipid extraction was conducted on all fish using a final ratio of 1.8:2:2 for water to chloroform to methanol as per Bligh and Dyer, with the following modifications: chloroform contained 50 mg/L butylated hydroxytoluene (Sigma-Aldrich Canada Ltd., Oakville, ON, Canada) to reduce oxidation of fatty acids (Weber *et al.* 2003), samples were centrifuged instead of filtered and samples were dried at room temperature under a gentle stream of nitrogen. Lipid weight was determined in the fish sample (mg lipid per gram of fish carcass) using a gravimetric technique where triplicate aliquots of the chloroform fraction were evaporated in pre-weighed glass vials under a nitrogen atmosphere to prevent lipid oxidation.

3.3.2.3 Triglyceride assay

Whole body triglycerides were determined in the unextracted fraction of the same fish carcass homogenized in ×2 water using a modification of the McGowan *et al.* (1983) method (Weber *et al.* 2003). An aliquot of the fish homogenate was added to an equal volume of 0.2 M sodium citrate. The sample was homogenized on ice, placed in a heating block at 100ºC for five minutes and immediately returned to ice. All samples were centrifuged for five minutes at 500 *g* prior to assaying in order to remove insoluble materials such as connective tissue from the liquid fraction.

To determine liver triglycerides, the frozen piece of fish liver was weighed, added to ×3 volume of ice-cold 0.2 M sodium citrate, minced with scissors and homogenized on ice in a glass mortar with a motorized Teflon pestle. The sample was capped, placed in a heating block at 100°C for five minutes and immediately returned to ice. Triglycerides were then determined in the liver tissue as per the triglyceride assay described earlier.

3.3.2.4 RNA/DNA assay

Tail muscle was weighed (0.010-0.020 g) and added to ice-cold TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0) plus 0.2 M NaCl. Tissue was finely minced with scissors, homogenized with a Tissue Tearor (2 × 10 seconds), 5 µl of 1% sodium dodecyl sulphate was added and the sample was shaken and incubated at 65°C for two hours. Samples were centrifuged at 5000 *g* for ten minutes at 4°C, the supernatant was removed and an aliquot was taken for subsequent protein assay. Ice-cold isopropanol and 3 M sodium acetate were added to the remainder of supernatant and the sample was stored overnight at -20°C to allow precipitation of nucleic acids. After centrifugation at 15,000 *g* for 30 minutes, the supernatant was removed, TE buffer was added to the pellet, the sample was incubated at 65°C for 1 hour and stored overnight at 4°C to ensure complete resuspension of nucleic acids. The following day, RNA and DNA concentrations and resulting RNA/DNA ratio were determined using a modified dual fluorescent dye method (Clemmesen 1988, Weber *et al.* 2003). Calf thymus DNA and yeast RNA were used as standards.

3.3.2.5 Protein assay

An aliquot of the tail muscle homogenate prepared for nucleic acid determination was used to determine muscle protein concentration. A modification of the Lowry *et al.*

(1951) protein assay (BioRad DC protein assay, BioRad, Hercules, CA, USA) was used with bovine serum albumin as the standard. Duplicate 5 µl samples were read on a 96-well microplate at 750 nm absorbance.

3.3.3 Statistical analyses

Two-way analysis of variance (ANOVA) was used to analyze data, with lake and season as factors. Data were transformed (log10 or square root) if parametric assumptions were not met. When data were missing (i.e. fall 2003 burbot), one-way ANOVA was performed to compare lakes within fall and spring and *t*-tests were used to compare mean values for a lake in fall versus spring. Mann-Whitney Rank Sum test was used if data failed parametric assumptions for *t*-tests. Pairwise multiple comparisons were performed using Tukey's post-hoc test following significant one- or two-way ANOVA. Results were considered significant if $p < 0.05$. Data are reported as mean ± standard error of the mean (SEM). Principal component analysis (PCA) was conducted on $\log_{10}$ transformed data using all morphometric and biochemical endpoints.

Intra-assay variability was calculated for biochemical assays by determining the coefficient of variation (%CV = standard deviation/mean*100) for six replicates of a pooled sample measured in the same assay. Inter-assay variability was determined by calculating the %CV among six replicates of the same pooled sample performed in two separate assays.

3.4 Results

3.4.1 Abiotic environment

Basic water chemistry data were collected at study lakes and are presented in Table 3.1. Temperature was similar among lakes, however all other measured variables

were quite different between reference and exposure lakes. Conductivity, total hardness, ammonia and nitrate values were higher in both the low and high exposure lakes compared to reference. In general, water chemistry variables were elevated in the high exposure lake compared to the low exposure lake. In addition, pH was lower in exposure lakes compared to the reference lake.

Table 3.1 Water chemistry variables for lakes at Key Lake uranium operation. Data are means ± SEM for combined fall and spring values. Hardness, ammonia and nitrate values are from Golder (2005) collected in fall 2004.

Variable	**Lake**		
	Reference	Low Exposure	High Exposure
Dissolved oxygen (mg/L)	10.0 ± 0.2	10.0 ± 0.1	9.5 ± 0.2
Temperature (°C)	15.6 ± 1.0	14.9 ± 1.0	14.9 ± 0.8
pH	6.7 ± 0.1	5.8 ± 0.6	5.3 ± 0.1
Conductivity (µS/cm)	15.2 ± 2.3	582.0 ± 73.8	719.3 ± 126.0
Total Hardness (mg/L)	4	221	317
Ammonia (as N) (mg/L)	0.03	0.19	2.1
Nitrate (mg/L)	< 0.04	1.4	4.1

3.4.2 Sample size, age, stomach contents

In the fall collection, a total of 38 northern pike were collected from the three study lakes and 12 burbot were collected from the two exposure lakes, approximately two weeks before ice-on. No burbot were collected from the reference lake in the fall. During the spring sampling period, approximately two weeks after ice-off, a total of 39 northern pike and 33 burbot were collected from the reference and exposure lakes. Electrofishing seconds were recorded in the spring and the catch per unit effort (number of fish caught per electrosecond) for each lake was 0.00099 for the reference lake, 0.0061 for the low exposure lake and 0.0033 for the high exposure lake. Fish were aged and verified to be YOY (i.e. age-0 in fall 2003 and age-1 in spring 2004), using otoliths for

burbot, and cleithra and scales for pike. In the spring sampling period, 18 spottail shiners were collected from the reference lake and 16 shiners were collected from the low exposure lake. Shiners were immediately frozen and transported as described above. The spottail shiners were not aged.

Invertebrates were the only type of food present in burbot stomachs; 69% of all burbot had prey items in their stomachs and of these 100% were invertebrates. The northern pike fed on both invertebrates and spottail shiners; 71% of all pike stomachs contained food, and of these, 38% had recently consumed shiners and 62% had recently consumed invertebrates. There were no shiners present in the high exposure lake, as evidenced by stomach content analysis and extensive electrofishing effort during fish collection. In the spring, 54% of burbot collected from the reference site had retrievable stomach content, whereas burbot from the low and high exposure lakes had prey items in 91% and 89% of all individuals, respectively. The average weight of stomach contents from burbot collected in the spring also varied for fish from the reference (0.07 ± 0.02 g), low (0.34 ± 0.08 g) and high (0.26 ± 0.09 g) exposure lakes. The majority of invertebrates present in burbot stomachs collected at the high exposure lake were chironomids (Order Insecta, Family Chironomidae).

3.4.3 Morphometric results

3.4.3.1 Northern pike

Northern pike were of similar length, weight and condition factor in reference and exposure lakes in both fall and spring (Table 3.2). There were no significant differences in length, weight or condition factor when comparing pike from high or low exposure lakes to the reference lake for each season. There was an overall significant ($p < 0.01$)

seasonal difference in length. In the low exposure lake, the pike were significantly longer in the spring compared to the previous fall. There were no significant differences in weight or condition factor of pike within the reference and high exposure lake between fall and spring.

Table 3.2 Morphometric (weight, total length, condition factor) and biochemical (total body lipids, muscle RNA/DNA ratio, muscle protein) variables determined in young-of-the-year northern pike (*Esox lucius*). Fish were collected at Key Lake uranium operation, Saskatchewan, Canada from David Lake (reference) which is located upstream of uranium milling inputs and Unknown Lake (high exposure and Delta Lake (low exposure) which are downstream of uranium milling input, in fall 2003 and spring 2004. Refer to Figure 3.1 for locations of study sites. Data shown are mean ± standard error of the mean. Condition factor = (weight/total length^3)100.

	REFERENCE		LOW EXPOSURE		HIGH EXPOSURE	
	Fall 2003	**Spring 2004**	**Fall 2003**	**Spring 2004**	**Fall 2003**	**Spring 2004**
Sample size	12	10	15	15	11	14
Weight (g)	17.30 ± 1.58	21.53 ± 1.87	22.69 ± 2.58	31.02 ± 4.15	19.50 ± 2.02	22.69 ± 1.94
Total length (cm)	14.29 ± 0.46	15.42 ± 0.40	15.57 ± 0.58	17.44 ± 0.71†	14.76 ± 0.49	15.75 ± 0.41
Condition factor	0.58 ± 0.01	0.57 ± 0.01	0.57 ± 0.01	0.53 ± 0.01	0.59 ± 0.01	0.56 ± 0.01
Total body lipids (%)	0.739 ± 0.04	1.15 ± 0.08††	1.57 ± 0.16***	1.80 ± 0.10**	1.14 ± 0.16*	1.64 ± 0.12*††
Muscle RNA/DNA ratio	2.59 ± 0.53	2.17 ± 0.49	2.52 ± 0.34	2.74 ± 0.46	2.41 ± 0.32	2.07 ± 0.24
Muscle protein (mg/g)	28.83 ± 2.68	32.61 ± 2.90	26.35 ± 1.77	27.30 ± 1.49	26.27 ± 1.57	29.18 ± 1.69

* Significantly different from reference lake in the same season (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$)

† Significantly different within a lake comparing fall and spring values ($^{\dagger}p < 0.05$, $^{\dagger\dagger}p < 0.01$, $^{\dagger\dagger\dagger}p < 0.001$)

3.4.3.2 Burbot

Overall, the morphometric endpoints for YOY burbot were different between reference and exposure lakes and from fall to spring within the exposure lakes (Table 3.3). Body weights of burbot were greater in the spring compared to the fall in both the low and high exposure lakes ($p < 0.05$ for both lakes). Within the spring, burbot collected from both the low and high exposure lakes had significantly greater body weights than fish from the reference lake ($p < 0.05$ and $p < 0.01$, respectively). Compared to fall values, burbot collected in the spring were significantly longer in the low exposure lake ($p < 0.01$) and high exposure lake ($p < 0.05$). Comparing burbot collected from all lakes in the spring, fish from both the low and high exposure lakes were significantly longer than reference fish ($p < 0.001$ and $p < 0.05$, respectively). In the spring, burbot collected from the high exposure lake had a significantly greater condition factor compared to fish collected from the reference lake ($p < 0.05$) and low exposure lake ($p < 0.001$). There was a seasonal difference in condition factor for burbot collected from the low exposure lake: condition factor was lower in the spring compared to the fall ($p < 0.001$; Table 3.3).

Table 3.3 Morphometric (weight, total length, condition factor) and biochemical (total body lipids, muscle RNA/DNA ratio, muscle protein) variables determined in young-of-the-year burbot (*Lota lota*). Fish were collected at Key Lake uranium operation, Saskatchewan, Canada from David Lake (reference) which is located upstream of uranium milling inputs and Unknown Lake (high exposure and Delta Lake (low exposure) which are downstream of uranium milling input, in fall 2003 and spring 2004. Refer to Figure 3.1 for locations of study sites. Data shown are mean ± standard error of the mean. Condition factor = (weight/total length^3)100.

	REFERENCE		LOW EXPOSURE		HIGH EXPOSURE	
	Fall 2003	**Spring 2004**	**Fall 2003**	**Spring 2004**	**Fall 2003**	**Spring 2004**
Sample size	0	13	6	11	6	9
Weight (g)	n/a	5.01 ± 0.66	5.80 ± 0.54	9.40 ± 0.98*†	4.65 ± 0.61	10.04 ± 1.81**†
Total length (cm)	n/a	9.24 ± 0.42	9.63 ± 0.29	11.8 ± 0.40***††	8.67 ± 0.47	11.1 ± 0.59*†
Condition factor	n/a	0.60 ± 0.02	0.64 ± 0.04	0.56 ± 0.01†††	0.70 ± 0.06	0.67 ± 0.02*
Total body lipids (%)	n/a	2.16 ± 0.13	1.13 ± 0.06	3.02 ± 0.35††	1.14 ± 0.04	3.89 ± 0.28***††
Muscle RNA/DNA ratio	n/a	8.24 ± 0.48	6.64 ± 0.34	5.94 ± 0.59*	7.61 ± 0.65	9.96 ± 0.54*
Muscle protein (mg/g)	n/a	24.97 ± 1.58	27.29 ± 1.78	20.85 ± 1.13††	25.99 ± 2.50	21.94 ± 0.87

* Significantly different from reference lake in the same season (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$)

† Significantly different within a lake comparing fall and spring values ($^{\dagger}p < 0.05$, $^{\dagger\dagger}p < 0.01$, $^{\dagger\dagger\dagger}p < 0.001$)

n/a = data not available

3.4.3.3 Spottail shiners

There were no differences in weight, length and condition factor for spottail shiners between the reference and low exposure sites (data not shown).

3.4.4 Biochemical results

3.4.4.1 Northern pike

Two-way ANOVA indicated significant differences in total body lipids among lakes and seasons ($p < 0.001$). Post-hoc tests revealed that in both fall and spring, YOY pike collected from the high and low exposure lakes had significantly greater total body lipids compared to pike collected from the reference lake (Table 3.2). Pike collected from the high exposure lake had greater total body lipids than reference pike in fall ($p < 0.05$) and spring ($p < 0.05$). Compared to pike from the reference lake, fish collected from the low exposure lake had greater total body lipids in the fall ($p < 0.001$) and spring ($p < 0.01$). Among seasons, pike collected from the reference and high exposure lakes had greater total body lipids in the spring compared to the fall ($p < 0.01$). When comparing results between the two exposure lakes, total body lipids were significantly lower ($p < 0.05$) in the fall in pike collected from the high exposure lake compared to fish from the low exposure lake.

There were significant differences in northern pike total body triglycerides among lakes ($p < 0.001$) but not seasons (Figure 3.2A). Total body triglycerides were significantly greater in pike collected from the low exposure lake compared to fish from the reference lake in both fall ($p < 0.01$) and spring ($p < 0.001$). Total body triglycerides were significantly greater in pike collected from the high exposure lake compared to the reference lake in spring only ($p < 0.001$). Similar to total body triglycerides, liver

triglycerides in northern pike were overall significantly different among lakes ($p < 0.01$) but not seasons (Figure 3.3A). Liver triglycerides were significantly greater in pike collected from the low exposure lake in the fall ($p < 0.01$) and significantly greater in pike from the high exposure lake in the spring compared to reference pike ($p < 0.05$). Comparing liver triglycerides between fish from the two exposure lakes, in the fall the high exposure pike had significantly lower ($p < 0.05$) liver triglycerides compared to fish collected from the low exposure lake.

Muscle RNA/DNA ratio in northern pike was not significantly different between lakes within a season or within lakes over the two seasons (Table 3.2). Protein content was not different in tail muscle from northern pike among lakes or seasons (Table 3.2).

3.4.4.2 Burbot

In the spring, YOY burbot collected from the high exposure lake had significantly greater total body lipids compared to burbot collected from the reference lake ($p < 0.001$; Table 3.3). In addition, burbot collected from both low and high exposure lakes had significantly greater total body lipids in the spring compared to the fall ($p < 0.01$). Total body triglycerides in burbot followed a similar trend as total body lipids. In the spring, burbot from the high exposure lake had significantly greater total body triglycerides compared to reference burbot ($p < 0.01$). Burbot had greater total body triglycerides in both the low ($p < 0.001$) and high ($p < 0.01$) exposure lakes in the spring compared to the previous fall (Figure 3.2B). Although there was a trend for higher liver triglycerides in burbot collected from the exposure lakes compared to the reference lake (Figure 3.3B), there was no statistically significant difference in this biochemical endpoint between lakes in the spring ($p = 0.062$). However, liver triglycerides were significantly higher in

the spring compared to the fall for burbot collected from both low ($p < 0.001$) and high ($p < 0.01$) exposure lakes.

Muscle RNA/DNA ratio measured in burbot collected from the low exposure lake was significantly lower ($p < 0.05$) than both reference and high exposure burbot in the spring (Table 3.3). Burbot from the high exposure lake had a significantly higher ($p < 0.05$) RNA/DNA ratio compared to reference fish (Table 3.3). Muscle protein measured in burbot from the low exposure lake was lower ($p < 0.01$) in the spring compared to the fall (Table 3.3).

Except where noted (northern pike total body lipids; burbot condition factor and RNA/DNA ratio), there were no statistically significant differences in any morphometric or biochemical endpoint between the low and high exposure lakes in either fall or spring.

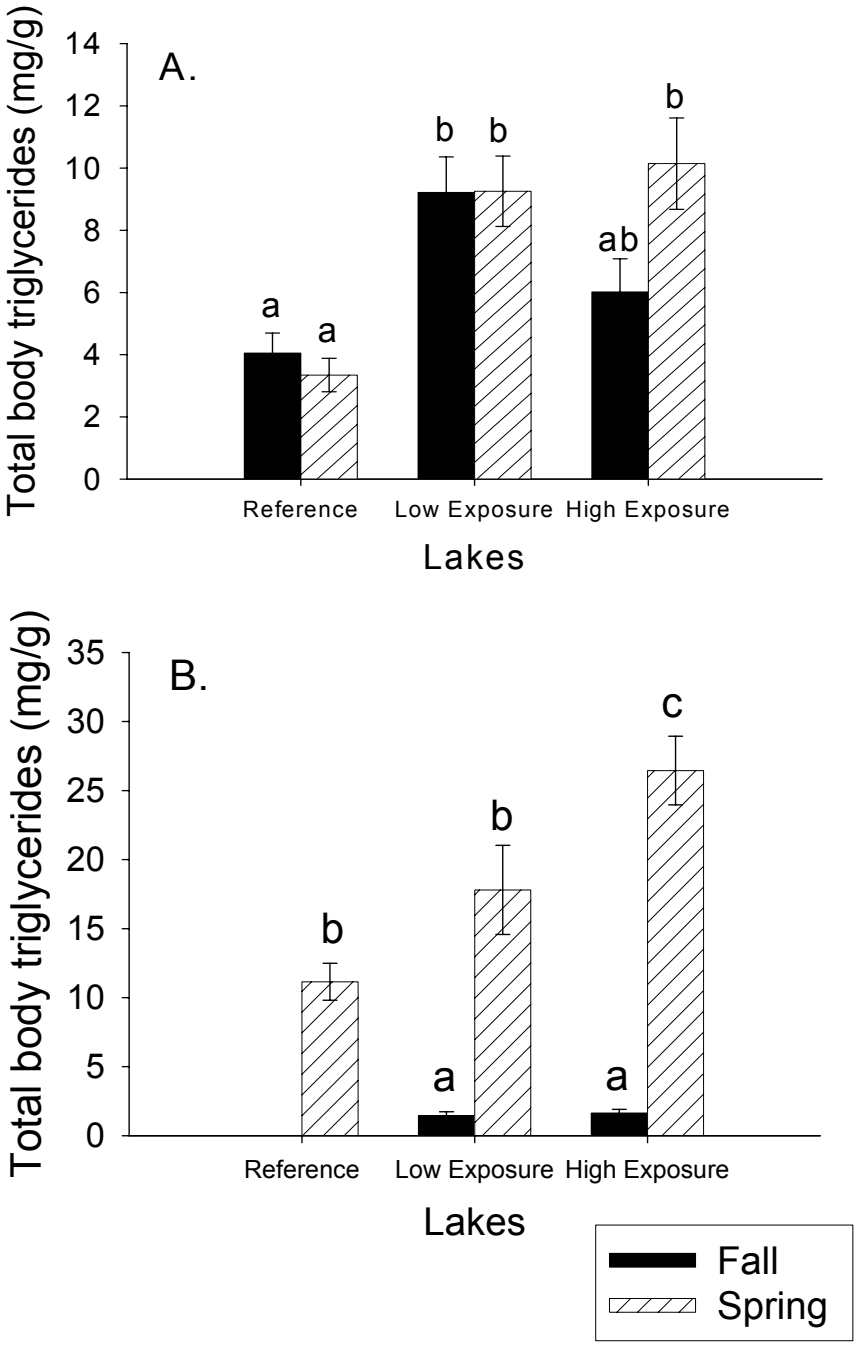

Figure 3.2 Total body triglycerides (mg triglyceride per gram fish tissue) in young-of-the-year northern pike (*Esox lucius*) (A) and burbot (*Lota lota*) (B) collected in fall 2003 and spring 2004 from a reference lake and two lakes receiving uranium milling effluent at Key Lake uranium operation, Saskatchewan, Canada. Data shown are mean ± standard error of the mean. Sample sizes range from 6-15 individuals per species per lake in fall and 10-15 individuals per species per lake in spring. Bars without letters in common are significantly different ($p < 0.05$) between lakes within a season and within a lake over two seasons using 2-way analysis of variance followed by Tukey's post-hoc test.

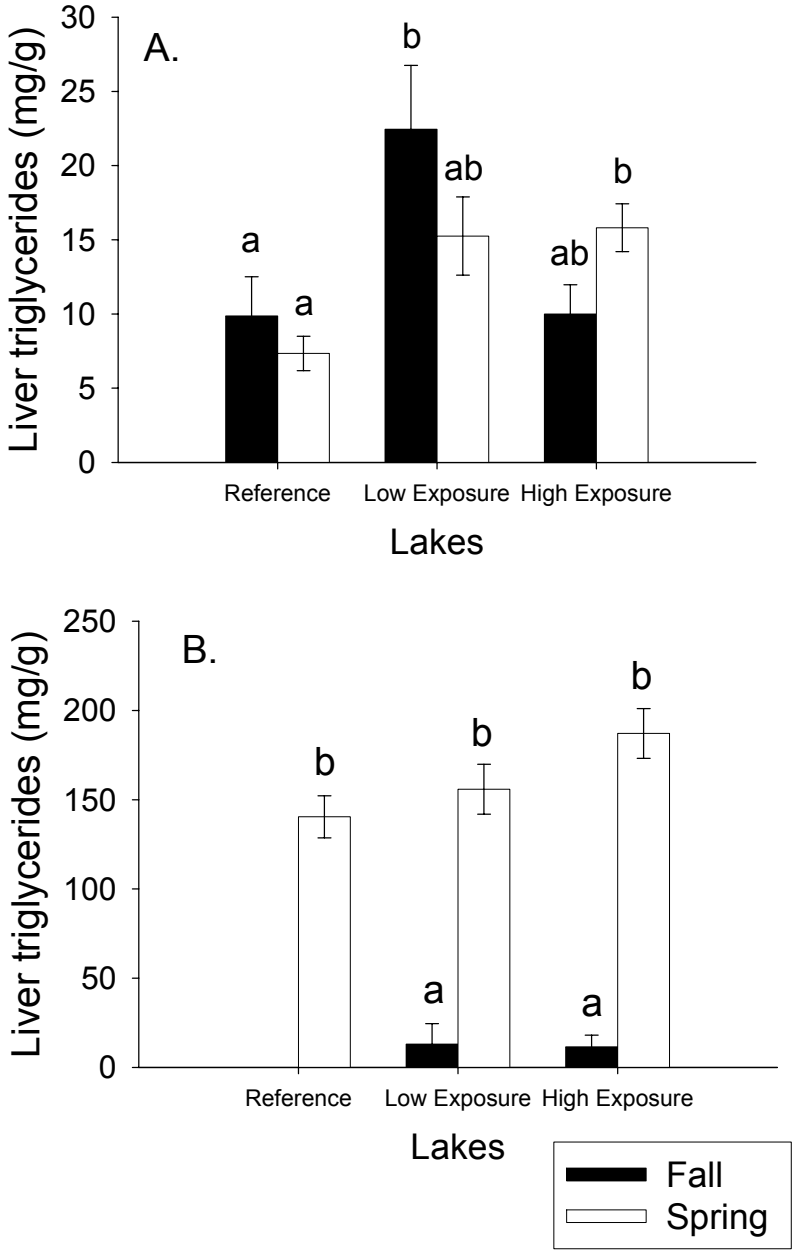

Figure 3.3 Liver triglycerides (mg triglyceride per gram liver) in young-of-the-year northern pike (*Esox lucius*) (A) and burbot (*Lota lota*) (B) collected in fall 2003 and spring 2004 from a reference lake and two lakes receiving uranium milling effluent at Key Lake uranium operation, Saskatchewan, Canada. Data shown are mean ± standard error of the mean. Sample sizes range from 6-15 individuals per species per lake in fall and 10-15 individuals per species per lake in spring. Bars without letters in common are significantly different ($p < 0.05$) between lakes within a season and within a lake over two seasons using 2-way analysis of variance followed by Tukey's post-hoc test.

3.4.4.3 Spottail shiners

Total body triglycerides were significantly greater in spottail shiners collected from the low exposure lake compared to reference lake in the spring ($p < 0.001$); Figure 3.4).

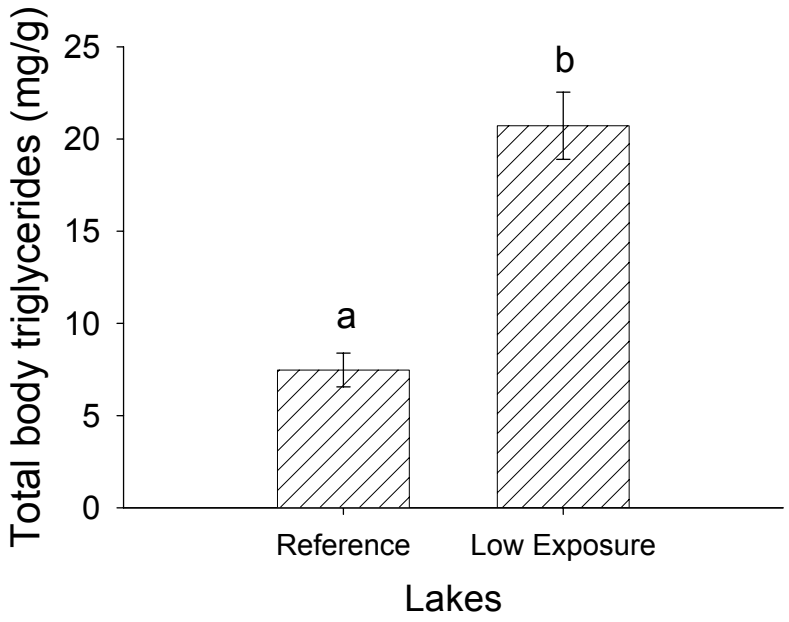

Figure 3.4 Total body triglycerides (mg triglyceride per gram fish tissue) in spottail shiners (*Notropis hudsonius*) collected in the spring 2004 from a reference lake and a lake receiving uranium milling effluent at Key Lake uranium operation, Saskatchewan, Canada. Data shown are mean ± standard error of the mean. Sample sizes were 18 shiners from the reference lake and 16 shiners from the low exposure lake. Bars without letters in common are significantly different ($p < 0.05$) using a *t*-test.

3.4.5 Principal component analysis

All morphometric (weight, length, condition factor) and biochemical (total body lipids, total body triglycerides, liver triglycerides, muscle RNA/DNA ratio and muscle protein) endpoints for individual northern pike and burbot were analyzed using principal component analysis (PCA). For northern pike, the first two principal components explained 57.4% of the variance in the data. Important individual variables for northern pike principal component 1 were weight (most positive weighting) and protein (lowest positive weighting). Principal component 2 was related to protein (positive) and RNA/DNA ratio (most negative) (Table 3.4 A). For burbot, the first two principal components explained 64.6% of the variance. Important individual variables for burbot principal component 1 were total body lipids (most positive weighting) and protein (most negative weighting), principal component 2 was related to RNA/DNA ratio (positive) and length (negative) (Table 3.4 B). For both species, weight, length, total body lipids, total body triglycerides and liver triglycerides were closely correlated with the first principal component.

Table 3.4 Northern pike (*Esox lucius*) (A) and burbot (*Lota lota*) (B) morphometric (weight, length, condition factor) and biochemical (total body lipids, total body triglycerides, liver triglycerides, muscle RNA/DNA ratio, muscle protein) variables identified by principal component analysis. For each axis, the largest positive and lowest positive or negative component loadings are in bold. Condition factor = (weight/total length^3)100.

A.

Component	Principal Component	
	1	**2**
Variance explained by components	3.37	1.22
Percent of total variance explained	42.1	15.3
Weight (g)	**0.841**	-0.347
Liver triglycerides (mg/g)	0.820	0.298
Length (cm)	0.814	-0.308
Total body triglycerides (mg/g)	0.801	0.289
Total body lipids (%)	0.784	0.150
Condition factor	0.247	-0.233
Protein (mg/g)	0.093	**0.364**
RNA/DNA ratio	**0.035**	**-0.792**

B.

Component	Principal Component	
	1	**2**
Variance explained by components	3.70	1.46
Percent of total variance explained	46.3	18.3
Total body lipids (%)	**0.926**	0.256
Total body triglycerides (mg/g)	0.910	0.296
Liver triglycerides (mg/g)	0.802	0.265
Length (cm)	0.780	**-0.489**
Weight (g)	0.763	-0.394
RNA/DNA ratio	0.112	**0.788**
Condition factor	-0.148	0.442
Protein (mg/g)	**-0.386**	0.164

Scores for the first two principal components, grouped by both lake and season, for the northern pike and burbot were used to construct Figure 3.5. There was no distinct clustering for northern pike when plotted by lake or season (Figure 3.5A1 and 3.5A2) or for burbot when plotted by lake (Figure 3.5B1). However, burbot scores grouped by season showed distinct clustering (Figure 3.5B2).

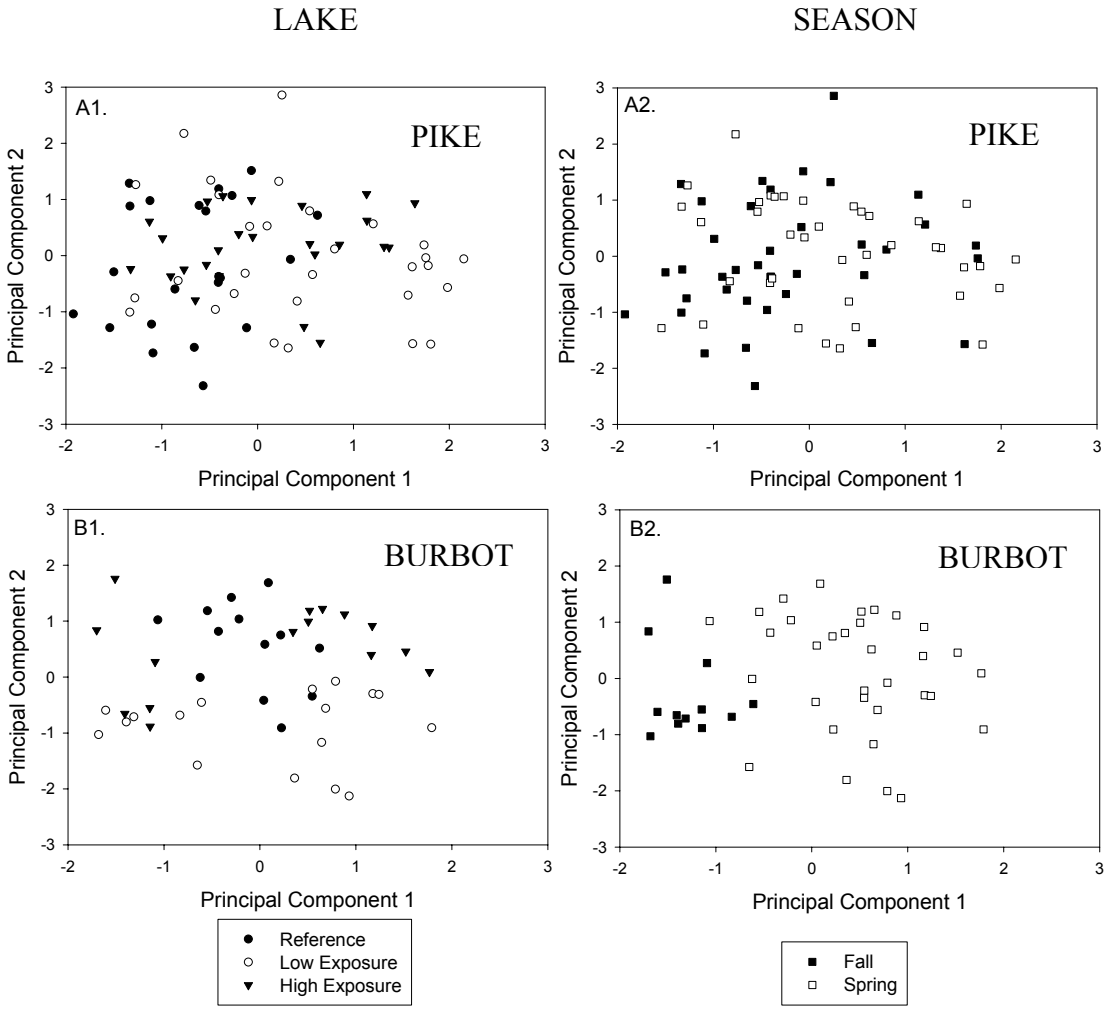

Figure 3.5 Principal component analysis of all morphometric (weight, length, condition factor) and biochemical (total body lipids, total body triglycerides, liver triglycerides, muscle RNA/DNA ratio, muscle protein) endpoints for young-of-the-year northern pike (*Esox lucius*) (A) and burbot (*Lota lota*) (B). Components are grouped by lakes (1) and by season (2).

3.4.6 Assay performance

Both intra- and inter-assay coefficients of variation were below 10% for all assays. Intra-assay variability was 4.6%, 3.5%, 3.7%, 3.0% and 4.1% for the RNA/DNA ratio, protein, total body lipids, total body triglycerides and liver triglyceride assays, respectively. Inter-assay variability was 8.7%, 5.0%, 5.6%, 4.1% and 4.5% for the RNA/DNA ratio, protein, total body lipids, total body triglycerides and liver triglyceride assays, respectively (data not shown).

3.5 Discussion

3.5.1 Abiotic environment

Young-of-the-year northern pike and burbot inhabiting the low and high exposure lakes were experiencing a much different environment, in terms of water chemistry, compared to the fish from the reference lake. Exposure lakes were characterized by higher conductivity and hardness, and lower pH. Various trace metals, including As, Mo, Ni, Se and U were reported to be consistently elevated in the exposure lakes compared to reference lakes near the uranium operation (Pyle *et al*. 2001, Golder 2005). Aside from possible metal and ion effects, there also existed a potential nutrient effect, with increased nitrogen (in the form of ammonia and nitrate) in the uranium milling effluent receiving lakes.

3.5.2 Site differences in lipids and triglycerides

I hypothesized that fishes inhabiting exposure lakes would have lower total body lipids and triglycerides compared to fish from the reference lake in both seasons, due to impacts of environmental contaminants on fish energy storage and metabolism (Calow 1991, Adams 1999, Congdon *et al*. 2001, Levesque *et al*. 2002). However, YOY

northern pike and burbot collected from exposure lakes generally exhibited greater total body lipids and triglycerides compared to reference fish in either season. The observed effects were consistent in both fish species over two seasons. The higher total lipids and triglycerides in exposed pike and burbot suggests that these fish may be in better overall condition than reference fish. Northern pike from exposure sites generally had higher liver triglyceride content compared to reference fish, with no difference between seasons. In contrast, burbot exhibited clear seasonal differences in liver triglyceride content, with greater liver triglycerides in the spring compared to fall and no differences in this biochemical endpoint in burbot from exposure and reference lakes. Overall, liver triglyceride values followed similar trends to total body triglycerides, indicating that total body triglyceride levels are closely related to liver triglyceride concentrations for both species. Trace metal exposure has varying effects on lipid dynamics (Adams 1999). Katti and Sathyanesan (1984) found that cadmium exposure increased liver lipid levels in catfish (*Clarias batrachus*). In contrast, Levesque *et al*. (2002) reported significantly lower liver triglyceride content in yellow perch (*Perca flavescens*) from a Cd, Zn and Cu contaminated lake compared to reference fish during two sampling periods (fall and summer).

Many northern freshwater systems are nitrogen and phosphorous limited, and elevated levels of these nutrients may increase phytoplankton biomass in a lake, which may influence the biomass and productivity of higher trophic levels such as fish (Dillon *et al*. 2004). In the present study, nitrogen (ammonia and nitrate) was higher in the exposure lakes compared to the reference lake. However, elevated phosphorous has not been reported downstream of effluent release, with recent water chemistry data indicating

that total phosphorous levels are < 10 μg/L in both the low exposure and reference lakes (K.T. Himbeault, personal communication 2006). The Redfield ratio explains that when nutrients are not limiting, the element ratio of N to P in phytoplankton is approximately 16:1 (Redfield *et al*. 1963). At Key Lake uranium operation, the ratio of nitrogen (ammonia) to total phosphorous in the reference lake is approximately 3:1. Since this ratio is less than 16:1, primary productivity in the system may be nitrogen limited. The input of nitrogen into the system from the uranium milling effluent increased the N:P ratio to approximately 19:1 in the low exposure lake and 210:1 in the high exposure lake indicating that these lakes are no longer nitrogen depleted, but are now phosphorous limited. Data here indicates that an increase in nitrogen alone has the potential to increase productivity in lakes receiving milling effluent at Key Lake uranium operation.

Boreal lakes experiencing nutrient (N and P) enrichment as a result of fire regimes can have higher macroinvertebrate biomass compared to reference lakes (Scrimgeour *et al*. 2001). Fish energy stores depend on the food supply available during the summer growing season, which is ultimately linked to lake productivity (Eckmann 2004). If uranium milling effluent increased productivity of exposure lakes, this may explain the higher triglyceride levels in YOY fish collected in the fall and spring sampling periods compared to the reference fish. A concurrent study observed higher total macroinvertebrate, including midges of the insect family Chironomidae, density in the high exposure lake, but not the low exposure lake, compared to the reference lake in fall 2003 (E. Robertson, unpublished data). Higher macroinvertebrate densities in exposure lakes was supported by burbot stomach content analysis in the spring, where approximately half of the burbot from the reference lake had empty stomachs while the

majority of individuals from the low and high exposure lakes had recently ingested invertebrates, mainly chironomids. In addition, the average mass of YOY burbot stomach contents was much lower in fish from the reference site compared to fish from both exposure sites. However, macroinvertebrate density varied among study lakes (high exposure > low exposure = reference; E. Robertson, unpublished data), suggesting that the observed site differences in lipids and triglycerides of YOY fish in fall and spring may not entirely be a result of differences in prey quantity in the study lakes.

Differences in prey quality between the low exposure and reference lakes were also noted. Total body triglycerides in spottail shiners followed a similar trend as in northern pike, with higher total body triglycerides in shiners collected from the low exposure lake compared to shiners from the reference lake. This suggests that uranium milling effluent may have an indirect, food web based impact on burbot and northern pike energy reserves: if available prey possess higher energy content (quality), then predatory YOY fishes should gain more lipids and triglycerides than reference fish. However, shiners were not present in the high exposure lake and YOY burbot were not preying on shiners in any lake, as evidenced by stomach content analysis. Thus, changes in fish triglycerides may not be solely related to milling effluent, as increases in these energy storage endpoints were not necessarily exposure-dependent when comparing low and high exposure sites. Other studies have concluded that differences in fish condition have been the result of indirect contaminant effects through an aquatic food web (Iles and Rasmussen 2005). Improved survival of age-0 fish in nutrient enriched systems is generally attributed to decreased starvation due to increased food availability and decreased predation due to increased growth (Grant and Tonn 2002). Overall, a

combination of increased prey quantity and quality in the present study may explain the observed higher lipid and triglyceride content in burbot and pike collected from exposure lakes.

3.5.3 Seasonal differences in lipids and triglycerides

Not only can overwinter mortality of young fish vary with species, population density, prey abundance and predator density (Pratt and Fox 2002), evidence here suggests that lipid depletion following a long, northern winter may not always occur and may also vary with these ecological variables. Contrary to logic and various papers on overwintering (e.g. Cunjak 1988, Lemly 1996, Berg and Bremset 1998, Post and Parkinson 2001, Biro *et al*. 2004), effluent exposed burbot consistently had higher total body lipids, total body triglycerides and liver triglycerides in the spring compared to the previous fall. The concept that fishes must rely on stored energy to survive winter months is related to low food availability (Johnson and Evans 1991, Foy and Paul 1999) as well as decreased food digestion associated with low temperatures (Toneys and Coble 1980). However, Hurst and Conover (1998) noted that many details related to winter feeding ecology of the majority of fish species remain unstudied. Indeed, various reports have challenged the idea that fish are unable to feed or assimilate energy from food during winter (Sogard and Olla 2000, Biro *et al*. 2004, Parrish *et al*. 2004). Bauer and Schlott (2004) reported that common carp (*Cyprinus carpio*) were relatively active in the winter, despite previous beliefs that the fish were inactive. McCollum *et al*. (2003) found that winter food availability regulated the energetic condition of age-0 white crappies (*Pomoxis annularis*) entering the spring. Biro *et al*. (2004) reported that fed YOY rainbow trout (*Oncorhynchus mykiss*) in a laboratory setting under simulated winter

conditions were capable of doubling their lipid content over a 100 day period. Similarly, juvenile Atlantic salmon (*Salmo salar*) provided with shelter from predators in a setting with high food availability experienced increased growth and survival over the winter, as there was an advantage for fish to expend energy to feed for growth and weight maintenance (Parrish *et al*. 2004). Clearly, lipid depletion following winter does not occur with all fish species and may vary depending on various ecological variables including foraging strategy, predator density, prey abundance and competition.

Total body triglycerides, a more ecologically relevant measure of energy storage than total body lipids, only exhibited a seasonal (fall to spring) increase in YOY burbot and not in northern pike. Eckmann (2004) found that YOY perch (*Perca fluviatilis*) had decreased lipid levels after the winter, whereas ruffe (*Gymnocephalus cernuus*) were actually higher in lipids following winter. This was attributed to differences in predation strategy, where ruffe use sensory ability to forage and perch are a visually oriented predator (Eckmann 2004). Although both species in the present study are carnivorous, pike are pelagic carnivores and burbot tend to forage in the benthic area (Scott and Crossman 1973). This difference in feeding niche was supported by stomach content analysis as northern pike, but not burbot, consumed spottail shiners, a pelagic prey item. Burbot locate prey with olfactory and tactile cues by touching the substrate with their pelvic fins and barbels (Hinkens and Cochran 1988), whereas pike are visual predators (Scott and Crossman 1973). Since winter feeding conditions are characterized by low light due to ice cover and photoperiod, a species that relies primarily on vision for feeding will experience a reduction in foraging success compared to a sensory and olfactory predator, as assumed in the present study and in Eckmann (2004). Hofmann

and Fischer (2003) reported that juvenile burbot exhibited a two-fold increase in food conversion efficiency when moving from 20°C to 5-6°C and therefore, the need for food is lower at lower temperatures. If food is available and burbot are foraging well with their sensory system, any food obtained will thus be converted efficiently to energy storage or growth during the cold winter months and may explain the seasonal differences in lipids and triglycerides observed in burbot in the present study.

Evidence generated in the present study suggests that fishes in these freshwater systems may not be relying completely on stored energy to survive the winter. It appears that the key element of Lemly's (1996) winter stress syndrome hypothesis, that fish respond to low water temperatures and short photoperiod with reduced feeding, may not be occurring with YOY northern pike and burbot. Thus, YOY northern pike and burbot may not be vulnerable to winter stress syndrome, as their feeding ecology and life history characteristics suggest that these fish can feed and consequently maintain or increase energy stores during winter. In addition, Lemly's (1996) idea that winter stress syndrome results as the combination of cold temperatures and a metabolic stressor would increase an individual's susceptibility to overwinter mortality. However, the effects of winter temperatures on metabolism must be considered. Since fish are poikilothermic, their metabolic rate and many of their physiological functions are fundamentally influenced by temperature (Fry 1971). Perhaps during winter, when temperatures are low, any impacts of a metabolic stressor in exposure sites would be less than that expected at warmer temperatures. Elevated ammonia and low pH were not found to impact liver and gill protein turnover in rainbow trout during winter (Morgan *et al*. 1998), although similar studies at warmer temperatures found alteration in tissue protein synthesis (Wilson *et al*.

1996, Linton *et al*. 1997). These differences in effects of sublethal exposures dependent on temperature may thus be related to low metabolic rates associated with low temperatures (Morgan *et al*. 1998).

3.5.4 Overwinter mortality

Although it is possible that burbot were gaining lipids and triglycerides during winter, the possibility of size-dependent overwinter mortality must be considered. Since smaller fish have a higher mass-specific metabolic rate and lower energy density than larger fish, size-dependent overwinter mortality may occur (Peters 1983, Post and Parkinson 2001, McCollum *et al*. 2003). Numerous laboratory and field studies with various fish species have established that smaller YOY fish indeed suffer higher mortality over the winter than larger individuals (Toneys and Coble 1980, Johnson and Evans 1991, Post and Evans 1989, Griffiths and Kirkwood 1995, Foy and Paul 1999, Gotceitas *et al*. 1999, Kristiansen *et al*. 2000, Sogard and Olla 2000, Grant and Tonn 2002, Biro *et al*. 2004). In addition, size-dependent predation can regulate winter mortality, as smaller fish are more vulnerable to gape-limited predators (Werner and Gilliam 1984, Kristiansen *et al*. 2000).

In the present study, mortality of smaller burbot with reduced energy stores during winter could potentially contribute to the observed seasonal increase in total body lipids and triglycerides. In addition, the burbot collected in spring from exposure lakes had greater lengths compared to the previous fall, further suggesting that size-dependent overwinter mortality may have occurred. Several studies have reported that size-dependent overwinter mortality was responsible for an increase in average length or mass of individuals in spring compared to the previous fall (Post and Evans 1989, Griffiths and

Kirkwood 1995, Grant and Tonn 2002). However, size-dependent mortality is not a universal phenomenon for all fish species. For instance, size-dependent overwinter mortality may not occur in natural walleye (*Stizostedion vitreum*) populations (Pratt and Fox 2002). Immature Atlantic salmon parr exhibited growth in length during winter, which was not a result of size-dependent mortality since overall cohort survival was estimated to be 98% (Parrish *et al*. 2004). If burbot in this study were not succumbing to size-dependent overwinter mortality, then their seasonal increase in total length may be a result of winter growth. Neither temperature growth thresholds nor minimum lipid requirements for overwinter survival in YOY burbot are known. However, some studies support the occurrence of fish growth during winter. Age-0 cod (*Gadus morhua*) achieved some positive growth under winter temperatures (Gotceitas *et al*. 1999), juvenile burbot were reported to have a growth rate of approximately 0.005 cm/day under fed conditions at 7.8°C (Hofmann and Fischer 2003) and perch were shown to grow at temperatures < 10°C (Karås 1990).

As an indication of size- or energy-dependent overwinter mortality, I compared the variability in my data in the fall and spring fish collections. Calculation of the coefficient of variation (standard deviation/mean) for weight, length and total body lipids indicated there was higher variability in all of these endpoints in spring compared to fall for both northern pike and burbot. If only individuals in the best condition preferentially survived the overwinter period, I would expect much lower variability in spring, since phenotypic variability in the population would have been reduced. In addition, catch per unit effort was higher in the low and high exposure lakes in the spring compared to the reference lake. This suggests that overwinter mortality was not significantly greater in

the sites receiving uranium milling effluent since there was a higher catch per unit effort at the exposure lakes compared to the reference lake in the spring. However, since electrofishing uses an electrical current to stun fish, the efficiency of electrofishing varies with water conductivity. Estimating population abundance may have been biased with electrofishing sampling, since high water conductivity at the exposure sites may have increased the catch rates. Although data gathered here indirectly supports the possibility of overwinter growth in burbot, further investigations into the life history and overwintering behaviour of this species is required.

3.5.5 Site and season differences in growth

In fishes, growth is related to various environmental factors including food availability, toxicant exposure and temperature. Muscle RNA to DNA ratio provides an estimate of short-term (hours to days) growth (Clemmesen 1988). While DNA per cell remains relatively constant, the amount of RNA is proportional to the amount of cellular protein synthesis, providing an estimate of growth. Total protein content provides a measure of longer-term growth (days to weeks) and to a lesser extent, energy storage. Protein synthesis in an active tissue such as caudal muscle reflects recent growth and therefore nutritional status of the individual fish. I hypothesized that YOY fish would grow faster in the reference lake compared to exposure lakes, since increased metabolic activity associated with inhabiting a contaminated environment may channel a greater proportion of available energy into metabolism and away from growth. However, I found that the YOY pike and burbot did not exhibit any exposure-dependent site differences in muscle RNA/DNA ratio or muscle protein in either season. Since fishes at

exposure sites had high levels of stored energy, their growth would not likely be reduced relative to fishes inhabiting the reference lake.

It is interesting to note that although YOY pike in the exposure sites had higher lipids and triglycerides, an increase in growth measured with muscle RNA/DNA ratio or muscle protein was not observed in this species. Further investigation is required to determine if this combination of factors could indicate metabolic disruption, where although energy was available, it did not appear to be allocated to growth. Overall, biochemical estimates of fish growth in this study did not change in response to uranium milling effluent exposure.

3.5.6 Other energetic considerations

Possible food web changes are only one of numerous environmental and biological variables that can influence energy allocation and growth in wild fish. A discussion of bioenergetics must consider energy expenditures related to predation. Through personal observation during fish collection, I observed fewer large, adult northern pike in the relatively small high exposure lake compared to the larger low exposure and reference lakes. Fish with low predation risk can employ an active feeding regime and forage more optimally (Post and Parkinson 2001), which may further explain the higher lipids found in fish from the high exposure lake. Field based experiments have shown that the presence of a predator will reduce growth and survival of YOY fish (Landry *et al*. 1999, Biro *et al*. 2003). Larger fish are more likely to survive than smaller fish due to size-dependent predation (Kristiansen *et al*. 2000). As mentioned previously, this is because small fish are more vulnerable to gape-limited predators (Werner and

Gilliam 1984) and larger fish have relatively more energy and therefore, would be in better condition to actively escape predators (McCollum *et al*. 2003).

3.5.7 Morphometric versus biochemical endpoints

Previous studies have reported that fish exposed to certain metals (Cd, Cu, Zn) have decreased condition factor relative to unexposed fish (Laflamme *et al*. 2000, Sherwood *et al*. 2000, Levesque *et al*. 2002, Rajotte and Couture 2002). However, lower condition factor in fishes exposed to metals in uranium milling effluent was not observed in the present study. In fact, burbot from the high exposure lake had higher condition factor compared to reference fish in the spring. Overall, the northern pike were similar in morphometric indices among lakes, while the burbot had greater lengths and weights in the exposure lakes compared to burbot collected from the reference lake. Although there were no site differences observed in spring burbot using biochemical measures of growth (muscle RNA/DNA ratio or muscle protein), there were site differences in total length, suggesting that body length is a more reliable indicator of growth than the biochemical endpoints used in this study.

Using principal component analysis, I observed a close relationship between total body lipids, total body triglycerides, liver triglycerides, weight and length in both northern pike and burbot. For the northern pike, there appeared to be a slight grouping of variables by lake: reference fish and high exposure fish were similar along principal component 1 and the low exposure fish were slightly shifted to the right, indicating relatively higher lipids, triglycerides, weight and length. However, results for pike grouped by season revealed little to no separation of fish comparing fall versus spring along either principal component 1 or 2 and there were no clear seasonal differences in

variability of all morphometric and biochemical endpoints. The burbot variables grouped by lake exhibited more range along principal component 1 for the exposure lakes compared to the more central reference lake. However, the reason for this becomes clear when seasonal differences are considered. Burbot seasonal grouping showed distinct clustering along principal component 1. Since the burbot exhibited strong seasonal differences in lipids, triglycerides, weight and length, these results were not surprising. Even though the results from PCA suggest that weight and length correlated closely with total body lipids, triglycerides and liver triglycerides, it is important to note that for the northern pike, there were no observed site differences in weight and length. However, there were clear site differences in lipids and triglycerides, suggesting that although closely correlated the biochemical measures of condition detected site differences that were not noted with morphometric endpoints. This makes sense, since before changes in weight, length and condition factor occur, changes in biochemical composition should become apparent (De Boeck *et al*. 1997).

3.6 Conclusions

To evaluate changes in fish physiology due to environmental stress, employment of a suite of biochemical measures is recommended. Results of this study highlight the value of using certain biochemical measures of condition. For instance, in northern pike there were no differences in the standard morphometric measures of condition such as weight, length or condition factor among study lakes. However, I found significant differences in lipids and triglycerides in pike among lakes and seasons. Thus, exposure to uranium milling effluent had an influence on YOY fish that may not be detected with traditional morphometric measurements. Since lipids, including triglycerides, are

important for estimating fish condition they continue to be valuable assessment tools in fisheries research. However, I found that muscle RNA/DNA ratio and muscle protein concentration did not detect relevant site or season differences for either species. Perhaps growth measured morphometrically with total length may be more relevant than variations in relatively short term growth rates measured with RNA/DNA ratio or protein, particularly in fish that have survived beyond the fry stage (Weber *et al*. 2003).

Increased lipids and triglycerides in both species, as well as weight and length in burbot, following exposure to uranium milling effluent may be an indirect effect via food web enrichment, characterized by higher prey quantity and quality in combination with winter growth or overwinter mortality of individuals with lower energy stores. In a study in metal contaminated lakes, Iles and Rasmussen (2005) suggested that indirect, food web mediated effects may have a relatively higher impact on yellow perch than direct, physiological effects. In addition, the energetic costs associated with predation risk may also have contributed to the observed site differences in energy storage. However, direct effects of metal exposure on fish physiology can have energetic costs (Campbell *et al*. 2003) and thus can not be ruled out. Continuing research in this area is focusing on the relative importance of direct versus indirect causes for the observed differences in bioenergetics of juvenile fishes exposed to metal mining discharges.

CHAPTER 4
4.0 OVERWINTER CHANGES IN MORPHOMETRIC AND BIOCHEMICAL ENDPOINTS IN NORTHERN PIKE (*ESOX LUCIUS*), BURBOT (*LOTA LOTA*) AND SLIMY SCULPIN (*COTTUS COGNATUS*) EXPOSED TO URANIUM MINING AND MILLING EFFLUENT

4.1 Abstract

Fishes exposed to uranium mining and milling effluent inhabit an environment with higher levels of metals and ions compared to fishes inhabiting reference sites. Using morphometric and biochemical endpoints, the health of northern pike (*Esox lucius*), burbot (*Lota lota*) and slimy sculpin (*Cottus cognatus*) was assessed downstream of McClean Lake uranium operation in northern Saskatchewan, Canada. Overwinter survival was estimated by examining total body lipids and total body triglycerides in the fall and spring and there was no consistent site or season response in these energetic endpoints for the three fish species. Only muscle RNA/DNA ratio and muscle protein concentration exhibited clear seasonal differences, with a consistent decrease in these growth indices in fishes in the spring relative to the previous fall at both exposure and reference sites.

4.2 Introduction

Maintenance of a healthy fish population is primarily influenced by recruitment of new individuals into the population, and recruitment of new individuals into a population is closely linked to survival of juvenile fish beyond their first year. For fishes in temperate and northern environments, the first winter represents a major challenge to young-of-the-year (YOY) survival and future recruitment into the population (Hurst and

Conover 1998). Cold temperatures, ice cover and winter photoperiod can contribute to overwinter mortality in YOY fishes via starvation, osmoregulatory failure or the presence of anoxic water conditions.

During the first year of life, it is important for fishes to allocate energy to growth and lipid storage. Body size has an important influence on an animal's energetic requirements, its potential for resource exploitation and its susceptibility to natural enemies (Werner and Gilliam 1984). Size-selective overwinter mortality has been noted to occur in a variety of YOY fish species, where smaller fish are more susceptible to winter mortality than larger fish (Oliver *et al.* 1979, Toneys and Coble 1980, Henderson *et al.* 1988, Post and Evans 1989, Johnson and Evans 1991, Griffiths and Kirkwood 1995, Foy and Paul 1999, Gotceitas *et al.* 1999, Kristiansen *et al.* 2000, Sogard and Olla 2000, Grant and Tonn 2002, Biro *et al.* 2004). Lipids are a primary source of energy and are important for both fitness and survival of fish (Adams 1999). Fish that obtain fat reserves before winter have a higher probability of winter survival (Thompson *et al.* 1991, Lemly 1996, Post and Parkinson 2001). Decreased lipid levels can lead to starvation as well as compromised osmoregulation (Lemly 1996, Adams 1999).

The combination of winter and an environmental contaminant, uranium mining and milling effluent, may further reduce the condition of YOY fishes. Uranium mining and milling effluent released into local aquatic systems results in elevated levels of various metals and ions. Energetically expensive changes in physiology or behaviour resulting from contaminant exposure will reduce the allocation of energy towards energy storage and growth, which are critical for winter survival and recruitment. Since winter

is, by itself, a physiologically stressful time for fish, the addition of any type of contaminant has the potential to significantly decrease overwinter survival.

Weight and length are common morphometric endpoints in fisheries research and provide an estimate of overall condition. Weight is a surrogate for energy storage and length is a measure of growth. Fulton's condition factor is a simple formula and is one of many used to evaluate the weight-length relationship (reviewed by Bolger and Connolly 1989). Fulton's condition factor (weight/length^3*100) converts a two-dimensional weight-length relationship into a single statistic that provides a simple indication of the well-being of a fish, since at a given length, a fish with higher weight is considered to be in better condition. Organ weights, such as livers or gonads, can be related to body weight in a hepato-somatic (HSI) or gonado-somatic index (GSI) (organ weight/total weight*100), respectively. Liver weight is generally a measure of energy storage and gonad weight in sexually mature individuals is a commonly used measure of reproductive status. In general, variation in the HSI reflects mobilization or accumulation of lipid in the liver. In addition, the HSI is commonly used as a biomarker of contaminant exposure (Goede and Barton 1990, Facey *et al.* 2005). Since the liver is very important for detoxification, exposure to contaminants can lead to hypertrophy (an increase in size) or hyperplasia (an increase in the number of cells) or both (Goede and Barton 1990, Hinton and Lauren 1990). Therefore, determining morphometric endpoints in fishes will provide information on growth, energy storage, reproductive potential and response to toxicants.

Before changes in morphometric endpoints occur, changes in biochemical composition should become apparent (De Boeck *et al.* 1997). Lipids are a primary source of energy and are important for both fitness and survival of fish (Adams 1999).

Total lipid extraction, although commonly used, does not distinguish among different lipid classes. However, measurement of total body triglycerides provides a more ecologically and physiologically relevant endpoint than total lipids, since triglycerides are the predominant form of stored energy in fish (Henderson and Tocher 1987, Sheridan 1988, Sargent *et al.* 1989, Jobling *et al.* 1998). Muscle RNA/DNA ratio provides an estimate of short-term growth (hours to days) based on the concept that although DNA remains relatively constant per cell, RNA will vary depending on the amount of protein synthesis. Muscle protein content indicates longer-term growth rates (days to weeks). During times of starvation and metabolic stress, fishes will also utilize muscle protein as an energy source.

Given the high potential for winter mortality, survival during the first winter for fish can determine future year-class strength for a population. Young-of-the-year fish experience a trade-off between energy allocated to growth versus energy allocated to storage (Adams 1999, Post and Parkinson 2001). Small differences in body size can have a large effect on survival through various factors such as increased relative metabolism, osmoregulatory failure, increased risk of predation or decreased ability to exploit resources. Sufficient lipid stores prior to winter are also important for fish survival and eventual recruitment. Clearly, overwinter survival represents a major challenge to YOY fishes, particularly at northern latitudes where winter is long and cold.

To assess potential impacts of uranium mining and milling effluent at McClean Lake uranium operation, Saskatchewan, Canada, various morphometric and biochemical endpoints were measured in fall and spring in three fish species. Morphometric endpoints included weight, length, condition factor, hepato-somatic index and gonado-

somatic index. Biochemical endpoints included total body lipids, total body triglycerides, muscle RNA/DNA ratio and muscle protein. I predicted that fishes exposed to uranium mining and milling effluent would have decreased growth and energy storage relative to fish from reference sites in both fall and spring. In addition, I predicted fish would have depleted lipids and triglycerides at all sites following winter. To investigate these hypotheses, YOY fishes were collected just prior to and immediately following winter. At each sampling period, fish growth was estimated using length, muscle RNA/DNA ratio and muscle protein. Energy storage and energy allocation in the fishes was assessed with body weight, condition factor, HSI, GSI as well as total body lipids and total body triglycerides.

4.3 Materials and methods

4.3.1 Site description and fish collection

The McClean Lake uranium operation (58°23'N, 103°48'W) is located in northern Saskatchewan, Canada. The operation began mining uranium in 1995 and initiated milling in 1999. The Sink/Vulture Treated Effluent Management System is the single, common facility for the controlled release of all waste water generated at the McClean Lake uranium operation (COGEMA Resources Inc. 2003).

Northern pike (*Esox lucius*) and burbot (*Lota lota*) were collected downstream of uranium mining and milling effluent discharge in Kewen Lake (exposure lake, Figure 4.1). Konner Lake was the reference lake for burbot and Indigo Lake was used as the reference lake for the collection of northern pike (Figure 4.1). Slimy sculpin (*Cottus cognatus*) were collected at two locations along Collins Creek: an exposure area downstream of mining and milling activities and a reference area upstream of all mining

and milling inputs (Figure 4.1). Water quality variables and trace element concentrations at reference and exposure sites from 2004-2005 are provided in Table 4.1.

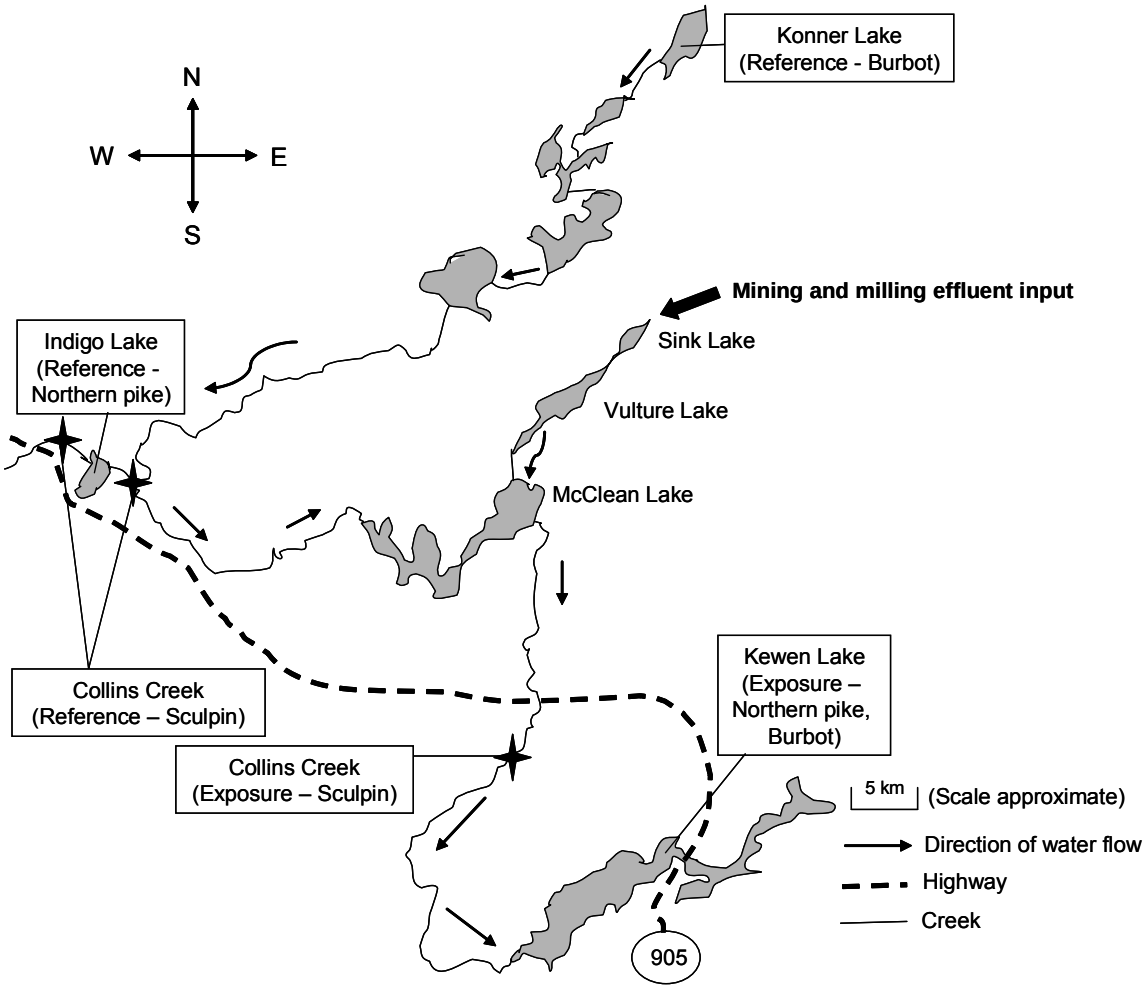

Figure 4.1 Map of watershed at McClean Lake uranium operation, Saskatchewan, Canada (58°23'N, 103°48'W) where lakes and creeks are receiving treated uranium mining and milling effluent. The direction of water flow in creeks is shown with arrows and lakes are shown in grey. A section of Saskatchewan highway 905 is shown with a dashed line. Reference and exposure sites where fishes were sampled in fall 2004 and spring 2005 are identified in boxes.

All fish were collected using a backpack electrofishing unit in fall 2004 and spring 2005. Fish were captured, held for less than 1.5 hours and over-anesthetized with MS-222 (3-aminobenzoic acid); total length and wet weight recorded and ageing structures (cleithra and scales) removed from pike. Fish were immediately stored on dry

ice until transport back to the laboratory -80°C freezer at the Toxicology Centre, University of Saskatchewan.

4.3.2 Laboratory analyses

Burbot and sculpin were aged using otoliths; northern pike were aged using cleithra and scales. Fish tails including a portion of caudal muscle were cut from each fish and immediately returned to the -80°C freezer for subsequent RNA/DNA ratio and protein determinations. Each carcass was dissected as follows. Stomach contents were removed, weighed, and identified as either invertebrate or vertebrate (fish) prey items. Weight of the stomach contents was subtracted from the initial weight of the fish and the resulting value was used for subsequent calculations involving fish weight. Liver and gonads (if present) were weighed and hepato-somatic and gonado-somatic indices calculated as (organ weight/body weight * 100%).

4.3.2.1 Total body lipids

The remaining carcass was weighed, added to ×2 volume of nanopure water, finely minced with scissors, homogenized (3 × 30 seconds) using a Tissue Tearor (BioSpec Products Inc., Bartlesville, OK, U.S.A.) and a 4 ml aliquot of the resulting homogenate was used for Bligh and Dyer solvent lipid extraction (Bligh and Dyer 1959). The chloroform-methanol lipid extraction was conducted on all fish using a final ratio of 1.8:2:2 for water to chloroform to methanol as per Bligh and Dyer (1959), with the following modifications: chloroform contained 50 mg/L butylated hydroxytoluene (Sigma-Aldrich Canada Ltd., Oakville, ON, Canada) to reduce oxidation of fatty acids (Weber *et al.* 2003), samples were centrifuged instead of filtered and samples were dried at room temperature under a gentle stream of nitrogen. Lipid weight was determined in

the fish sample (mg lipid per gram of fish carcass) using a gravimetric technique where triplicate aliquots of the chloroform fraction were evaporated in pre-weighed glass vials under a nitrogen atmosphere to prevent lipid oxidation.

4.3.2.2 Triglyceride assay

Whole body triglycerides were determined in the unextracted fraction of the same fish carcass homogenized in ×2 water using a modification of the McGowan *et al.* (1983) method (Weber *et al.* 2003). An aliquot of the fish homogenate was added to an equal volume of 0.2 M sodium citrate. The sample was homogenized on ice, placed in a heating block at 100ºC for five minutes and immediately returned to ice. All samples were centrifuged for five minutes at 500 *g* prior to assaying in order to remove insoluble materials such as connective tissue from the liquid fraction.

4.3.2.3 RNA/DNA assay

Tail muscle was weighed (0.010-0.020 g) and added to ice-cold TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0) plus 0.2 M NaCl. Tissue was finely minced with scissors, homogenized with a Tissue Tearor (2 × 10 seconds), 5 µl of 1% sodium dodecyl sulphate was added and the sample was shaken and incubated at 65°C for two hours. Samples were centrifuged at 5000 *g* for ten minutes at 4°C, the supernatant was removed and an aliquot was taken for subsequent protein assay. Ice-cold isopropanol and 3 M sodium acetate were added to the remainder of supernatant and the sample was stored overnight at -20°C to allow precipitation of nucleic acids. After centrifugation at 15,000 *g* for 30 minutes, the supernatant was removed, TE buffer was added to the pellet, the sample was incubated at 65°C for 1 hour and stored overnight at 4°C to ensure complete resuspension of nucleic acids. The following day, RNA and DNA concentrations and

resulting RNA/DNA ratio were determined using a modified dual fluorescent dye method (Clemmesen 1988, Weber *et al.* 2003). Calf thymus DNA and yeast RNA were used as standards.

4.3.2.4 Protein assay

An aliquot of the tail muscle homogenate prepared for nucleic acid determination was used to determine muscle protein concentration. A modification of the Lowry *et al.* (1951) protein assay (BioRad DC protein assay, BioRad, Hercules, CA, USA) was used with bovine serum albumin as the standard. Duplicate 5 µl samples were read on a 96-well microplate at 750 nm absorbance.

4.3.3 Statistical analyses and assay performance

Two-way ANOVA was used with site and season as the two factors, followed by Tukey's post-hoc test as required for YOY fishes. Data were analyzed using two-way ANCOVA with site and season as the two factors and age as the covariate when fishes varied in age (i.e. fish collected were not strictly young-of-the-year), followed by Tukey's post-hoc test as required. If data were not normally distributed, they were transformed prior to statistical analysis. If there was a significant interaction between the two factors in the two-way ANOVA, *t*-tests were used to compare fish between sites and seasons separately. The Mann-Whitney Rank Sum test was used if data for *t*-test failed parametric assumptions. Results were considered significant if $p < 0.05$ for all tests and results are reported as mean ± standard error of the mean (SEM). Principal component analysis (PCA) was conducted on log10 transformed data using all morphometric and biochemical endpoints for each fish species separately.

Intra-assay variability was calculated for biochemical assays by determining the coefficient of variation (%CV = standard deviation/mean*100) for six replicates of a pooled sample measured in the same assay. Inter-assay variability was determined by calculating the %CV among six replicates of the same pooled sample performed in two separate assays.

4.4 Results

4.4.1 Abiotic environment

Water chemistry data were only available for one reference lake, Konner Lake. Water chemistry variables did not differ greatly between reference and exposure lakes, although differences in water chemistry between the reference and exposure sections of the creek were evident (Table 4.1). Water from the exposure lake and the exposure section of the creek was higher than reference sites in conductivity, total dissolved solids, total hardness, ammonia, sulphate, arsenic and molybdenum.

Table 4.1 Water quality data for study lakes and creeks at McClean Lake uranium operation, Saskatchewan, Canada. Results are mean values of monthly samples from May 2004 to November 2005 (AREVA Resources Inc. 2006). Exposure section of Collins Creek is located downstream of McClean Lake and the reference area of Collins Creek is upstream of Indigo Lake. Refer to Figure 4.1 for location of all sites. Values preceded by the < symbol represent the detection limit for the specific analyte.

Analyte	Units	Exposure Sites		Reference Sites	
		Collins Creek	**Kewen Lake**	**Collins Creek**	**Konner Lake**
Conductivity	µS/cm	124.0	69.1	22.5	14
pH		6.5	6.6	6.5	6.6
Total dissolved solids	mg/L	85.8	51.1	28.1	19
Total Hardness	mg/L	41.8	21.7	7	5
Total P	mg/L	<0.01	<0.01	<0.01	<0.01
Ammonia (as N)	mg/L	0.089	0.054	0.041	0.060
Sulphate	mg/L	39.0	16.7	0.6	0.3
Radium-226	Bq/L	0.007	0.006	0.006	0.007
Arsenic	µg/L	0.35	0.23	0.19	0.1
Cadmium	µg/L	<0.001	<0.001	<0.001	<0.001
Copper	mg/L	<0.001	<0.001	<0.001	<0.001
Iron	mg/L	0.41	0.23	0.55	0.17
Lead	mg/L	<0.002	<0.002	<0.002	<0.002
Mercury	µg/L	<0.05	<0.05	<0.05	<0.05
Molybdenum	mg/L	0.026	0.012	<0.001	<0.001
Nickel	mg/L	0.0015	<0.001	<0.001	<0.001
Selenium	mg/L	0.00012	0.00013	0.00019	0.0001
Uranium	µg/L	<0.1	<0.1	<0.1	<0.1
Vanadium	mg/L	<0.001	<0.001	<0.001	<0.001
Zinc	mg/L	<0.005	<0.005	<0.005	<0.005

4.4.2 Sample size, age, stomach contents

A total of 49 northern pike, 45 burbot and 37 slimy sculpin were collected at the study lakes and creeks at McClean Lake uranium operation in fall and spring combined (Tables 4.2, 4.3 and 4.4). All northern pike and burbot were young-of-the-year, age-0 in the fall and age-1 the following spring. Slimy sculpin ranged in age from 0 to 7 years (Table 4.4) and therefore ANCOVA with age as a covariate was used to analyze data for this species.

Stomach content analysis for northern pike at all sites and seasons revealed that 43% of individuals had recently consumed food. Of the fish with stomach contents, 38% had consumed invertebrates and 62% had eaten fish. Based on stomach contents, 78% of all burbot had recently consumed food and of these individuals, 97% had consumed invertebrates and 3% had consumed fish. Most slimy sculpin (89%) had recently consumed prey items prior to collection. Of these fish, 97 % consumed invertebrates and one fish (3%) in the spring season had consumed fish eggs.

In the fall, 43% of northern pike from the reference lake and 50% of pike from the exposure lake had stomach contents, which weighed 0.31 ± 0.2 g and 0.26 ± 0.07 g, respectively. In the spring, 64% of northern pike from the reference lake and 14% of pike from the exposure lake had stomach contents, which weighed 0.098 ± 0.03 g and 0.35 ± 0.03 g, respectively. In the fall, 64% of burbot from the reference lake and 83% of burbot from the exposure lake had stomach contents, which averaged 0.028 ± 0.008 g and 0.094 ± 0.04 g in weight, respectively. In the spring, 75% of burbot from the reference lake and 92% of burbot from the exposure lake had recently consumed food, which weighed 0.16 ± 0.06 g and 0.12 ± 0.02 g, respectively. In the fall, 100% of slimy sculpin

from the reference site and 67% of sculpin from the exposure site had recently consumed food which weighed 0.084 ± 0.008 g and 0.017 ± 0.007 g, respectively. In the spring, 100% of slimy sculpin from the reference site and 92% of sculpin from the exposure site had recently consumed food, which weighed 0.25 ± 0.2 g and 0.063 ± 0.02 g, respectively.

4.4.3 Northern pike

For northern pike, neither body weights nor total lengths were statistically different between lakes or within lakes over seasons (Table 4.2). Condition factor was significantly different ($p = 0.046$) between seasons, however no individual differences were detected for either exposure or reference fish when comparing fall and spring values. Hepato-somatic index (Figure 4.2A) was significantly different ($p < 0.001$) between seasons, with higher HSI in spring compared to fall for pike from both the reference lake ($p < 0.001$) and exposure lake ($p < 0.01$).

For the biochemical measures of condition, northern pike total body lipids (Table 4.2) and total body triglycerides (Figure 4.3A) were not statistically different between lakes or seasons. Northern pike muscle RNA/DNA ratio (Table 4.2) was not statistically different comparing fish collected from the reference and exposure lakes, however, results were significantly ($p < 0.01$) different between seasons. For pike from the exposure lake, muscle RNA/DNA ratio was significantly lower ($p < 0.01$) in the spring compared to the fall. Similar to muscle RNA/DNA ratio, fish muscle protein concentrations (Figure 4.4A) were not statistically different between lakes, however results were significantly ($p < 0.01$) different between seasons. For pike from both the

reference and exposure lakes, muscle protein values were significantly lower ($p < 0.05$ for both) in the spring compared to the previous fall.

Table 4.2 Morphometric (weight, total length, condition factor) and biochemical (total body lipids, muscle RNA/DNA ratio) variables determined in young-of-the-year northern pike (*Esox lucius*). Fish were collected at McClean Lake uranium operation, Saskatchewan, Canada from Indigo Lake (reference) which is located upstream of uranium mining and milling inputs and Kewen Lake (exposure) which is downstream of uranium mining and milling inputs, in fall 2004 and spring 2005. Refer to Figure 4.1 for locations of study sites. Data shown are mean ± standard error of the mean. Condition factor = (weight/total length^3)100.

	REFERENCE		EXPOSURE	
	Fall 2004	**Spring 2005**	**Fall 2004**	**Spring 2005**
Sample size	7	14	14	14
Weight (g)	7.50 ± 1.72	7.23 ± 0.99	7.15 ± 0.79	8.67 ± 1.12
Total length (cm)	11.01 ± 0.90	11.15 ± 0.54	11.05 ± 0.38	11.88 ± 0.49
Condition factor	0.50 ± 0.01	0.49 ± 0.01	0.51 ± 0.01	0.48 ± 0.01
Total body lipids (%)	1.10 ± 0.08	1.06 ± 0.06	1.09 ± 0.07	1.05 ± 0.04
Muscle RNA/DNA	2.43 ± 0.48	1.98 ± 0.24	2.73 ± 0.33	1.50 ± 0.16††

†† Significantly different ($p < 0.01$) within lake over two seasons as determined using 2-way analysis of variance followed by Tukey's post-hoc test

4.4.4 Burbot

Young-of-the-year burbot weight and total length varied significantly ($p < 0.05$) between lakes. However, pairwise comparisons did not detect any differences in weight. In the fall, the mean length of burbot from the exposure lake was significantly higher ($p < 0.05$) than the length of fish collected from the reference lake (Table 4.3). Using two-way ANOVA, there was a significant interaction between lake and season for burbot condition factor and HSI. Therefore, *t*-tests were used to examine differences between means for these endpoints. In the spring, burbot from the exposure lake had significantly higher ($p < 0.001$) condition factor compared to fish collected from the reference lake. In addition, burbot collected from the exposure lake had a significantly higher ($p < 0.01$) condition factor in spring compared to fall (Table 4.3). In the fall, hepato-somatic index was significantly lower ($p < 0.05$) in burbot collected from the exposure lake compared to fish from the reference lake (Figure 4.2B). In the spring, the opposite was found and burbot from the exposure lake had significantly higher ($p < 0.001$) HSI than burbot collected from the reference lake (Figure 4.2B). Also, burbot collected at the exposure lake had significantly higher ($p < 0.001$) HSI in spring compared to fall.

There was a significant interaction between lake and season for YOY burbot total body lipids as well as total body triglycerides. Therefore, *t*-tests were used to examine differences between means for these endpoints. Total body lipids were significantly higher in burbot collected from the exposure lake in the spring compared to burbot collected from the reference lake ($p < 0.001$). Total body lipids were also significantly higher ($p < 0.01$) in YOY burbot collected from the exposure lake in spring compared to fall (Table 4.3). Total body triglycerides (Figure 4.3B) followed a similar trend to total

body lipids. In the spring, YOY burbot from the exposure lake had higher ($p < 0.001$) total body triglycerides compared to burbot collected from the reference lake. Total body triglycerides were significantly higher ($p < 0.001$) in burbot collected from the exposure lake in spring compared to fall.

Both burbot muscle RNA/DNA ratio and muscle protein content were significantly different between seasons ($p < 0.05$ and $p < 0.001$, respectively), although differences between lakes were not significant for either endpoint (Table 4.3 and Figure 4.4B). Burbot collected from the reference lake had a significantly lower ($p < 0.05$) muscle RNA/DNA ratio in spring compared to fall. Muscle protein values for burbot collected from the reference lake were significantly lower ($p < 0.01$) in spring compared to fall. Muscle protein values for burbot collected from the exposure lake were significantly lower ($p < 0.05$) spring compared to fall (Figure 4.4B).

Table 4.3 Morphometric (weight, total length, condition factor) and biochemical (total body lipids, muscle RNA/DNA ratio) variables determined in young-of-the-year burbot (*Lota lota*). Fish were collected at McClean Lake uranium operation, Saskatchewan, Canada from Konner Lake (reference) which is located upstream of uranium mining and milling inputs and Kewen Lake (exposure) which is downstream of uranium mining and milling inputs, in fall 2004 and spring 2005. Refer to Figure 4.1 for locations of study sites. Data shown are mean ± standard error of the mean. Condition factor = (weight/total length^3)100.

	REFERENCE		EXPOSURE	
	Fall 2004	**Spring 2005**	**Fall 2004**	**Spring 2005**
Sample size	14	12	6	13
Weight (g)	4.82 ± 1.22	6.53 ± 1.18	7.14 ± 1.29	9.00 ± 1.23
Total length (cm)	8.71 ± 0.78	10.38 ± 0.71	10.98 ± 0.83*	11.11 ± 0.57
Condition factor	0.55 ± 0.02	0.52 ± 0.01	0.50 ± 0.01	0.61 ± 0.02***††
Total body lipids (%)	1.38 ± 0.15	1.29 ± 0.15	1.38 ± 0.21	2.71 ± 0.22***††
Muscle RNA/DNA	5.11 ± 0.50	3.38 ± 0.62†	5.10 ± 0.97	4.23 ± 0.52

* Significantly different from reference within season as determined using 2-way analysis of variance followed by Tukey's post-hoc test (*$p < 0.05$, ***$p < 0.001$)

† Significantly different within lake over two seasons as determined using 2-way analysis of variance followed by Tukey's post-hoc test ($^{\dagger}p < 0.05$, $^{\dagger\dagger}p < 0.01$)

4.4.5 Slimy sculpin

Sculpin collected from the reference creek were significantly older than sculpin collected from the exposure section of the creek in spring ($p < 0.05$), but not in the fall ($p = 0.056$) (Table 4.4). Sculpin were not significantly different in age between seasons for fish collected at either the reference or the exposure section of the creek.

Sculpin age varied significantly with weight ($p < 0.001$), length ($p < 0.001$), HSI ($p < 0.001$) and total body triglycerides ($p < 0.01$) and therefore, ANCOVA with age as a covariate was used to analyze these data. Sculpin weight was significantly different between sites ($p < 0.05$) and seasons ($p < 0.01$). In the spring, sculpin collected from the exposure section of the creek weighed less than reference fish ($p < 0.01$). As well, sculpin collected from the exposure section of the creek weighed less ($p < 0.001$) in the spring compared to fall (Table 4.4).

Sculpin total length was significantly different between sites ($p < 0.01$) and seasons ($p < 0.05$). Sculpin were significantly shorter ($p < 0.01$) in the exposure section of the creek compared to fish from the reference section of the creek in the spring. Sculpin collected from the exposure section of the creek were significantly shorter ($p < 0.01$) in spring compared to fall (Table 4.4).

Slimy sculpin condition factor was significantly different between sites ($p < 0.001$) and seasons ($p < 0.001$). Specifically, sculpin collected from both reference and exposure sites had significantly lower ($p < 0.01$) condition factor in spring compared to fall. Also, in both seasons, condition factor for sculpin from the exposure section of the creek was lower ($p < 0.01$) compared to sculpin collected from the reference section of

the creek. Although slimy sculpin hepato-somatic index varied with age ($p < 0.001$), HSI was not different between sites or seasons (Figure 4.2C).

Measurable gonads were present in all sculpin collected at the reference section of the creek in fall and spring. However, at the exposure section of the creek only 7 of 9 sculpin in the fall and 4 of 12 sculpin in the spring had measurable gonads. Comparing sculpin with measurable gonads, the average gonado-somatic index for sculpin from the reference site were similar in the fall (2.99 ± 0.59) and the spring (2.32 ± 0.29). Sculpin with measurable gonads collected in the fall from the exposure section of the creek had a mean GSI of 0.65 ± 0.31. In the spring, the group of sculpin with measurable gonads collected from the exposure section of the creek had a large range in gonado-somatic index (7.00 ± 4.96). Slimy sculpin GSI did not vary significantly with age. Two-way ANOVA was not used, because there was a significant interaction between site and season. *T*-tests were used or Mann-Whitney Rank Sum Test if normality failed. For fish with gonads collected at the exposure section of the creek, GSI was significantly higher ($p < 0.05$) in spring compared to fall. In the fall, GSI for fish with measurable gonads was significantly higher ($p < 0.01$) in fish collected from the reference site compared to fish collected from the exposure section of the creek.

Total body lipids for slimy sculpin were significantly different between seasons ($p < 0.001$). Sculpin collected from the reference site had significantly lower ($p < 0.05$) total body lipids in spring compared to fall. Sculpin collected from the exposure section of the creek were significantly lower ($p < 0.001$) in total body lipids in spring compared to fall (Table 4.4).

Using ANCOVA, total body triglycerides were significantly different between sites ($p < 0.01$) and seasons ($p < 0.001$). Slimy sculpin from both reference and exposure sites had lower ($p < 0.001$) total body triglycerides in spring compared to fall (Figure 4.3C). In addition, in the spring, total body triglycerides were lower in sculpin collected from the exposure site compared to sculpin collected from the reference site (Figure 4.3C).

There was a significant interaction between site and season for sculpin muscle RNA/DNA ratio, and therefore, *t*-tests were used to compare these endpoints. Muscle RNA/DNA ratio for sculpin collected from the reference site was significantly lower ($p < 0.05$) in spring compared to fall. Similarly, muscle RNA/DNA ratio for sculpin collected from the exposure section of the creek was significantly lower ($p < 0.05$) in spring compared to fall (Table 4.4).

Muscle protein (Figure 4.4C) was significantly different between sites ($p < 0.01$) and seasons ($p < 0.05$). Muscle protein concentration for slimy sculpin collected from the reference site was significantly lower ($p < 0.05$) in spring compared to fall. In the spring, muscle protein for sculpin collected from the reference site was significantly lower ($p < 0.001$) than muscle protein content for sculpin collected at the exposure site.

Table 4.4 Ages and morphometric (weight, total length, condition factor) and biochemical (total body lipids, muscle RNA/DNA ratio) variables determined in slimy sculpin (*Cottus cognatus*). Fish were collected at McClean Lake uranium operation, Saskatchewan, Canada from two areas of Collins Creek: a reference site upstream of all uranium mining and milling inputs and an exposure site downstream of uranium mining and milling effluent input, in fall 2004 and spring 2005. Refer to Figure 4.1 for locations of study sites. Data shown are mean ± standard error of the mean. Condition factor = (weight/total length^3)100.

	REFERENCE		EXPOSURE	
	Fall 2004	**Spring 2005**	**Fall 2004**	**Spring 2005**
Sample size	7	9	9	12
Age (years)	2.71 ± 0.61	3.44 ± 0.67	1.33 ± 0.60	1.92 ± 0.40
Weight (g)	9.55 ± 0.96	10.13 ± 1.40	5.56 ± 1.87	3.71 ± 1.18**†††
Total length (cm)	9.00 ± 0.29	9.53 ± 0.50	7.31 ± 0.67	6.43 ± 0.66**††
Condition factor	1.29 ± 0.03	1.13 ± 0.06††	1.11 ± 0.03**	0.98 ± 0.02**††
Total body lipids (%)	2.95 ± 0.50	1.96 ± 0.24†	2.74 ± 0.29	1.57 ± 0.11†††
Muscle RNA/DNA ratio	2.74 ± 0.36	1.77 ± 0.21†	2.57 ± 0.50	3.94 ± 0.43*

* Significantly different from reference within season as determined using 2-way analysis of variance or 2-way analysis of covariance followed by Tukey's post-hoc test (*$p < 0.05$, **$p < 0.01$)

† Significantly different within site over two seasons as determined using 2-way analysis of variance or 2-way analysis of covariance followed by Tukey's post-hoc test ($^{\dagger}p < 0.05$, $^{\dagger\dagger}p < 0.01$, $^{\dagger\dagger\dagger}p < 0.001$)

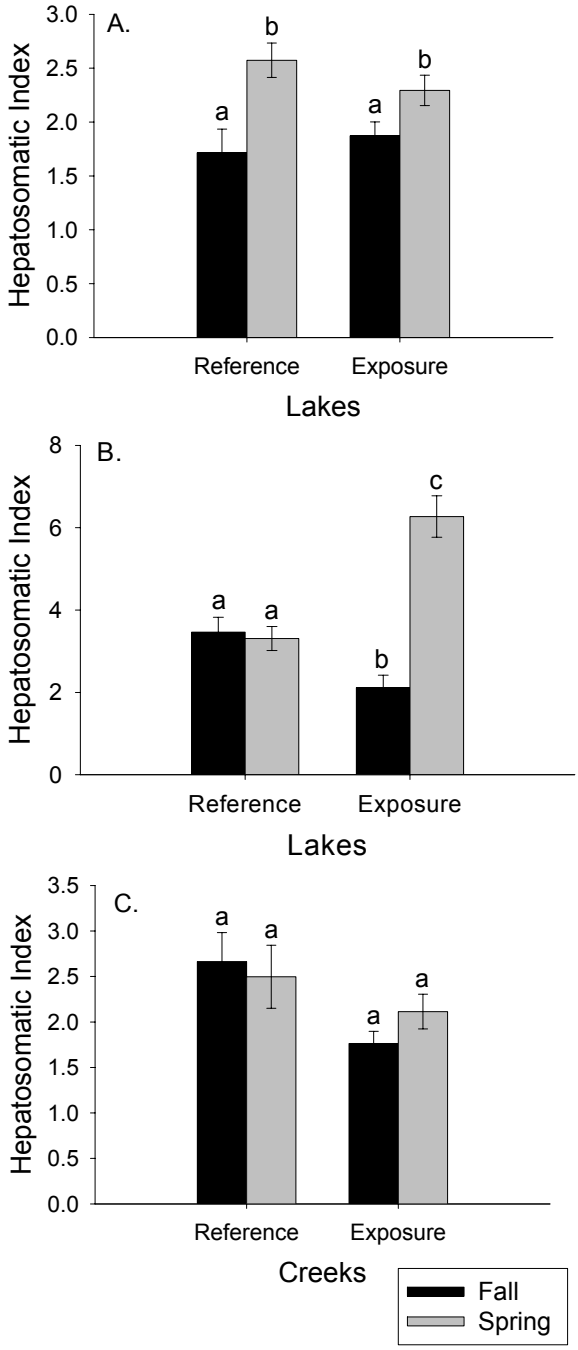

Figure 4.2 Hepato-somatic index (liver weight/body weight*100) for young-of-the-year northern pike (*Esox lucius*; A), young-of-the-year burbot (*Lota lota*; B) and slimy sculpin (*Cottus cognatus*; C) collected in fall 2004 and spring 2005 from reference sites upstream of all uranium mining and milling inputs and exposure sites downstream of uranium mining and milling effluent input at McClean Lake uranium operation, Saskatchewan, Canada. Data shown are mean ± standard error of the mean. Sample sizes range from 6-14 individuals per species per site in fall and 9-14 individuals per species per site in spring. Bars without letters in common are significantly different ($p < 0.05$) between sites within a season and within a site over two seasons using 2-way analysis of variance followed by Tukey's post-hoc test.

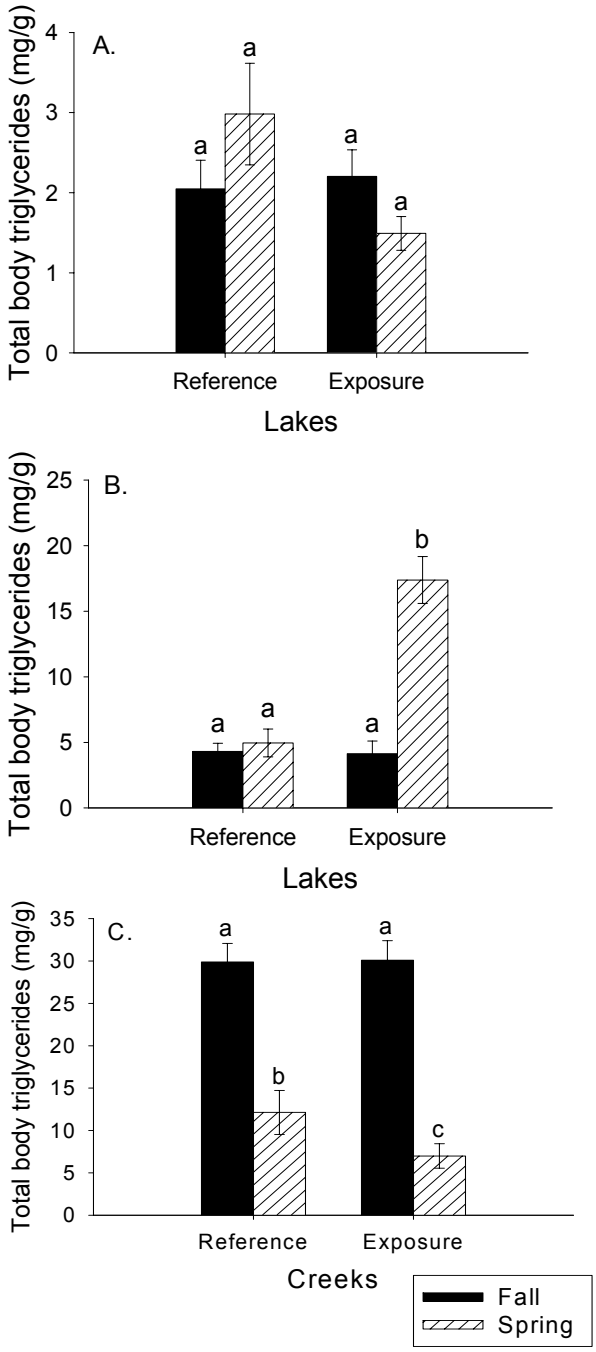

Figure 4.3 Total body triglycerides (mg triglyceride per g fish tissue) for young-of-the-year northern pike (*Esox lucius*; A), young-of-the-year burbot (*Lota lota*; B) and slimy sculpin (*Cottus cognatus*; C) collected in fall 2004 and spring 2005 from reference sites upstream of all uranium mining and milling inputs and exposure sites downstream of uranium mining and milling effluent input at McClean Lake uranium operation, Saskatchewan, Canada. Data shown are mean ± standard error of the mean. Sample sizes range from 6-14 individuals per species per site in fall and 9-14 individuals per species per site in spring. Bars without letters in common are significantly different ($p < 0.05$) between sites within a season and within a site over two seasons using 2-way analysis of variance or 2-way analysis of covariance followed by Tukey's post-hoc test.

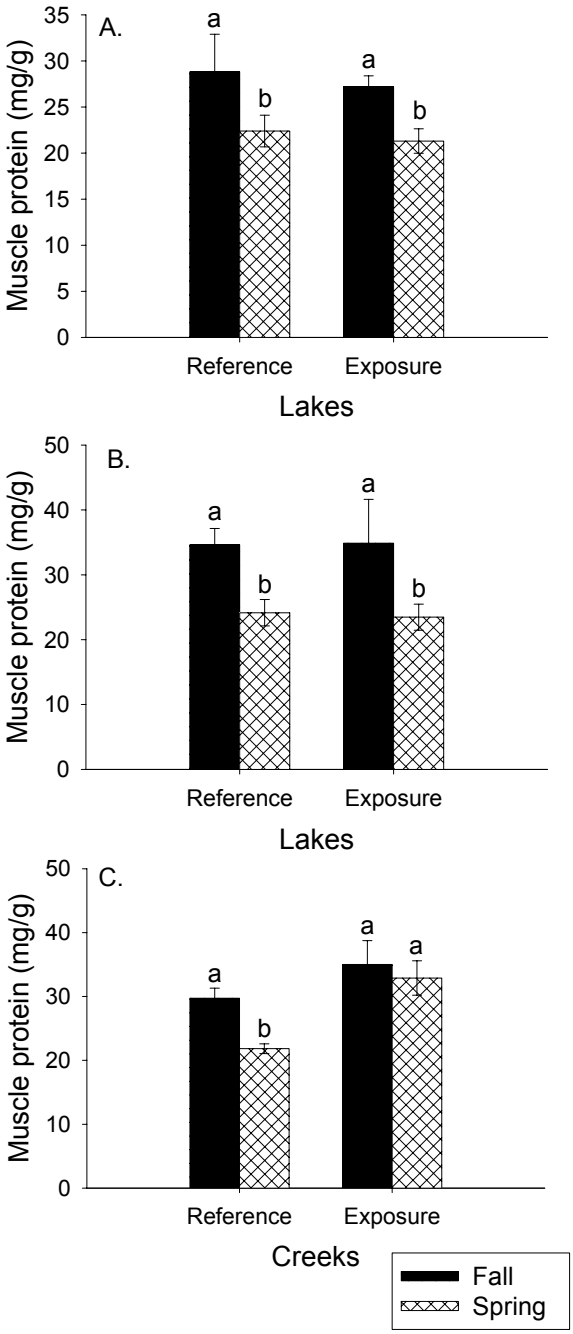

Figure 4.4 Muscle protein (mg protein per g fish muscle) for young-of-the-year northern pike (*Esox lucius*; A), young-of-the-year burbot (*Lota lota*; B) and slimy sculpin (*Cottus cognatus*; C) collected in fall 2004 and spring 2005 from reference sites upstream of all uranium mining and milling inputs and exposure sites downstream of uranium mining and milling effluent input at McClean Lake uranium operation, Saskatchewan, Canada. Data shown are mean ± standard error of the mean. Sample sizes range from 6-14 individuals per species per site in fall and 9-14 individuals per species per site in spring. Bars without letters in common are significantly different ($p < 0.05$) between sites within a season and within a site over two seasons using 2-way analysis of variance followed by Tukey's post-hoc test.

4.4.6 Principal component analysis

Morphometric (weight, length, condition factor and hepato-somatic index) and biochemical (total body lipids, total body triglycerides, muscle RNA/DNA ratio and muscle protein) endpoints for individual northern pike, burbot and slimy sculpin were analyzed using principal component analysis. For northern pike, the first two principal components explained 53.2% of the variance in the data. Important individual variables for northern pike principal component 1 were totally body triglycerides (most positive weighting) and length (lowest positive weighting). Principal component 2 was related to condition factor (positive) and HSI (most negative) (Table 4.5).

For burbot, the first two principal components explained 71.9% of the variance. Important individual variables for burbot principal component 1 were total body lipids (most positive weighting) and protein (most negative weighting), principal component 2 was related to condition factor (positive) and length (negative) (Table 4.6).

For slimy sculpin, the first two principal components explained 67.1% of the variance. Important individual variables for burbot principal component 1 were weight (most positive weighting) and muscle RNA/DNA ratio (most negative weighting), principal component 2 was related to totally body lipids (positive) and HSI (negative) (Table 4.7).

Table 4.5 Northern pike (*Esox lucius*) morphometric and biochemical variables analyzed using principal component analysis. For each axis, the largest positive and lowest positive or negative component loadings are in bold.

Component	Principal Component	
	1	2
Variance explained by components	2.7	1.5
Percent of total variance explained	34.5	18.7
Total body triglycerides	**0.67**	0.18
Total body lipids	0.54	-0.11
Condition factor	0.53	**0.60**
Muscle protein	0.45	0.48
Muscle RNA/DNA ratio	0.36	0.50
Hepato-somatic index	0.29	**-0.58**
Weight	-0.79	0.41
Length	**-0.84**	0.33

Table 4.6 Burbot (*Lota lota*) morphometric and biochemical variables analyzed using principal component analysis. For each axis, the largest positive and lowest positive or negative component loadings are in bold.

Component	Principal Component	
	1	2
Variance explained by components	3.7	2.0
Percent of total variance explained	47.1	24.8
Total body lipids	**0.91**	0.16
Total body triglycerides	0.86	0.41
Weight	0.79	-0.52
Hepato-somatic index	0.74	0.48
Length	0.72	**-0.62**
CF	0.43	**0.66**
Muscle RNA/DNA ratio	-0.24	0.64
Muscle protein	**-0.54**	0.23

Table 4.7 Slimy sculpin (*Cottus cognatus*) morphometric and biochemical variables analyzed using principal component analysis. For each axis, the largest positive and lowest positive or negative component loadings are in bold.

Component	Principal Component	
	1	**2**
Variance explained by components	3.5	1.9
Percent of total variance explained	43. 5	23.6
Weight	**0.92**	-0.25
Length	0.88	-0.34
Condition factor	0.79	0.43
Total body triglycerides	0.67	0.62
Total body lipids	0.51	**0.64**
Hepato-somatic index	0.26	**-0.59**
Muscle protein	-0.34	0.48
Muscle RNA/DNA ratio	**-0.57**	0.39

Scores for the first two principal components, grouped by both lake and season, for the northern pike, burbot and slimy sculpin were used to construct Figure 4.5. There was no distinct clustering for northern pike when plotted by lake (Figure 4.5A1) however, a seasonal clustering was evident (Figure 4.5A2), with the pike collected in the spring clustering towards the negative end of principal component 2. PCA for burbot grouped by lake (Figure 4.5B1) was very similar to that for burbot grouped by season (Figure 4.5B2). Site and season differences in burbot morphological and biochemical endpoints were grouped along principal component 1. Similar to the burbot, results for the slimy sculpin showed similar clustering of fish for both site and season along principal component 1 (Figure 4.5C1 and 4.5C2).

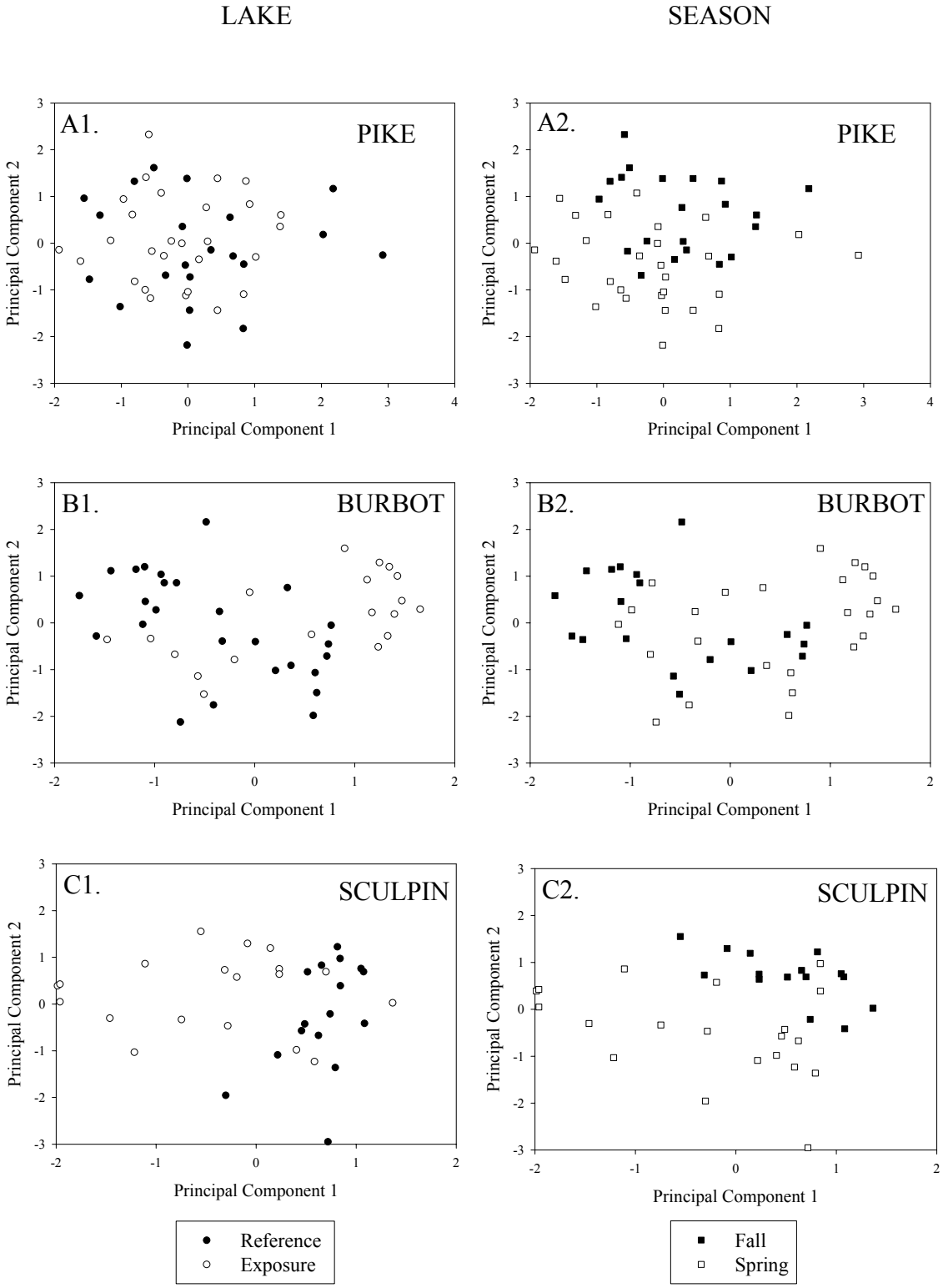

Figure 4.5 Principal component analysis of all morphometric (weight, length, condition factor, hepato-somatic index) and biochemical (total body lipids, total body triglycerides, muscle RNA/DNA ratio, muscle protein) endpoints for young-of-the-year northern pike (*Esox lucius*) (A), burbot (*Lota lota*) (B) and slimy sculpin (*Cottus cognatus*) (C) Components are grouped by lakes (1) and by season (2).

4.4.7 Assay performance

Both intra- and inter-assay coefficients of variation were below 10% for all assays. Intra-assay variability was 4.6%, 3.5%, 3.7%, 3.0% and 4.1% for the RNA/DNA ratio, protein, total body lipids, total body triglycerides and liver triglyceride assays, respectively. The inter-assay variability was 8.7%, 5.0%, 5.6%, 4.1% and 4.5% for the RNA/DNA ratio, protein, total body lipids, total body triglycerides and liver triglyceride assays, respectively (data not shown).

4.5 Discussion

4.5.1 Abiotic variables

Water chemistry variables revealed that although there were site differences in conductivity, total dissolved solids, hardness and sulphate, other variables including nutrients, radionuclides and metals did not differ greatly between reference and exposure sites.

4.5.2 Seasonal differences in growth

Consistent seasonal trends in short- and long-term growth were observed in all species at almost all sites: both muscle RNA/DNA ratio and muscle protein concentration followed similar trends and were significantly lower in the spring compared to the previous fall. Environmental factors such as food availability (Clemmesen 1988, Raae *et al.* 1988, Steinhart and Eckmann 1992, Buckley *et al.* 1999, Weber *et al.* 2003), toxicant exposure (Kearns and Atchison 1979) and temperature (Fry 1971, Buckley *et al.* 1999) can all impact biochemical indices of growth in fish.

Numerous studies have found that fed fish have higher RNA/DNA ratios than fish deprived of food (Clemmesen 1988, Raae *et al.* 1988, Steinhart and Eckmann 1992,

Weber *et al.* 2003). Similar to RNA/DNA, tissue protein concentration also tends to be higher in fed fish than in food deprived individuals (Smith 1981, Steinhart and Eckmann 1992, McLaughlin *et al.* 1995, Weber *et al.* 2003). Proteins can also be exploited as an energy source, although they tend to be utilized only under conditions of severe stress (Benton *et al.* 1994). Since there was not a trend across all species for lower total body lipids and triglycerides in the spring compared to the fall, the observed decreased fish growth in spring was not likely the result of starvation. Although environmental contamination can impact biochemical indices of growth (Kearns and Atchison 1979), in this study it was not likely that exposure to uranium mining and milling effluent resulted in decreased muscle RNA/DNA and muscle protein. Since seasonal differences in muscle RNA/DNA ratio and muscle protein content were observed in fish collected from both exposure and reference sites, mining and milling contamination does not appear to be responsible for the changes in fish growth.

Since all fish in this study were collected a short time after ice-off in the spring, water temperatures may not have reached an optimum level for growth to be initiated. Temperature is a factor in fish growth as it governs the rate of chemical reactions, metabolic requirements and rate of digestion (Fry 1971). Freshwater fish are poikilothermic and therefore their metabolic rate and many of their physiological functions are fundamentally influenced by temperature. Buckley *et al.* (1999) found that temperature and food availability determine growth rates in fish. Previous studies examined effects of temperature on protein turnover in fish and found that, providing food is not limiting, protein synthesis is greater at warmer water temperatures (Watt *et al.* 1988, Mathers *et al.* 1993). Beamish *et al.* (1996) found lower muscle protein levels in

lake sturgeon (*Acipenser fulvescens*) in March, relative to June, July, August and October. This is similar to the post-winter decrease in fish growth measured using muscle RNA/DNA ratio and muscle protein observed in this study. Low spring temperatures, an environmental factor affecting all sites, were likely responsible for the seasonal differences in muscle RNA/DNA ratio and muscle protein measured in the fishes at McClean Lake uranium operation.

4.5.3 Total body lipids and triglycerides

I hypothesized that there would be a decrease in both total body lipids and triglycerides following winter in all species from both the reference and exposure sites. I also hypothesized that total body lipid and triglyceride content would be lower in fish from exposure sites in both seasons compared to fish from references sites. However, trends in total body lipids and triglycerides were quite variable between species and did not support these initial hypotheses in all cases.

4.5.3.1 Northern pike

Young-of-the-year northern pike did not exhibit seasonal changes in either total body lipids or total body triglycerides. More specifically, overwinter depletion of lipids and triglycerides was not observed in pike collected from either the reference or exposure lakes, suggesting that this species may not be susceptible to overwinter starvation in this aquatic system. In addition, since there were no differences in these biochemical endpoints between sites, there does not appear to be any effect of uranium mining and milling effluent on total body lipid or triglyceride content in YOY northern pike at the lake sampled at McClean Lake uranium operation.

4.5.3.2 Burbot

Total body lipids and triglycerides in YOY burbot followed a similar pattern, highlighting the similarity of these two endpoints in this species. Site differences were observed as burbot from the exposure lake had significantly higher lipids and triglycerides compared to the reference site in the spring. In addition, there was a seasonal change in lipids and triglycerides in YOY burbot in the exposure lake. However, this seasonal change was not an overwinter depletion of lipids and triglycerides. Rather, YOY burbot from the exposure site had increased lipid and triglyceride content in the spring relative to the previous fall, which was contrary to what I had expected. Principal component analysis of burbot morphological and biochemical data show that burbot collected from the exposure site in spring were clustered together at the positive end of principal component 1, indicating that burbot with high total body lipid content were distinctive using PCA.

From an ecotoxicological perspective, reasons for the site differences in burbot total body lipids and triglycerides are unclear. Juvenile burbot in this watershed primarily feed on benthic invertebrates, as evidenced by stomach content analysis. In general, benthic invertebrate abundance is high during the winter (Moore 1980), as many insects emerge in the spring after overwintering as immature aquatic forms. Since burbot feed using their sensory system (Hinkens and Cochran 1988), reduced light associated with northern winters would not impact their sensory foraging ability. However, if burbot were feeding in the winter, why did this pattern of increased energy stores in the spring only occur in the exposure site? Perhaps there were habitat differences between the reference and exposure site that allowed for YOY burbot in the exposure lake to

obtain food throughout the winter, thereby increasing lipid and triglyceride content by the spring. In this study, there were no site differences in nitrogen (as ammonia) or total phosphorous between the reference lake and the exposure lake. The possibility of increased food availability at the exposure lake compared to the reference lake, if present, would therefore not be related to nutrient enrichment. Size-selective overwinter mortality may have been higher in the exposure site, resulting in fish with higher lipid content surviving into the spring. Overwinter mortality of smaller, lower-energy individuals has been noted in a variety of fish species under winter conditions (Sogard and Olla 2000, Pratt and Fox 2002, Biro *et al.* 2004) resulting in spring survivors with high lipid content. However, an increase in burbot length from fall to spring at the exposure site was not noted, suggesting that overwinter mortality was not the primary mechanism of increased lipids in the spring. Eckmann (2004) suggested that some fish species can gain lipids during winter without significant size-selective overwinter mortality. There may be some impact of the uranium mining and milling effluent on YOY burbot energy storage possibly through increased feeding during the winter at the exposure site and/or increased size-selective overwinter mortality at the exposure site.

4.5.3.3 Slimy sculpin

Total body lipids and total body triglycerides in slimy sculpin were lower in the spring relative to the fall for fish collected at both exposure and reference sites. This pattern follows my initial hypothesis that fish rely on stored energy to survive northern winters and will have depleted lipid and triglyceride levels in the spring relative to the fall. Many studies on various fish species have found that fishes have lower lipid or triglyceride levels in the spring following a temperate or northern winter (Toneys and

Coble 1980, Cunjak 1988, Lemly 1996, Berg and Bremset 1998, Sogard and Olla 2000, Post and Parkinson 2001). Although fish from both reference and exposure sites had decreased lipids and triglycerides following winter, this difference was much more apparent in fish at the exposure section of the creek, suggesting that uranium mining and milling effluent may be an additional metabolic stressor for fishes at this site during winter resulting in winter stress syndrome (Lemly 1993, Lemly 1996). Out of the three indigenous fish species sampled at McClean Lake uranium operation, only the slimy sculpin appear to support Lemly's winter stress syndrome with lower lipid and triglyceride levels in the spring relative to the previous fall for fish inhabiting a site receiving uranium mining and milling effluent. For sexually mature sculpin, many of which were collected at the reference site, the decrease of lipids in the spring may correspond to loss of lipids via gonadal production, particularly for the females.

4.5.4 Morphometric endpoints

4.5.4.1 Northern pike

There were no site differences in northern pike weight, length, condition factor or HSI in either fall or spring. However, seasonal differences in HSI were observed in YOY northern pike, with an increase in HSI in the spring compared to the previous fall in fish from both reference and exposure lakes. It is interesting to note that a similar seasonal pattern in total body triglycerides was not noted in northern pike. This suggests that although total amounts of stored energy (total body triglyceride content) in northern pike did not vary significantly from fall to spring, the location of stored energy within the fish may have varied seasonally. In the fall, most stored energy in northern pike would have been allocated to long-term energy storage sites, such as adipose tissue (Tocher 2003), as

a fish prepared for winter. During the winter months, some of this stored energy was likely utilized; however, pike total body triglycerides were not lower in the spring relative to the fall. In the spring, a greater proportion of triglycerides may have been located in the liver (higher HSI), the short-term energy storage site in most freshwater fish (Tocher 2003). The increase in pike HSI in the spring was likely the result of increased spring feeding.

HSI can vary in response to contaminant exposure as hypertrophy or hyperplasia results from detoxification (Goede and Barton 1990, Hinton and Lauren 1990). However since there were no differences in pike HSI comparing fish from the reference and exposure sites, it does not appear that uranium operations effluent influenced YOY northern pike liver size. Results from principal component analysis show grouping of northern pike into fall and spring along principal component 2. This pattern corresponds to the seasonal difference in pike HSI described above.

Overall, uranium mining and milling effluent did not appear to impact YOY northern pike growth or energy storage at the McClean Lake uranium operation. There were no site differences in any biochemical or morphometric endpoints comparing fish from the reference and exposure sites in either fall or spring. Seasonal differences in certain endpoints (muscle RNA/DNA ratio, muscle protein, HSI) were not related to effluent exposure.

4.5.4.2 Burbot

Winter survival is known to increase with fish length, assuming that overwinter mortality is regulated by starvation (Johnson and Evans 1991, McCollum *et al.* 2003). Since burbot collected from the exposure lake were longer than fish from the reference

lake in the fall, then theoretically burbot from the exposure lake may have had a better chance at overwinter survival. Interestingly, both HSI and condition factor varied between sites and seasons. More specifically, HSI and condition factor were higher in burbot from the exposure site compared to both the reference fish in the same season and the exposure fish the in fall. The hepato-somatic index is strongly related to condition factor in juvenile Atlantic cod (*Gadus morhua*) (Lambert and Dutil 1997, Couture *et al.* 1998). My results support this relationship in YOY burbot, a freshwater gadoid. Site and seasonal patterns in burbot HSI followed similar trends as total body lipids and triglycerides, with increases in all endpoints in the spring relative to the fall in burbot from the exposure lake as well as site differences in spring, with higher values for these endpoints in burbot from the exposure lake relative to burbot from the reference lake. Since the liver is the primary long-term energy storage site in gadoids (Sargent *et al.* 1989, dos Santos *et al.* 1993), it is reasonable that an increase in liver lipid content, including the storage lipid (triglycerides) content, would be concurrent with an increase in liver weight.

4.5.4.3 Slimy sculpin

Dubé *et al.* (2005) found that YOY slimy sculpin experienced decreased survival and growth with increasing concentrations of metal mining effluent from a Zn-Pb-Cu mine. Previous studies have reported that fish exposed to metals have decreased condition factor relative to unexposed fish (Laflamme *et al.* 2000, Sherwood *et al.* 2000, Levesque *et al.* 2002, Rajotte and Couture 2002). The population of slimy sculpin in this study contained a mixture of adults and juveniles and many morphometric (weight, length, condition factor) and biochemical (total body triglycerides) endpoints varied

significantly with age. Sculpin were shorter, weighed less and had a lower condition factor in the exposure section of the creek compared to fish from the reference site, although site differences in these endpoints were related to fish age, not contaminant exposure. I collected older, larger fish at the reference site in both fall and spring relative to fish collected at the exposure site. This is clearly presented in the PCA plots for slimy sculpin. Fish collected at the exposure site were clustered to the negative end of principal component 2, indicating this group of fish were low in weight, length and condition factor. In order to better assess the health of slimy sculpin populations at McClean Lake uranium operation, larger sample sizes are required in order to obtain fish samples with a similar age distribution at both reference and exposure sites.

4.5.5 Morphometric versus biochemical endpoints

Differences between morphometric and biochemical endpoints were not consistent in all species. For instance, although there were site differences in morphometric endpoints in slimy sculpin, these changes did not correspond to changes in HSI, total body lipids or total body triglycerides. In contrast, northern pike morphometric endpoints and energy storage endpoints (total body lipids and triglycerides) were not different between sites or seasons. Although total length was not different in northern pike, there were differences in the biochemical endpoints employed to estimate growth (RNA/DNA ratio, muscle protein) suggesting that for this species, total length is a more relevant measure of growth than muscle RNA/DNA ratio or muscle protein.

From a monitoring standpoint, it is interesting to note that although burbot weight was not different between seasons or sites, there were differences in HSI, total body lipids and total body triglycerides. Clearly, the non-lethal morphometric endpoint of

weight did not detect changes in energy storage that were noted with other endpoints. Beamish *et al.* (1996) found that in lake sturgeon, the loss of stored energy during periods of low food availability is largely compensated with water uptake, thereby buffering any potentially significant changes in condition factor. In fish, it is possible that changes in body weight may not be detected, even though changes in lipid or triglyceride content have occurred. However, since determining HSI, total body lipids and totally body triglycerides requires destructive sampling, these endpoints should only be employed when a fish population can recover from the loss of a portion of the population for scientific sampling.

4.6 Conclusions

Although the McClean Lake operation is releasing uranium mining and milling effluent, there appeared to be little effect on morphometric and biochemical endpoints in northern pike, burbot or slimy sculpin in the sites examined. Since the operation has been releasing effluent for less than 10 years, there is little historical contamination at these sites. As the operation continues to run, there may be an increase in contamination at these exposure sites in the aquatic environment and impacts on local fishes should continue to be monitored. Also, although biochemical endpoints measured in these fish may detect subtle changes in condition, results from this study suggest that species have different natural response patterns for energy allocation. Knowledge of a fish species' life history must be considered when interpreting morphometric or biochemical results. In terms of basic biological information, all three species followed similar trends in growth endpoints of muscle RNA/DNA ratio and muscle protein, indicating that these endpoints change similarly in all species from fall to spring. Altered energy storage was

occurring in certain fish species inhabiting sites downstream of uranium mining and milling effluent input at McClean Lake operation, however, further research is required to determine possible causes of these changes.

CHAPTER 5
5.0 GENERAL DISCUSSION

5.1 Lipids and triglycerides measurement methods

Despite the wide variety of lipid measurement methods employed in fisheries studies, triglycerides represent a more relevant type of lipid to measure, as it is a direct measure of stored energy. The Bligh and Dyer (1959) method for lipid extraction is not only time consuming and results in worker exposure to chloroform, but evidence presented in this study showed that the technique is limited when fish have low lipid values. The results of Chapter 2 tie in with Chapters 3 and 4, as total body lipids (gravimetric) and triglycerides in the unextracted whole body fraction were used for the analysis of fish lipids and triglycerides at Key Lake and McClean Lake operations. Future studies in fisheries may benefit from the data presented in Chapter 2, as the measurement of triglycerides in a non-solvent extracted fraction is an ecologically and physiologically relevant, rapid and reliable technique.

5.2 Basic biological information

Basic biological information on fishes in boreal freshwater systems was gathered during this research through fish collections at reference sites. Total body lipids, total body triglycerides, muscle RNA/DNA ratio and muscle protein have not been examined in slimy sculpin, YOY burbot or YOY northern pike in northern Saskatchewan or elsewhere in the boreal ecosystem. Values for these biochemical measures of condition and growth were similar within each species, when comparing fish collected at reference sites between the two uranium operations. I observed species differences in total body

lipids, total body triglycerides, liver triglycerides, muscle RNA/DNA ratio in fish collected from reference sites (Table 5.1). Muscle protein content was the only biochemical endpoint with similar values for all species (Table 5.1).

Table 5.1 Biochemical indices of growth and bioenergetics in young-of-the-year northern pike (*Esox lucius*) (*n* = 21-43), young-of-the-year burbot (*Lota lota*) (*n* = 13-39) and slimy sculpin (*Cottus cognatus*) (*n* = 16) collected from reference sites upstream of uranium mining and milling activities at Key Lake and McClean Lake uranium operations, Saskatchewan, Canada in fall (2003 and 2004) and spring (2004 and 2005). Data are mean ± standard error of the mean.

	Northern pike	Burbot	Slimy sculpin
Total body lipids (%)	1.0 ± 0.04	1.6 ± 0.1	2.4 ± 0.3
Total body triglycerides (mg/g)	3.2 ± 0.3	6.7 ± 0.8	19.9 ± 2.8
Liver triglycerides (mg/g)	8.7 ± 1.5	140.4 ± 11.8	n/d
Muscle RNA/DNA ratio	2.3 ± 0.2	5.6 ± 0.4	2.1 ± 0.2
Muscle protein (mg/g)	27.6 ± 1.4	28.2 ± 1.4	25.3 ± 1.3

n/d - not determined

Also, species differences in behaviour were hypothesized in this investigation. Young-of-the-year burbot were likely feeding throughout the winter, as evidenced by higher lipid and triglyceride concentrations in the spring compared to the fall at both uranium operations. Differences in fish ecology were noted, particularly in terms of diet. Spottail shiners were not common prey items in the burbot and benthic invertebrates prevailed as the most common food source for YOY burbot. However, northern pike fed on a mixture of small fish and invertebrates. Both of these results support basic ecological literature (van Vliet 1964, Scott and Crossman 1973) on foraging behaviour of juveniles of these species. Sculpin of all ages fed on benthic invertebrates in this study, which agrees with existing literature on the feeding behaviour of this small bodied fish species (van Vliet 1964).

Results for biochemical measures of condition were all within ranges obtained for other fish species and age classes (Table 5.2).

Table 5.2 Range in biochemical indices of growth and bioenergetics in young-of-the-year fishes (northern pike, burbot, slimy sculpin) collected from reference and exposure sites at Key Lake and McClean Lake uranium operations, Saskatchewan, Canada in fall (2003 and 2004) and spring (2004 and 2005). Data represent minimum to maximum values for each endpoint.

	Northern pike	Burbot	Slimy sculpin
Total body lipids (%)	0.5-2.8	0.6-5.4	0.8-5.4
Total body triglycerides (mg/g)	0.5-22.8	0.7-37.8	1.5-42.6
Liver triglycerides (mg/g)	1.4-52.1	0.9-242.0	n/d
Muscle RNA/DNA ratio	0.9-7.0	1.2-12.7	0.9-6.1
Muscle protein (mg/g)	8.5-52.8	15.5-66.0	16.7-54.7

n/d - not determined

For example, age-0 rainbow trout range in total lipid content from 1.5-7.0% lipid by wet weight (Post and Parkinson 2001, Biro *et al*. 2004). Wild age-0 roach (*Rutilus rutilus*) range from 3-12% fat and wild age-0 perch have been reported to range from 3-8% fat (Griffiths and Kirkwood 1995). Percent lipid in young-of-the-year walleye ranges from 6-25%, varying greatly with predation pressure (Pratt and Fox 2002) and percent lipid for YOY walleye pollock (*Theragra chalcogramma*) ranges from 7-27%, varying with food availability (Sogard and Olla 2000). Total body lipid levels for wild juvenile bluegill were 12-14% lipid in late summer (Lemly 1993). Total body triglycerides compose approximately 0.5-5 mg/g in juvenile coho salmon (Heintz *et al*. 2004). Liver triglyceride content in adult yellow perch, a relatively lean fish, ranges from 5 mg/g in summer to 2.8 mg/g in fall (Levesque *et al.* 2002) and triglycerides compose 35-64 mg/g in cod livers (dos Santos *et al*. 1993).

Muscle RNA/DNA ratio ranges from approximately 1.5-4.0 in starved and fed larval rainbow trout and 2.8-6.5 in starved and fed juvenile fathead minnows (Weber *et al.* 2003). Muscle RNA/DNA ratio ranges from 2-10 in juvenile common carp (De Boeck *et al*. 1997), from 2-8 in 18 day old turbot (*Scophthalmus maximus*) larvae (Clemmesen 1988) and from 1.5-4.5 in larval whitefish (*Coregonus* spp.) (Steinhart and Eckmann 1992). Muscle protein concentration ranges from approximately 30-45 mg/g in starved and fed larval rainbow trout and 28-38 mg/g in starved and fed juvenile fathead minnows (Weber *et al.* 2003). Muscle protein in wild juvenile lake sturgeon is approximately 10-20% of total muscle composition (Beamish *et al*. 1996). Muscle protein for two month old common carp is approximately 110 mg/g or 11% (De Boeck *et al*. 1997). Clearly, there are species differences in all of the biochemical endpoints measured in this study. Numerous other biotic and abiotic variables can influence these endpoints for example age, sampling season, reproductive state, prey availability, water temperature, parasite load or nutrient limitation.

It is important to note that different methods can be used to evaluate biochemical endpoints of condition in fish. For instance lipids can be measured gravimetrically, following a chloroform-methanol lipid extraction (Griffiths and Kirkwood 1995, Sogard and Olla 2000, Biro *et al*. 2004, Eckmann 2004) or a hexane lipid extraction (Lemly 1993). Lipids can also be measured using a Soxhelet apparatus using petroleum ether as a solvent (Pratt and Fox 2002). Fish triglycerides can be determined using a chromatographic technique (dos Santos *et al*. 1993, Lochmann *et al*. 1995, Jobling *et al*. 1998, Heintz *et al*. 2004) or an enzymatic assay (Weber *et al*. 2003). RNA/DNA ratio can be determined fluorometrically (Clemmesen 1988, Steinhart and Eckmann 1992,

Weber *et al*. 2003) or RNA content can be determined using UV spectrophotometry and DNA content determined fluorometrically (De Boeck *et al*. 1997). Protein content can be determined spectrophotometrically, using a method similar to Lowry *et al*. (1951) (Raae *et al*. 1988, De Boeck *et al*. 1997, Weber *et al*. 2003) or following protein nitrogen content determination using the Kjeldahl method (Beamish *et al*. 1996).

Employment of different methods in different laboratories may result in different values for biochemical endpoints such as lipids, triglycerides, muscle RNA/DNA ratio and muscle protein. Although the accuracy of my results is not easily assessed due to lack of preexisting species- and fish age-specific data, the precision of all biochemical endpoints measured in this study was high, with both inter- and intra-assay variability being less than 10%.

5.3 Differences between operations

Water chemistry revealed that the exposure sites at Key Lake operation were much more impacted in terms of metal, salt and ion levels than those at McClean Lake. Differences in metals, salts and nutrients (nitrogen) at the exposure sites between the two uranium operations may be responsible for the differences in the fish endpoints determined at each operation. Key Lake has been operating for a longer period than McClean Lake (> 20 years versus < 10 years) and therefore there has been a longer history of contamination. In addition, the exposure sites at Key Lake operation were much closer to the effluent input than the exposure sites chosen at McClean Lake operation (approximately 5 km at Key Lake versus approximately 15 km at McClean Lake). At McClean Lake operation, there was little difference between the reference and

exposure sites while at Key Lake operation, exposure lakes were quite different from the reference lake in terms of water quality.

The most consistent response at both uranium operations was higher total body lipids, total body triglycerides and morphometric measures in burbot from the exposure site in the spring. There was both a seasonal increase in these endpoints and a site difference, with an increase in all endpoints in burbot collected at the exposure lakes compared to fish from reference sites (Tables 5.3 and 5.4).

Table 5.3 Summary of results for morphometric (weight, length, condition factor), bioenergetic (total body lipids, total body lipids, liver triglycerides) and growth (muscle RNA/DNA ratio, muscle protein concentration) endpoints for fishes collected at lakes receiving uranium milling effluent at Key Lake uranium operation, Saskatchewan, Canada showing either an increase (↑), decrease (↓) or no change (-) in endpoints relative to fish from the reference site, for both fall and spring. Values for burbot and spottail shiners in the fall were not determined in this study.

Endpoints	Northern pike		Burbot	Spottail shiners
	Fall	Spring	Spring	Spring
Morphometric	-	-	↑	-
Bioenergetic	↑	↑	↑	↑
Growth	-	-	-	n/d

n/d - not determined.

Table 5.4 Summary of results for morphometric (weight, length, condition factor, hepato-somatic index, gonado-somatic index), bioenergetic (total body lipids, total body triglycerides) and growth (muscle RNA/DNA ratio, muscle protein concentration) endpoints at McClean Lake uranium operation, Saskatchewan, Canada showing either an increase (↑), decrease (↓) or no change (-) in endpoints relative to fish from the reference site, for both fall and spring.

	Northern pike		Burbot		Slimy sculpin	
Endpoints	Fall	Spring	Fall	Spring	Fall	Spring
Morphometric	-	-	↑↓	↑	↓	↓
Bioenergetic	-	-	-	↑	-	-
Growth	-	-	-	-	-	↑

The site and season differences in burbot condition at both uranium operations may be the result of a response to a common toxicant or environmental characteristic resulting from effluent inputs at both uranium operations. Given the differences in water quality in exposure lakes between the uranium operations (exposure sites at Key Lake higher in metals, salts and ions compared to sites at McClean Lake), burbot may be sensitive to a low level of contamination resulting in overwinter mortality of smaller, lower energy individuals. This sensitivity would be related to a contaminant present at a minimum concentration in exposure lakes at both uranium operations. Alternatively, effluent from both uranium operations could somehow be stimulating overwinter growth and accumulation of lipids and triglycerides in YOY burbot. At Key Lake uranium operation, I hypothesized that increased nitrogen could be stimulating prey quality and/or quantity. However, an increase in nitrogen was not observed in the exposure sites at McClean Lake operation. Further research at these sites is required in order to determine the precise cause of the site and season differences in burbot morphometric and bioenergetic endpoints. Basic information on YOY burbot is required to better understand the observed effects at these uranium operations. Information on YOY burbot overwinter feeding ecology, minimum water temperature required for growth and species sensitivity to the observed metal and salt levels in the exposure sites at the operations would aid in determining potential causes of the observed biological effects.

Despite site and season differences in burbot at the exposure sites, similar results were not observed in northern pike. Site differences in pike total body lipids and total body triglycerides were noted at Key Lake in both fall and spring, however there were no differences in these endpoints in pike at McClean Lake uranium operation. There was a

discrepancy in the species responses, with a similar site difference in the burbot at both operations and different site responses in YOY northern pike between the different uranium operations. Therefore, to further estimate the cause(s) of the effects and differences in response between the two operations, basic biological information on YOY northern pike is required, similar to that required for YOY burbot.

Although I found a consistent decrease in fish muscle RNA/DNA ratio and muscle protein in spring compared to fall at all study sites at McClean Lake uranium operation, a similar seasonal trend was not noted at Key Lake. This difference between operations was likely due to a discrepancy in spring sampling time. McClean was sampled immediately after ice left the lakes (2 days) while spring sampling was conducted at Key Lake approximately 2 weeks after ice-off. This difference in post-winter sampling time between uranium operations could explain the differences in biochemical endpoints of growth for fishes. Perhaps fishes at Key Lake were able to initiate seasonal growth by the time of spring sampling, due to increased water temperatures.

5.4 Problems with winter stress syndrome hypothesis

During winter, fish metabolism is decreased due to the seasonal decrease in temperature (Fry 1971). Therefore, theoretically, the potential impact of a metabolic stressor could be reduced during winter. Lemly (1993, 1996) did not discuss this confounding factor associated with the winter stress syndrome hypothesis. The hypothesis of winter stress syndrome has only been tested in the laboratory and I have found that quantifying winter stress syndrome in a field setting was not practical. The complex effluent from uranium operations may have multifaceted effects on biota, much

more complicated than the effect of a single metabolic stressor alone. At Key Lake operation, I found that the uranium effluent inputs may be responsible for nutrient enrichment in the system, possibly increasing prey quality and quantity. Also, potential indirect effects of an environmental contaminant are not considered in the theory of winter stress syndrome. Since environmental contamination can act directly and indirectly on biota (Congdon *et al.* 2001), both mechanisms of action must be considered when predicting potential impacts of environmental contamination on fishes.

Lemly (1996) notes that basic knowledge of life history characteristics and feeding ecology, particularly for YOY fish, would allow identification of potentially vulnerable fish species in temperate regions of the world. Unfortunately, there are few studies with direct observation and concrete conclusions regarding feeding ecology in juvenile fishes. The idea that most fish remain motionless and do not feed during the winter has come into question for a variety of species (Sogard and Olla 2000, Bauer and Schlott 2004, Biro *et al.* 2004, Eckmann 2004, Parrish *et al.* 2004). Fishes in northern Saskatchewan may indeed feed during winter when food is available, although more detailed observational studies are required to verify this. Monitoring problems are associated with trying to quantify overwinter mortality in a field setting. It was difficult to distinguish between effects of energy-dependent overwinter mortality or overwinter feeding and growth with the study design employed here.

5.5 Usefulness of morphometric and biochemical endpoints

5.5.1 Muscle RNA/DNA and protein

Growth can be limited by temperature, food availability, toxicant exposure and other forms of stress. Overall, I found that fish muscle RNA/DNA ratio and muscle

protein were not very useful at detecting differences in growth related to effluent exposure. The RNA/DNA ratio has been shown to be useful in revealing difference in growth of larval fish (Clemmesen 1988, Raae *et al*. 1988, Steinhart and Eckmann 1992), since this lifestage is characterized by rapid exponential growth (Buckley *et al.* 1999). The fishes collected in this study had already passed the time of rapid growth as larvae and had entered the plateau of growth rates experienced by juveniles. Kearns and Atchison (1979) found that in YOY yellow perch the RNA/DNA ratio reflected growth differences in midsummer but these differences were reduced by late summer (September). In this study, any differences in potentially high midsummer growth rates for YOY northern pike, YOY burbot and slimy sculpin may have been muted prior to our late fall sampling period. Overall, I found that RNA/DNA ratio and muscle protein concentration was more useful at detecting differences in growth related to water temperature, not environmental contamination. However, since growth is very important for young fish, this physiological process is not easily compromised. Changes in muscle RNA/DNA ratio or muscle protein would be relatively late compared to reductions in bioenergetic endpoints such as lipids and triglycerides (Weber *et al*. 2003). In addition, since fishes in this study were not greatly affected by effluent exposure (McClean Lake) or had high levels of stored energy at exposure sites (Key Lake), it is not surprising that decreases in fish muscle RNA/DNA ratio or muscle protein were not noted.

5.5.2 Lipids and triglycerides

Although the YOY fishes inhabiting exposure lakes at Key Lake uranium operation had higher total body lipids and triglycerides in both seasons, the environmental relevance of this difference is not clear. The population level effects of

this exposure-induced change in fat content will vary depending on the mechanism responsible for the change. Since the study design of my Master's research was not to determine cause of the observed effects, I can only hypothesize about the potential mechanisms responsible for the site differences in energetic endpoints in fishes. Metabolic disruption and inability to metabolize lipids and triglycerides could be a direct effect of the uranium operation. Increased food availability due to nutrient enrichment represents a potential indirect effect of the operation. Higher mortality rates of individuals with lower energy stores could result from both direct and indirect effects of the effluent on fishes. Any of these mechanisms alone or in combination may be responsible for the increased lipids and triglycerides in burbot and northern pike at Key Lake operation and burbot at McClean Lake operation. However, metabolic disruption or higher mortality rates of lower energy individuals would result in detrimental population level effects for these indigenous fish species. If nutrient enrichment at Key Lake operation was responsible for the site and seasonal changes in lipid content, future population dynamics may not be negatively impacted.

5.5.3 Morphometric endpoints

Although previous environmental monitoring programs have relied on simple morphometric endpoints, there has been an increased emphasis in research to investigate more subtle, biochemical endpoints in ecotoxicology (Beamish *et al*. 1996, Buckley *et al*. 1999, Levesque *et al*. 2002, Rajotte and Couture 2002, Couture and Rajotte 2003, Weber *et al*. 2003). However, in certain situations in my research, I have found that morphometric endpoints may be more suitable to estimate fish condition compared to biochemical endpoints. For instance, burbot HSI is closely related to lipid content of this

species, since the liver is the primary energy storage site of lipids in gadoids. Also, as noted above, the biochemical growth rates in fish muscle estimated with RNA/DNA ratio and muscle protein were poor indicators of contaminant exposure. Total length may be a more accurate and ecologically relevant estimate of growth than biochemical growth measures of muscle RNA/DNA ratio and muscle protein for the age classes of the fish species examined in this investigation.

5.6 Conclusions

Novel information on biochemical endpoints in indigenous fish species collected at reference sites in northern Saskatchewan revealed species differences in total body lipids, total body triglycerides, liver triglycerides and muscle RNA/DNA ratio. Muscle protein values were relatively similar for the fish species examined. Different fish species have different morphometric and biochemical responses to uranium mining and milling effluent exposure. Although certain morphometric and biochemical endpoints changed in response to effluent exposure, other endpoints were not found to change in response to environmental contamination. Exposure sites at two different uranium operations have dissimilar water quality data and therefore, morphometric and biochemical response of fishes varied between uranium operations. Further research in these systems is required to determine causes of the observed effects and to quantify future population stability and year-class strength.

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