ECOTOXICOLOGICAL ASSESSMENT OF JUVENILE NORTHERN PIKE INHABITING LAKES DOWNSTREAM OF A URANIUM MILL

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By

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ABSTRACT

Previous studies on fishes exposed to effluent from the Key Lake uranium mill in northern Saskatchewan have demonstrated elevated lipids in young-of-the-year pike (*Esox lucius*), deformities in larval pike and decreased survival of fathead minnows (*Pimephales promelas*). The objectives of this thesis were to evaluate possible factors that could be contributing to altered bioenergetics of juvenile northern pike inhabiting lakes receiving effluent from the Key Lake operation and to examine the effects of effluent exposure on biomarkers of oxidative stress and histopathology of target organs. Although glycogen and triglycerides stores were significantly greater in pike from exposure lakes compared to the reference, triglycerides stores of juvenile pike prey items showed no overall differences among lakes. Measures of parasitism, however, were negatively correlated with pike bioenergetics thereby reflecting a possible energetic cost of parasitism on reference lake fish. The degree of infection by intestinal parasites and gill monogeneans was greatest in reference pike and intermediate in low exposure pike, whereas high exposure pike harboured no parasites.

Arsenic, nickel and selenium are elevated in lakes downstream of the Key Lake mill and have been shown to be associated with increased reactive oxygen species (ROS) in biological systems causing oxidative stress. The potential for oxidative stress was assessed in pike liver and kidney using several biomarkers. Overall, the concentrations of total, reduced and oxidized glutathione and the ratio of oxidized to reduced glutathione did not differ significantly among exposure and reference pike. The activity of glutathione peroxidase was greater in high exposure than reference liver whereas, contrary to predictions, lipid peroxidation was greater in reference than exposure pike tissues. Histopathological evaluations revealed greater kidney and gill pathology in reference lake pike, whereas for liver, hepatocyte morphology differed among lakes without

any clear signs of pathology. Trace metal analyses of muscle showed that eight elements (arsenic, cobalt, copper, iron, molybdenum, selenium, thallium, uranium) were significantly elevated in exposure pike. These results provide only limited evidence of oxidative stress in exposure pike tissues and no evidence of histopathology despite indications that metals are bioaccumulating in tissue.

Overall, the results from this thesis suggest that the health and condition of juvenile northern pike living downstream of the Key Lake uranium mill may not be compromised by effluent exposure.

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Maman, Dad and Jen - thanks for being there!

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LIST OF ABBREVIATIONS

AETE Aquatic Effects Technology Evaluation

ANCOVA Analysis of covariance

ANOVA Analysis of variance

CCME Canadian Council of Ministers of the Environment

GPx glutathione peroxidase

GSH reduced glutathione

GSSG oxidized glutathione

HAE 4-hydroxyalkenal

HSI hepatosomatic index

K Fulton's condition factor

MDA malondialdehyde

ROS reactive oxygen species

SEM standard error of the mean

SSWQO Saskatchewan Surface Water Quality Objectives

PREFACE

Chapters 2 and 3 of this thesis are organized as manuscripts for publication in scientific journals. Therefore, there is some repetition of introductions, materials and methods throughout and abstracts for each data chapter are included.

Chapter 2 was submitted to Ecotoxicology and Environmental Safety on September 4th 2007 and Chapter 3 will be submitted to Aquatic Toxicology in the coming months.

CHAPTER 1
1.0 GENERAL INTRODUCTION

1.1 Uranium production in Canada and the Key Lake uranium mill

According to 2004 statistics, the value of metal mining production in Canada is dominated by nickel, gold, copper, iron, zinc, and uranium. The quantity of uranium produced in that year amounted to 11 548 tonnes (Statistics Canada, 2004) while the estimated known resources remaining to be exploited totaled approximately 432 000 tonnes (Vance, 2004). Currently, the Athabasca basin of northern Saskatchewan holds the country's only uranium production operations since mining ended in Ontario in the 1990s (Vance, 2004). The world's richest uranium ore is extracted at the McArthur River mine and trucked to the Key Lake operation (Cameco Corporation, Saskatoon, SK, Canada) (57°13' N, 105°38' W; Figure 1.1) for milling. The Key Lake uranium mill has been in operation since 1982 and is expected to continue processing ore until at least the year 2020 (Conor Pacific, 2000). Data available up until 2004 places the Key Lake uranium mill at the forefront in terms of global uranium production with 7200 tonnes of uranium produced in 2004 (Vance, 2004).

The process of milling begins with an aqueous slurry of mineralized waste rock mixed with uranium ore from the McArthur River mine that is 2 to 4% triuranium octoxide (U_3O_8) and ends with the production of yellow cake that is graded to be approximately 99% U_3O_8 (Rodgers, 2000). The yellow cake is packaged into drums and shipped to various locations around the world for refinement (Rodgers, 2000). Milling involves several steps, one of which extracts

uranium, molybdenum, arsenic and other metals[1] and ions from an aqueous solution derived from the slurry. Other phases of the process involve the addition of nitrogenous compounds to the aqueous solution, including amine, ammonium sulphate and anhydrous ammonia. These compounds are required to extract and precipitate the uranium and to adjust the pH (Rodgers, 2000). Additional chemicals used as part of milling include sulphuric acid, kerosene and isodecanol.

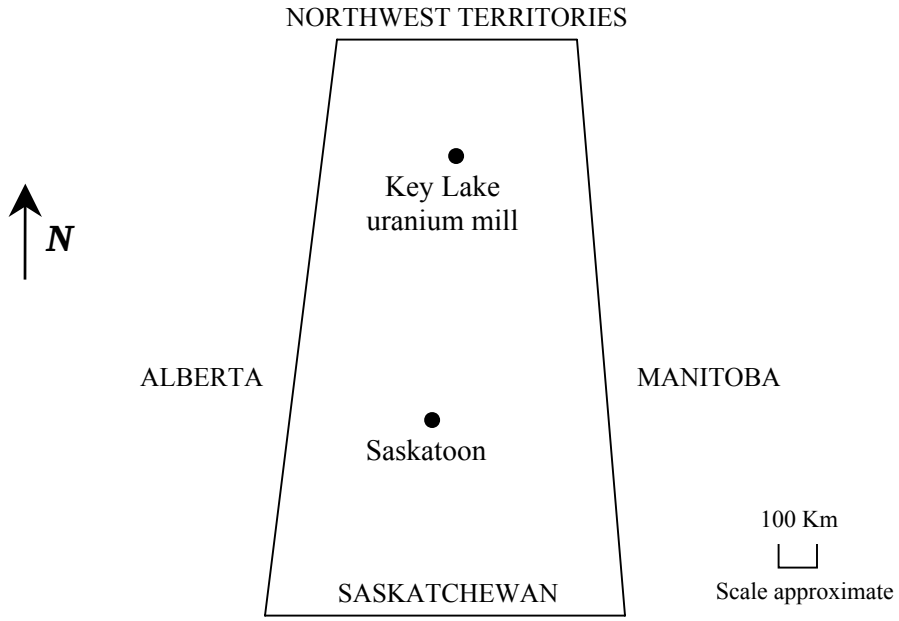

Figure 1.1 Map of Saskatchewan, Canada showing the approximate locations of Saskatoon and the Key Lake uranium mill.

The milling effluent is treated on-site by passing it through a bulk neutralization circuit that precipitates metals and adjusts the pH (Rodgers, 2000). Following bulk neutralization,

[1] Use of the term "metal" throughout this thesis refers to both metals and elements, such as arsenic and selenium, that are categorized as metalloids.

the effluent flows to ponds where it is monitored and tested until it is deemed acceptable for release into the David Creek drainage (Figure 1.2) at a rate of approximately 6 000 m^3/d (Muscatello et al., 2006). If the effluent in the holding ponds fails water quality testing, it undergoes a second round of treatment (Rodgers, 2000). Despite the treatment process, lakes downstream of the Key Lake uranium mill effluent discharge contain elevated concentrations of molybdenum, arsenic, selenium, nickel, ions, ammonia, and nitrates (Golder, 2005). The first lake of the drainage to receive

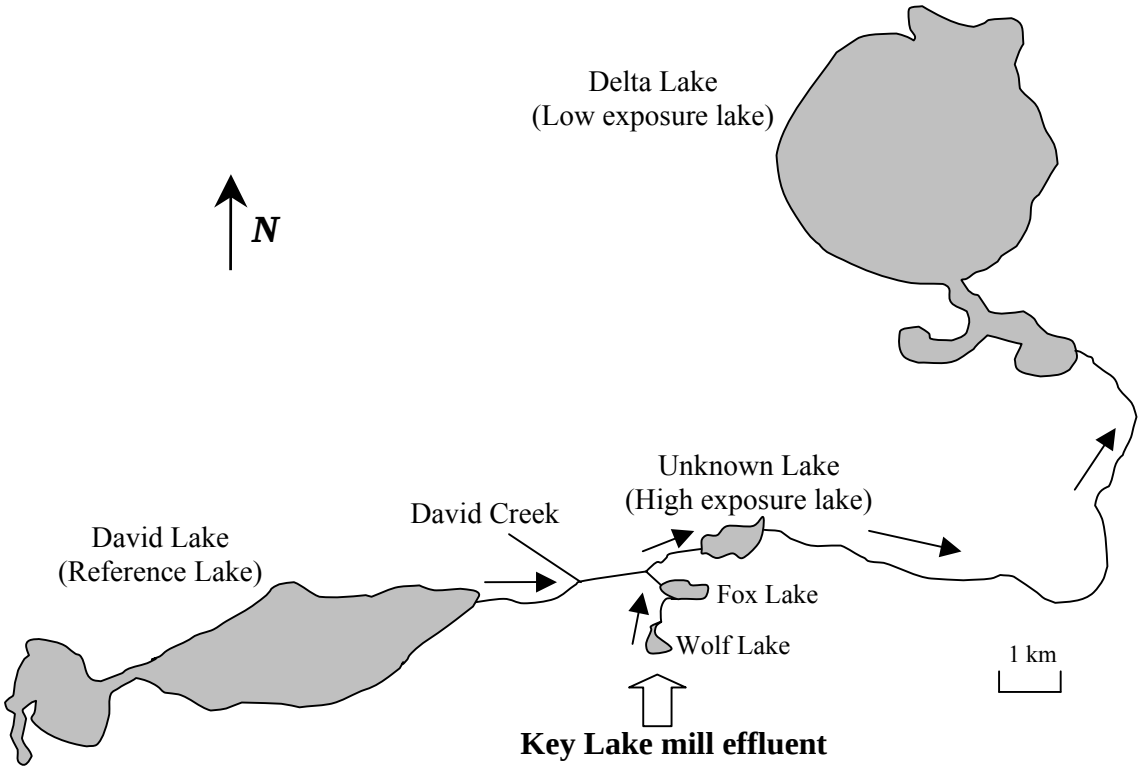

Figure 1.2 Map of lakes near the Key Lake uranium mill including the three study lakes (Reference, High and Low exposure lakes). The small arrows indicate the direction of the current.

effluent is Wolf Lake, followed by Fox, Unknown and Delta Lakes. The concentration of effluent in the lakes ranges from approximately 72% in Wolf Lake to approximately 28% in Delta Lake (Robertson, 2006). In accordance with the concentration gradient of effluent,

Unknown Lake will be referred to as the High exposure lake and Delta Lake as the Low exposure lake throughout this thesis.

Due to the elevated concentration of effluent downstream of the Key Lake operation discharge, there is concern that effluent exposure could be compromising the health and sustainability of the aquatic ecosystems and resident fish populations. In 2004, a comprehensive study of the abiotic and biotic components of lakes receiving effluent from the Key Lake operation was undertaken in order to meet requirements set out by the Metal Mining Effect Regulations under the *Fisheries Act* (Golder, 2005). This study took place a year before the field work for this thesis was undertaken and therefore provides timely information. The water quality variables of Unknown and Delta Lakes include elevated concentrations of ions, metals, total ammonia, nitrate, dissolved solids as well as elevated hardness and a depressed pH (Unknown Lake only) compared to David Lake (Golder, 2005). Although guideline values set out by provincial and federal regulatory bodies are reported for less than half of the measured water quality variables, a few were observed to exceed these limits.

The Canadian Council of Ministers of the Environment (CCME) Environmental Quality Guidelines and the Saskatchewan Surface Water Quality Objectives (SSWQO) are either "narrative or numerical values" (Saskatchewan Environment, 1997, p. 5) developed to protect aquatic life such as fish (Saskatchewan Environment, 1997; CCME, 1999). The paragraph that follows discusses some of the water quality variables of lakes downstream of the Key Lake uranium mill in relation to CCME guideline and SSWQO values.

The pH at Unknown Lake (pH 5.0) was below the recommended limit of 6.5 set out by the CCME and SSWQO whereas total ammonia at Unknown Lake (2.1 mg/L) exceeded the SSWQO limit of 2.0 mg/L. In terms of metals, the concentration of arsenic at Unknown Lake (3.8 µg/L)

was close to the CCME guideline of 5.0 µg/L (Golder, 2005). Molybdenum at Unknown and Delta Lakes surpassed the CCME limit (0.073 mg/L) and selenium exceeded the CCME limit of 1.0 µg/L at Unknown Lake (3.0 µg/L) (Golder, 2005) but not Delta Lake (1.0 µg/L) (Muscatello et al., in press).

Concentrations of metals in sediment are also reported in Golder (2005) although compared to water quality variables, fewer guidelines are presented and these only include CCME Interim Sediment Quality Guidelines and Probable Effects Levels. An Interim Sediment Quality Guideline differs from other CCME Sediment Quality Guidelines in that the proposed values are developed based on incomplete scientific data. The Probable Effects Level represents the concentration of a contaminant in sediment above which "significant and immediate hazards to exposed organisms" (CCME, 1995, p. 19) are likely to occur.

Due to potential problems with the analytical process, 2004 sediment values for lakes near the Key Lake uranium mill may not be entirely accurate (K. Himbeault, personal communication). However, results from 2001 (reported as dry weight) are also available (Golder, 2005) and are presumably more reliable. Only arsenic and cadmium were detected at concentrations that exceed CCME values. Arsenic concentrations in Unknown (363 µg/g) and Delta Lake (79 µg/g) sediments exceed both the Interim Sediment Quality Guideline (5.9 µg/g) and the Probable Effects Level (17 µg/g) set out by CCME. Arsenic concentration in David Lake sediment (13 µg/g) exceeds the Interim Sediment Quality Guidelines by approximately a factor of 2 but is below the Probable Effects Level. Concentrations of cadmium in David (1.6 µg/g) and Delta (2.1 µg/g) Lake sediments are greater than the Interim Sediment Quality Guidelines (0.6 µg/g) but not the Probable Effects Level (3.5 µg/g) whereas the concentration in Unknown Lake (<0.5 µg/g) is below both CCME values.

In terms of biota, results from Golder (2005) on macroinvertebrates demonstrated some significant differences between lakes receiving uranium milling effluent and David Lake on standard estimates of community health such as indices of biodiversity (Golder, 2005). The exposure lakes were reported to have higher biodiversity than David Lake, however no significant differences were observed for macroinvertebrate density. Several studies on the effects of the uranium milling effluent on fishes have also been undertaken (Pyle et al., 2001; Klaverkamp et al., 2002; Golder, 2005; Muscatello et al. 2006; Bennett and Janz, 2007). Recent work on northern pike (*Esox lucius*) exposed to water collected from lakes downstream of the effluent discharge have reported teratogenic effects on developing fry (Muscatello et al., 2006) while a seven-day *in situ* toxicity test using fathead minnows (*Pimephales promelas*) found a 70% higher mortality of fish held in lakes receiving Key Lake effluent versus David Lake (Pyle et al., 2001). Bennett and Janz (2007) observed elevated hepatic triglycerides and whole body lipids in young-of-the-year pike collected from exposure lakes.

In light of the observed effects of Key Lake uranium milling effluent on fish survival, development and bioenergetics, the present research will further investigate impacts on bioenergetics and biochemical and tissue level targets of toxicity in juvenile northern pike.

1.2 Bioenergetics

1.2.1 Fish energy stores

Two of the most important energy storage macromolecules in animals, neutral lipids and glycogen, serve a variety of physiological and ecological functions in fish. Neutral lipids are located in liver and muscle tissue of fish, subcutaneously and in association with pyloric ceca and intestines (Mommsen, 1998). In addition to being a source of energy, neutral lipids serve as pigments and essential growth factors (Lovell, 1998) and are required for basic ontogenic processes such as reproduction (Reznick and Braun, 1987). Throughout development, storage of

neutral lipids tends to be higher in older fish compared to fish that are young and rapidly growing (Jobling, 1994). Lipids and glycogen are also essential for surviving periods of low or no food availability such as north temperate winters (Ince and Thorpe, 1976; Lemly, 1993a; Post and Parkinson, 2001). Under conditions of starvation, fish have been shown to utilize hepatic and muscle glycogen and lipids preferentially over muscle protein stores while glycogen stores are more readily depleted than lipids (Ince and Thorpe, 1976).

Glycogen, the storage form of glucose, is mostly located in the liver of animals but can also be found at lower concentrations in muscle tissue (Nelson and Cox, 2005). One of the key functions of glycogen is to supply a quick source of energy in response to a stressor (Campbell et al., 1999). The release of catecholamines from fish chromaffin tissue in the head kidney (Jobling, 1994) mobilizes glycogen into glucose as part of the secondary stress response (Wedemeyer et al., 1990; Brown, 1993; Varanka et al., 2001). Glucose can then fuel the anaerobic bursts of activity that would be needed to escape a stressful stimulus such as a predator (Wedemeyer et al., 1990) or for capturing prey. Glucose is also used to produce ATP anaerobically when the water of ice-covered lakes becomes hypoxic in winter (Jobling, 1994) and is a preferred substrate for producing ATP via cellular respiration in nervous tissue and blood cells (Hemre et al., 2002).

Fish exposure to contaminants including metals (Förlin et al., 1986; Varanka et al., 2001; Levesque et al., 2002; Teh et al., 2004) and pesticides (Nivedhitha et al., 1998) can deplete energy stored in the form of lipids and glycogen. This effect can be compounded, putting additional pressure on survival, in fish that are simultaneously exposed to natural stressors such as winter (Lemly, 1993a). A recent study by Bennett and Janz (2007) examined the possible effects of effluent from the Key Lake uranium mill on energy stores and overwinter survival of

resident young-of-the-year northern pike. Contrary to predictions, pike from the exposure lakes had greater lipid stores compared to the reference. One of the goals of the present study, therefore, was to examine possible factors that could explain the greater energy stores in exposure pike; factors included food web enrichment and energy dynamics associated with parasitism.

1.2.2 Food web enrichment

Two nutrients are essential to the productivity of aquatic ecosystems: phosphorus and nitrogen (Brönmark and Hansson, 2005). Primary producers (e.g. algae), require phosphorus and nitrogen for building proteins, nucleic acids and other macromolecules. Since they form the base of food webs (Figure 1.3), the productivity of primary producers drives the productivity of organisms at higher trophic levels such as macroinvertebrates and fishes (Dillon et al., 2004). Previous studies have demonstrated increased productivity of food webs in waterbodies experimentally enriched with either phosphorus (Peterson et al., 1985) or nitrogen (Sanford et al., 2005).

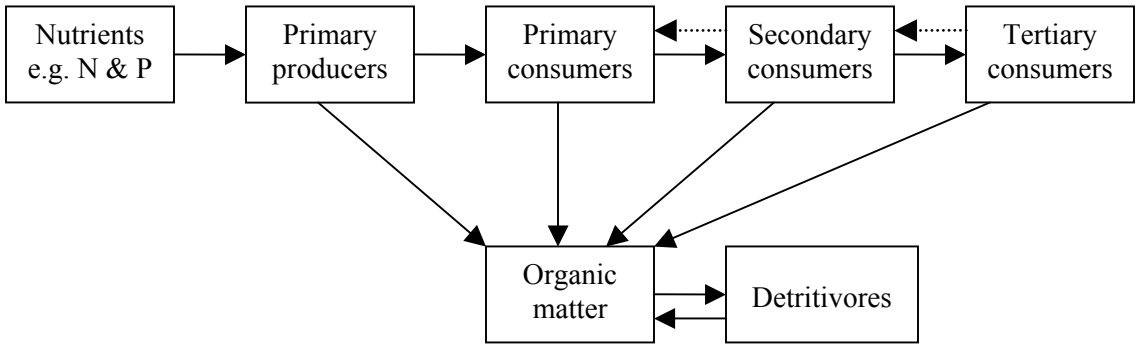

Figure 1.3 Simplified food web modified from Morris et al. (2005). Required flows of energy are indicated by solid arrows whereas dotted arrows show potential energy flows.

The nitrogen present in uncontaminated aquatic ecosystems originates from the breakdown of proteins and nucleic acids from decaying organic matter (Larsen, 1980) and from molecular

nitrogen (N_2) and other nitrogen compounds present in the atmosphere (Wetzel, 1983). Water chemistry and bacterial activity cycle nitrogen between forms that are either available or unavailable to primary producers such as algae and other aquatic plants (Wetzel, 1983). Nitrogen in the forms of nitrate and ammonium favour primary productivity since they are readily taken up and incorporated into plant material (Campbell et al., 1999)

In temperate waters, productivity is typically limited by phosphorus rather than nitrogen (Dillon et al., 2004) although exceptions to the rule do exist (Moss et al., 1994). The relative abundance of phosphorus in a number of lakes studied by Moss et al. (1994), where productivity is nitrogen limited, is a natural occurrence related to the mineralogy of the catchment area. Bennett and Janz (2007) suggested that a similar situation characterized by nitrogen limitation may exist for lakes near the Key Lake operation. Unlike the lakes studied by Moss et al. (1994), however, the lakes at Key Lake contain relatively low concentrations of phosphorus (less than 10 ug/L; Bennett and Janz, 2007). Nevertheless, low phosphorus does not preclude the possibility that nitrogen is sufficiently low so as to be the element that is limiting primary productivity. According to A. Redfield (1890-1983), algal growth is sustainable at a ratio of nitrogen to phosphorus in the water that is 16:1 (the Redfield ratio) (Brönmark and Hansson, 2005). David Lake, which has been used as reference in previous studies (Muscatello et al., 2006; Bennett and Janz, 2007) has a ratio of nitrogen to phosphorus that is approximately 3:1, suggesting a system that is nitrogen rather than phosphorus limited (Bennett, 2006).

1.2.3 Ontogeny of pike nutrient acquisition

Northern pike are omnivorous carnivores (Scott and Crossman, 1973) that typically occupy the top trophic level of aquatic food webs in waterbodies where they are present. A carnivorous lifestyle is initiated with the onset of exogenous feeding of larval pike following yolk sac absorption (Scott and Crossman, 1973). Whereas the early life stages of pike feed on

zooplankton, a number of macroinvertebrates and forage fish constitute some of the main prey items of juvenile and adult pike (Beaudoin et al., 1999). Macroinvertebrates that have been collected from the stomachs of northern pike include caddisfly larvae (Trichoptera), fly and midge larvae (Diptera), and dragonfly larvae (Anisoptera) (Beaudoin et al., 1999); all three taxonomic groups occur in temperate freshwater lentic systems (Hilsenhoff, 1991; Wiggins, 1996). In terms of habitat, fly and midge larvae can be found burrowed in the sediment (Merritt and Cummins, 1984), caddisfly larvae live at the water/sediment interface (Hilsenhoff, 1991) while dragonfly larvae are either at the water/sediment interface or clinging to the surface of macrophytes (Merritt and Cummins, 1984). Waterboatman (Heteroptera) are also abundant in north temperate ecosystems (Hilsenhoff, 1991) and could conceivably be pike prey items. These insects have been dubbed "true water bugs" (Merritt and Cummins, 1984, p. 231) since they spend almost their entire lives in water (Hilsenhoff, 1991) and are often observed swimming with a characteristic rowing motion (Merritt and Cummins, 1984). In terms of trophic classification, dragonfly larvae and waterboatman are mainly carnivorous, caddisfly larvae are herbivores and carnivores (Merritt and Cummins, 1984) and larval dipterans are omnivores feeding on detritus, algae and invertebrates including other larval dipterans (Merritt and Cummins, 1984).

In addition to macroinvertebrates, pike commonly consume fish such as spottail shiners (*Notropis hudsonius*), a forage fish that is abundant in Saskatchewan waters (Scott and Crossman, 1973). Spottail shiners are omnivores that feed on aquatic insect larvae and algae but may rely more heavily on the latter (Scott and Crossman, 1973).

1.2.4 Parasitism and host bioenergetics

As shown in Figure 1.3, food webs are comprised of several different trophic levels that can be divided into two broad categories: producers and consumers. Producers synthesize organic

material from inorganic sources (e.g. plants) whereas consumers feed on other organisms as a source of organic material (e.g. pike). Animals living a parasitic lifestyle can be classified as consumers despite their atypical feeding regime. Instead of killing and consuming their prey, parasites acquire nutrients from their host in a manner that does not typically threaten host survival. Due to their uncharacteristic feeding behaviour, parasites have often been overlooked in studies on food web dynamics even though they play an important role in the flow of energy in aquatic ecosystems (Wood, 2007). The paragraphs that follow will discuss some of the reasons why they are important and how they may be affecting the bioenergetics of other consumers in food webs such as pike.

Parasites are exceedingly commonplace in terrestrial and aquatic ecosystems to the point that virtually all animals are parasitized (Marcogliese, 2005). Pike are susceptible to infection by 138 different species (Scott and Crossman, 1973) such as cestodes, nematodes and monogeneans (Dick and Choudhury, 1996). Parasitization can occur throughout a fish's life span, including juvenile life stages (Landsberg, 1998; Bhuthimethee, 2005), and most internal organs as well as the entire external body surface present possible sites of infection (Hoffman, 1967). Parasitic modes of life can be divided into two categories: direct and indirect. An indirect life cycle requires one or more intermediate hosts, in addition to a final host where sexual reproduction takes place (Whitfield, 1993), whereas a direct life cycle entails only one host. Species of parasites belonging to the Class Cestoda typically have an indirect life cycle where the adult cestode, also called a tapeworm, infects the intestines of the final host (Smyth, 1994). Certain species of nematodes also colonize the intestines of fish (Hoffman, 1967) and most have an indirect life cycle (Williams and Jones, 1994). Members of the Class Monogenea (monogenetic trematodes or monogeneans), live on the external surface of fish, particularly the gills

(Bychowsky, 1961), and possess a direct life cycle (Smyth, 1994). Species of parasites differ, not only in how they complete their life cycle, but also in how they acquire host nutrients. Cestodes lack an alimentary canal and rely on diffusion and active transport across their tegument for the uptake of organic molecules including proteins (Whitfield, 1993), amino acids, glucose (Chappell, 1993), and fatty acids (Smyth, 1994) from the intestine of their final host. Nematodes on the other hand, possess an alimentary canal (Whitfield, 1993) and consume the semi-liquid substance found in their host's gastrointestinal tract (Smyth, 1994). Similar to nematodes, monogeneans also have an alimentary canal (Bychowsky, 1961) and species that infect fish gills feed on blood, epithelial cells and gill mucous (Smyth, 1994). It is evident from these modes of nutrient acquisition that parasitism could incur a direct energetic cost to their host. In addition, a certain amount of energy would presumably be required on the part of the host for repair of damaged tissues and to mount an immune response against the infection (Mackenzie et al., 1995). Suboptimal foraging behaviour such as increased prey handling time (Barber and Huntingford, 1995) and reduced feeding (Khan, 1988) have been observed in fish infected by parasites and could also deplete energy stores.

There appears to be only a small number of research articles in the literature associating parasitic burden with depleted glycogen and lipid stores in animals. For studies on fish, wild Atlantic herring (*Clupea harengus*) heavily infected with *Ichthyophonus hoferi* tended to be emaciated and have drastically reduced lipid stores (Rahimian, 1998), Atlantic cod (*Gadus morhua*) experimentally infected with a parasitic copepod had reduced hepatic lipids (Khan et al., 1990) and wild bluegill sunfish (*Lepomis macrochirus*) had decreased whole body neutral lipids with increased parasite load (Neff and Cargnelli, 2004). For studies on other animals, feral willow ptarmigan *(Lapogus lapogus*) infected with intestinal cestodes did not differ from

uninfected birds in terms of the concentration of neutral fats in pectoral and leg muscle (Thomas, 1986) while wild frogs (*Rana tigernia*) showed either increased or decreased concentrations of hepatic glycogen and lipids depending on the species of parasite and whether the infection was by one parasite or many (Kameswari et al, 1979). In addition to effects on lipid and glycogen stores, parasitism can affect indirect measures of energetics such as decrease condition factor (Neff and Cargnelli, 2004), activity (Lopez, 1999) and fecundity (Heins and Baker, 2003; Neff and Cargnelli, 2004).

Host-parasite relationships are complex and can be influenced by a number of natural and anthropogenic factors including contaminant exposure. Contaminants can adversely affect parasites in three ways: 1) toxicity to invertebrates and fish can mean a complete loss or a lower density of intermediate and final hosts; 2) direct exposure of the parasite to contaminants via free-living stages (Mackenzie, 1999); 3) changes to host physiology leading to a sub-optimal habitat for parasites (e.g. hydrocarbons in bile affecting intestinal cestodes) (Khan and Kiceniuk, 1983; Mackenzie, 1999). A number of studies have shown the negative effects of metals on parasite survival, infectivity (Pietrock and Marcogliese, 2003), reproduction (Riggs et al., 1987), population dynamics (Munkittrick and Dixon, 1988) and species diversity (Broeg et al., 1999). In lakes receiving effluent from the Key Lake uranium mill, any one of the causal factors listed above could be coming into play and leading to a decrease in parasitism in juvenile pike. If the assumption that parasitism incurs an energetic cost to their host is correct, and that pollution decreases parasitic infections, then the greater energy stores observed in exposure lake pike (Bennett and Janz, 2007) may be explained, at least in part, by a decrease in parasitic infection.

1.3 Oxidative stress

Reactive oxygen species (ROS) are molecules derived from oxygen that are produced naturally as by-products of metabolism and aerobic respiration (Kelly et al., 1998; Pessayre et

al., 2004; Valavanidis et al., 2006) and as cell signaling molecules (Dröge, 2002). If present in excess however, they may be detrimental to proper cell function (Shi et al., 2004) and viability (Manzl et al., 2004). Concentrations of ROS are normally kept in check by antioxidant defense mechanisms comprised of a number of low molecular weight molecules and enzymes. Exposure to certain contaminants can cause elevated concentrations of ROS due to enzymatic and non-enzymatic reactions that directly generate ROS (e.g. cytochrome P450 reductase activity and redox cycling, respectively) and/or by interfering with the antioxidant defense system, thereby indirectly increasing ROS (Kelly et al., 1998). When antioxidant defenses can no longer keep ROS concentrations to within a non-toxic range, major biological macromolecules such as DNA, proteins and the phospholipids of membranes can be oxidatively damaged. This situation, where ROS overwhelm antioxidant defenses leading to subcellular damage, is called oxidative stress (Kelly et al., 1998). Metals have been shown to cause an increase in ROS through a variety of mechanisms including redox cycling and disruptions to antioxidant defenses (Halliwell and Gutteridge, 1999; Ercal et al., 2001).

One of the key low molecular weight molecules that is part of the antioxidant defense system is reduced glutathione. This molecule is present in the cytosol, mitochondria and nucleus of virtually all types of cells (Boelsterli, 2003). Reduced glutathione can neutralize ROS directly or enzymatically via glutathione peroxidase thereby becoming oxidized in the process. Glutathione reductase uses NADPH as reducing equivalents to regenerate the reduced form of glutathione (Boelsterli, 2003). The main enzymes directly involved in the antioxidant defense system are glutathione peroxidase, catalase and superoxide dismutase. Glutathione peroxidase has a selenocysteine moiety at its active site that is oxidized upon reacting with inorganic and organic peroxides (Matés, 2000). Reduced glutathione provides the reducing equivalents necessary for

regenerating the active site of the enzyme. There are different forms of glutathione peroxidase and most can be found in the cytosol, mitochondria (Rodriguez et al., 2004) and nucleus (Halliwell and Gutteridge, 1999). One type of glutathione peroxidase, phospholipid hydroperoxide glutathione peroxidase, is closely associated with cell membranes and is essential for neutralizing lipid peroxides (Halliwell and Gutteridge, 1999). Measures of reduced and oxidized glutathione and the activity of glutathione peroxidase are common biomarkers of exposure of oxidative stress. Since ROS effectively oxidizes and damages cell membrane phospholipids, lipid peroxidation is a typical biomarker of effect of oxidative stress (Halliwell and Gutteridge, 1999). As a result of oxidative damage, cell membranes become less fluid and more permeable (Kelly et al., 1998) and cell death may ensue (Das, 1999).

In mammalian studies, ROS and oxidative stress are linked to a number of diseases including cancer (Kelly et al., 1998), atherosclerosis, cataracts, rheumatoid arthritis, and neurodegenerative disorders (Niki et al., 2005). Studies using fish have demonstrated reduced growth (Fontagné et al., 2006), survival (Peña-Llopis, 2003) and lesions to DNA (Kelly et al., 1998) that were associated with oxidative stress. Although oxidative stress may not be the primary mechanism behind some of these effects (Pandey et al., 2003), oxidative damage to biological macromolecules signals the importance of ROS and oxidative stress as a mechanism of toxic action of contaminants such as metals.

1.4 Histopathology

An organism's response to contaminant exposure can be assessed at a number of different levels of biological organization (Figure 1.4). Histopathological changes in tissues are biomarkers of effect and exposure that integrate responses to contaminants at the biochemical, molecular and cellular level (Hinton and Laurén, 1990). The response time for histopathological biomarkers can be indicative of chronic exposure to sublethal concentrations of contaminants

(Bernet et al., 1999), a factor which is relevant to studies on fish inhabiting metal-mining contaminated waters. Furthermore, histopathological alterations in organs and tissues can vary in severity according to the basic toxicological tenets of dose and duration of exposure (Mallat, 1985; Cooley et al., 2000; Thophon et al., 2003; Roy and Bhattacharya, 2006). However, a given tissue lesion cannot usually be ascribed to a specific contaminant (Hinton and Laurén, 1990).

Numerous fish studies have demonstrated that exposure to aqueous or dietary contaminants, such as metals, cause histopathological lesions to kidney and liver tissues (Levesque et al., 2003; Thophon et al., 2003; Teh et al., 2004). The vulnerability of these organs to toxic damage stems from their role in the accumulation, metabolism and excretion of contaminants (Boelsterli, 2003). Gills are also susceptible to lesions due to their large surface area, constant direct contact with contaminants present in the water column (Bernet et al., 1999) and for being involved in the uptake of contaminants (Liao et al., 2004).

Similar to biomarkers of oxidative stress, histopathological lesions have been associated with traditional ecotoxicological endpoints including survival, growth and reproduction (Wajsbrot et al., 1993; Ankley et al., 2001; Cerqueira and Fernandes, 2002). Although histopathological changes to gonads can more readily be related to effects on reproduction, liver lesions may also indicate impaired reproduction given the importance of this organ in producing vitellogenin, a yolk-protein precursor that is incorporated into fish eggs (Braunbeck et al., 1990).

Large, comprehensive monitoring programs in the United States and in Europe have used histopathology to assess the health status of marine fish (Au, 2004). One of the outcomes

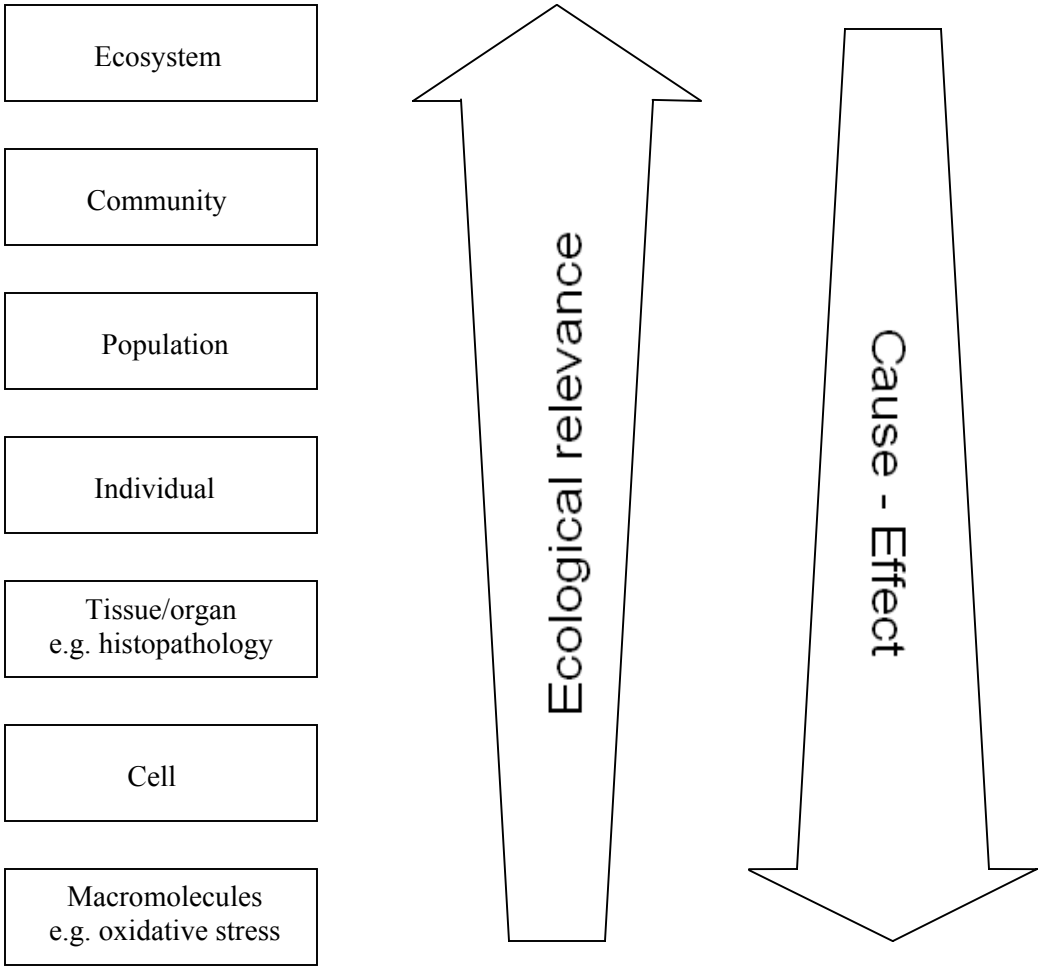

Figure 1.4 Organismal responses to a contaminant at different levels of biological organization. Modified from Munkittrick and McCarty (1995).

from these studies was the establishment of a causal link between contaminant exposure and histopathological alterations to fish organs and tissues (Au, 2004). In Canada, the Aquatic Effects Technology Evaluation (AETE) program, a collaborative effort between the mining industry and federal and provincial governments, considered histopathology for incorporation into monitoring studies on metal mining effluents (GlobalTox, 1997). Other recent studies on freshwater fishes inhabiting metal-mining contaminated waters in Canada, include histopathology of liver, kidney and gills in yellow perch (*Perca flavescens*) collected from the mining region of Rouyn-Noranda, QC (Campbell et al., 2003) and a study on histopathological

lesions to liver of lake trout (*Salvelinus namaycush*) living downstream of an iron-ore mine in Newfoundland (Payne et al., 2001) .

1.5 Hypotheses and research objectives

As a continuation of the research by Bennett and Janz (2007) investigating the bioenergetics of juvenile fish inhabiting lakes near the Key Lake uranium mill, I assessed whether food web enrichment due to nitrogenous compounds and/or a decrease in parasitism could explain the elevated energy stores in juvenile pike from exposure lakes. I hypothesized that the increased concentration of total ammonia and nitrates in exposure lakes compared to reference are causing an enrichment of the food web that translates into prey of higher quality (i.e. higher fat content) for juvenile pike. Another factor that could be affecting pike bioenergetics is parasitism. Parasites can incur an energetic cost to their host, however, rates of parasitic infection can decrease in fish inhabiting contaminated ecosystems. Therefore, I hypothesized that pike from exposure lakes may have greater energy stores compared to reference due to reduced parasitism.

Arsenic, nickel and selenium are elevated in water and sediment of lakes receiving effluent from the Key lake uranium mill. These metals can increase concentrations of ROS and cause oxidative stress in biological systems. I hypothesized that exposure to these metals will cause an increase in ROS and oxidative stress in target organs of pike. This was assessed by evaluating biomarkers of oxidative stress in pike liver and kidney. Oxidative stress and other mechanisms associated with constituents of the complex effluent from the Key Lake uranium mill could be affecting the microscopic structure of tissues and organs. I hypothesized that histopathological lesions to liver, kidney and gills will be greater in exposure lake pike compared to reference.

The objectives of this thesis are as follows:

(i) Quantify triglycerides and glycogen in juvenile northern pike liver and muscle

(ii) Determine the concentration of triglycerides in pike prey items

(macroinvertebrates and a forage fish)

(iii) Determine the abundance and prevalence of intestinal and gill parasites

(iv) Assess the levels of reduced and oxidized glutathione and lipid peroxidation in liver and kidney and glutathione peroxidase activity in liver

(v) Evaluate histopathological changes in liver, kidney and gills

(vi) Analyze metal concentrations in muscle

CHAPTER 2
2.0 ALTERED BIOENERGETICS AND PARASITISM IN JUVENILE NORTHERN PIKE (*ESOX LUCIUS*) INHABITING LAKES DOWNSTREAM OF A URANIUM MILL

2.1 Abstract

A previous study on young-of-the-year northern pike (*Esox lucius*) demonstrated elevated energy stores in fish inhabiting lakes receiving effluent from the Key Lake uranium mill in northern Saskatchewan, Canada. The objective of this study was to evaluate possible factors that could be contributing to altered bioenergetics of juvenile northern pike. Although significantly elevated muscle glycogen, muscle triglycerides, liver glycogen and liver triglycerides was demonstrated among exposure and reference lakes, triglycerides stores of aquatic insects and spottail shiners (*Notropis hudsonius*) that are prey items of juvenile pike showed no overall differences among lakes. Measures of parasitism, on the other hand, were negatively correlated with pike bioenergetics thereby reflecting a possible energetic cost of parasitism on reference lake fish. The degree of infection, as measured by the abundance and biomass of intestinal parasites and the abundance of monogeneans on pike gills, was greatest in reference fish and intermediate in low exposure pike, whereas high exposure fish harboured no parasites.

2.2 Introduction

Environmental contaminants can disrupt the normal functioning of a number of fundamental biological processes, one of which concerns the exchange and utilization of energy by organisms in an ecosystem, a phenomenon termed bioenergetics. The bioenergetics of aquatic ecosystems can be affected by industrial effluents directly, by disrupting metabolic pathways in exposed organisms (Levesque et al., 2002), and indirectly by altering community structure and/or

function (Culp et al., 2003). Organisms exposed to contaminants can experience depleted energy stores presumably due to the metabolic costs associated with detoxification (Adams, 1999) and/or repair of damaged cells and tissues (Campbell et al., 2003). In support of this hypothesis, reduced levels of lipids (Munkittrick and Dixon, 1988; Levesque et al., 2002) and glycogen (Teh et al., 2004) have been observed in fish exposed to environmental contaminants such as metals. Levesque et al. (2002) reported that yellow perch (*Perca flavescens*) chronically exposed to metals in the field had lower hepatic lipid levels compared to reference fish whereas juvenile splittail (*Pogonichthys macrolepidotus*) exposed to selenium in their diet exhibited reduced hepatic glycogen stores (Teh et al., 2004).

An earlier study from our lab considered whether or not young-of-the-year northern pike (*Esox lucius*) chronically exposed to uranium milling effluent from the Key Lake operation in northern Saskatchewan experienced depleted energy stores and, if so, whether this deficiency was compromising their capacity to survive their first winter (Winter Stress Syndrome proposed by Lemly, 1993a). Instead of having depleted energy stores, however, pike from exposure lakes demonstrated elevated stores of total body lipids and triglycerides compared to pike from a clean reference lake (Bennett and Janz, 2007). These results were contrary to expectations for the reasons mentioned above. Despite an important body of evidence for contaminant-associated energy depletion (Munkittrick and Dixon, 1988; Levesque et al., 2002; Teh et al., 2004), other studies that report elevated lipid (Ribeiro et al., 2005) and glycogen stores in fish (De Boeck et al., 1997) exposed to contaminants do exist in the literature.

The objective of the present study was to investigate some of the factors that may be contributing to the elevated energy stores in pike inhabiting lakes receiving uranium milling effluent from the Key Lake operation. A number of different possible contributing factors exist;

including a greater quantity or quality of prey items in the receiving lakes, a metabolic disruption in the utilization of energy stores in pike exposed to the uranium milling effluent, a reduction in parasite load, etc.

Prey of higher quality (i.e. higher energy content) may be available due to the presence of nitrates and ammonia/ammonium in the receiving lakes (Golder, 2005) that could be causing a nutrient enrichment of the food web (i.e. a bottom-up effect) (Dillon et al., 2004). To further investigate this possibility, the energy stores of juvenile pike as well as some of their main vertebrate and invertebrate prey items, representing different trophic levels, were examined in this thesis.

Another potential explanation for the differences in energy stores in pike inhabiting exposure and reference lakes could be linked to parasitic infection. Parasites are ubiquitous in ecosystems (Marcogliese, 2005) and depend almost entirely on one or more hosts for the completion of their lifecycle. The energetic cost of parasitic infection is evidenced by the variety of nutrients parasites directly consume from their host. Cestodes (Cestoda), for instance, actively absorb amino acids, glucose (Chappell, 1993), fatty acids (Smyth, 1994) and whole proteins (Whitfield, 1993) from their host's alimentary canal. Other parasites, such as monogeneans (Monogenea), attach themselves to the external surfaces of fish and deplete host energy stores by consuming blood, mucous, and epithelial cells (Chappell, 1993). Parasitic infections can also incur an indirect energetic burden that is associated with the repair of damaged tissues and with the activation of an immune response against the infection (Mackenzie et al., 1995).

Similar to other animals in nature, parasites can be sensitive to the presence of contaminants and rates of host infection can either increase (Broeg et al., 1999; Hecker and Karbe, 2005) or decrease (Riggs et al., 1987; Munkittrick and Dixon, 1988) depending on the type of

environmental deterioration in question (Lafferty, 1997). A decrease in a parasite population can occur indirectly through the removal of a host species, by reducing host density and also due to direct toxicity to the parasites themselves (see review by Pietrock and Marcogliese, 2003).

The predictions for the present study were that: 1) energy stores (glycogen and triglycerides) of juvenile northern pike will be elevated in fish collected from lakes receiving uranium milling effluent (paralleling results reported by Bennett and Janz, 2007); 2) pike prey items will also have higher energy stores (triglycerides) due to the possible food web enrichment effects of nitrogenous compounds present in the effluent; 3) a lower abundance of parasites will be seen in pike from the exposure lakes compared to the reference; and 4) parasitism will be negatively correlated with pike bioenergetics.

2.3 Materials and Methods

2.3.1 Study site

Samples were collected from three lakes that are in close proximity to the Key Lake operation in northern Saskatchewan (57°13' N, 105°38' W). Unknown Lake is located approximately two km downstream of the uranium mill effluent discharge and has a surface area of 0.12 km^2 and a mean depth of 0.47 m while Delta Lake is approximately 10 km from the discharge and has a surface area of 2.85 km^2 and a mean depth of 2.3 m. The concentration of effluent in the receiving environment ranges from approximately 72% in Wolf Lake to approximately 28% in Delta Lake (Robertson, 2006). In accordance with the concentration gradient of effluent in the lakes, Unknown Lake will be referred to as the High exposure lake and Delta Lake as the Low exposure lake from this point onward. David Lake, the reference lake, has a surface area of 1.4 km^2 and a mean depth of 1.9 m. It is located upstream of the effluent discharge and does not receive contaminants from any known source. All three lakes are part of the same catchment and are connected by David Creek.

2.3.2 Field collections

Juvenile northern pike entering their second year of life (1+) were collected between June 16 and 23, 2005. Four, eight and seven pike were captured in the morning (i.e. 8:30 to 12:00) from the high, low and reference lakes, respectively, whereas after noon (i.e. 12:00 to 19:30), five pike were caught from each of the high exposure and reference lakes and four from the low exposure lake. Totals amounted to nine pike from the high exposure lake and 12 from both the low exposure and reference lakes. To facilitate collection, pike were stunned using a Smith-Root backpack electrofisher (Smith-Root, Vancouver, WA, USA) then captured with dip nets. The fish were kept in small coolers filled with lake water prior to being sacrificed with a lethal dose of 3-aminobenzoic acid ethyl ester solution (MS 222, Sigma-Aldrich, Oakville, ON, Canada) and dissected on-site. Tissues collected were kept on dry ice throughout the duration of the field sampling (eight days) then transferred to a -80˚C freezer upon return to the University of Saskatchewan, Saskatoon, SK, Canada.

Dissections included removal and sectioning of the liver into six portions; five portions were placed into separate cryovials and frozen and one portion was fixed in Bouin's fixative. Five of the portions, including the one fixed in Bouin's, were retained for biochemical and histopathological analyses (Chapter 3). Prior to sectioning, livers were rinsed with a buffer solution of Tris-KCl to remove any bile that may have leaked from the gall bladder and gently blotted dry with a Kimwipe®. Due to difficulties obtaining an accurate liver weight in the field, weights were obtained from each individual portion upon return to the University of Saskatchewan and added to obtain total liver weight. The head of each fish was excised from the rest of the body and frozen in a Whirlpak® bag. Similarly, gastrointestinal tracts were excised, placed in bags, and frozen. Carcasses were bagged and frozen for subsequent removal of a small

piece of white muscle from a location above the lateral line and anterior to the dorsal fin to be used in glycogen and triglycerides determinations.

Collection of macroinvertebrates and spottail shiners (*Notropis hudsonius*) took place from August 27 to September 2, 2005. Macroinvertebrates were collected using a 15 cm^2 Ekman grab and a dip net whereas shiners were sampled using a Smith-Root boat electrofisher equipped with a 2.5 hp gas powered pulsator. Samples were bagged in Whirlpaks® or placed in cryovials and kept on dry ice for the duration of the field sampling before transferring to a -80˚C freezer at the University of Saskatchewan. A subsample of macroinvertebrates was preserved in 10% formalin for taxonomic identification.

Water quality variables such as specific conductivity (µS/cm), temperature (˚C), dissolved oxygen (mg/L), and pH were measured at each lake at a depth of approximately one meter using a YSI 650 Multi-parameter Display System equipped with a 600 QS probe (Yellow Springs, OH, USA).

2.3.3 Northern pike morphometrics and ageing

Prior to dissections, weights (mg) and total lengths (cm) were recorded from each pike. As an indication of general body condition, Fulton's condition factor ($K = weight/length^3*100$) was calculated. The hepatosomatic index (liver weight/total body weight*100) was also calculated. The weight used in the calculations was corrected for the stomach content weight (see below). Cleithra were retained from each fish for age determination by a fisheries technician with the Ontario Federation of Anglers and Hunters (Peterborough, ON, Canada).

2.3.4 Laboratory analyses

2.3.4.1 Tissue and prey item preparation

The bioenergetic status of the pike was assessed by determining triglycerides and glycogen levels from a small sample of dorsal white muscle (up to 300 mg) and liver (approximately 50

mg). Triglycerides concentrations in whole-body spottail shiners and macroinvertebrates most of which are known prey items of pike (Beaudoin et al., 1999), were also analyzed. Macroinvertebrates, identified to the lowest possible taxonomic group, included: midge and fly larvae (Diptera), caddisfly larvae (Trichoptera, Phryganeidae), dragonfly larvae (Odonata, Anisoptera), and waterboatman (Heteroptera, Corixidae). Similar to vertebrates, triglycerides are an important storage form of energy in insects (Meier et al., 2000).

Pike tissues and whole-body prey items were homogenized prior to assaying using either a Tissue Tearor (Fisher Scientific, Houston, TX, USA), for pike muscle and all prey items, or a teflon mortar and glass pestle (Wheaton Science Products, Millville, NJ, USA) in the case of pike liver. Samples were homogenized in ice-cold 0.2 M sodium citrate buffer (EMD Chemicals Inc., Gibbstown, NJ, USA) and kept on ice. Homogenates were heated at 95° C for four minutes in order to inactivate amylase, an enzyme that catalyzes the breakdown of glycogen. Although the concentration of glycogen was only determined in pike tissues, prey item homogenates were also heated for consistency. When necessary, fly and midge larvae, caddisfly larvae and waterboatman replicates were obtained by pooling individuals to obtain a mass of at least 60 mg. In order to remove particles of exoskeleton that could interfere with assay performance, all macroinvertebrate homogenates were centrifuged for 20 to 30 seconds at 6 000 x g in a picofuge (VWR, West Chester, PA, USA). Aliquots of the homogenates were kept frozen at -80˚ C until analysis. Both the triglycerides and glycogen assays were carried out using a SpectraMAX 190 spectrophotometer (Molecular Devices Corporation, Sunnyvale, CA, USA).

2.3.4.2 Triglycerides assay

The triglycerides assay was performed using a modified version of a serum triglycerides kit prepared by Sigma (Saint Louis, MO, USA); the kit is based on a method developed by McGowan et al. (1983) and has been previously validated in our laboratory for work on fish

tissues (Weber et al., 2003; Bennett and Janz, 2007). Prior to performing the assay, all samples, except for homogenates of fly and midge larvae, were diluted to fall within the range of the standard curve. The standard curve was developed using glycerol and calibrated to yield the concentration of triglycerides as triolein equivalents, triolein being the most widespread form of triglycerides in fish (Weber et al., 2003).

2.3.4.3 Glycogen assay

Glycogen was determined using purified Type IX bovine liver glycogen (Sigma-Aldrich) as a standard (0.05-20 µg/ml for standard curve) and a method by Gómez-Lechón et al. (1996) adapted for fish (Weber et al., in press).

2.3.5 Parasitic examinations

Gills and intestines from each fish were examined for parasites under an Olympus SZ61 stereomicroscope (Olympus, Center Valley, PA, USA). All four gill arches from the right side of the head were removed and examined for monogeneans; the total abundance of which was then recorded per pike. The gastrointestinal tract of each pike was also examined for intestinal parasites. The total abundance of intestinal parasites was recorded as well as a breakdown by number of cestodes and nematodes (Nematoda), since only these two taxonomic groups were present. The abundance of cestodes was determined by counting scoleces (cestode attachment organ) whereas nematodes were counted directly since they were not susceptible to breakage. Prevalence and abundance were calculated according to Bush et al. (1997). The total weight of intestinal parasites per fish was measured as well as the weight of cestodes and nematodes separately. Weights were measured to five decimal places using a Sartorius BP211D scale (Sartorius North America Inc., Edgewood, NY, USA). Prior to weighing, parasites were dissected out into a petri dish of distilled water and gently blotted dry on Kimwipes®.

2.3.6 Stomach contents

At the time of thawing the gastrointestinal tracts for parasitic examination, stomach contents were removed, weighed and prey items identified to the lowest possible taxonomic group.

2.3.7 Statistical analyses

All data were tested for normality with the Shapiro-Wilk test and for equality of variance with the Levene test using SYSTAT 11 (SPSS Inc., Chicago, IL, USA). Data that did not meet the assumptions were log10 or square-root transformed. Significant differences among the three lakes were tested by one-way ANOVA followed by a Tukey test, as appropriate, or by a t-test when comparisons were made between two lakes. Data that did not meet the assumptions of normality and homogeneity of variance following transformation were tested using Kruskal-Wallis one-way ANOVA followed by a Dunn's test as appropriate.

Significant correlations between parasitic infection and measures of pike bioenergetics were tested using pooled data from all three lakes. Pearson product-moment correlation was used for data that were normally distributed and linear otherwise, Spearman rank correlation was used. The respective Pearson product-moment correlation coefficients (r) and Spearman rank correlation coefficients (r_s) were also calculated. Since the abundance and biomass of nematodes made up only a small percentage of the total intestinal parasite data (0.7% and 0.3% of total, respectively), statistical analyses involving intestinal parasites were conducted on the pooled data. Separate t-tests for the low exposure and reference lakes comparing cestode abundance and biomass data against pooled cestode and nematode abundance and biomass data did not yield any significant differences thereby further justifying this approach. All statistical analyses were performed using SigmaStat 3.1 (SPSS Inc., Chicago, IL, USA) with an alpha value set at 0.05.

For both the triglycerides and glycogen assays, six determinations of a pooled sample were used to assess intra-assay variability whereas the same pooled sample was run six more times on

a separate occasion to evaluate inter-assay variability. Samples that had a coefficient of variation greater than 10% between duplicate wells were re-analyzed.

2.4 Results

2.4.1 Abiotic environment

Details of the abiotic variables measured on site at the time of northern pike collection in June 2005, water quality variables collected in 2004 and reported by Golder (2005), and selenium concentrations from Muscatello et al. (in press), are outlined in Table 2.1. Several variables distinguish the exposure lakes from the reference in terms of water quality. Conductivity and total hardness ($CaCO_3$) were greater at both exposure lakes compared to the reference whereas pH was depressed only at the high exposure lake. Nitrates and total ammonia were approximately one or more orders of magnitude greater at the exposure lakes compared to the reference lake with the concentration of total ammonia exceeding Saskatchewan Surface Water Quality Objectives (SSWQO) at the high exposure lake.

Table 2.1 Water quality variables collected in June 2005 for lakes receiving effluent from the Key Lake uranium mill (low and high exposure) and one reference lake. Total hardness, total ammonia, nitrate and concentrations of arsenic, molybdenum, nickel and uranium are from Golder (2005). Concentrations of selenium are from Muscatello et al. (in press). Saskatchewan Surface Water Quality Objectives (SSWQO) and Canadian Council of Ministers of the Environment (CCME) guidelines are also presented.

Variable	Lake			SSWQO	CCME
	Reference	Low exposure	High exposure		
Dissolved oxygen (mg/L)	7.3	10.9	9.4		
Temperature (°C)	17.4	16.7	17.1		
pH	6.4	7.1	5.3	6.5 to 8.5	6.5 to 9
Conductivity (µS/cm)	21	423	683		
Total hardness (as $CaCO_3$) (mg/L)	4	221	317		
Total ammonia (mg/L)	0.03	0.2	2.1	1.9 to 2.0[a]	10 to >100[a]
Nitrate (mg/L)	< 0.04	1.4	4.1		13
Arsenic (µg/L)	0.1	0.9	3.8	50	5
Molybdenum (µg/L)	< 0.1	126	108	700	73
Nickel (µg/L)	< 0.1	2.2	6.6	25 to 100‡	25 to 150‡
Selenium (µg/L)	0.1	1	3	10	1
Uranium (µg/L)	< 0.1	< 0.1	0.4	11 to 218‡	

[a] Guideline is temperature and pH dependent. Total ammonia ($NH_3 + NH_4$) values shown correspond to the range in pH and temperature measured in the lakes during fall 2004.

‡ Guideline is hardness dependent and increases with hardness.

2.4.2 Stomach contents

The number of pike from each lake that had prey items in their stomachs was 9 out of 12 for the reference lake, 11 out of 12 for the low exposure lake, and 3 out of 9 for the high exposure lake. There were some similarities in the stomach contents among the exposure and reference lakes. Dragonfly larvae were present in the stomachs of pike from all three lakes. The low exposure and reference lakes also had spottail shiners in their stomachs (5 out of 12 and 1 out of 12, respectively) whereas fish from the high exposure lake only had invertebrates (i.e. dragonfly larvae and one unidentifiable arthropod). One fish from the low exposure lake had 9 larval pike in its stomach while leeches were found in the stomachs of 4 pike that were all from the reference lake. Mean stomach contents (wet weight) differed significantly between the low (mean ± SEM; 0.83 ± 0.16 g) and high (0.10 ± 0.03 g) exposure lakes only ($p = 0.018$). The mean stomach content weight for pike from the reference lake was intermediate between the two exposure lakes (0.44 ± 0.21 g).

2.4.3 Morphometrics

Measures of weight, total length, condition factor, and hepatosomatic index did not differ significantly among lakes ($p > 0.05$; Table 2.2).

Table 2.2 Weight (g), total length (cm), condition factor (weight/length^3*100), and hepatosomatic index (HSI = liver weight/body weight*100) of 1+ northern pike (*Esox lucius*) collected from two lakes receiving effluent from the Key Lake uranium mill (low and high exposure; n = 12 and n = 9, respectively) and one reference lake (n = 12). Data are presented as means ± SEM. There were no significant differences among lakes for any of the morphometric measures ($p > 0.05$).

Lake	Weight (g)	Total length (cm)	Condition factor	HSI
Reference	21.1 ± 2.2	15.9 ± 0.6	0.51 ± 0.01	0.88 ± 0.04
Low exposure	20.1 ± 2.8	15.7 ± 0.7	0.50 ± 0.02	0.84 ± 0.05
High exposure	15.8 ± 1.6	14.4 ± 0.5	0.52 ± 0.01	0.96 ± 0.08

2.4.4 Bioenergetics

2.4.4.1 Northern pike bioenergetics

The results show that 1+ northern pike living in lakes downstream of the Key Lake uranium mill effluent have greater energy stores than pike from the uncontaminated reference lake (Figure 2.1). Concentrations of triglycerides, the main storage form of lipids in vertebrates, were significantly greater in liver ($p < 0.01$) and muscle ($p < 0.05$) in pike from the exposure lakes compared to the reference (Figure 2.1A). Liver triglycerides levels were significantly higher for both exposure lakes compared to the reference whereas no significant difference was found between the two exposure lakes. Although an overall significant difference for muscle triglycerides were detected by one-way ANOVA ($p = 0.041$), the Tukey post-hoc test showed no pairwise differences between the lakes.

A trend similar to triglycerides concentrations was seen with glycogen levels measured in liver and muscle (Figure 2.1B). In the case of liver glycogen, only pike from the high exposure lake had greater glycogen levels compared to both the reference and low exposure lakes ($p < 0.001$). The sample size for muscle glycogen in the low exposure and reference pike was $n = 11$ rather than $n = 12$ since one fish from each of the two lakes contained undetectable levels of glycogen. Results showed significantly greater levels of muscle glycogen for both exposure lakes compared to the reference ($p < 0.01$) while no significant difference was found between the exposure lakes (Figure 2.1B).

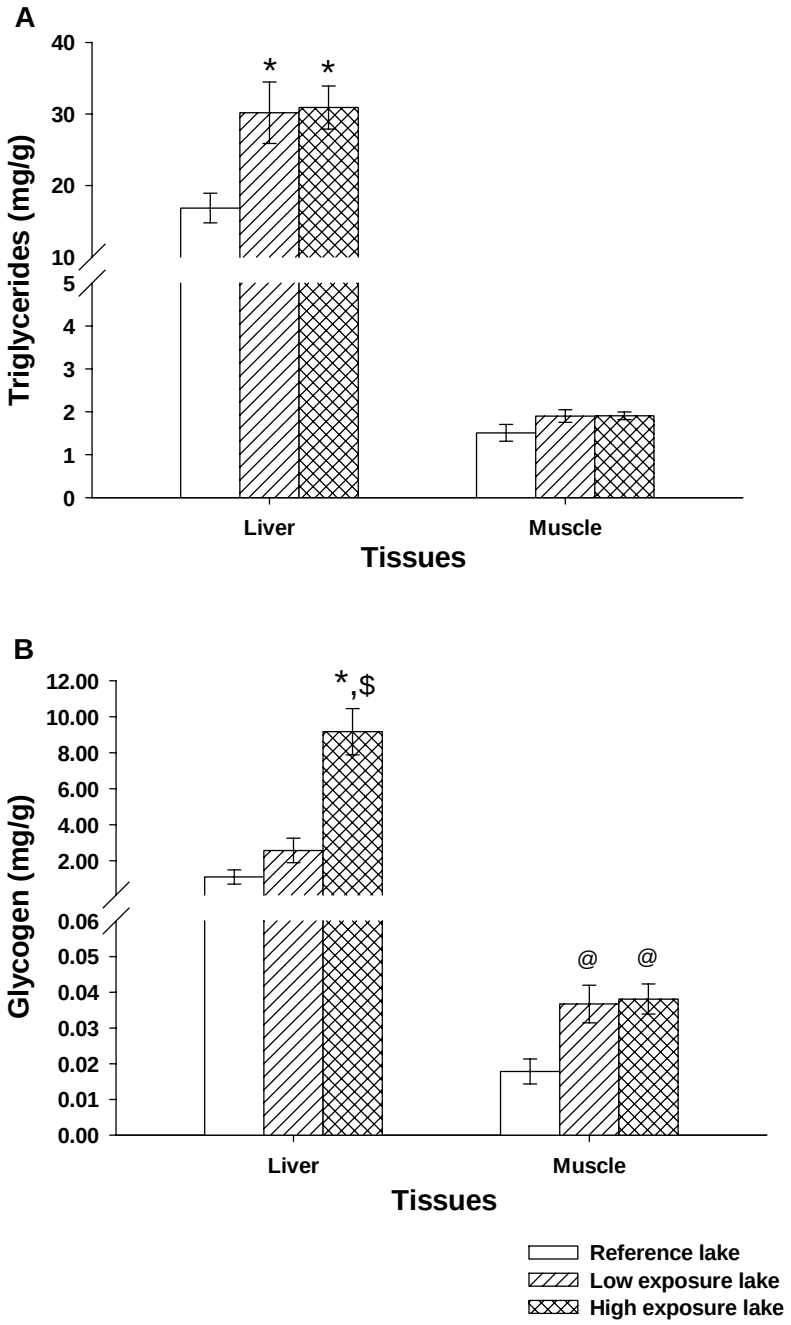

Figure 2.1 Triglycerides (A) and glycogen (B) (mg/g) in liver and muscle tissues of 1+ northern pike (*Esox lucius*) collected from two lakes receiving effluent from the Key Lake uranium mill (low and high exposure; n = 12 and n = 9, respectively) and one reference lake (n = 12). Muscle glycogen, however, was n = 11 for the low exposure and reference lakes. Data are means ± SEM with significant differences ($p < 0.05$) compared to the reference indicated by * for liver and @ for muscle. A significant difference among exposure lakes is shown by $ for liver glycogen. One-way ANOVA of muscle triglycerides showed a significant difference among lakes ($p = 0.041$) however, the post-hoc comparisons lacked the power to detect where the difference lay.

2.4.4.2 Prey items bioenergetics

Overall, whole-body triglycerides levels in the prey items did not differ significantly among lakes (Figure 2.2). The only exception was for waterboatman collected from the low exposure lake which had significantly less triglycerides (6.46 ± 0.51 mg/g) compared to the high exposure (30.7 ± 2.6 mg/g) and reference (36.3 ± 3.4 mg/g) lakes. The waterboatman from the low exposure lake, however, were consistently smaller than the others (about half the size) which may explain the difference in triglycerides concentrations. No spottail shiners were collected at the high exposure lake, therefore comparisons were only between the reference and low exposure lakes. Reference lake shiners were significantly greater in fork length ($p < 0.001$) and weight ($p < 0.001$) (6.4 ± 0.1 cm and 3.0 ± 0.2 g, respectively) compared to shiners from the low exposure lake (4.9 ± 0.2 cm and 1.2 ± 0.2 g fork length and weight, respectively). Of all the prey items, only spottail shiners demonstrated a noticeable increase in triglycerides in the exposure lake (45.5 ± 4.1 mg/g) compared to the reference (36.6 ± 1.2 mg/g) but the difference was not significant ($p = 0.093$). An attempt to control for the possible effect of size on triglycerides concentrations by performing an ANCOVA with triglycerides as the dependent variable and weight or length as covariate was unsuccessful since the data did not meet the assumption of equality of regression slopes.

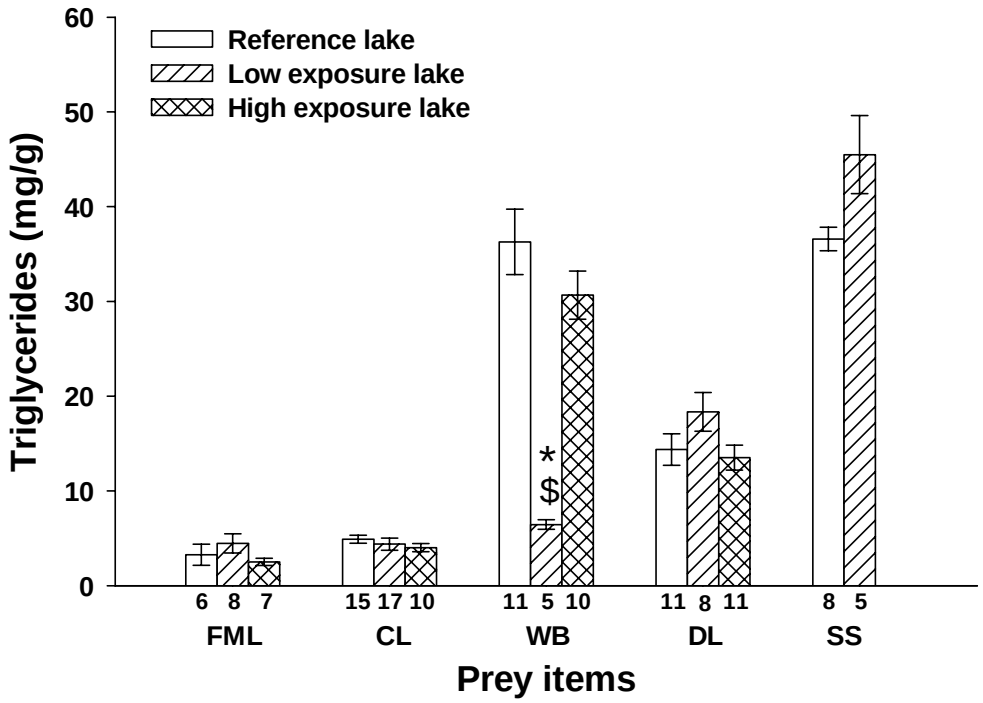

Figure 2.2 Triglycerides (mg/g) in fly and midge larvae (FML), caddisfly larvae (CL), waterboatman (WB), dragonfly larvae (DL), and spottail shiners (SS) collected from two lakes receiving effluent from the Key Lake uranium mill (low and high exposure) and one reference lake. Data are means ± SEM. For each prey item, a significant difference ($p < 0.05$) compared to reference is indicated by * and by $ for a significant difference between exposure lakes. The number beneath each bar is the number of replicates.

2.4.5 Parasitism

2.4.5.1 Parasite prevalence, abundance and biomass

The prevalence of both monogenean and intestinal parasite infection differed markedly between lakes. All pike from the reference lake were infected with both types of parasites whereas no pike from the high exposure lake were infected. The low exposure lake showed an intermediate prevalence of infection with 83% and 67% for monogenean and intestinal parasites, respectively. Pike from the reference lake not only supported the highest prevalence of infection but also showed the greatest mean abundance of monogeneans and intestinal parasites (Figure 2.3). The reference lake had a significantly higher ($p < 0.001$) mean abundance of intestinal

parasites (92.7 ± 30.6) than the low (5.8 ± 2.8) and high (0 ± 0) exposure lakes (Figure 2.3A). Although the mean intestinal parasite biomass was 2.5 times greater at the reference lake (76.3 ± 16.0 mg) compared to the low exposure lake (30.6 ± 12.2 mg), the difference was not statistically significant ($p = 0.053$). Similar to mean intestinal parasite abundance, the mean abundance of monogeneans was significantly greater ($p < 0.001$) for the reference lake (96.0 ± 20.7) compared to the high (0 ± 0) but not the low exposure lake (25.1 ± 11.7) (Figure 2.3B). No parasites were found in the stomachs of most fish except for one pike from the low exposure lake that had one parasite in its stomach (Trematoda). This observation was noted but the datum was not included in any of the analyses.

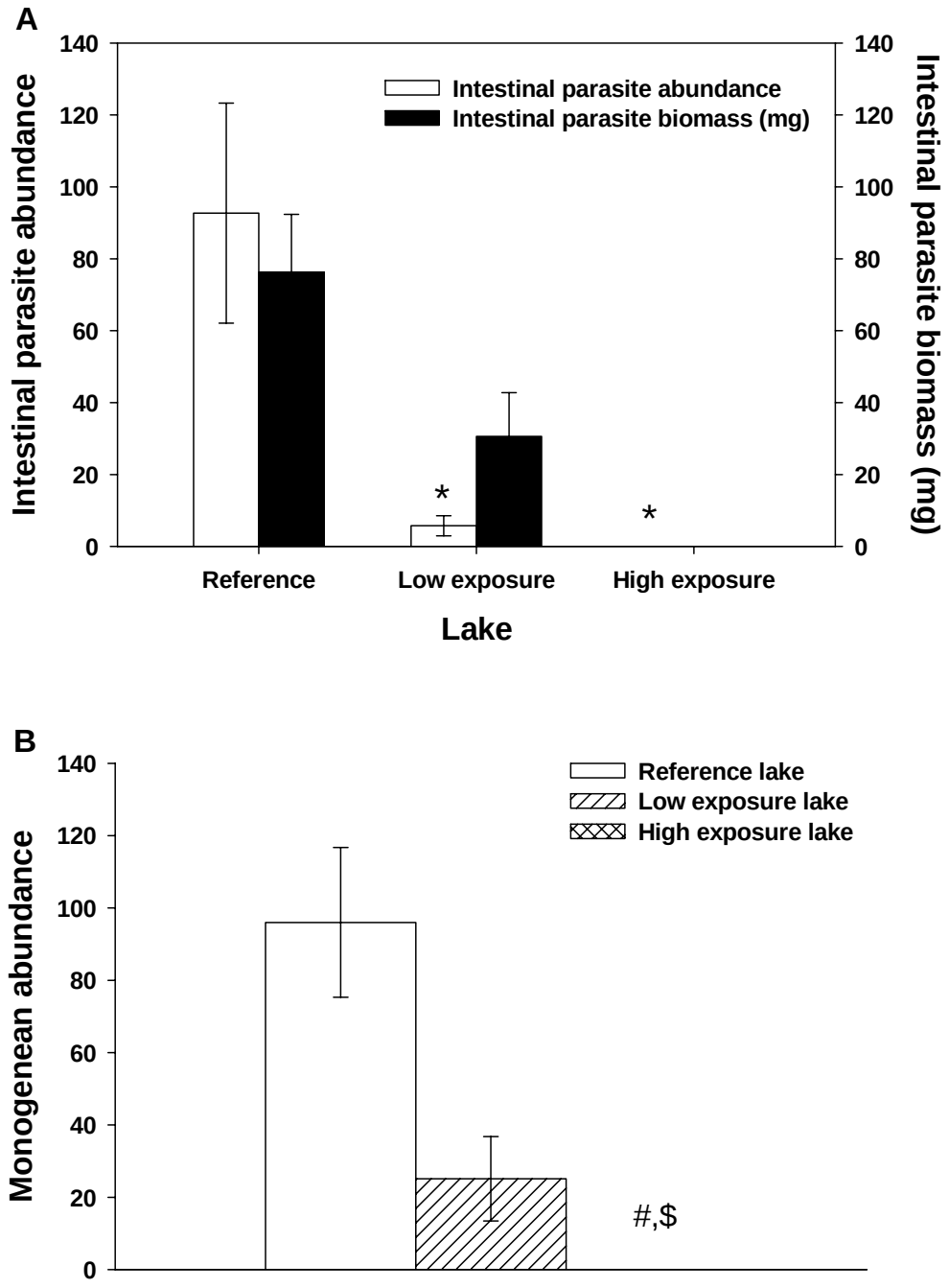

Figure 2.3 Intestinal parasite abundance and biomass (mg) (A) and abundance of monogeneans (B) obtained from 1+ northern pike (*Esox lucius*) collected from two lakes receiving effluent from the Key Lake uranium mill (low and high exposure; n = 12 and n = 9, respectively) and one reference lake (n = 12). The figure shows mean ± SEM and significant differences ($p < 0.05$) compared to reference are indicated by * for intestinal parasite abundance and # for monogenean abundance. Intestinal parasite biomass did not differ significantly among lakes. Significant differences among exposure lakes is shown by $ for monogenean abundance.

2.4.5.2 Relationship between pike bioenergetics and parasitism

Intestinal parasite abundance showed a significant negative correlation with all measures of pike bioenergetics (Table 2.3 and Figure 2.4) whereas the number of monogeneans (Table 2.3 and Figure 2.5) was significantly negatively correlated with pike liver triglycerides and glycogen levels but not muscle triglycerides and glycogen. Intestinal parasite biomass, was negatively correlated with all measures of pike bioenergetics but was only significant for muscle glycogen ($p = 0.005$).

In terms of pike morphometrics, intestinal parasite abundance was significantly positively correlated with total length ($r_s = 0.48$, $p = 0.005$) and weight ($r_s = 0.46$, $p = 0.007$) as was monogenean abundance ($r_s = 0.52$, $p = 0.002$; $r_s = 0.50$, $p = 0.004$ for total length and weight, respectively). Similarly, intestinal parasite biomass correlated positively with total length and weight but this was not significant ($p = 0.45$ and $p = 0.76$, respectively). Condition factor correlated negatively with monogenean ($r_s = -0.21$, $p = 0.24$) and intestinal parasite ($r_s = -0.14$, $p = 0.42$) abundance as well as with intestinal parasite biomass ($r = -0.35$, $p = 0.13$). Hepatosomatic index also correlated negatively with monogenean ($r_s = -0.22$, $p = 0.21$) and intestinal parasite ($r_s = -0.29$, $p = 0.11$) abundance and intestinal parasite biomass ($r = -0.28$, $p = 0.24$). For both condition factor and hepatosomatic index, however, these correlations were not significant ($p > 0.05$).

Table 2.3 Correlations between parasitic infection and measures of bioenergetics in 1+ northern pike (*Esox lucius*) collected from two lakes receiving effluent from the Key Lake uranium mill (low and high exposure; n = 12 and n = 9, respectively) and one reference lake (n = 12). Correlations with muscle glycogen were n = 11 for the low exposure and reference lakes. Data shown are correlation coefficients (Pearson product-moment coefficients *r* are in bold and Spearman rank coefficients r_s are in normal font) with p values in parentheses. Significant correlations are indicated by * ($p < 0.05$).

	Parasitic infection		
Bioenergetics in 1+ northern pike	Monogenean abundance	Intestinal parasite abundance	Intestinal parasite biomass
Liver triglycerides	-0.40 (0.022) *	-0.48 (0.005) *	**-0.24** (0.30)
Liver glycogen	-0.77 (<0.001) *	-0.79 (<0.001) *	-0.35 (0.12)
Muscle triglycerides	-0.23 (0.20)	-0.36 (0.04) *	**-0.30** (0.19)
Muscle glycogen	-0.34 (0.064)	-0.58 (<0.001) *	**-0.63** (0.005) *

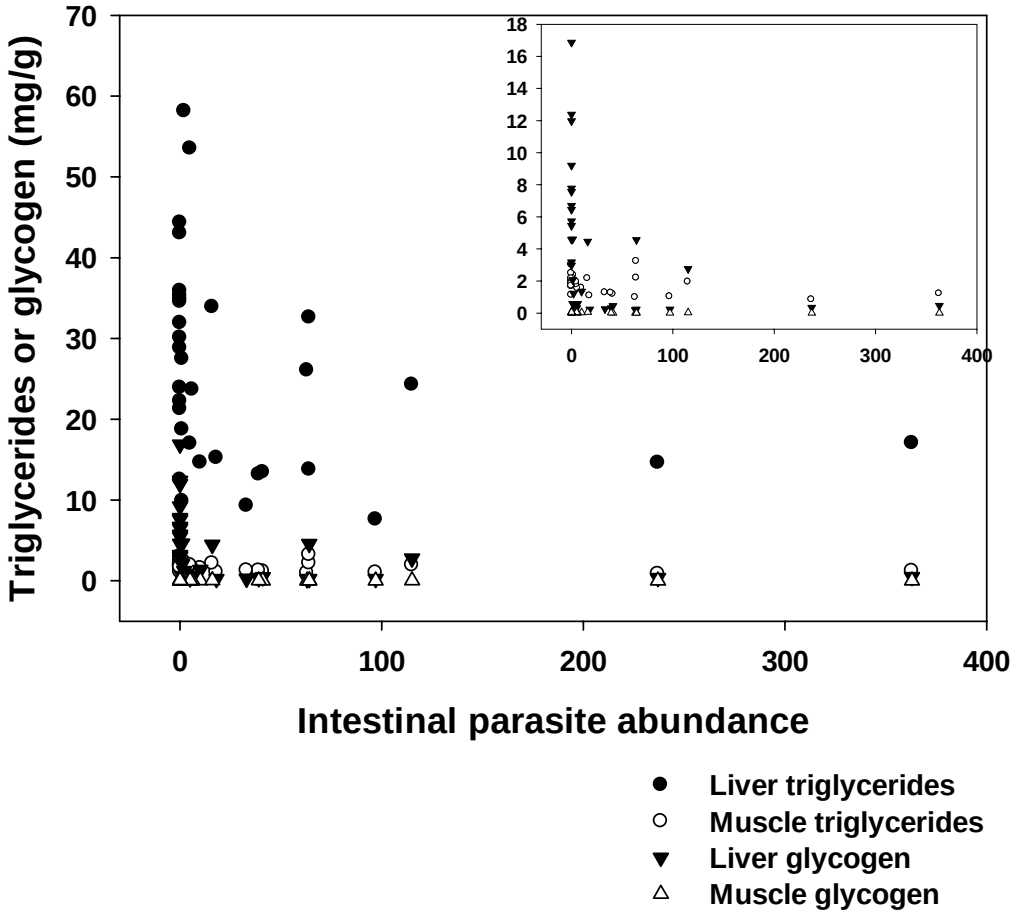

Figure 2.4 Scatter plots of triglycerides and glycogen concentrations in liver and muscle against abundance of intestinal parasites from 1+ northern pike (*Esox lucius*) collected from two lakes receiving effluent from the Key Lake uranium mill (low and high exposure; n = 12 and n = 9, respectively) and one reference lake (n = 12). Muscle glycogen, however, was n = 11 for the low exposure and reference lakes. Inset shows scatter plots of muscle triglycerides and liver and muscle glycogen against parasite abundance.

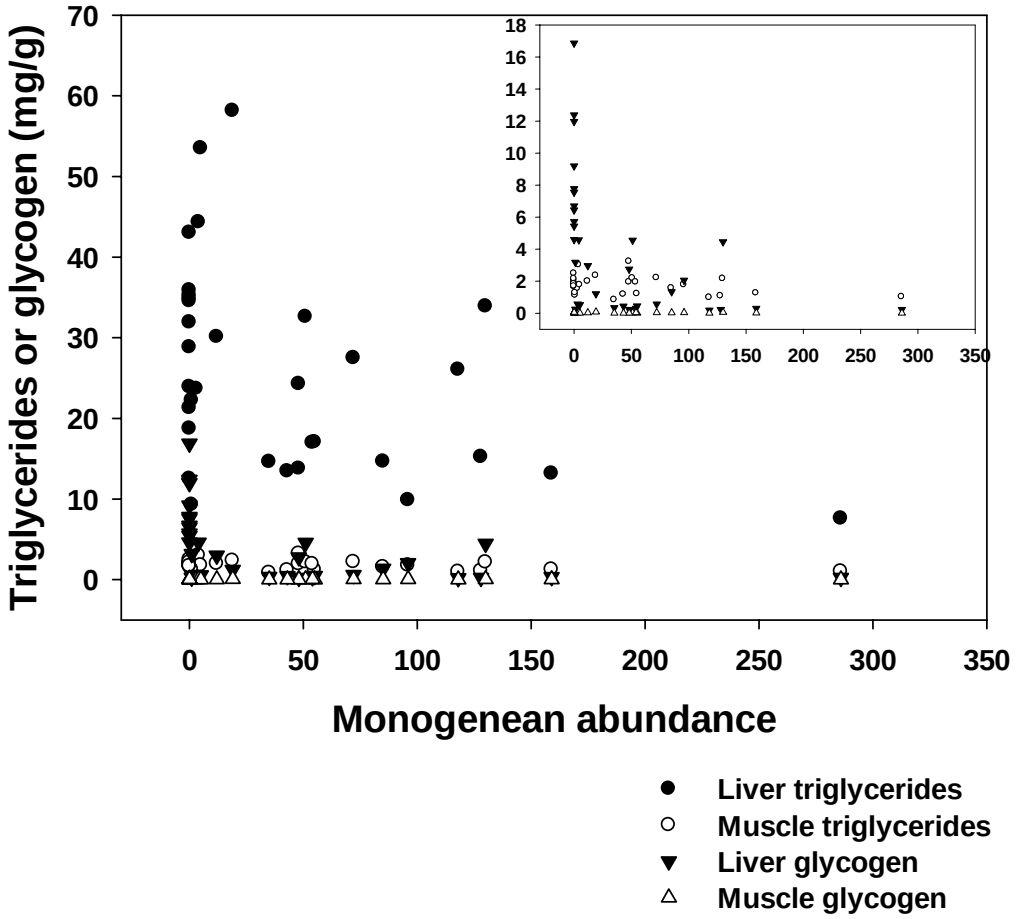

Figure 2.5 Scatter plots of triglycerides and glycogen concentrations in liver and muscle against abundance of monogeneans from 1+ northern pike (*Esox lucius*) collected from two lakes receiving effluent from the Key Lake uranium mill (low and high exposure; n = 12 and n = 9, respectively) and one reference lake (n = 12). Muscle glycogen, however, was n = 11 for the low exposure and reference lakes. Inset shows scatter plots of muscle triglycerides and liver and muscle glycogen against parasite abundance.

2.4.6 Assay performance

Intra- and inter- assay variability was below 10% for both the glycogen and triglycerides assays. Triglycerides intra- and inter-assay variability on a pooled sample was 2.0% and 2.1%, respectively while glycogen intra- and inter-assay variability was 2.5% and 3.0%, respectively.

2.5 Discussion

2.5.1 Northern pike bioenergetics

Juvenile northern pike (1+) living in lakes receiving effluent from the Key Lake uranium mill in northern Saskatchewan demonstrated elevated triglycerides and glycogen stores, which, according to the present study, does not appear to be due to a nutrient enrichment of exposure lake food webs compared to the ecologically similar reference lake. The bioenergetics of pike from this study support the findings of Bennett and Janz (2007) who reported elevated total body triglycerides in young-of-the-year pike and other fishes living in lakes receiving effluent from the Key Lake uranium mill. Hepatic triglycerides and muscle glycogen were significantly greater in both exposure lakes compared to reference while hepatic glycogen was significantly elevated in the high exposure lake fish compared to the low exposure and reference lakes. Muscle triglycerides showed a significant difference among lakes by one-way ANOVA, however, Tukey's post hoc multiple comparison test did not detect a significant difference. According to Zar (1999), most post-hoc tests lack the power of one-way ANOVA at detecting a significant difference when one exists. This may be especially true in the present case since the ANOVA p value ($p = 0.041$) was close to alpha. An examination of the means and standard errors, however, indicate that the significant difference was in the direction of greater muscle triglycerides concentration for one or both of the exposure lakes versus the reference.

Other research on energy stores in fishes share some similarities and differences with results from the present study. Wild-caught sardines (*Sardinella gibbosa*) had on average 32.6 mg/g muscle triglycerides (Chaijan et al., 2006), a level that is approximately 30 times greater than in pike, whereas triglycerides in liver of sexually immature gilthead seabream (*Sparus auratus*) showed a mean of 34.7 µmol/g (or 30.7 mg/g assuming a molecular weight of 885.4 g/mol for triglycerides as triolein) (Sangiao-Alvarellos et al., 2006), which is very close to liver

triglycerides in pike from the exposure lakes. As for glycogen, Levesque et al. (2002) found levels in liver of yellow perch collected from metal-contaminated and reference lakes that were close to those seen in pike with values ranging from 4.5 to 11.0 mg/g. The levels of muscle glycogen reported in the literature were at least an order of magnitude higher (Nivedhitha et al., 1998) than concentrations measured in this study, indicating that muscle may not be important for glycogen storage in juvenile pike.

2.5.2 Prey items bioenergetics

Overall, juvenile pike invertebrate and vertebrate prey items did not differ among lakes in triglycerides levels. The results, therefore, do not support the hypothesis that fish from exposure lakes have greater energy stores because they are consuming a higher quality diet. The only exception was for waterboatman from the low exposure lake where a marked decrease in triglycerides compared to both the reference and high exposure lakes was seen whereas the reference and high exposure lakes did not differ. Waterboatman from the low exposure lake were also notably smaller than those collected from the other two lakes, thereby confounding interpretation of the results. Another notable difference in the triglycerides of prey items was with low exposure lake spottail shiners that had 24% greater whole body triglycerides compared to the reference although the difference was not significant. These results are consistent with Bennett and Janz (2007) who reported significantly greater whole body triglycerides in shiners from the low exposure lake compared to reference (approximately a 180% increase). Since the percent lipid content of fish has been shown to be size-dependent with small individuals having lower lipid levels than large individuals (Borcherding et al., 2007), the lack of a significant difference in the present study may reflect the difference in size of the fish between the two lakes where the low exposure shiners were significantly smaller than the reference. Although there was no significant difference in spottail shiner triglycerides in the present study, the significantly

elevated triglycerides reported in previous work (Bennett and Janz, 2007) could help explain the higher energy stores in low exposure lake pike. This explanation does not hold for high exposure pike bioenergetics since no spottail shiners appear to inhabit this lake (this thesis and Bennett and Janz, 2007). The absence of spottail shiners at the high exposure lake could be due to the lack of suitable habitat for this species owing to the lake's small size.

2.5.3 Nutrient enrichment and productivity

Nutrient enrichment of waterbodies has been more commonly associated with agricultural fertilizers (Gafner and Robinson, 2007), discharge from waste water treatment plants (DeBruyn et al., 2003) and pulp and paper mill effluents (Culp et al., 2003) than with discharges from metal-mining operations. Inputs of nitrogen and phosphorus from anthropogenic sources serve as nutrients to phytoplankton, the primary producers of aquatic ecosystems and thus the lowest trophic levels of aquatic food webs. The increase in primary productivity that results from nutrient enrichment (Peterson et al., 1985) has been linked to increases in the productivity of animals higher up the food web such as macroinvertebrates and fishes (Dillon et al., 2004). DeBruyn et al. (2003) showed an increased productivity of macroinvertebrate primary consumers downstream of sewage effluent discharge, while pulp and paper mill effluent increased periphyton biomass and the abundance of riverine larval and emerged aquatic insects (Culp et al., 2003). These effluents, as well as agricultural run-offs, are high in both phosphorus and nitrogen. A study by Sanford et al. (2005) examining the sole addition of nitrogenous compounds to freshwater ecosystems also observed evidence of nutrient enrichment. The addition of ammonium nitrogen to constructed wetlands was related to a significant increase in the abundance of larval mosquitoes and chironimids compared to the unsupplemented control (Sanford et al., 2005).

2.5.4 Abiotic environment of lakes near the Key Lake uranium mill

Contrary to the afore-mentioned studies, those looking at metal-mining effluents have typically reported impoverished food webs (Sherwood et al., 2000, 2002a; Iles and Rasmussen, 2005) as well as possible metabolic disruptions in fishes leading to depleted energy stores (Lohner et al., 2001; Levesque et al., 2002); observations that clearly do not reflect a nutrient enrichment effect. Similar to other water bodies receiving metal mining effluents, the lakes downstream of the Key Lake uranium mill discharge are characterized by elevated levels of metals, high conductivity, hardness, and a slightly depressed pH. In terms of metals, arsenic, molybdenum, nickel, selenium, and uranium among others, have been detected in water, sediment and fish tissues at greater concentrations in exposure lakes compared to reference (Pyle et al., 2001; Golder, 2005; Muscatello et al., 2006). Samples of muscle from pike collected for this study were analyzed for 22 trace metals as part of work examining oxidative stress and histopathology (Chapter 3). Over half the metals were elevated in one or both of the exposure lakes compared to the reference with eight of these being significantly greater.

In addition to metals, lakes receiving effluent from the Key Lake uranium mill demonstrate elevated levels of nitrogenous compounds (total ammonia and nitrate). Despite this fact, overall concentrations of nutrients in the form of both nitrogen and phosphorus tend to be relatively low for exposure and reference lakes. Total phosphorus is less than 10 µg/L (Bennett and Janz, 2007), a level that would suggest an oligotrophic system (Dillon et al., 2004), whereas nitrogen is also very low in comparison to levels of total nitrogen reported for 496 Ontario lakes (Dillon et al., 2004). Regardless of the relatively low nitrogen and phosphorus content, the ratio of these two nutrients at the reference lake points toward a nitrogen-limited system (Bennett, 2006) rather than one that is phosphorus-limited as is the case with most temperate freshwater ecosystems (Dillon et al., 2004). Therefore, the possibility of a nutrient enrichment of food webs in exposure

lakes compared to reference cannot be ruled out. Furthermore, potential benefits associated with added nutrients in the form of nitrogen could outweigh any possible detrimental effects of metals on food webs (Sherwood et al., 2000, 2002a; Iles and Rasmussen, 2005) and fish (Lohner et al., 2001; Levesque et al., 2002) bioenergetics.

2.5.5 Contaminants and elevated energy stores in fish

Toxicological studies with fish have demonstrated not only decreases (Campbell et al., 2003) but also increases (De Boeck et al., 1997; Nivedhitha et al., 1998; Lohner et al., 2001; Dorval et al., 2005; Ribeiro et al., 2005) in energy stores following exposure to contaminants including metals. Hepatic lipid and muscle glycogen stores were elevated in eels (*Anguilla anguilla*) collected from a coastal wetland contaminated with polycyclic aromatic hydrocarbons, organochlorine pesticides and heavy metals (Ribeiro et al., 2005). The authors of this study concluded that the accumulation of hepatic lipids may in part be due to sub-cellular injuries that were observed in histopathological analyses. The effects of contaminants on the bioenergetic status of fish was also seen in the form of elevated glycogen in muscle following carbamate fungicide exposure (Nivedhitha et al., 1998) and in liver due to sublethal copper concentrations (De Boeck et al., 1997) and chronic exposure to selenium-laden fly ash (Lohner et al., 2001). The biochemical and endocrine metabolic pathways regulating glycogen and lipid metabolism are complex and interrelated (Jobling, 1994; Strydom et al., 2006). Elevated energy stores have been associated with decreased plasma thyroxine, triiodothyronine and cortisol (Dorval et al., 2005) and impairment of enzymes involved in energy metabolism such as Krebs cycle enzymes (Strydom et al., 2006) could possibly be a contributing factor. Biochemical and endocrine measures in juvenile northern pike collected from exposure and reference lakes at Key Lake showed no differences in serum triiodothyronine and thyroxine concentrations and no difference in serum cortisol levels among lakes following a stress challenge. Serum glucose levels

following a stressor, however, was significantly lower in exposure fish compared to the reference (D. Janz, unpublished data). This points towards a possible impaired ability in exposure fish to mobilize glycogen into glucose following a stressor and may explain the elevated glycogen stores seen in this thesis. Elevated hepatic glycogen stores following a stress challenge has also been reported for yellow perch chronically exposed to metals (Gravel et al., 2005).

Another possible explanation behind the elevated bioenergetics of exposure pike is that the greater ionic strength of exposure waters (see Table 2.1 conductivity and total hardness) is reducing the energetic costs associated with ionoregulation (Wood et al., 2007). In other words, pike living in the reference lake may be expending more energy relative to exposure pike in order to recover ions lost to the environment (Takei, 1993).

2.5.6 Morphometrics

In terms of morphometrics, there were no significant differences among lakes in pike total length, weight, condition factor, and hepatosomatic index. These findings are similar to morphometric results for fry (Muscatello et al., 2006) and young-of-the-year (Bennett and Janz, 2007) pike from Key Lake but do not support results with adult female pike where those from exposure lakes had a significantly lower condition factor compared to the reference (Muscatello et al., 2006). In a study by Munkittrick and Dixon (1988) no difference in morphometrics was seen in young adult fish but a difference did emerge in older adults with those from metal-contaminated sites weighing less and being shorter. Other than the possible effects of metals (De Boeck et al., 1997; Sherwood et al., 2000; Levesque et al., 2002; Iles and Rasmussen, 2005), another major determinant of growth in poikilotherms, temperature (Jobling, 1994), varied only slightly among lakes (Golder, 2005; Bennett and Janz, 2007; this study) and could therefore help explain the lack of a difference in size.

Elevated energy stores would typically indicate that exposure pike are in better condition than reference pike (Adams, 1999) and could have a greater chance of surviving the winter when food resources are limited (Lemly, 1993a). However, the fish were collected in late spring/early summer, a time when energy could be allocated to growth rather than storage which raises the possibility that exposure and reference pike are under different energy allocation regimes.

2.5.7 Other food web mediated factors

Although the present study did not show evidence of nutrient enrichment in terms of elevated fat content in the prey, there could be other food web mediated effects due to nutrient enrichment. For instance, a greater abundance of organisms has been attributed to nutrient enrichment (Dillon et al., 2004). A study of benthic macroinvertebrates by Golder (2005), however, did not find a significant difference in macroinvertebrate density among exposure and reference lakes at the Key Lake uranium mill site. Another possibility is that prey items are larger rather than more abundant, as was seen with phosphorus loading of a tundra river which led to an increase in the size of aquatic insects (Peterson et al., 1985). Access to prey that are of a suitable size, rather than just sufficiently abundant, is important for fish to gain maximum energy benefits (Sherwood et al., 2002b). For young growing pike, there are probable energetic benefits with switching from invertebrate prey items to fish, once gape-size limitations are overcome. Therefore, the energetic cost of relying more on invertebrates and less on fish as prey items (Sherwood et al., 2002b), such as might be occurring in the high exposure lake, may be too great to be compensated for by invertebrates of larger size or a greater abundance of invertebrates. This is yet another important reason why the observed elevated energy stores is counter-intuitive, especially in the high exposure lake where species of forage fish such as spottail shiners appear to be absent (Bennett and Janz, 2007; this study). The results of the stomach content analyses, where only invertebrate prey items were detected in the high exposure

lake pike along with the absence of spottail shiners, supports the assertion that the high exposure lake lacks a diverse fish community. On the other hand, the lower diversity could mean reduced predation and competition with heterospecifics. There may also be less competition with conspecifics since an observed higher rate of deformity in exposure fry (Muscatello et al, 2006) could mean reduced survival and lower abundance of juvenile pike. The toll that predator avoidance and competition for resources with conspecifics and heterospecifics takes on the energy budget of juvenile pike could therefore be diminished if fish density and diversity is lower in the high exposure lake. As for the low exposure lake, further research is required to better characterize the fish community.

2.5.8 Parasitism and pike bioenergetics

Among the many possible explanations underlying the elevated energy stores in pike collected from lakes receiving effluent from the Key Lake uranium mill, this thesis also considered the potential energetic costs of parasitism. The rates of infection, as measured by prevalence and abundance, clearly followed the contamination gradient with the high exposure lake pike having no intestinal parasites and monogeneans, the low exposure lake pike having an intermediate rate of infection and the reference lake pike being 100% infected. Similarly, the mean abundance of parasites was greatest in reference lake pike, followed by the low and then the high exposure lake fish. In reference lake pike, the mean abundance of parasites was comparable to levels found in previous studies reporting intestinal parasites (Waite et al., 1990) and monogeneans (Wlasow et al., 2003) in wild-caught northern pike from uncontaminated lakes.

The prediction that parasitism could be influencing pike triglycerides and glycogen stores is supported by the negative correlations between parasitic infection and all measures of pike bioenergetics. In particular, intestinal parasite abundance may play an important role in

modulating pike bioenergetics since this measure of parasitism was significantly negatively correlated with all measures of pike bioenergetics. The abundance of monogeneans was significantly negatively correlated with pike triglycerides in both muscle and liver whereas intestinal parasite biomass, presumably the more biologically relevant measure of the energetic cost of parasitism to its host, was only significantly negatively correlated with muscle glycogen. The correlation between intestinal parasite biomass and muscle glycogen, however, had one of the greatest strengths of association as seen with the relatively high correlation coefficient (r_s = -0.63).

Studies using fish that report evidence of energy depletion by parasites as reflected in the concentrations of host energy storage macromolecules, such as glycogen and lipids, are not common in the literature. Rahimian (1998) observed that wild Atlantic herring (*Clupea harengus*) heavily infected with *Ichthyophonus hoferi* had drastically reduced lipid stores, Khan et al. (1990) reported reduced hepatic lipids in Atlantic cod (*Gadus morhua*) experimentally infected with a parasitic copepod, and Neff and Cargnelli (2004) showed that wild bluegill sunfish (*Lepomis macrochirus*) had decreased whole body neutral lipids with increased parasite load. Indirect measures of the energetic cost of parasitism on hosts have also been reported. Reduced activity of fish infected with monogeneans (Lopez, 1999) and a significant negative correlation between parasite density and condition factor in infected bluegill sunfish (*Lepomis macrochirus*) (Neff and Cargnelli, 2004) were interpreted to possibly be a result of depleted energy stores. Neff and Cargnelli (2004) further postulated a negative impact on paternity of infected fish as a result of the energetic burden of parasitic infection. In this study, correlations of parasitism with indirect measures of energy such as pike size, condition factor, and hepatosomatic index were only significant for parasite abundance versus pike total length and

weight. Instead of negative correlations, as was seen with pike bioenergetics and as would be expected if the parasites are presenting an energetic cost to their host, the correlations were positive. This suggests that morphometrics may not serve as good indicators of the energetic cost of parasitism in juvenile pike.

2.5.9 Parasitism and contaminants

Parasites are ubiquitous in nature (Marcogliese, 2005) and have been suggested to be useful indicator species of contaminant-impacted ecosystems (Mackenzie et al., 1995) and impaired food webs (Marcogliese et al., 2006). Numerous studies have shown the sensitivity of parasites to contaminants (Pietrock and Marcogliese, 2003). Certain parasites may be vulnerable partly due to their ability to accumulate contaminants such as metals (Sures et al., 1997). Cestodes, for example, have been shown to accumulate metals to levels that can exceed host tissue concentrations by several orders of magnitude (Sures et al., 1997). A study by Riggs et al. (1997) of approximately 3 000 fishes collected from a selenium-contaminated cooling reservoir showed that exposure to selenium was related to a decrease in abundance of the cestode *Bothriocephalus acheilognathi*. Other intestinal parasites, such as acanthocephalans, can also decrease in number in wild fish populations chronically exposed to metals (Munkittrick and Dixon, 1988). Parasites that are in direct contact with the environment such as monogeneans and the free-living stages of some intestinal parasites may be especially vulnerable to contaminants (Pietrock and Marcogliese, 2003).

The results of pike parasitic infection in the present study can be explained in one of several ways. A few species of cestodes and nematodes that infect pike require a fish intermediate host, such as spottail shiners, to complete their life cycle. Therefore, the absence of intestinal parasites in the high exposure fish may be an indirect effect owing to the lack of an intermediate host species. Since cestodes and numerous nematode species have a free-living stage, a potential

direct toxic action of the uranium milling effluent on the parasites is also a possibility. The vast majority of monogenean species, on the other hand, complete their life cycle with only one host. Their absence at the high exposure lake could more easily signify a direct toxic action of metals especially since these parasites live on the external surface of their host. To maintain their populations, all parasites need to eventually reproduce and infect new hosts. Therefore, another factor that may be contributing to the lower rate of infection of both intestinal parasites and monogeneans in the exposure lake pike could be a lower density of host populations. Host density is especially critical since the free-living transmission stage of some parasites is relatively short-lived (i.e. a matter of hours) (Mackenzie et al., 1995).

2.6 Conclusions

Juvenile northern pike inhabiting lakes receiving effluent from the Key Lake uranium mill have been shown in this study and in earlier work (Bennett and Janz, 2007) to contain elevated stores of energy storage macromolecules such as triglycerides and glycogen. Although the receiving waters are relatively elevated in nutrients in the form of nitrogen, the results of this study do not support the hypothesis that the greater energy stores in pike are due to food web enrichment. This is evidenced by the lack of significant differences among exposure and reference lakes in the concentrations of triglycerides in pike invertebrate and vertebrate prey items. There is, however, a possible effect of parasitism on pike bioenergetics as seen with the significant negative correlations between intestinal parasite and monogenean abundance and intestinal parasite biomass and certain measures of pike bioenergetics. In general, parasitism will not cause drastic changes in host energy reserves to the point of emaciation and disease due to nutrient deficiencies (Chappell, 1993), but more subtle effects, such as changes in triglycerides and glycogen reserves, may occur. Other factors that could be affecting pike bioenergetics, such

as metabolic and endocrine modulation by metals and ions cannot be ruled out and could be part of future investigations.

CHAPTER 3
3.0 ASSESSMENT OF OXIDATIVE STRESS AND HISTOPATHOLOGY IN JUVENILE NORTHERN PIKE (*ESOX LUCIUS*) INHABITING LAKES DOWNSTREAM OF A URANIUM MILL

3.1 Abstract

Lakes receiving effluent from the Key Lake uranium mill in northern Saskatchewan contain elevated metals, some of which are reported in the literature to be associated with increased reactive oxygen species (ROS) in cells and tissues causing oxidative stress. The potential for oxidative stress was assessed in juvenile northern pike (*Esox lucius*) collected from two exposure (high and low) and one reference lake near the Key Lake operation. Overall, the concentrations of total, reduced and oxidized glutathione and the ratio of oxidized to reduced glutathione did not differ significantly among exposure and reference pike liver and kidney, with the exception of low exposure pike kidney that had significantly elevated oxidized glutathione and ratio of oxidized to reduced glutathione. The concentration of by-products of lipid peroxidation (malondialdehyde and 4-hydroxyalkenal) was significantly elevated in reference versus high and low exposure kidney and in reference versus high exposure liver. The activity of the antioxidant enzyme glutathione peroxidase was greater in high exposure than reference liver. Histopathological evaluations of liver, kidney and gills showed greater kidney and gill pathology in reference lake pike whereas, for liver, hepatocyte morphology differed among lakes without any clear signs of pathology. Trace metal analyses of muscle showed that eight elements (arsenic, cobalt, copper, iron, molybdenum, selenium, thallium, uranium) were significantly elevated in exposure pike. These results provide only limited evidence of oxidative stress in

exposure pike tissues and no evidence of histopathology despite indications that metals, most notably arsenic and selenium, are bioaccumulating in tissue.

3.2 Introduction

Effluents from metal mining operations have demonstrated adverse effects on aquatic organisms that are often due to the toxicity of metals discharged into receiving waters (Levesque et al., 2003; Muscatello et al., 2006). The Key Lake uranium mill in northern Saskatchewan has been releasing metal contaminated effluents into a nearby system of lakes since 1982 and is expected to continue for the next several years (Conor Pacific, 2000). Past studies at the Key Lake operation have reported developmental abnormalities in larvae originating from resident northern pike (*Esox lucius*) populations (Muscatello et al., 2006) and an elevated incidence of mortality in fathead minnows (*Pimephales promelas*) during in situ toxicity testing (Pyle et al., 2001); the authors concluded exposure to metals as the causative factor. One of the mechanisms of toxic action of metals is via an increase in the concentrations of reactive oxygen species (ROS) in cells (Kelly et al., 1998). Reactive oxygen species are molecules derived from oxygen (Livingstone, 2001) that act as oxidants capable of damaging major biological macromolecules such as DNA, proteins and the phospholipids of cell membranes (Matés, 2000) which can ultimately lead to cell death. Hydrogen peroxide, superoxide anion (Shi et al., 2004) and hydroxyl radical (Halliwell and Gutteridge, 1999) are a few ROS that can be produced by metals. Reactive oxygen species also occur naturally in cells due to aerobic respiration and enzymatic activity and are known to fulfill important biological functions, such as cell signaling (Dröge, 2002; Wu et al., 2004). Due to the harmful potential of ROS, however, there exists a battery of antioxidant defense mechanisms for keeping concentrations to below toxic levels. If excessive ROS are produced as a result of exposure to contaminants such as metals, defense mechanisms can be overwhelmed and cellular damage may ensue. The imbalance between ROS production

and neutralization by antioxidant defenses, and the resulting damage to cellular macromolecules, has been termed "oxidative stress" (Kelly et al, 1998).

Arsenic, nickel and selenium have been reported to be elevated in sediment, water and fish tissues from lakes receiving effluent from the Key Lake uranium mill (Pyle et al., 2001; Klaverkamp et al., 2002; Golder, 2005; Muscatello et al., 2006). Elevated concentrations of hepatic and renal selenium, arsenic and nickel in white suckers (*Catostomus commersoni*) (Klaverkamp et al., 2002) as well as elevated concentrations of selenium in adult pike liver and kidney (Muscatello et al., 2006) have been observed. Intracellular reactions involved in the metabolism of inorganic arsenicals, arsenite and arsenate, can increase the concentrations of superoxide anion, hydrogen peroxide and hydroxyl radical (Liu et al., 2001; Kumagai and Pi, 2004; Shi et al., 2004). Arsenic can also indirectly contribute to elevated ROS through interactions with antioxidant defenses such as binding to reduced glutathione, converting reduced glutathione to oxidized glutathione as well as inhibiting the activities of glutathione reductase, an enzyme that reduces oxidized glutathione (Halliwell and Gutteridge, 1999; Vahter, 2002; Allen and Rana, 2004; Shi et al., 2004).

Reduced glutathione, a tripeptide (γ-glutamyl-cysteinyl-glycine), is normally present in tissue at relatively high concentrations (Wu et al., 2004) and is a key component of antioxidant defense mechanisms (Sayeed et al., 2003; Shi et al., 2004). Upon reacting with ROS, reduced glutathione is transformed into an inactive oxidized form that is subsequently reduced by glutathione reductase under normal conditions. Similar to arsenic, selenium can also promote the conversion of reduced glutathione into its oxidized form. This is accomplished through interactions with reduced glutathione rather than by interfering with glutathione reductase, as can occur with arsenic (Hoffman, 2002). There is also evidence demonstrating that organic and

inorganic forms of selenium oxidize reduced glutathione and generate superoxide anion in the process (Spallholz and Hoffman, 2002; Palace et al., 2004). Nickel is another metal that has been reported to be elevated downstream of the Key Lake uranium mill (Pyle et al., 2001; Golder, 2005) that can produce ROS (Chen et al., 2003; Kang et al., 2005). Since nickel is a transition metal and therefore able to change oxidation states (Ni^{2+} to Ni^{3+} and vice-versa), it can generate hydroxyl radical through the Fenton reaction (Kasprzak, 1991; Halliwell and Gutteridge, 1999).

An additional goal of this study was to determine whether or not juvenile northern pike inhabiting lakes receiving contaminated effluent from the Key Lake operation demonstrate histopathological lesions to liver, kidney and gills, known target organs of metal exposure (Levesque et al., 2003; Thophon et al., 2003; Teh et al., 2004). Histopathological lesions to cells and tissues reflect the concerted effects of multiple different mechanisms of action rather than only a few, as is typically the case with biochemical endpoints. Furthermore, whereas certain measures of oxidative stress are biomarkers of exposure, histopathological lesions are biomarkers of effects. A number of histopathologies in fish exposed to sublethal concentrations of metals are reported in the literature (Förlin et al., 1986; Paris-Palacios et al., 2000; Levesque et al., 2003; Teh et al., 2004). Teh et al. (2004) fed juvenile Sacramento splittails (*Pogonichthys macrolepidotus*) selenium in their diet and detected several hepatic alterations such as single cell necrosis, glycogen depletion and fatty vacuolar degeneration. Field studies have also reported histopathologies in fish chronically exposed to metal mining effluents. Levesque et al. (2003) observed structural changes in gills, thyroid and kidney of yellow perch (*Perca flavescens*) inhabiting lakes containing elevated levels of zinc, lead, cadmium, copper, and nickel.

In the present thesis, oxidative stress was evaluated in juvenile northern pike by measuring the levels of reduced and oxidized glutathione, the ratio of oxidized to reduced glutathione and the activity of glutathione peroxidase, a seleno-enzyme that neutralizes ROS such as organic and hydrogen peroxides (Matés, 2000). The extent of lipid peroxidation was also analyzed since ROS can attack and oxidize the fatty acid side-chains of phospholipids causing cell membrane dysfunction. Histopathological changes to liver, kidney and gills were assessed as an indication of the health status of juvenile northern pike exposed to the uranium milling effluent. Finally, in order to verify that metals in the water are in fact bioavailable to pike, the concentrations of trace metals in muscle were measured.

3.3 Materials and Methods

3.3.1 Study site

The Key Lake uranium mill (Cameco Corporation, Saskatoon, SK, Canada), located in northern Saskatchewan (57°13' N, 105°38' W) releases approximately 6 000 m^3 of treated effluent per day into a nearby system of lakes. Sampling took place at three lakes located in close proximity to the Key Lake operation. Two of the study lakes are located downstream of the effluent discharge and follow the contamination gradient (i.e. high and low exposure). Unknown Lake, located approximately 2 km downstream of the uranium mill discharge will be referred to as the high exposure lake whereas Delta Lake, the low exposure lake, is approximately 10 km from the discharge. David Lake, the reference lake, is located upstream of the effluent discharge and does not receive contaminants from any known source. All three lakes are part of the same catchment and are connected by David Creek.

3.3.2 Field collections

Juvenile northern pike entering their second year of life (1+) were collected between June 16 and 23, 2005. Nine pike were obtained from the high exposure lake and 12 from both the low

exposure and reference lakes. Pike were stunned using a Smith-Root backpack electrofisher (Smith-Root, Vancouver, WA, USA) and captured with dip nets. They were kept in small coolers filled with lake water prior to being sacrificed with a lethal dose of 3-aminobenzoic acid ethyl ester solution (MS 222, Sigma-Aldrich, Oakville, ON, Canada) and dissected on-site.

Dissections included removal and sectioning of the liver into 6 portions; 5 portions, retained for biochemical analyses, were placed into separate cryovials and immediately frozen on dry ice and one portion was kept for histopathological analyses. Prior to sectioning, livers were rinsed with a buffer solution of Tris-KCl to remove any bile that may have leaked from the gall bladder and gently blotted dry with a Kimwipe®. One liver portion frozen in a cryovial was used for bioenergetic analyses (see Chapter 2). All tissues collected for histopathology were fixed in Bouin's fixative for approximately 24 hours, washed three times in 70% ethanol, and stored in 70% ethanol until processing. Tissues for biochemical analyses were kept on dry ice throughout the duration of the field sampling (eight days) then transferred to a -80˚C freezer upon return to the University of Saskatchewan. Other dissections included removing the trunk kidney which was sectioned in two. One half was placed in a cryovial and frozen on dry ice while the other half was fixed for histopathological analyses. The gills from the left side of the head were removed and fixed for histopathological analyses.

Weight (mg) and total length (cm) were recorded from each fish prior to dissection. Due to difficulties obtaining an accurate liver weight in the field, weights were obtained from each individual portion upon return to the University of Saskatchewan and added to obtain total liver weight. Cleithra were removed and sent to the Ontario Federation of Anglers and Hunters (Peterborough, ON, Canada) for age determination by a fisheries technician. Water quality variables such as specific conductivity (µS/cm), temperature (˚C), dissolved oxygen (mg/L), and

pH were measured once at each lake at a depth of approximately one meter using a YSI 650 Multi-parameter Display System equipped with a 600 QS probe (Yellow Springs, OH, USA). Results for body weight, condition factor, hepatosomatic index (HSI), total length and water quality variables are reported in Chapter 2.

3.3.3 Laboratory analyses

3.3.3.1 Glutathione

Concentrations of reduced and oxidized glutathione were determined using a colourimetric assay kit purchased from Cayman Chemical (Ann Arbor, MI, USA) and based on methods developed by Tietze (1969) and Griffith (1980). The assay involves reacting reduced glutathione with 5,5'-dithiobis-2-nitrobenzoic acid (DTNB or Ellman's reagent) and measuring the resultant increase in absorbance at 405 nm spectrophotometrically. Prior to assaying, liver and kidney samples were homogenized in a buffer of 0.4 M 2-(N-orpholino) ethanesulphonic acid, 0.1 M phosphate and 2 mM EDTA (pH 6.0) and appropriately diluted to fall within the range of the standard curve which was developed using reduced glutathione. In order to minimize interference by other thiols that may be present in the sample, the kinetic rather than the endpoint method was used for the assay. The concentration of total glutathione (reduced plus oxidized) was determined by converting all of the oxidized glutathione present in the sample to its reduced form via glutathione reductase. A slightly different procedure, which involves excluding reduced glutathione from the assay reaction by derivatizing it with 2-vinylpyridine, was performed to determine the concentration of oxidized glutathione alone. The concentration of reduced glutathione and the ratio of oxidized to reduced glutathione was calculated from the results obtained for total and oxidized glutathione. The concentrations of reduced and oxidized glutathione can provide information on whether or not the cells are experiencing oxidative stress or are compensating by inducing reduced glutathione and/or exporting oxidized glutathione out

of cells. Calculating the ratio of oxidized to reduced glutathione also provides useful information due to the importance of the relative concentration of these two forms of glutathione in determining oxidative stress (i.e. an elevated ratio suggests oxidative stress).

3.3.3.2 Glutathione peroxidase activity

The activity of glutathione peroxidase was determined in liver using a kit purchased from Cayman Chemical and based on a method by Paglia and Valentine (1967). Liver samples used for the assay were homogenized in a buffer solution of 50 mM Tris-HCl (pH 7.5), 5 mM EDTA and 1 mM of dithiothreitol. In the assay, glutathione peroxidase present in the sample catalyzes the conversion of cumene hydroperoxide into an alcohol and water. The catalytic site, which becomes oxidized in the process, is reduced by reduced glutathione which is in turn reduced by NADPH. The activity of glutathione peroxidase is therefore proportional to the oxidation of NADPH and was calculated using the extinction coefficient of NADPH to NADP+ which is 0.00373 μM^{-1}. Cumene hydroperoxide, reduced glutathione and NADPH were included with the kit and were added to each sample for the assay. In order to have an estimate of background absorbance, one sample blank from each lake was run. The procedure for the sample blank was identical to the other samples except that the addition of cumene hydroperoxide was omitted. A positive control of bovine erythrocyte glutathione peroxidase was also included with each assay run.

3.3.3.3 Lipid peroxidation

Lipid peroxidation in kidney and liver samples was determined by measuring the concentrations of malondialdehyde and 4-hydroxyalkenal, aldehyde products of lipid peroxidation. The assay was performed using a commercial kit from Oxis International Incorporated (Foster City, CA, USA) and is described in detail in Esterbauer et al. (1991). In summary, the chromogen, n-methyl-2-phenylindole, reacts with one molecule of either

malondialdehyde or 4-hydroxyalkenal to produce a colour with an absorbance at 586 nm. Liver and kidney were homogenized in a buffer solution of 20 mM Tris-HCl (pH 7.4) and in 10 μL per mL of homogenate of 0.5 M butylated hydroxytoluene in acetonitrile (Sigma). Butylated hydroxytoluene acts as an antioxidant and prevents the generation of new lipid peroxides during homogenization. Samples were also homogenized under low light to minimize photooxidation. Prior to assaying, samples were appropriately diluted in buffer to fall within the range of the standard curve. Malondialdehyde was used as standard since it is typically present in tissues at a 10 times greater concentration than 4-hydroxyalkenal. Sample blanks were performed on three samples to determine the degree of absorbance that was not due to malondialdehyde and 4-hydroxyalkenal. These samples were assayed in the same manner as the others except that the chromogen was omitted from the reaction mixture.

3.3.3.4 Protein

Protein concentrations in liver and kidney samples were determined following the procedure of Lowry et al. (1951) using bovine serum albumin as standard.

All assays, except for the lipid peroxidation assay, were performed on a SpectraMAX 190 spectrophotometer (Molecular Devices Corporation, Sunnyvale, CA, USA). Determinations of malondialdehyde and 4-hydroxyalkenal as measures of lipid peroxidation was performed using a Beckman Coulter DU 640 spectrophotometer (Beckman Coulter, Inc., Fullerton, CA, USA).

3.3.4 Histopathology

Liver, kidney and gills were dehydrated in a graded series of ethanol, paraffin-embedded and sectioned to a thickness of 5 μm. Sections were mounted onto slides and stained with haematoxylin and eosin. Semi-quantitative histopathological analyses were performed using an Olympus model Vanox-T light microscope (Olympus America Incorporated, Melville, NY, USA) whereas an Olympus model BH-2 light microscope mounted with a Zeiss 7.2 megapixels

Cyber-shot camera (Carl Zeiss Inc., Thornwood, NY, USA) and Image-Pro Discovery software version 4.5 (Media Cybernetics Inc., Silver Spring, MD, USA) were used for quantitative analyses. All histopathological examinations were performed in a blind manner to minimize the risk of experimenter bias.

3.3.4.1 Liver

The focus of the liver histopathological evaluations were parenchymal hepatocytes since these cells are responsible for accumulating and metabolizing contaminants such as metals and are the most abundant cell type in the teleost liver, constituting approximately 85% of the total volume (Hinton and Laurén, 1990). Semi-quantitatively and quantitatively scored endpoints were determined from a preliminary examination of slides. Cytoplasmic vacuolation was semi-quantitatively scored on a scale from - to +++ (- no vacuolation; + slight vacuolation; ++ moderate vacuolation; +++ maximum vacuolation). Scoring was done on four replicate fields of view at 400X magnification. The transsectional area of 30 hepatocytes was also measured at 1000X magnification. Hepatocytes chosen for quantifying area were those that had a complete and clearly visible cell membrane. A number of different fields of view at 1000X magnification were examined for each individual pike liver slide until 30 hepatocytes had been measured.

3.3.4.2 Kidney

Similar to liver, a preliminary examination of kidney was performed to determine the endpoints to be analyzed. Quantitative measures obtained from four replicate fields of view at 400X magnification included: number of pyknotic and fragmented nuclei and number of dilated tubules. A dilated tubule was defined as any tubule, proximal or distal, having a dilated lumen and low epithelial cell height. Most dilated tubules also had one or more epithelial cells with pyknotic or fragmented nuclei and tended to be stained more darkly purple. Quantifying the number of pyknotic and fragmented nuclei occasionally required viewing very small nuclei at

1000X magnification. Semi-quantitative scoring of acellular glomerular spaces and the thickness of Bowman's capsule were obtained from 10 renal corpuscles per kidney at 400X magnification. Renal corpuscles used in the evaluations were the first 10 encountered, starting from one end of the slide, that were artifact free, sectioned approximately through the center of the corpuscle and that were completely developed (as opposed to newly formed, "nascent" corpuscles that stain dark blue). A scale from - to +++ was used to assess the extent of acellular glomerular spaces (- no empty spaces; + slight amount of empty spaces; ++ moderate amount of empty spaces; +++ maximum empty spaces). Acellular glomerular spaces were a result of either capillary dilation or cell death. The thickness of Bowman's capsule was also semi-quantitatively scored (- capsule barely visible; + slightly thickened; ++ moderately thickened; +++ maximally thickened).

3.3.4.3 Gills

Evaluation of the gills included two measures that reflect its functions in exchanging gases and ions with the ambient water: primary filament epithelial padding height and secondary lamellar width. Four measurements of epithelial padding height were taken from four different primary filaments at the half-way point between two adjacent lamellae. Secondary lamellae width was measured across the center of the cell body of the first pillar cell encountered starting from the bottom of the lamellae. Pillar cells morphology consists of extensions that project away from the cell body which delineate capillary channels (Takashima and Hibiya, 1995). The location for measurements was chosen at pillar cells rather than at capillaries since the lumen of the latter can sometimes change size (Takashima and Hibiya, 1995) and possibly increase variability of measurements. The primary filaments chosen for epithelial padding height and lamellar width were the first four encountered starting from one end of the gill arch that were artifact free and sectioned in such a way that the secondary lamellae were visible. Measurements

of primary filament epithelial padding height and secondary lamellar width were done at 1000X magnification.

3.3.5 Trace metal analyses

Samples of muscle tissue were removed from frozen pike carcasses using nitric acid-washed teflon coated instruments and a scalpel. Samples were heated at 60° C for 24 hours in nitric acid washed scintillation vials. The dried muscle samples were sent to Prairie Diagnostic Services, Saskatoon, SK, Canada for analysis of trace metal concentrations which were reported as dry weight. Analyses were performed on an ICP-MS (X series, Thermo Electron Corporation, Waltham, MA, USA) with a dynamic reaction cell attached and a detection limit of < 0.3 µg/g. Quality assurance and quality control was evaluated using standard reference materials.

3.3.6 Statistical analyses

All data were tested for normality with the Shapiro-Wilk test and for equality of variance with the Levene test using SYSTAT 11 (SPSS Inc., Chicago, IL, USA). Data that did not meet the assumptions were log10 or square-root transformed. Data that were normally distributed and exhibited homogeneity of variance were tested for significant differences among lakes by one-way ANOVA followed by a Tukey test, as appropriate. Data that violated the assumptions following transformation as well as semi-quantitative histopathological data were tested using Kruskal-Wallis one-way ANOVA followed by a Dunn's test as appropriate. Correlation between hepatic glutathione peroxidase activity and the concentration of selenium in muscle was tested for significance using Spearman rank correlation (r_s). All statistical analyses were carried out using SigmaStat 3.1 (SPSS Inc., Chicago, IL, USA) software with an alpha value of 0.05.

For the lipid peroxidation assay, six determinations of a pooled sample were used to assess intra-assay variability whereas the same pooled sample was run six more times on a separate occasion to evaluate inter-assay variability. Intra-assay variability was also assessed for the

glutathione assay. For all assays, samples that had a coefficient of variation greater than 10% between duplicate absorbance readings were re-analyzed.

3.4 Results

3.4.1 Biomarkers of oxidative stress

3.4.1.1 Glutathione

Overall, measures of glutathione in liver and kidney tissues indicated that juvenile northern pike inhabiting lakes receiving effluent from the Key Lake operation may be experiencing limited oxidative stress (Figures 3.1, 3.2 and 3.3). Concentrations of total glutathione, reduced and oxidized glutathione and the ratio of oxidized to reduced glutathione in liver did not differ significantly among lakes ($p > 0.05$) (Figures 3.1 and 3.3). For kidney, pike from the low exposure lake had significantly greater oxidized glutathione (mean ± SEM; 0.196 ± 0.014 μmol/g) compared to the reference lake (0.135 ± 0.011) (Figure 3.2A) and a significantly elevated ratio of oxidized to reduced glutathione (0.365 ± 0.048 μmol/g) compared to the reference (0.240 ± 0.019 μmol/g) and high exposure (0.249 ± 0.026 μmol/g) lakes (Figure 3.2B). A trend towards greater total glutathione in liver and kidney of both exposure lakes was observed but was not significant.

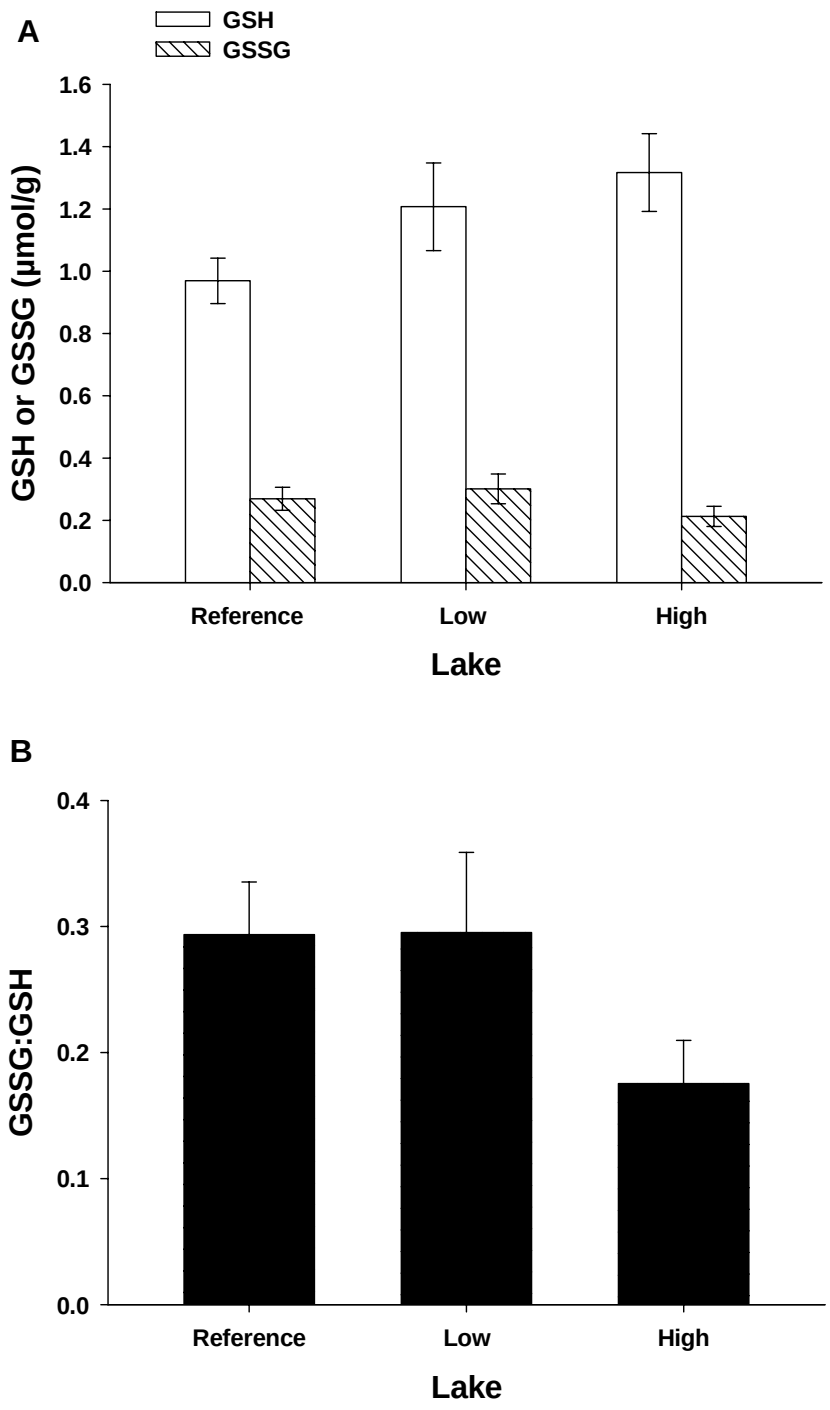

Figure 3.1 Reduced (GSH) and oxidized (GSSG) glutathione (µmol/g) (A) and GSSG:GSH (B) in liver of 1+ northern pike (*Esox lucius*) collected from two lakes receiving uranium milling effluent (low and high exposure; n = 12 and n = 9, respectively) and one reference lake (n = 12). Data are expressed as means ± SEM.

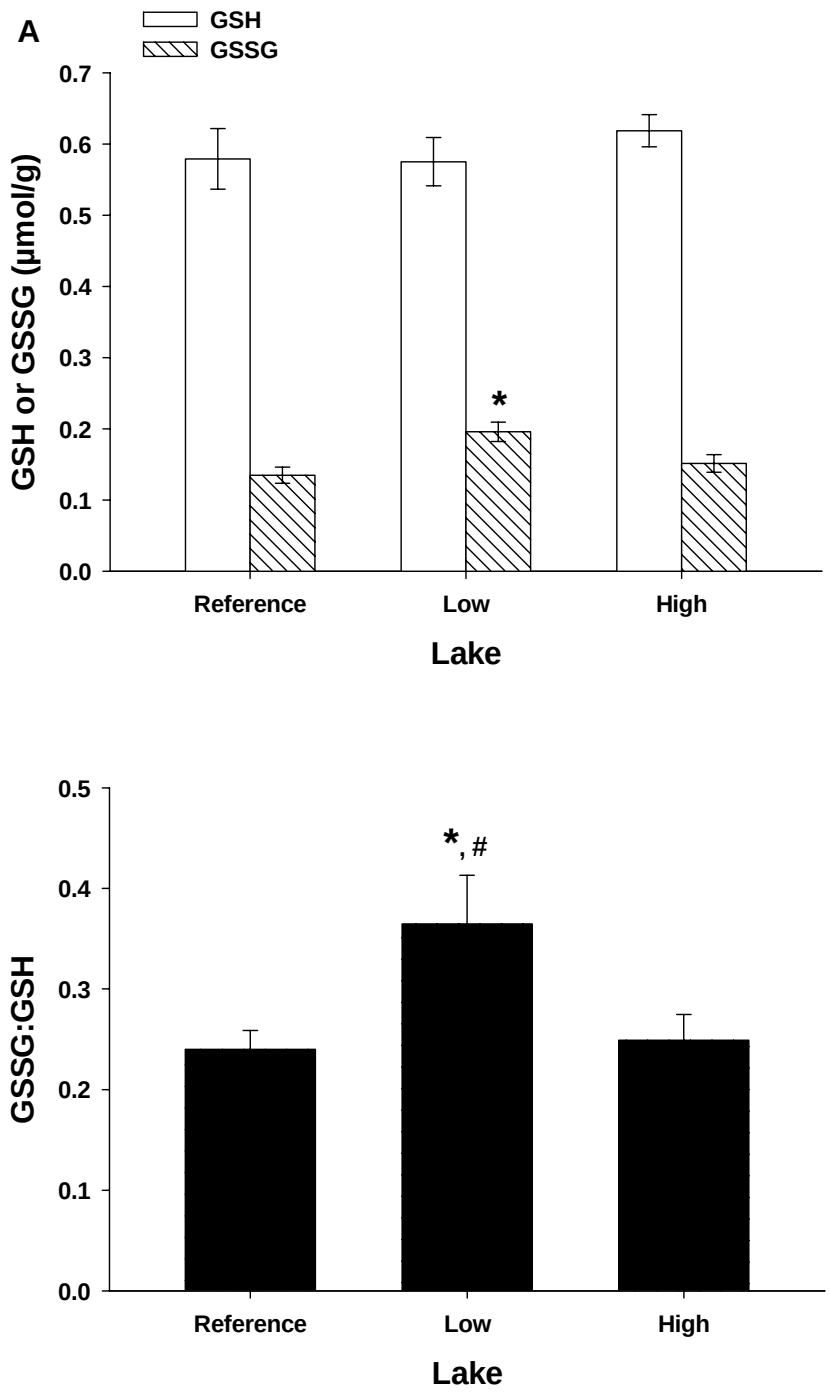

Figure 3.2 Reduced (GSH) and oxidized (GSSG) glutathione (µmol/g) (A) and GSSG:GSH (B) in kidney of 1+ northern pike (*Esox lucius*) collected from two lakes receiving uranium milling effluent (low and high exposure; n = 12 and n = 9, respectively) and one reference lake (n = 12). Data are expressed as means ± SEM. * shows a significant difference from the reference and # a significant difference from the high exposure lake using one-way ANOVA followed by Tukey's test ($p < 0.05$).

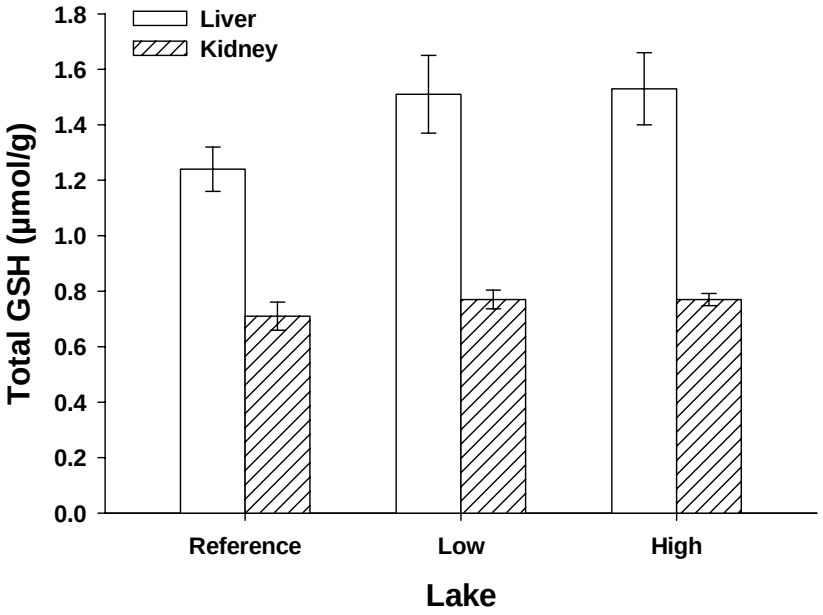

Figure 3.3 Total glutathione (GSH) (µmol/g) in liver and kidney of 1+ northern pike (*Esox lucius*) collected from two lakes receiving uranium milling effluent (low and high exposure; n = 12 and n = 9, respectively) and one reference lake (n = 12). Data are expressed as means ± SEM.

3.4.1.2 Glutathione peroxidase

Due to a technical oversight while performing the glutathione peroxidase activity assay, the concentration of protein was not determined for four samples from each of the reference and low exposure lakes and three samples from the high exposure lake. In these cases, glutathione peroxidase activity was normalized using the mean protein concentration calculated from the protein concentrations obtained from the remaining samples for each lake.

Liver glutathione peroxidase activity was significantly greater in pike collected from the high exposure lake ($p = 0.007$; 1.52 ± 0.19 µmol NADPH/min/mg protein) compared to the reference (0.935 ± 0.09 µmol NADPH/min/mg protein) but not the low exposure lake (1.25 ± 0.08 µmol NADPH/min/mg protein) (Figure 3.4). A significant positive correlation existed between the concentrations of selenium in muscle (see page 79) and hepatic glutathione peroxidase activity ($p = 0.012$; $r_s = 0.43$) (Figure 3.5).

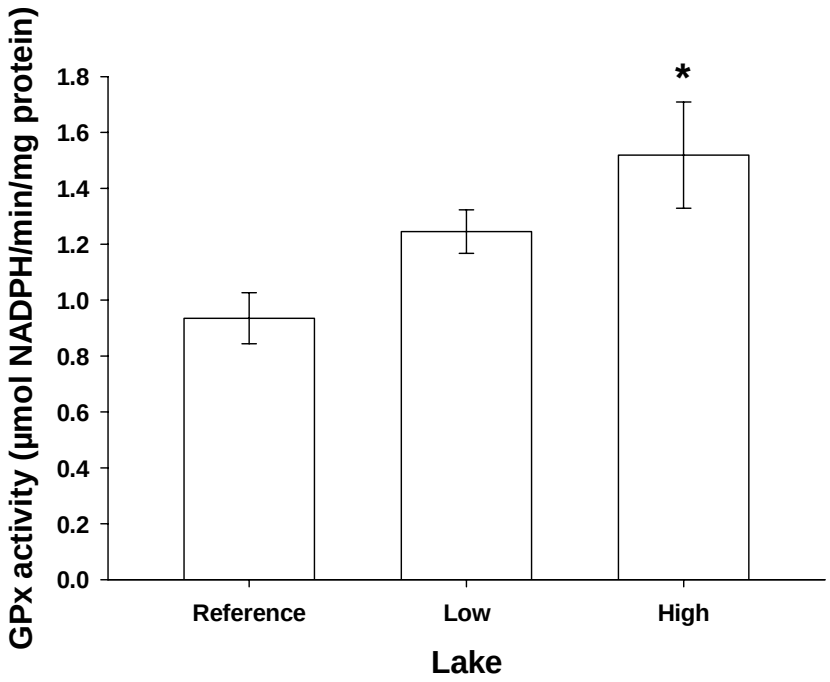

Figure 3.4 Glutathione peroxidase (GPx) activity (µmol NADPH/min/mg protein) in liver of 1+ northern pike (*Esox lucius*) collected from two lakes receiving uranium milling effluent (low and high exposure; n = 12 and n = 9, respectively) and one reference lake (n = 12). Data are expressed as means ± SEM. * indicates a significant difference from the reference lake using one-way ANOVA followed by Tukey's test ($p < 0.05$).

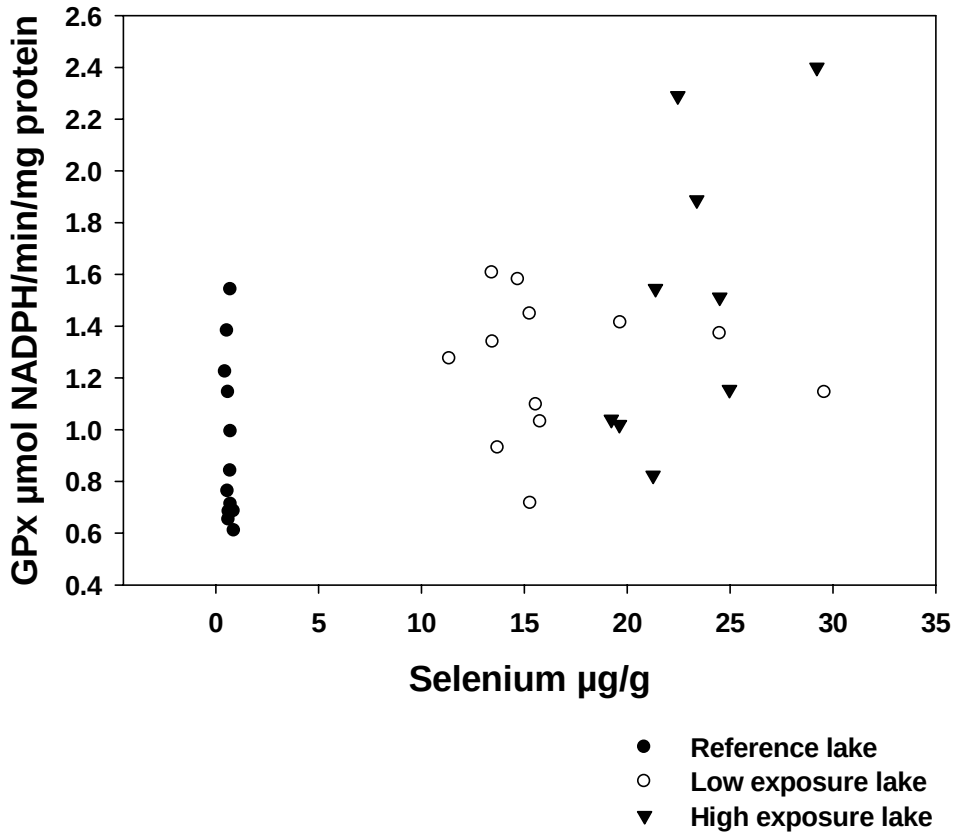

Figure 3.5 Scatter plot of glutathione peroxidase (GPx) activity (µmol NADPH/min/mg protein) in liver versus selenium concentration (µg/g dry weight) in muscle of 1+ northern pike (*Esox lucius*) collected from two lakes receiving uranium milling effluent (low and high exposure; n = 12 and n = 9, respectively) and one reference lake (n = 12). A significant positive correlation was detected by Spearman rank correlation (r_s = 0.43; p = 0.012).

3.4.1.3 Lipid peroxidation

Concentrations of malondialdehyde and 4-hydroxyalkenal, molecules derived from lipid peroxidation, were significantly greater in pike liver (p = 0.03) from the reference (0.195 ± 0.03 nmol/mg protein) compared to the high (0.128 ± 0.04 nmol/mg protein) but not the low (0.116 ± 0.01 nmol/mg protein) exposure lakes (Figure 3.6). Malondialdehyde and 4-hydroxyalkenal concentrations in kidney were also significantly greater (p = 0.002) for the reference (0.208 ± 0.01 nmol/mg protein) compared to both the low (0.171 ± 0.009 nmol/mg protein) and high (0.156 ± 0.009 nmol/mg protein) exposure lakes (Figure 3.6).

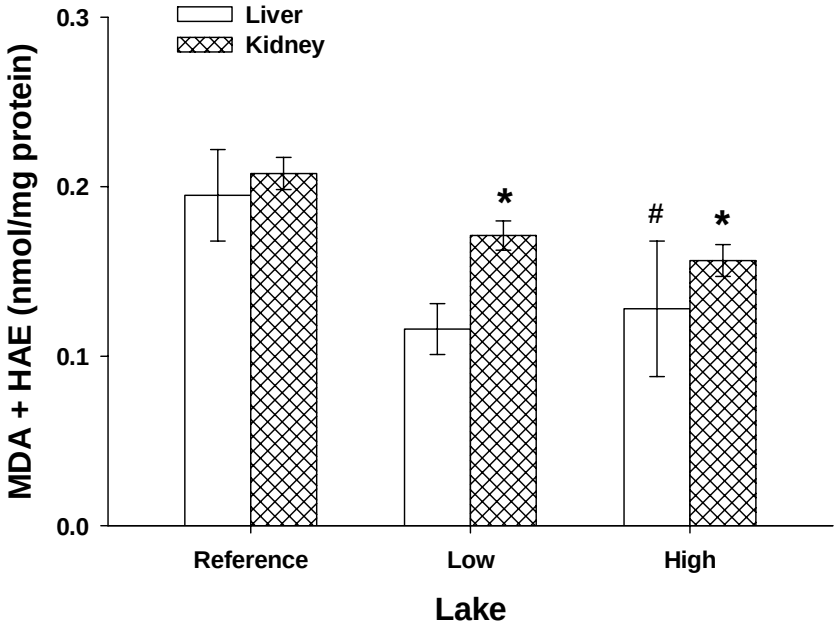

Figure 3.6 Concentrations of malondialdehyde (MDA) and 4-hydroxyalkenal (HAE) (nmol/mg protein), indirect measures of lipid peroxidation, in liver and kidney of 1+ northern pike (*Esox lucius*) collected from two lakes receiving uranium milling effluent (low and high exposure; n = 12 and n = 9, respectively) and one reference lake (n = 12). Data are expressed as means ± SEM. # indicates a significant difference from reference for liver using Kruskal-Wallis one-way ANOVA on Ranks followed by Dunn's test. * indicates a significant difference from reference for kidney using one-way ANOVA followed by Tukey's test.

3.4.2 Assay performance

Intra-assay variability was 3.5% and 3.0% for determinations of total and oxidized glutathione, respectively. The lipid peroxidation assay intra-assay variability was 4.5% and 8.7% for inter-assay variability. An intra-assay variability of 5.2% and an inter-assay variability of 4.8% was obtained for the protein assay. Intra- and inter-assay variability was not determined for the glutathione peroxidase assay however all samples were run within a three hour period from a single preparation of reagents, thereby minimizing possible variability. The lipid peroxidation assay sample blanks for kidney and liver showed that absorbance due to molecules other than malondialdehyde and 4-hydroxyalkenal was contributing approximately 15 to 20% to the actual sample absorbance. The only exception was for one kidney sample blank that

comprised only 1.3% of the actual sample absorbance. With respect to the glutathione peroxidase activity assay, absorbance due to molecules other than NADPH contributed approximately 2 to 5% of the actual sample absorbance.

3.4.3 Histopathology

3.4.3.1 Liver

The degree of hepatocyte vacuolation was significantly greater for the high exposure lake ($p < 0.001$) compared to the reference and low exposure lakes (Figure 3.7 and Table 3.1). Hepatocyte area followed the same trend with the high exposure lake (6.8 ± 0.3 μm) being greater than the low exposure lake and the low exposure (5.9 ± 0.4 μm) greater than the reference lake (5.2 ± 0.2 μm), albeit that only the high exposure and reference lakes differed significantly ($p = 0.008$).

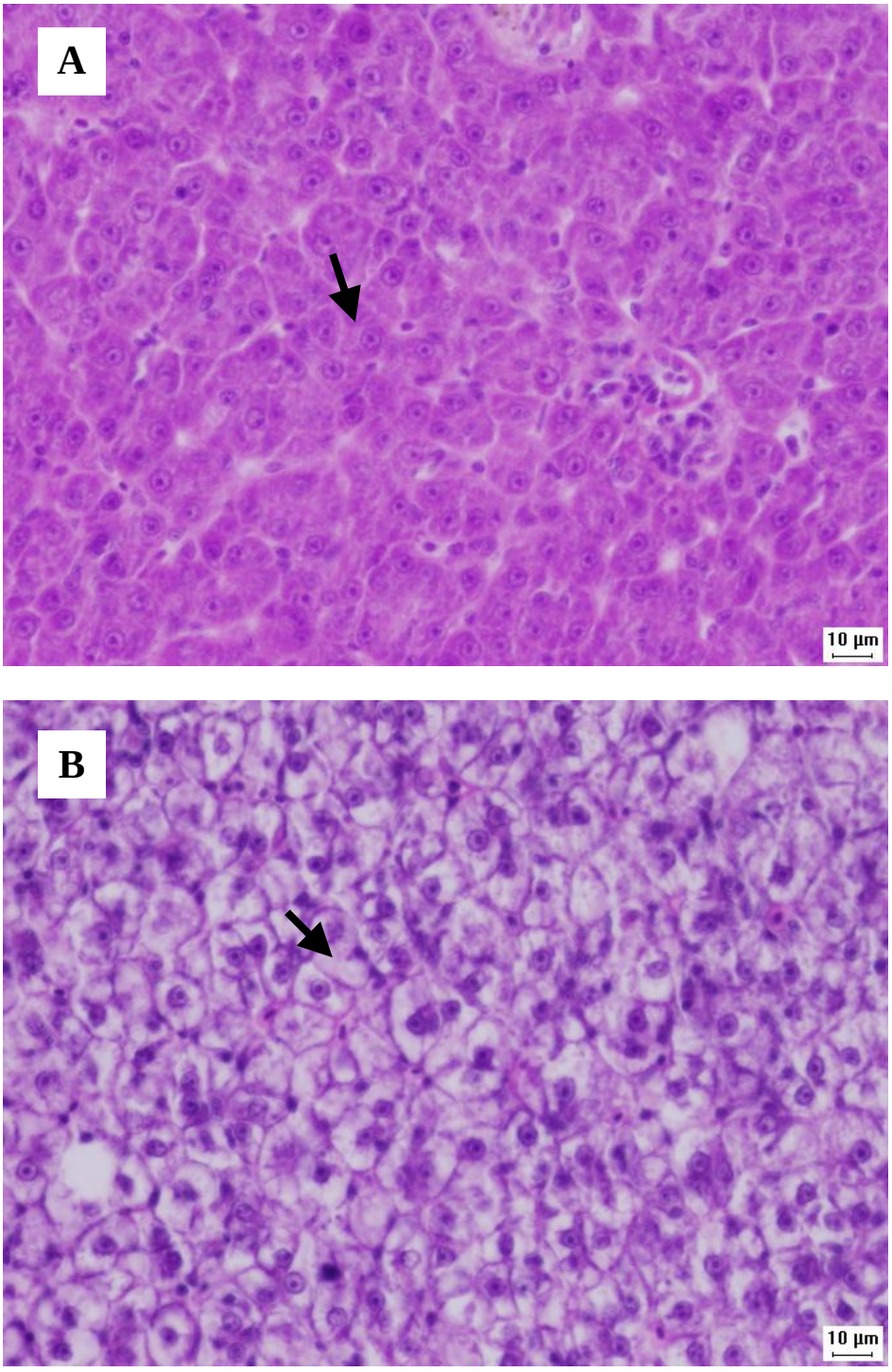

Figure 3.7 Hepatocytes from the livers of 1+ juvenile northern pike (*Esox lucius*) collected from a reference lake (A) and a lake receiving effluent from the Key Lake uranium mill (high exposure lake) (B). Livers were stained with haematoxylin and eosin and are shown at approximately 400X magnification. Arrows point to the cytoplasm of hepatocytes which represent a score of "–" or no vacuolation (A) and "+++" or maximum vacuolation (B).

3.4.3.2 Kidney

Significant differences among lakes were found for both quantitative measures of kidney histopathology: number of pyknotic and fragmented nuclei ($p < 0.001$) and number of dilated tubules ($p = 0.003$) (Figure 3.8 and Table 3.1). The number of pyknotic and fragmented nuclei was significantly greater for the reference (23 ± 10 per field of view) compared to the low (1.9 ± 0.9 per field of view) but not the high (6.1 ± 2 per field of view) exposure lakes. The number of dilated tubules followed the same trend with the reference (1.8 ± 0.6 per field of view) being significantly greater than the low (0.083 ± 0.04 per field of view) but not the high (0.6 ± 0.3 per field of view) exposure lakes. The semi-quantitative measure of capsule thickness was significantly greater for the reference compared to the high exposure lake ($p = 0.013$) (Figure 3.9 and Table 3.1), while the semi-quantitative evaluation of acellular glomerular spaces did not differ significantly among lakes ($p = 0.072$) (Figure 3.8 and Table 3.1).

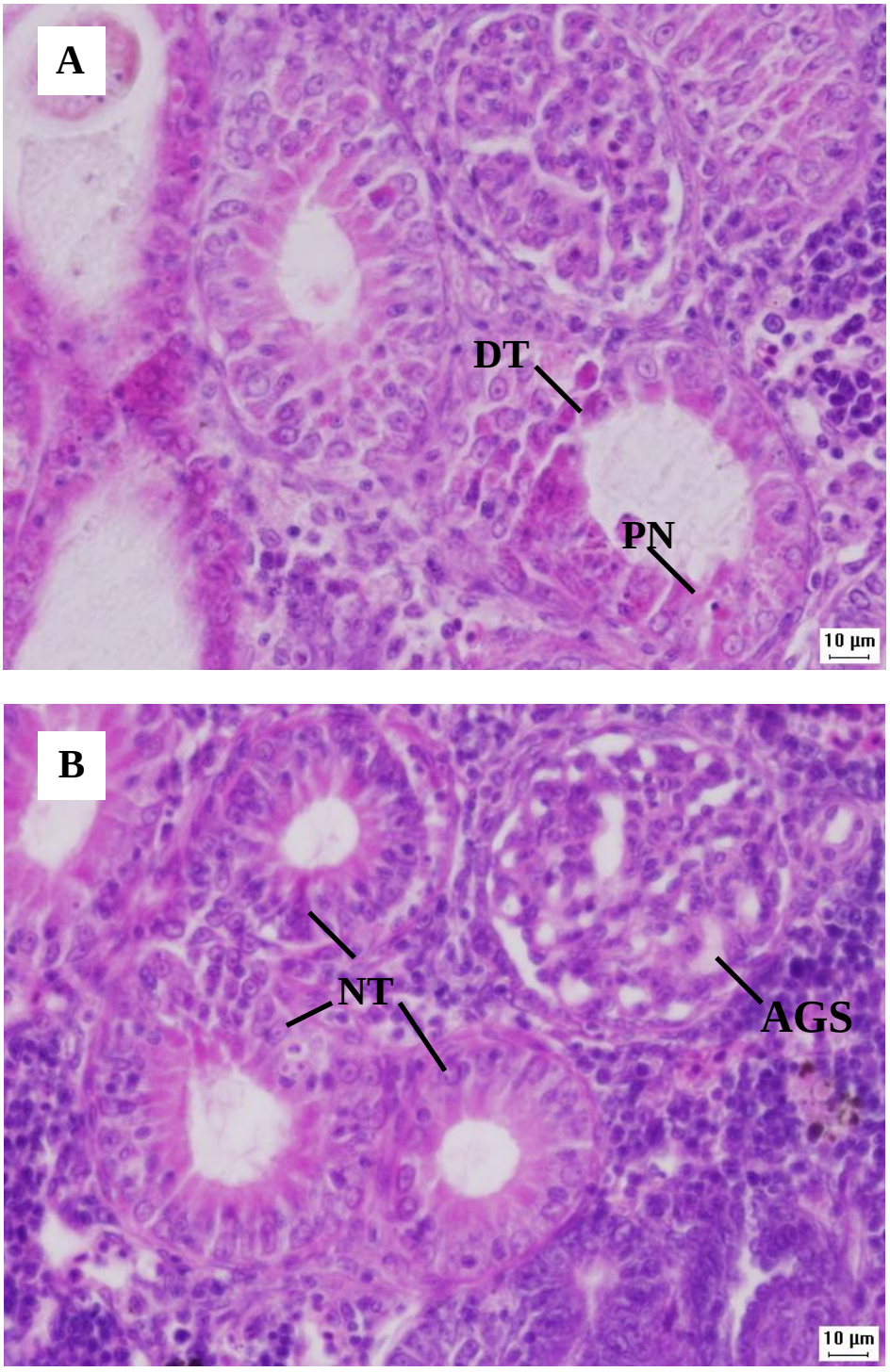

Figure 3.8 Tubules and glomerular corpuscles from the kidneys of 1+ juvenile northern pike (*Esox lucius*) collected from a reference lake (A) and a lake receiving effluent from the Key Lake uranium mill (high exposure lake) (B). Kidneys were stained with haematoxylin and eosin and are shown at approximately 400X magnification. DT: tubule with dilated lumen; PN: pyknotic nucleus; NT: normal tubules; AGS: acellular glomerular space.

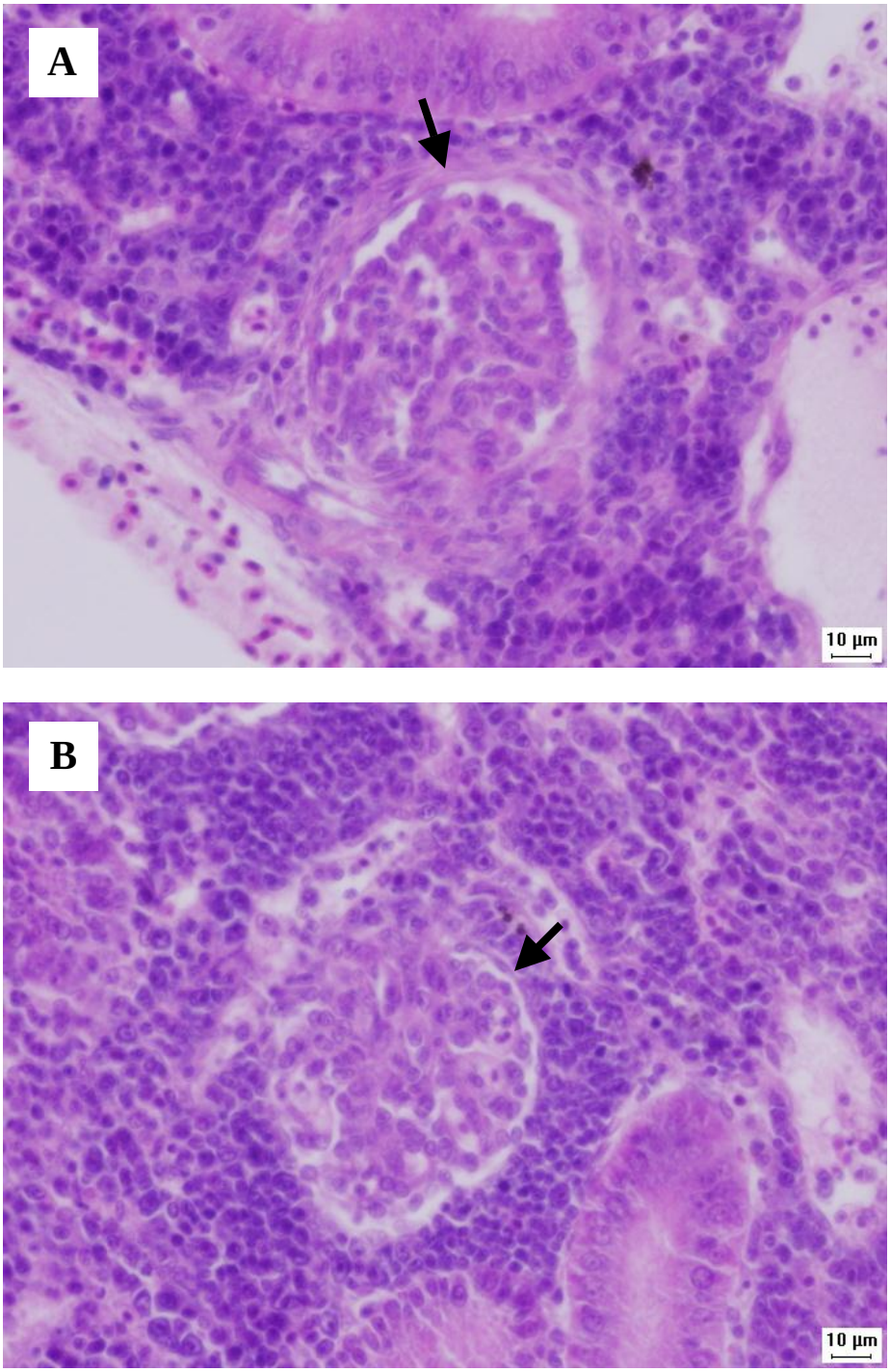

Figure 3.9 Glomerular corpuscles from the kidneys of 1+ juvenile northern pike (*Esox lucius*) collected from a reference lake (A) and a lake receiving effluent from the Key Lake uranium mill (low exposure lake) (B). Kidneys were stained with haematoxylin and eosin and are shown at approximately 400X magnification. Arrows point to Bowman's capsules which represent a score of "+++" or maximally thickened (A) and "–" or barely visible (B) in terms of thickness.

3.4.3.3 Gills

Primary filament epithelial padding height was significantly greater ($p < 0.001$) for the reference (33 ± 1 μm) compared to the low (24 ± 2 μm) and high (22 ± 2 μm) exposure lakes. The width of the secondary lamellae, on the other hand, did not differ significantly among lakes ($p = 0.25$) (Figure 3.10 and Table 3.1).

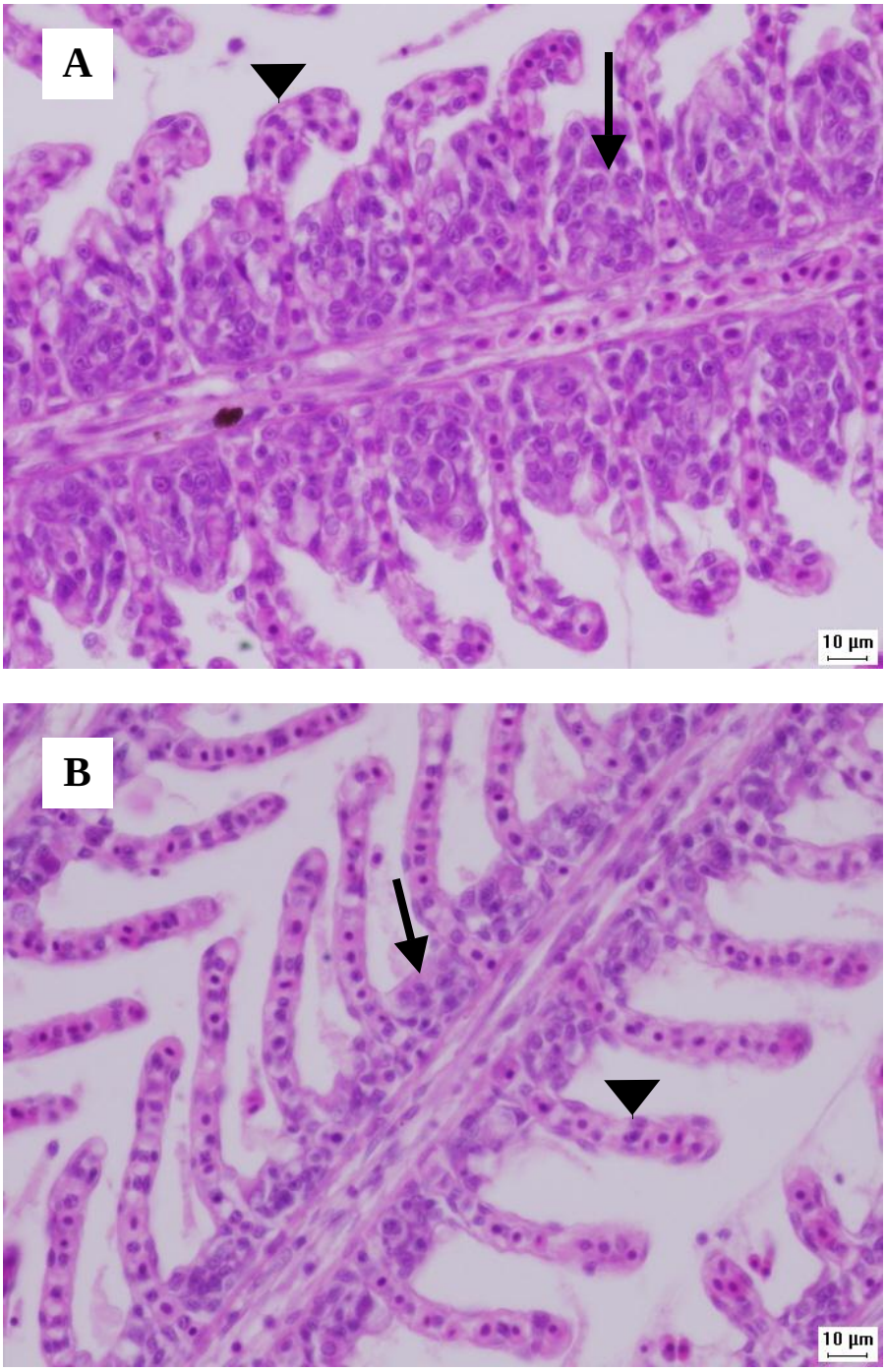

Figure 3.10 Gill primary filaments and secondary lamellae from 1+ juvenile northern pike (*Esox lucius*) collected from a reference lake (A) and a lake receiving effluent from the Key Lake uranium mill (high exposure lake) (B). Gills were stained with haematoxylin and eosin and are shown at approximately 400X magnification. Arrows point to the primary filament epithelial paddings and arrow heads point to secondary lamellae.

Table 3.1 Histopathological evaluations of liver, kidney and gills of 1+ northern pike (*Esox lucius*) collected from two lakes receiving uranium milling effluent (low and high exposure; n = 12 and n = 9, respectively) and one reference lake (n = 12). Quantitative data are expressed as means ± SEM. Semi-quantitative results are based on calculated means and are presented according to the scoring system (- none, + slight, ++ moderate, +++ maximum). Results with a "/" indicate that the mean lies between two scores. Different letters denote significant differences using one-way ANOVA followed by Tukey's test for quantitative data that met the assumptions of one-way ANOVA or using Kruskal-Wallis one-way ANOVA on ranks followed by Dunn's test for semi-quantitative data and quantitative data that did not meet the assumptions of one-way ANOVA ($p < 0.05$).

Histopathological endpoint	Lakes		
	Reference	**Low exposure**	**High exposure**
Liver			
Hepatocyte area (μm^2)	5.2 ± 0.2[a]	5.9 ± 0.4 [a,b]	6.8 ± 0.3 [b]
Hepatocyte vacuolation	- / + [a]	+ [a]	++ /+++ [b]
Kidney			
Number of pyknotic and fragmented nuclei	23 ± 10 [a]	1.9 ± 0.9 [b]	6.1 ± 2 [a,b]
Number of dilated tubules	1.8 ± 0.6 [a]	0.083 ± 0.04 [b]	0.58 ± 0.3 [a,b]
Acellular glomerular spaces	+ / ++ [a]	+ / ++ [a]	+ / ++ [a]
Capsular thickness	+ / ++ [a]	- / + [a,b]	- / + [b]
Gills			
Padding height (μm)	33 ± 1 [a]	24 ± 2 [b]	22 ± 2 [b]
Lamellar width (μm)	12 ± 0.5 [a]	12 ± 0.4 [a]	11 ± 0.6 [a]

3.4.4 Trace metal analyses

Of the 22 metals analyzed in muscle tissue, 12 were significantly different among lakes (reported as μg/g dry weight). Six metals (arsenic, copper, iron, molybdenum, selenium, and thallium) were significantly elevated for both exposure lakes compared to the reference ($p < 0.05$) (Table 3.2). Cobalt and uranium were significantly greater than the reference for the low and high exposure lakes, respectively. Selenium and arsenic were the only metals significantly

different among all three lakes and that also followed the contamination gradient (i.e. from the lowest concentration at the reference lake to the greatest at the high exposure lake). Conversely, reference lake values were significantly greater than one or both exposure lakes for bismuth, manganese and strontium (Table 3.2). Beryllium was significantly different only between the low and high exposure lakes and nickel, although not significantly different among lakes, was greater at both exposure lakes compared to the reference. Beryllium was the only metal that was below the detection limit for some samples. In these cases, half the value of the detection limit was used in the statistical analyses.

Table 3.2 Twenty-two trace metals detected in muscle of 1+ northern pike (*Esox lucius*) collected from two lakes receiving uranium milling effluent (low and high exposure; n = 12 and n = 9, respectively) and one reference lake (n = 12). Data that met the assumptions of one-way ANOVA were analyzed by one-way ANOVA followed by Tukey's test for multiple comparisons otherwise, Kruskal-Wallis one-way ANOVA on Ranks followed by Dunn's test was used. Data are expressed as means ± SEM (dry weight) and significant differences ($p < 0.05$) are indicated by different letters.

		Lakes		
Trace metal	**Unit**	**Reference**	**Low exposure**	**High exposure**
As	µg/g	0.133 ± 0.013^{a}	0.770 ± 0.079^{b}	4.28 ± 0.29^{c}
Ba	µg/g	45.7 ± 2.2^{a}	47.7 ± 1.2^{a}	47.1 ± 2.4^{a}
Be	ng/g	$2.09 \pm 0.68^{a,b}$	1.88 ± 0.73^{a}	4.85 ± 1.1^{b}
Bi	ng/g	8.68 ± 0.66^{a}	1.66 ± 0.56^{b}	3.72 ± 1.2^{b}
Cd	ng/g	24.5 ± 12^{a}	7.18 ± 1.6^{a}	8.20 ± 1.1^{a}
Co	ng/g	12.4 ± 1.2^{a}	20.0 ± 1.5^{b}	14.3 ± 1.3^{a}
Cr	µg/g	0.274 ± 0.0087^{a}	0.295 ± 0.013^{a}	0.296 ± 0.013^{a}
Cu	µg/g	1.02 ± 0.037^{a}	1.25 ± 0.058^{b}	1.29 ± 0.050^{b}
Fe	µg/g	8.38 ± 0.28^{a}	10.8 ± 0.72^{b}	11.3 ± 0.80^{b}
Mg	µg/g	1500 ± 10^{a}	1520 ± 16^{a}	1500 ± 15^{a}
Mn	µg/g	1.39 ± 0.059^{a}	1.03 ± 0.058^{b}	0.860 ± 0.052^{b}
Mo	µg/g	0.0527 ± 0.0043^{a}	0.325 ± 0.13^{b}	0.433 ± 0.072^{b}
Ni	µg/g	0.428 ± 0.062^{a}	0.438 ± 0.069^{a}	0.650 ± 0.11^{a}
Pb	µg/g	0.501 ± 0.14^{a}	0.330 ± 0.10^{a}	0.205 ± 0.027^{a}
Sb	ng/g	8.86 ± 3.1^{a}	10.5 ± 5.2^{a}	8.36 ± 1.9^{a}
Se	µg/g	0.682 ± 0.036^{a}	16.9 ± 1.5^{b}	22.9 ± 1.0^{c}
Sn	ng/g	53.0 ± 6.1^{a}	67.6 ± 11^{a}	87.4 ± 14^{a}
Sr	µg/g	5.03 ± 0.30^{a}	1.26 ± 0.077^{b}	1.46 ± 0.12^{b}
Tl	ng/g	32.6 ± 3.1^{a}	131 ± 9.0^{b}	162 ± 20^{b}
U	ng/g	3.39 ± 0.89^{a}	2.85 ± 0.39^{a}	5.68 ± 0.90^{b}
Va	µg/g	0.112 ± 0.017^{a}	0.132 ± 0.017^{a}	0.107 ± 0.025^{a}
Zn	µg/g	51.7 ± 12^{a}	33.9 ± 5.2^{a}	29.4 ± 2.3^{a}

3.5 Discussion

The results from the present study suggest that juvenile northern pike inhabiting lakes receiving effluent from the Key Lake uranium mill are experiencing limited oxidative stress and no histopathological lesions due to effluent exposure. Arsenic, selenium and nickel, metals that can cause oxidative stress, were reported to be elevated in water and sediment of lakes receiving uranium milling effluent (Golder, 2005) while arsenic and selenium but not nickel were significantly elevated in pike muscle (this thesis). Iron and copper, metals that can also increase intracellular concentrations of ROS (Halliwell and Gutteridge, 1999) were also significantly elevated in exposure pike muscle. However, the concentrations of these metals in water and sediment did not follow any clear trend with respect to the contamination gradient and were either greater or lower in exposure lakes compared to the reference (Golder, 2005).

Key Lake uranium milling effluent is a complex mixture of metals, ions, ammonia and nitrates, contaminants that can cause histopathological lesions to fish liver, kidney and gills (Wood, 2001; Ptashynski et al., 2002; Teh et al., 2004). The discussion that follows considers possible reasons for the lack of response of pike tissues on the biochemical and histopathological measures evaluated as part of this study.

3.5.1 Glutathione

In terms of oxidative stress, the overall response of the biomarkers did not indicate that ROS were present at toxic levels. Concentrations of total glutathione (reduced plus oxidized glutathione), reduced and oxidized glutathione and the ratio of oxidized to reduced glutathione did not differ significantly among lakes for kidney and liver. The only exception was for the low exposure pike kidney where oxidized glutathione and the ratio of oxidized to reduced glutathione were significantly elevated.

The concentrations of reduced and oxidized glutathione and the ratio of oxidized to reduced glutathione serve as biomarkers of oxidative stress due to the central role reduced glutathione plays in neutralizing ROS (Sayeed et al., 2003; Shi et al., 2004) and in maintaining redox homeostasis in cells (Shi et al., 2004). Reduced glutathione, present in virtually all cells (Stipanuk et al., 2006; Dickinson et al., 2003), is found at particularly high concentrations in kidney and liver (Halliwell and Gutteridge, 1999). The oxidized form of glutathione is normally kept at low levels since it is not useful in scavenging ROS and can disrupt the redox balance of cells (Masella et al., 2005). The ratio of oxidized to reduced glutathione is also normally kept at low levels and has been reported to be less than 0.01 in rat liver and approximately 0.2 in the liver of wild-caught pike (Koss et al., 1991). Cellular redox homeostasis, which is largely governed by the ratio of oxidized to reduced glutathione, is important in regulating cell functions including a number of signaling pathways (Wu et al., 2004).

There are a number of mechanisms by which metals can alter glutathione metabolism and, as a consequence, the concentration that is available for reacting with ROS. Metal binding to glutathione's sulfhydryl group (Shi et al., 2004) blocks its ability to reduce ROS whereas binding to the active sites of γ-glutamylcysteine synthetase, the rate-limiting enzyme for the synthesis of reduced glutathione (Dickinson et al., 2003), and of glutathione reductase, the enzyme that reduces oxidized glutathione (Halliwell and Gutteridge, 1999), will deplete concentrations of reduced glutathione. In the case of liver, reduced glutathione-metal complexes are exported from cells into bile, further decreasing intracellular glutathione (Gyurasics et al., 1991). Excess oxidized glutathione (Spallholz et al., 2004) is also exported from cells as a means of maintaining redox homeostasis, thereby removing a pool of reduced glutathione that would otherwise be available through the action of glutathione reductase on oxidized glutathione

(Hasspieler et al., 1994; Halliwell and Gutteridge, 1999). Finally, certain metals participate in mechanisms that produce ROS causing an increase in oxidized glutathione (Shi et al., 2004) and possible oxidative damage to enzymes required for glutathione metabolism (Valavanidis et al., 2006). Of the mechanisms listed above, several are associated with arsenic, nickel and selenium exposure. However, since nickel was not found to be elevated in pike tissues, the discussion will focus on arsenic and selenium.

Although there exists a number of ways by which metals can adversely impact the effectiveness of glutathione as an antioxidant, under certain conditions of metal exposure, normal glutathione metabolism can be maintained and reduced glutathione induced (Pandey et al., 2003; Sayeed et al., 2003; Allen et al., 2004; Sanchez et al., 2005). In the present study, the overall lack of a significant change in the concentration of reduced and oxidized glutathione in liver and kidney of exposure pike suggests that either arsenic and selenium are not causing an increase in ROS or that ROS are being produced but cells are able to compensate and maintain glutathione homeostasis. Other studies have also reported a lack of effect on the concentrations of either total, reduced or oxidized glutathione in fish exposed to sublethal concentrations of metals that are known to cause oxidative stress. Ahmad et al. (2006) observed no change in the concentration of reduced glutathione in European eels (*Anguilla anguilla*) acutely exposed to 1/20 of the LC_{50} of aqueous chromium whereas Roy and Bhattacharya (2006) reported an initial decrease in the concentration of total glutathione in spotted snakehead (*Channa punctatus*) exposed to 1/20 LC_{50} of aqueous arsenic followed by a recovery to concentrations that did not differ from the control group. In terms of field studies, Giguère et al. (2005) reported no difference in total hepatic glutathione in yellow perch collected from lakes in the mining region of Rouyn-Noranda, Québec that are contaminated with cadmium, copper, nickel and zinc. The

lack of change in the concentrations of glutathione reported in this study, and others, indicate that the large intracellular pool of glutathione may be insensitive to the effects of metals under certain doses and durations of exposure.

Although most measures of glutathione in exposure pike did not significantly differ from the reference, low exposure pike kidney was an exception. The elevated concentration of oxidized glutathione and the higher ratio of oxidized to reduced glutathione may be a result of the combined effects of parasitic infection and effluent exposure. The gills and intestine of pike from the low exposure lake had a prevalence and intensity of parasitic infection that was greater than high exposure but lower than reference pike (Chapter 2). Although kidney was not examined for parasites in this study, it is reasonable to assume that this organ, which is commonly infected with parasites in pike (Hinck et al., 2007), may have also had a level of infection intermediate between the high exposure and reference pike. A study by Marcogliese et al. (2005) reported that fish exposed to both parasitic infection and contaminants had greater signs of oxidative stress than fish exposed to contaminants alone. A similar situation may therefore exist in the low exposure pike kidney since, compared to reference and high exposure pike, only these fish may be experiencing both exposure to uranium milling effluent and a relatively high level of parasitic infection.

3.5.2 Glutathione peroxidase

In addition to reduced glutathione, a limited number of enzymes directly participate in the antioxidant defense system (Halliwell and Gutteridge, 1999). One of these is glutathione peroxidase, an enzyme that catalyzes the reduction of organic and hydrogen peroxides (Matés, 2000). As an enzyme, it is susceptible to damage by ROS (Valavanidis et al., 2006) and can be inactivated by metal binding (Shi et al., 2004). In theory, reduced enzymatic activity implies that some ROS are not being quenched, thus predisposing cells to oxidative stress. On the other

hand, there is evidence in the literature that glutathione peroxidase can be induced in the presence of metals (Berntssen et al., 2000; Basha and Rani, 2003; Sanchez et al., 2005), presumably due to increased ROS production.

Although glutathione peroxidase is present in both kidney and liver of fish (Berntssen et al., 2000), only liver was assayed for glutathione peroxidase activity for this thesis since an insufficient amount of kidney tissue was available for analysis. The significantly elevated glutathione peroxidase activity observed in liver of high exposure pike compared to the reference can be explained in one of three ways: 1) sub-cellular lesions in reference pike liver are damaging and inactivating the enzyme; 2) the concentrations of ROS in exposure pike may be elevated to the point of inducing glutathione peroxidase but low enough that oxidative damage to the enzyme is largely averted; 3) the enzyme may be induced in response to elevated concentrations of selenium (Hilton et al., 1980; Wang and Lovell, 1997; Gan et al., 2002).

The first possible explanation is supported by the elevated lipid peroxidation in reference pike liver. Although lipid peroxidation is a common biomarker of oxidative stress, it is also a general response to tissue injury (Boelsterli, 2003) that arises from biological insults such as disease (Niki et al., 2005). Therefore, the lipid peroxides themselves and/or the processes that lead to lipid peroxidation, could be having an adverse effect on other subcellular components such as enzymes (Das, 1999). The second explanation suggests that there are elevated ROS in exposure pike livers but that cells are compensating by inducing glutathione peroxidase (Berntssen et al., 2000; Basha and Rani, 2003; Sayeed et al., 2003; Sanchez et al., 2005). Another observation in favour of a compensatory mechanism is that exposure pike liver exhibited a trend towards elevated concentrations of reduced glutathione, although the difference was not significant. The third possible explanation contrasts selenium's role as an antioxidant, through its incorporation

into glutathione peroxidase (Thomson, 2004), with its potential for causing oxidative stress (Miller et al., 2007). Whether the former or the latter prevails depends on the concentration of selenium taken up by an organism (Miller et al., 2007). Glutathione peroxidase is inducible in the presence of non-toxic levels of selenium (Thorarinsson et al., 1994; Wang and Lovell, 1997; Gan et al., 2002) while concentrations that exceed the nutritional range are toxic (Lemly, 1997) and can cause a reduction in glutathione peroxidase activity (Tallandini et al., 1996; Gan et al., 2002).

Even though selenium is present at low concentrations in exposure lake water, the results from muscle in this study and from a previous study reporting selenium concentrations in pike eggs and adult pike muscle, kidney, liver, and bone (Muscatello et al., 2006), demonstrate that selenium is bioavailable and bioaccumulating in pike tissues through a mainly dietary route of exposure (Lemly, 1993b). The concentration of 16.9 µg/g in low and 22.9 µg/g (dry weight) in high exposure pike muscle both exceed the proposed threshold concentration for fish health of 8 µg/g (Lemly, 1993b). Furthermore, selenium concentrations measured in pike prey items (Muscatello et al., in press) are greater than the dietary threshold of 3 µg/g reported to cause toxicity in fish (Lemly, 1993b) and are two orders of magnitude above the concentration in the food of experimentally-fed rainbow trout (*Oncorhynchus mykiss*) required for maximal plasma glutathione peroxidase activity (Hilton et al., 1980). According to the levels of selenium in reference lake prey items, reference pike may also be obtaining enough selenium through their diets to achieve maximal glutathione peroxidase activity (Hilton et al., 1980). If this assumption were true, then exposure pike may not have greater glutathione peroxidase activity than reference because of induction by selenium, but rather, one of the other two possible explanations could be more plausible.

3.5.3 Lipid peroxidation

In addition to changes in the antioxidant defense system, one of the hallmarks of oxidative stress is oxidative damage to biological macromolecules such as the phospholipids of cell membranes (Shi et al., 2004). In order to determine whether phospholipids are being oxidatively damaged in exposure pike due to ROS generated by metals, measures of lipid peroxidation were determined in liver and kidney. The results did not support predictions since lipid peroxidation was significantly lower in exposure compared to reference pike. Rainbow trout experimentally exposed to aqueous sodium selenite also showed a decrease in lipid peroxidation compared to control fish that could not be attributed to a protective effect of antioxidant defenses since induction was not observed (Miller et al., 2007). In a field study by Giguère et al. (2005), decreased lipid peroxidation was reported in liver of yellow perch inhabiting metal-mining contaminated lakes as compared to perch collected from reference lakes. The authors speculated that this was due to a hormetic effect where antioxidant defense systems are induced to levels above the minimum required to counter oxidative stress (Giguère et al., 2005).

The significant difference in lipid peroxidation in the present study could be driven by processes affecting reference rather than exposure pike. As mentioned previously, lipid peroxidation is a non-specific response following cellular injury (Boelsterli, 2003) and could arise as a result of mechanisms other than metal-mediated oxidative damage. Marcogliese et al. (2005) observed increased lipid peroxidation in the livers of fish infected with parasites that could have been due to ROS released by activated phagocytes (Whyte et al., 1989). Since reference pike showed the greatest prevalence and intensity of parasitic infection (Chapter 2), parasitism could be one of the causal factors behind the elevated lipid peroxidation.

3.5.4 Integrating biomarkers of oxidative stress

When interpreting biomarkers of oxidative stress, it is not sufficient to consider each biomarker in isolation since their biochemical mechanisms are interrelated. For instance, the lack of change in measures of glutathione while glutathione peroxidase activity and lipid peroxidation differed among lakes requires consideration. Reduced glutathione serves as co-factor for glutathione peroxidase and becomes converted to the oxidized form through enzymatic activity (Halliwell and Gutteridge, 1999). Therefore, increased glutathione peroxidase activity in exposure pike liver should correspond to an increase in the conversion of reduced glutathione to its oxidized form. Similarly, cellular defenses against lipid peroxidation consume reduced glutathione as reducing equivalents for ascorbic acid which then reduces vitamin E, one of the main antioxidants that protects membrane phospholipids (Di Mascio et al., 1991), as well as for glutathione peroxidase, which neutralizes lipid peroxides (Matés, 2000). Glutathione-s-transferase uses reduced glutathione as a co-factor for neutralizing the toxic by-products of lipid peroxidation (i.e. malondialdehyde and 4-hydroxyalkenal) (Martinez-Lara et al., 2002). The overall lack of response in measures of glutathione despite changes in glutathione peroxidase activity and lipid peroxidation could indicate that this biomarker of oxidative stress is relatively less sensitive than others, as has been suggested by Marcogliese et al. (2005). Although levels of glutathione were unaffected in exposure pike liver, there may still be a link between the elevated glutathione peroxidase activity and decreased lipid peroxidation. Glutathione peroxidase may be effectively quenching ROS before they cause damage to membrane phospholipids and neutralizing any lipid peroxides that do arise (Matés, 2000) thereby causing a lower concentration of lipid peroxidation in exposure pike compared to reference.

A study by Hoffman et al. (1989) on ducklings fed selenomethionine in their diets for 6 weeks reported concentrations of 4.8 μg/g selenium wet weight in liver (or 19.2 μg/g dry weight

assuming 75% moisture; Muscatello et al., 2006) and a significant increase in glutathione peroxidase activity compared to controls but no change in the concentrations of glutathione and lipid peroxidation. At higher hepatic selenium concentrations, 26.0 µg/g wet weight (or 104 µg/g dry weight), glutathione peroxidase activity was not different from controls while reduced glutathione was lower and lipid peroxidation was increased. The present study may reflect a similar situation where the concentration of selenium in tissues are at a level that only affects glutathione peroxidase activity and not other biomarkers of oxidative stress.

A study on arsenic's capacity for causing oxidative stress (Schlenk et al., 1997) reported no change in total glutathione and lipid peroxidation in liver of adult channel catfish subjected to 0.01, 0.1 or 1.0 mg/L of aqueous sodium arsenite or arsenate; concentrations that are several times greater than that reported for the exposure lakes (Golder, 2005). The lack of a response reported by Schlenk et al. (1997) may be due to the concentration being too low or because of the exposure duration of only 7 days, since concentrations of arsenic in liver, kidney, and muscle of fish have been shown to increase past this time period (Allen et al., 2004).

3.5.5 Histopathology

Histopathological analyses of kidney and gills corroborate with the findings on oxidative stress in that pike collected from exposure lakes did not demonstrate significant pathological lesions compared to the reference; in fact, the opposite was often observed. Histopathological observations of the liver, however, revealed morphological differences between exposure and reference pike without any clear signs of pathology.

3.5.5.1 Kidney

Reference pike kidney had a greater number of pyknotic and fragmented nuclei, dilated tubules and thickening of Bowman's capsule in comparison to exposure pike kidney. The extent of acellular glomerular spaces was the only histopathological measure that did not differ among

lakes. Pyknotic and fragmented nuclei, indicators of apoptotic and necrotic cell death (Myers and McGavin, 2007), were mostly observed in the epithelial cells of proximal and distal convoluted tubules and were rarely associated with other renal cells. The occurrence of dilated tubules appears to be a consequence of dead and dying epithelial cells while a thickening of Bowman's capsule can arise as a result of fibrosis (Weber et al., 2003) following irreversible injury to cellular and acellular components of the capsule (Ackermann, 2007). It is not known why exposure pike kidney were relatively free of histopathologies while reference pike showed a greater incidence of lesions. Differences between the populations in infectious diseases, such as parasites, is a possible explanation.

3.5.5.2 Liver

Hepatocytes from high exposure pike were distinguished from the reference by a greater degree of vacuolation and a larger transsectional area whereas low exposure hepatocytes did not differ from the reference on either of these measures. The observed vacuoles are essentially empty spaces since the process of fixing, dehydrating and staining the tissues for analysis would have removed their contents (Takashima and Hibiya, 1995). However, due to their characteristic shape, the vacuoles most likely contained neutral fats (J. Smits, personal communication). This assumption is supported by results from the bioenergetic analyses (Chapter 2) and by Bennett et al. (2007) where elevated concentrations of triglycerides were detected in exposure pike. Glycogen granules may also have been contained in the vacuoles since bioenergetic analyses also revealed elevated hepatic glycogen in exposure pike but preparation of tissues for histopathological analyses were not conducive to observing glycogen.

Abnormal accumulation of neutral lipids, such as triglycerides, and the formation of vacuoles in hepatocytes, is a common response of the liver to contaminant exposure (Peters et al., 1987; Köhler, 1990; Thophon et al., 2003; Teh et al., 2004; van Dyk et al., 2007) that arises from

perturbations in lipid metabolism (Teinen-Moslen, 2001). Elevated glycogen stores can also occur due to metabolic disruptions (Bhaskar and Govindappa, 1986) associated with exposure to metals (Sastry and Subhadra,1982; Thophon et al., 2003). Conversely, elevated neutral lipids and glycogen could also simply be of dietary origin. Regardless of the causative factor, elevated stores of neutral lipids in hepatocytes (i.e. steatosis) can be accompanied by pathological lesions such as necrosis (Köhler, 1990) and fibrosis (Pessayre et al., 2004) (i.e. steatohepatitis). Steatosis and steatohepatitis following contaminant exposure has been demonstrated in fish (Thophon et al., 2003; Teh et al., 2004) whereas the occurrence of these conditions due to elevated dietary fats has been reported in human subjects (Pessayre et al., 2004). Hepatic steatosis can persist indefinitely in some human subjects without being accompanied by pathological lesions such as necrosis and fibrosis (Pessayre et al., 2004). Alternatively, steatosis caused by contaminants or other factors can worsen and become pathological if lipid accumulation increases and/or persists over several months or years (Gresham, 1993).

In the present study, the majority of hepatocytes from exposure pike did not demonstrate a pronounced degree of vacuolation characterized by an atrophied nucleus that is displaced to the side of the cell (Peters et al., 1987; Cooley et al., 2000; van Dyk et al., 2007). The preservation of nuclear morphology, along with the absence of necrosis and fibrosis, suggests that the accumulation of lipids in pike from exposure lakes is relatively mild and may not be pathological. Pike in the exposure lakes may, however, eventually develop steatohepatitis since continued exposure to causative factors in the environment is plausible given the species' sedentary nature (Scott and Crossman, 1973).

In addition to increased vacuolation, high exposure and reference hepatocytes differed in transsectional area. The increased transsectional area of exposure hepatocytes is not reflected in

a greater hepatosomatic index, which again, suggests that the degree of vacuolation is not severe. An increase in the hepatosomatic index of fish due to steatosis has been previously reported in the literature (Martin and Black, 1998) while Cooley et al. (2000) reported no change in the hepatosomatic index despite a significant increase in hepatocyte area following contaminant exposure. The results from this study and that of Cooley et al. (2000) indicate that the hepatosomatic index lacks sensitivity in detecting changes in hepatocyte size.

3.5.5.3 Gills

Fish gills are in constant contact with substances in the ambient water and are therefore relevant indicators of contaminant exposure in fish (Mallatt, 1985; Caldwell, 1997; Syasina and Sokolovskii, 2001; Levesque et al., 2003; Thophon et al., 2003). The lakes downstream of the Key Lake uranium mill discharge contain a complex mixture of metals, ammonia and other ions that could be impacting gill morphology as well as its functions of gas and ion exchange, acid-base regulation and waste excretion (Wood, 2001).

Basic gill anatomy consists of four bony gill arches per opercular cavity that support a number of finger-like primary filaments. The primary filament is comprised of a cartilaginous support and blood vessels that are covered with a layer of mainly epithelial cells. Secondary lamellae, the structures ultimately responsible for blood-water exchange, branch off from the primary filament. Responses of the gills to contaminants in the environment are varied (see review by Mallat, 1985) and include hyperplasia of mucous, chloride and epithelial cells, necrosis, cellular hypertrophy (Mallat, 1985), clavate and shortened secondary lamellae (Syanisa and Sokolovski, 2001; Riba et al., 2004), epithelial lifting, edema, and excess mucous secretion (Mallat, 1985). Similar to other organs, histopathological alterations of gills are generally not specific to a particular contaminant (Mallat, 1985; Wood, 2001).

As part of this thesis, two measures that reflect the gills' function in blood-water exchange were quantified: primary filament epithelial padding height and secondary lamellar width. Thickening of the primary filament epithelial padding due to cellular hyperplasia, hypertrophy, edema, and/or excess mucous secretion could impair blood-water exchange by reducing the surface area of the secondary lamellae that is in contact with the water. As for the secondary lamellae, an increase in width because of edema, hypertrophy, hyperplasia and/or mucous (Wood, 2001) could interfere with blood-water exchange by increasing the diffusion distance. The possible presence of excess mucous could not be evaluated in this study since preparation of the gills for microscopic examination was not amenable to demonstrating mucous (Wood, 2001).

Levesque et al. (2003) observed an increase in epithelial padding height of the primary filament, greater blood-water diffusion distance and increased width of secondary lamellae in yellow perch chronically exposed to metals in the field. In the present study, the epithelial padding height was thicker in the reference fish compared to exposure and no significant differences were found in secondary lamellar width. The greater epithelial padding height of reference pike was unexpected and contrary to predictions. A notable characteristic of reference lake water is its very low ionic strength, a factor that has been shown to affect gill morphology (Wood, 2001). However, such alterations usually involve hyperplasia of chloride cells leading to a considerable increase in secondary lamellar width (Greco et al., 1996). Since secondary lamellar width was unaffected in this study, it is less likely that low ionic strength was having an effect on reference pike gills. Decreased primary filament epithelial padding height of exposure pike due to cell loss may not explain the significant difference in padding height either, since there did not appear to be noticeable gill cell death. Instead, differences in parasitic infection could again provide an explanation for the results since reference pike displayed a significantly

elevated prevalence and intensity of gill monogeneans compared to exposure pike (Chapter 2). Infection by these parasites has been shown to induce a hyperplastic response of primary filament epithelial cells (Dezfuli et al., 2007) and a greater padding thickness. Overall, results from this study do not support the hypothesis that exposure to Key Lake uranium milling effluent causes histopathological lesions to juvenile pike gills.

3.5.6 Metals and toxicity

Metals and contaminants in the environment can cause oxidative stress and histopathological lesions in tissues only if present in the environment for a sufficient duration, are bioavailable for uptake by the organism and are reaching biological targets at a high enough concentration. The overall lack of a response in the measures of oxidative stress and histopathology in exposure lake pike indicates that at least one of these criteria was not met. It is reasonable to assume that the first criterion was satisfied since the pike were likely inhabiting the effluent contaminated lakes throughout their entire lives (>1 year). On the other hand, there are reasons to presume that the second criteria was not met, especially with respect to nickel, and that the third criteria was not fulfilled.

The bioavailability of metals and other contaminants in the effluent could be decreased by the high water hardness, elevated total suspended solids and depressed pH of the exposure lakes (Pyle et al., 2001; Golder, 2005). Calcium is known to reduce the bioavailable concentration of metals in the water by competitively inhibiting their uptake by the gills (Wood, 2001; Niyogi and Wood, 2003) and by reducing gill permeability (Sprague, 1985). This protective effect is so important that water hardness is taken into consideration by regulatory bodies when establishing water quality guidelines for certain metals (CCME, 2006). Suspended solids in the water column are also protective by binding metals and reducing their bioavailability (Sprague, 1985). The depressed pH of the high exposure lake can have both protective and detrimental effects with

respect to metals. Protons can compete with metals for gill uptake (Niyogi and Wood, 2003) but an acidic pH can also shift the speciation of metals into the more toxic ionic form (Sprague, 1985) and release metals that are bound to sediment (McDonald and Wood, 1993). Although these factors generally apply to metals, it is unclear whether this would affect selenium and arsenic bioavailability since these elements are metalloids. The bioavailability of nickel, however, could be modulated by these factors. In addition to metals, elevated hardness and a depressed pH may confer protection from the elevated concentration of ammonia and ammonium of exposure lakes (Wood, 2001), contaminants that can cause histopathological lesions to organs (Banerjee and Bhattacharya, 1994; Wood, 2001).

Despite water quality variables that could be causing a reduction in the bioavailability of metals and contaminants to exposure pike, evidence that metals are taken up and bioaccumulating in tissues is demonstrated by eight of twenty-two metals analyzed in muscle being significantly elevated in exposure pike compared to reference. Of relevance to the potential for production of ROS leading to oxidative stress, arsenic, selenium, iron and copper were elevated in pike collected from exposure lakes. Liver is a better representation of concentrations of metals in the environment than most organs (Rajotte and Couture, 2002) and both kidney and liver generally accumulate metals to a greater extent than muscle (Suňer et al., 1999; Pedlar et al., 2002; Rajotte and Couture, 2002; Ribeiro et al., 2005; Muscatello et al., 2006). Therefore, while the concentrations of selenium, arsenic, copper and iron reported in muscle of exposure pike were higher compared to the reference, the concentrations in liver and kidney could be even greater. Copper and iron were elevated in exposure pike muscle even though these metals are generally not present at greater concentrations in exposure water and sediment compared to reference (Golder, 2005). Both these metals participate in Fenton

chemistry to produce hydroxyl radical, the most potent ROS that can be produced intracellularly (Halliwell and Gutteridge, 1999).

One of the possible reasons for the lack of response with the oxidative stress biomarkers in exposure pike liver and kidney could be attributed to antagonistic interactions between metals (Hoffman et al., 1992; Hamilton et al., 2002). A study by Hoffman et al. (1992) demonstrated that mallard ducklings fed selenomethionine in combination with sodium arsenate in their diets had greater concentrations of reduced glutathione, a lower ratio of oxidized to reduced glutathione, a lower concentration of lipid peroxidation and decreased histopathological lesions in liver compared to ducklings fed selenomethionine alone. The presence of selenium could also be affording protection from arsenic since it was found that the two will form an insoluble complex inside lysosomes before being expelled from cells (Hoffman, 2002). Antagonistic interactions would effectively decrease the concentrations of arsenic and selenium that are reaching sub-cellular targets of toxic action (i.e. antioxidant defense system) and generating ROS.

Another possible explanation for the lack of effects is that exposure pike have developed tolerance due to chronic contaminant exposure. Concomitant with kidney and liver being target organs of metals, metallothionein is highly inducible in these tissues (McDonald and Wood, 1993). Metallothionein induction in the presence of arsenic has been observed in fish (Roy and Bhattacharya, 2006) and could confer tolerance to this metal (Halliwell and Gutteridge, 1999). The mechanism of protection may involve metallothionein's capacity to neutralize ROS rather than direct binding of arsenic (Roy and Bhattacharya, 2006). As for selenium, Hilton et al. (1980) proposed that rainbow trout may be able to detoxify selenium by methylation or by incorporation into selenoproteins. This hypothesis was revisited by Thorarinsson et al. (1994)

who postulated that fish can be protected from selenium's toxicity through its incorporation into a large selenoprotein pool.

3.6 Conclusions

A comprehensive suite of oxidative stress biomarkers and histopathological analyses revealed no overall indications of oxidative stress or target organ toxicities in juvenile northern pike living downstream of the Key Lake uranium mill, and this despite significant bioaccumulation of several metals, most notably, arsenic and selenium.

CHAPTER 4
4.0 GENERAL DISCUSSION

4.1 Project rationale and summary

Recent field research on fish exposed to Key Lake uranium mill effluent have reported teratogenic defects in larval northern pike (Muscatello et al., 2006), reduced survival of fathead minnows (Pyle et al., 2001) and altered bioenergetics of young-of-the- year pike (Bennett and Janz, 2007). Elevated concentrations of certain metals, nutrients (i.e. ammonia and nitrates), and ions characterize the water chemistry of lakes receiving effluent from the Key Lake uranium mill (Golder, 2005). This thesis sought to further explore factors that may be affecting the bioenergetic status of juvenile pike and to determine possible toxicity of effluent exposure to target organs at the biochemical and tissue levels of biological organization.

In summary, the results demonstrated that juvenile pike collected from lakes receiving uranium milling effluent (high and low exposure lakes) have greater stores of triglycerides and glycogen in liver and muscle compared to reference pike (Table 4.1). Triglycerides are an important storage form of lipids in animals whereas glycogen is the storage form of carbohydrates (Nelson and Cox, 2005). Liver and muscle were analyzed since these tissues are involved in the storage of neutral lipids and glycogen in animals however, muscle may not be an important storage site for glycogen in juvenile northern pike (this study).

The lack of a significant difference among reference and exposure pike on length, weight, and condition factor suggests that the elevated available energy observed in exposure pike was being diverted away from growth and towards energy storage. The hypothesis that pike from exposure lakes had greater energy stores because they are consuming prey items of higher quality (i.e.

elevated fat in prey) was not supported by the results (Table 4.1). Therefore, food web enrichment due to the presence of elevated nutrients in the form of nitrogenous compounds may not be occurring in exposure lakes. The difference among lakes in the prevalence and abundance of intestinal and monogenean parasites supported the hypothesis that parasitic infection could be exerting an energetic cost on reference pike thereby providing a possible explanation for the relatively elevated energy stores of pike inhabiting the exposure lakes.

Table 4.1 Summary of results on bioenergetics and parasitism (Chapter 2) of 1+ northern pike (*Esox lucius*) as well as bioenergetics of pike prey items collected from two lakes receiving effluent from the Key Lake uranium mill (low and high exposure). Results that differed significantly from the reference lake are indicated by an arrow whereas those that did not differ are shown by a dash.

	Lake	
Variable	**Low exposure**	**High exposure**
Pike bioenergetics	— **or** ↑	— **or** ↑
Prey item bioenergetics	— **or** ↓	—
Pike parasitism	— **or** ↓	— **or** ↓

Due to the presence of contaminants such as metals in lakes receiving effluent from the Key Lake uranium mill (Golder, 2005), additional hypotheses examining possible effects at the biochemical and tissue level were investigated as part of this thesis. Arsenic, selenium and nickel can cause oxidative stress in biological systems (Spallholz and Hoffman, 2002; Chen et al., 2003; Shi et al., 2004) and have been reported to be elevated in water, sediment and/or fish tissues of the exposure lakes (Pyle et al., 2001; Klaverkamp et al., 2002; Golder, 2005; Muscatello et al., 2006). I therefore hypothesized that kidney and liver, target organs of metals, would demonstrate oxidative stress as measured by commonly used biochemical biomarkers. According to the results, pike from exposure lakes generally did not show signs of oxidative

stress compared to reference and in fact, the opposite was observed for measures of lipid peroxidation (Table 4.2). The absence of oxidative stress and histopathological lesions (Table 4.3) in target organs of exposure pike is opposite to what would be predicted given the significantly elevated concentrations of arsenic, selenium, copper, and iron (but not nickel) in muscle tissues, indicating that metals are bioavailable and accumulating in pike.

Table 4.2 Summary of results of biomarkers of oxidative stress (Chapter 3) of 1+ northern pike (*Esox lucius*) collected from two lakes receiving effluent from the Key Lake uranium mill (low and high exposure). Results that differed significantly from the reference lake are indicated by an arrow whereas those that did not differ are shown by a dash.

	Lake	
Oxidative stress biomarker	**Low exposure**	**High exposure**
Glutathione		
Liver	—	—
Kidney	— **or** ↑	—
Glutathione peroxidase activity		
Liver	—	↑
Lipid peroxidation ([MDA+HAE])		
Liver	—	↓
Kidney	↓	↓

Table 4.3 Summary of results on histopathological analyses (Chapter 3) of 1+ northern pike (*Esox lucius*) collected from two lakes receiving effluent from the Key Lake uranium mill (low and high exposure). Results that differed significantly from the reference lake are indicated by an arrow whereas those that did not differ are shown by a dash.

	Lake	
Target organ	**Low exposure**	**High exposure**
Liver	—	↑
Kidney	— **or** ↓	— **or** ↓
Gills	— **or** ↓	— **or** ↓

4.2 Bioenergetics

4.2.1 Triglycerides and glycogen versus other indicators of bioenergetics

In vertebrates, lipids, glycogen and also proteins can be broken down for the production of ATP (Nelson and Cox, 2005). An analysis of protein concentrations as a source of energy was excluded from the present thesis for a number of reasons. Although proteins have been suggested to be an important energy macromolecule in fish (Lovell, 1998), some authors propose that it may mainly be used under conditions of acute stress (Mayer et al., 1992). Furthermore, protein concentration may be an overly crude measure of available energy due to the multitude of other physiological functions assumed by proteins that could take precedence over catabolism for ATP. Bennett and Janz (2007) measured muscle protein concentration as an indicator of growth in young-of-the-year pike and burbot and reported no differences between fish from lakes receiving effluent from the Key Lake uranium mill and the reference lake. These results further justify excluding an analysis of protein concentration, since, compared to glycogen and lipids, this factor appears to be unaffected in fish inhabiting lakes near the Key lake uranium mill.

Common indicators used in monitoring of fish overall health and bioenergetic status include condition factor and hepatosomatic index (Busacker et al., 1990). The mining industry in

Canada is required under Environment Canada's Environmental Effects Monitoring program to assess the impacts of their effluent discharge on aquatic biota including fish (Environment Canada, 2002). As part of this program, fish weight, condition factor and/or weight-at-length and hepatosomatic index can be used as indicators of energy. Although these measures are fast and easy to obtain, they may be less sensitive than measuring the concentrations of energy storage macromolecules. This is evidenced by the results from this study where significant differences were detected among reference and exposure lake pike in the concentrations of glycogen and triglycerides while no differences were observed in condition factor and hepatosomatic index.

4.2.2 Bioenergetics: future research

There is a multitude of abiotic and biotic factors that influence fish bioenergetics, including quantity and quality of available prey items, predation pressure, intra- and interspecific competition, season, temperature, contaminants, age, reproductive status, and disease. This thesis examined the effects of prey quality and parasitism. However, another factor of toxicological relevance to pike inhabiting lakes downstream of the Key Lake uranium mill is the possibility that effluent exposure is disrupting metabolic processes and causing the observed increases in glycogen and triglycerides stores. To further explore this hypothesis, future research could examine the activity of enzymes involved in the anabolism and catabolism of neutral lipids and glycogen to determine whether these metabolic processes are disrupted. Another approach would be to evaluate overall energy utilization by pike at the whole-organism level. For example, techniques are available for estimating energy conversion efficiency (Sherwood et al., 2000), the efficiency with which energy from food is converted into growth, and respiration rate, the consumption of oxygen per unit time (McGeer et al., 2000). Studies using measures such as

these have provided evidence that metabolic rate increases in contaminant exposed fish (Sherwood et al., 2000).

In terms of biota, differences in the fish communities between exposure and reference lakes could have a bearing on predation pressure and competition for resources; factors that can incur a cost to an individual's overall energy budget. Rather than characterize the fish communities of these lakes, which would be a challenging endeavour, an indirect measure of this factor could be obtained by measuring the activity of lactate dehydrogenase in muscle. The activity of this glycolytic enzyme has been previously used as an indicator of activity levels of feral fish (Sherwood et al., 2002b). Therefore, if there are differences among the exposure and reference lakes in behaviours associated with predator avoidance and intra- and inter-specific competition, this might be reflected in the activity of lactate dehydrogenase.

4.3 Biomarkers of oxidative stress

The extensive body of mammalian and fish based research studying ROS production and oxidative stress following contaminant exposure (Liu et al., 2001; Chen et al., 2003; Giguère et al., 2005; Kang et al., 2005; Marcogliese et al., 2005; Miller et al., 2007) demonstrate that there are disadvantages and advantages with respect to using biomarkers of oxidative stress. One disadvantage is that biomarker responses do not always follow a consistent, easily interpretable pattern due to the number of factors that can affect cellular responses to ROS (Regoli et al., 2002). To help overcome this issue, it has been suggested that a number of biomarkers should be measured in different organs (Kelly et al., 1998), such as has been done in this thesis. In doing so, oxidative stress could be established with greater certainty and inferences on possible impairments to physiological functioning, health and fitness, more readily made. Furthermore, including a biomarker of effect, lipid peroxidation for example, gives an indication of whether cellular antioxidant mechanisms are compensating or being overwhelmed by a contaminant

induced increase in ROS. A review by Di Giulio et al. (1989) points to the value of using biomarkers of oxidative stress in aquatic toxicology biomonitoring. However, similar to other authors (Regoli et al., 2002), they discuss the need for research on how abiotic and biotic factors may influence the responses of biomarkers in aquatic organisms exposed to contaminants (Di Giulio et al., 1989).

A factor that relates to oxidative stress that was not included in this thesis is a measure of the concentration of ROS in tissue. This can be accomplished using a variety of methods that vary according to the ROS of interest. Electron spin resonance is a technique that can measure certain ROS in biological systems directly. Reactive oxygen species that are highly unstable can be reacted with another molecule (called a spin trap) to form a more stable product that can then be measured using electron spin resonance (Halliwell and Gutteridge, 1999). A fluorescent dye called dichlorofluorescin diacetate reacts with a number of different ROS in cells and tissues to form a compound that can be measured fluorimetrically (Halliwell and Gutteridge, 1999). Whether or not these techniques are feasible with samples collected from the field is doubtful due to the transient nature of ROS which can have a half-life of parts of a second to only a few hours (Kohen and Nyska, 2002). Since a delay between sample collection and laboratory analyses is often unavoidable with field studies, biomarkers such as those measured in the present study may be more relevant to research on oxidative stress using field samples.

4.4 Histopathology

Histopathological analyses of tissues have been used extensively in field and lab based research and monitoring of fish exposed to contaminants (Förlin et al., 1986; Paris-Palacios et al., 2000; Levesque et al., 2003; Thophon et al., 2003; Teh et al., 2004) and has the potential to provide a wealth of information. However, similar to biomarkers of oxidative stress, there are drawbacks as well as advantages to using this technique as part of toxicological research. Some

disadvantages include: mistaking artifacts for lesions, overlooking histopathological alterations that are present in tissues (Mallatt, 1985), confounding the effects of a contaminant with disease and normal physiological processes (Hinton and Laurén, 1990). There are however ways of averting or minimizing some of these issues. For instance, the confounding effect of artifacts can be controlled by processing and preparing samples in a standardized fashion and by doing so with the utmost care. With this thesis, fish dissection and preservation of tissues in the field was standardized as well as most steps of tissue processing for slide preparation.

The problem of failing to notice a lesion may be more common in the case of small sample sizes where less comparisons can be made between samples for detecting differences. However, important histopathological lesions that may be a better indication of impaired health then more subtle ones, should be detectable even by a researcher that has received a minimum amount of training or when working with a small sample size. The histopathological analyses for this study did show that lesions were present in tissues of pike. However, due to the relatively limited amount of time and training that could be devoted to this part of the thesis, it cannot be assumed that the analysis was completely thorough and some lesions may have been overlooked.

4.5 Development of tolerance and acclimation

Due to the lack of observed effects in terms of oxidative stress and histopathological lesions in exposure pike, in spite of accumulated metals in tissue, future research could examine whether or not pike have developed tolerance to the Key Lake uranium mill effluent. Tolerance occurs when exposure to a contaminant eventually results in a diminished response of a measured endpoint (Eaton and Klaassen, 2001). Controlled lab studies to determine if the pike have acclimated to the effluent would also be of interest. McDonald and Wood (1993) describe acclimation as occurring when chronic exposure to sublethal concentrations of a contaminant results in either a smaller physiological change following exposure to a greater contaminant

dose, an increase in the LT_{50} or an increase in the LC_{50}. Acclimation and tolerance to contaminants has been observed with oxidative stress (Kelly et al., 1998) and histopathological (Roy and Bhattacharya, 2006) endpoints measured in fish. If pike have developed tolerance or have acclimated to the uranium milling effluent, this might indicate it is not a suitable species for future monitoring studies where a more sensitive species should be used.

4.6 Integrating bioenergetics, oxidative stress and histopathology

Several associations can be made between the bioenergetic, parasitic, oxidative stress and histopathological variables measured in the juvenile pike collected for this thesis. Some of these relationships, such as the possible effects of parasitism on the histopathological lesions in reference pike gills and kidney and the hepatocyte vacuolation of exposure pike being due to elevated glycogen and triglycerides, have already been discussed in Chapter 3. Additional links, however, can be made. The overall lack of oxidative stress and histopathological lesions in exposure pike compared to reference may be contributing to the relatively elevated energy stores observed in exposure pike. Whether due to contaminants or disease processes, biochemical and tissue lesions such as those observed in reference pike could presumably deplete energy stores (Adams, 1999; Campbell et al., 2003).

In terms of oxidative stress, the fact that the liver of exposure lake pike contained elevated neutral lipids in the form of triglycerides while the levels of lipid peroxidation were lower than reference pike may appear counterintuitive. Triglycerides could be protected from oxidative damage for two reasons: 1) the most common form of triglycerides, triolein, is comprised of fatty acid side chains with only one unsaturated bond whereas fatty acids that are susceptible to oxidation are polyunsaturated (Ercal et al., 2001; Niki et al., 2005); and 2) the packaging of neutral lipids inside cells as lipid droplets (Nelson and Cox, 2005) may physically restrict access of ROS to the fatty acid side chains. Conversely, there is some evidence that increased stores of

neutral lipids lead to greater lipid peroxidation (Lettéron et al., 1996; Pessayre et al., 2004) which could be facilitated by the enzymatic release of fatty acids during normal metabolism (Halliwell and Gutteridge, 1999). Lettéron et al. (1996) suggested a causative relationship between drug induced steatosis in the livers of mice and increased lipid peroxidation. The authors hypothesized that oxidative damage to the accumulated neutral lipids in hepatocytes may be the cause for the progression from steatosis to steatohepatitis. However, the accumulation of lipids almost always appeared histologically in the form of microvesicular steatosis, an indication of impaired mitochondrial beta-oxidation of fatty acids (Lettéron et al., 1996). Therefore, they could not conclude unequivocally that the lipid peroxidation was due to increased lipids and not due to mitochondrial dysfunction.

It is generally assumed that lipid peroxidation reflects oxidative damage to membrane phospholipids (Kelly et al., 1998; Ercal et al., 2001; Valavanidis et al., 2006). This has been demonstrated experimentally where elevated concentrations of one of the by-products of lipid peroxidation, malondialdehyde, was detected in human erythrocytes exposed in vitro to hydrogen peroxide. Since human erythrocytes are essentially devoid of neutral lipids (Sherwood, 2001), the increased malondialdehyde was likely due to oxidation of membrane phospholipids. Additional evidence that membrane phospholipids are susceptible to oxidative damage is given by the presence of phospholipid hydroperoxide glutathione peroxidase, an enzyme that specifically neutralizes peroxidized membrane lipids (Halliwell and Gutteridge, 1999).

4.7 Conclusions

Several indicators of condition and health in juvenile northern pike living downstream of the Key Lake uranium mill effluent discharge were assessed as part of this thesis. The elevated stores of energetic macromolecules in pike supported results from earlier research (Bennett and

Janz, 2007). A possible contributing factor to the greater energy stores included decreased parasitism whereas the results did not support the hypothesis that pike were consuming fattier prey items due to a potential food web enrichment effect of elevated nitrogenous compounds. Arsenic, nickel and selenium are elevated in lakes downstream of the Key Lake uranium mill but only arsenic and selenium and not nickel were significantly greater in exposure pike tissue. Other metals that can cause oxidative stress and that were elevated in pike tissue included iron and copper. Even though metals are accumulating in pike, there were no clear signs of oxidative stress or histopathological lesions in exposure pike target organs compared to reference. Furthermore, contrary to predictions, reference lake pike did demonstrate some signs of oxidative stress and histopathology. In the future, research could examine whether this apparent lack of toxicity of Key Lake uranium mill effluent on juvenile northern pike extends into later life stages of pike.

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