EVALUATING DIETARY SELENIUM UPTAKE AND SPECIATION DOWNSTREAM OF A URANIUM PROCESSING MILL USING CAGED SMALL-BODIED FISH

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By

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ABSTRACT

The main objective of this study was to investigate small-bodied fish caging as an approach to evaluate selenium (Se) bioaccumulation and speciation in native fish species inhabiting lakes influenced by uranium (U) milling effluent in northern Saskatchewan, Canada. In contaminated environments freshwater fish show a high propensity to accumulate Se beyond levels needed for normal physiological function. Maternal transfer of elevated Se concentrations to offspring can cause deformities and reduced survival in fry, and in certain cases negatively impact the sustainability of native fish populations. This research included a caging validation study using wild, naïve (i.e., collected from a reference lake) lake chub (*Couesius plumbeus*) and spottail shiner (*Notropis hudsonius*), and three field based 21-day caging studies to investigate the dominance of the feeding pathway with respect to Se uptake and speciation in wild populations of northern small-bodied fish exposed to a gradient of Se. Three feeding regimes were used: an *in situ* benthic diet, a basal Se diet of *Chironomus dilutus* (1.5 µg Se/g dry weight) and a Se-spiked diet of *C. dilutus* (5.5 µg Se/g dry weight). Lake chub were identified as more suitable candidates for caging due to higher survival and condition factor at the completion of the *in situ* 21-day trial. The resulting Se bioaccumulation was compared among treatments as well as to wild small-bodied fish populations from the study area. Results from the caging experiments showed that caged lake chub exposed to natural and controlled diets with elevated Se had significantly greater whole-body Se concentrations after 21 days compared to fish caged in the reference lake. The results also showed that whole-body Se concentrations exceeded conservative Se thresholds, and approached the currently proposed USEPA regulatory threshold (7.91 µg/g dry weight) designed to protect fish species in only three weeks. The use of stable carbon (C), nitrogen (N), and sulphur (S) isotope ratios indicated that alternate benthic food

sources native to the exposure lake were consumed in conjunction with the controlled diets. Stable isotope analysis of both wild and caged lake chub indicated that the N and S isotopic signatures decreased with increasing Se exposure, representing differences in isotopic signatures of the food sources. Speciation results from caged lake chub indicated that Se substituted for S in methionine (i.e. selenomethionine) was the dominant Se species found in caged lake chub exposed to dietary sources of elevated Se. Overall, this research demonstrates that using caged native lake chub represents a useful biomonitoring approach to investigate patterns of Se bioaccumulation and speciation in fish.

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In this study all sampling and procedures for experiments involving animals were conducted in accordance with the Canadian Council on Animal Care (University of Saskatchewan Animal Care and Use Protocol 20030088).

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LIST OF ABBREVIATIONS

Δ = change
µg/g = micrograms per gram
µS/cm = microsiemens per centimetre
µg = microgram
µg/L = micrograms per litre
µm = micrometre
µM = micromolar
ANCOVA = analysis of covariance
ANOVA = analysis of variance
As = arsenic
ATSDR = Agency for Toxic Substances and Disease Registry
C = Carbon
$\delta^{13}C$ = Carbon stable isotope signature
$CaCO_3$ = calcium carbonate
CCME = Canadian Council of Ministers of the Environment
CLS = Canadian Light Source
cm = centimetre
d = day
DO = dissolved oxygen
EEM = Environmental Effect Monitoring
EPT = Ephemeroptera-Plecoptera-Trichoptera
eV = Energy
g = gram
HDPE = high density polyethylene
h = height
H' = Shannon Weaver Diversity Index Value
Hg = Mercury
HSI = hepatosomatic index
HXMA = Hard X-ray Micro-Analysis
ICP-MS = inductively coupled plasma-mass spectrometry
kg = kilogram
km = kilometres
LA-ICP-MS = Laser Ablation Inductively Coupled Plasma Mass Spectrometry
L = litre
m = metre
m^3/d = cubic metres per day
MDL = Method detection limit
mg = milligram
mg/kg = milligrams per kilogram
mg/L = milligrams per litre
mL = millilitre

mm = millimetre
MS-222 = tricaine methanesulfonate
n = number of samples
$\delta^{15}N$ = Nitrogen stable isotope signature
Na_2SeO_3 = sodium selenite
NSERC = Natural Sciences and Engineering Research Council of Canada
O_2 = oxygen
PCA = Principal Components Analysis
PVC = Poly Vinyl Chloride
ºC = degrees centigrade
r^2 = Coefficient of determination
R3Se+ = Trimethyl selenonium iodide
R-Se-R = Selenomethionine
R-Se-Se-R = Selenocystine
S = sulphur
S-Se-S = Seleno-bis-diglutathione
$\delta^{32}S$ = Sulphur stable isotope signature
Se = selenium
Se^0 = Solid elemental selenium
Se^{2-} = inorganic selenides
Se^{4+} = Selenite
Se^{6+} = Selenate
SeO_3^{2-} = Selenite
SeO_4^{2-} = Selenate
SE = standard error
SSRL = Stanford Synchrotron Radiation Lightsource
TOC = Total Organic Carbon
U = Uranium
U_3O_8 = Yellowcake
USEPA = United States Environmental Protection Agency
W = width
XAS = X-ray Absorption Spectroscopy

PREFACE

The 2nd and 3rd chapters of this thesis are organized as manuscripts for publication in scientific journals. As a result there is some repetition of introductions, materials and methods throughout this thesis. Chapter 2 was accepted for publication by Ecotoxicology and Environmental Safety on February 21st, 2011 and Chapter 3 was accepted for publication by Ecotoxicology and Environmental Safety on June 17th, 2011.

CHAPTER 1

1 GENERAL INTRODUCTION

1.1 Selenium in the environment

Selenium (Se) is a widely occurring natural element that is important to all forms of life in trace amounts. Selenium belongs to group VIA on the periodic table of elements sharing similar properties with elements such as sulphur (S). The properties shared by this group of elements are the cause for interactions and substitutability of Se and S (Reilly, 2006). Selenium commonly co-occurs with sulphide minerals and within the matrices of metal ores including uranium ore deposits (ATSDR, 2003). Selenium is not evenly distributed throughout the environment and may be found in high concentrations within some ore bodies and be absent in others. The release of Se into the environment occurs as a result of both natural processes and anthropogenic activities such as agriculture, mining, industry, power generation and oil and gas production (ATSDR, 2003; Reilly, 2006; Stewart et al., 2010). Beginning in the early 1980s evidence of anthropogenic Se contamination began to emerge in aquatic environments adjacent to several large industrial operations in the United States. These anthropogenic activities have the potential to reduce the quality of available habitat, species diversity and the structure of fish populations and communities (Lemly, 1993a).

1.2 Selenium cycling and speciation in aquatic environments

Selenium has a unique biogeochemistry compared to other elements. In aquatic environments aqueous Se is rarely found in its elemental form (Se^{0}) and instead exists in one of its main inorganic forms selenate (Se^{6+}), selenite (Se^{4+}) or inorganic selenides (Se^{2-}) (Gomez-Ariza et al., 1999). The elemental form of Se is insoluble and tends to precipitate out in sediment. However, the mobility of inorganic forms such as selenate may be enhanced by

complexation with cations such as sodium. The soluble inorganic forms of Se (selenate and selenite) are readily mobile under oxidising conditions and the organic forms, while volatile, are readily mobile under reducing conditions (ATSDR, 2003; USEPA, 2004; Simmons and Wallschläger, 2005). Under most conditions the dissolved fraction of Se approximates the total amount of Se in the water column (Cutter, 1989).

Selenium cycling in the aquatic environment is variable depending on the hydrology, chemistry and ecology of the system. In the aquatic environment Se partitioning and therefore its toxicity can be affected by a variety of water quality variables including pH, but primarily depends on microbial activity and the presence of organic matter (ATSDR, 2003; Simmons and Wallschläger, 2005). Lakes and other slow moving lentic water bodies have much higher rates of Se cycling than fast moving lotic systems due to the residence time of particles in the system (Lemly, 1999; Simmons and Wallschläger, 2005; Orr et al., 2006). Selenium in the water column can be taken up in biological processes, it can adsorb to sediments or organic carbon, inorganic forms can be reduced in the sediment to the elemental form, complex with other available metals, or small amounts may volatilise into the atmosphere (Bowie et al., 1996; Lemly, 1997, 1999; Simmons and Wallschläger, 2005). Selenium toxicity can also be reduced due to competition with sulphate, phosphate, and other trace metals including arsenic and mercury (ATSDR, 2003; USEPA, 2004; Simmons and Wallschläger, 2005).

1.2.1 Selenium uptake, bioaccumulation and biomagnification

Selenium is an essential micronutrient that is vital to biological systems in small amounts. Symptoms of Se deficiency in fish include lethargy, abnormal swimming patterns, growth reduction, liver degeneration, tissue vacuolisation, and mortality (Hilton et al., 1980; Bell et al.,

1986; Watanabe et al., 1997). However, the distinction between Se deficiency and toxicity in fish is relatively narrow. Selenium uptake from the water column by algae, bacteria, and fungi is the result of non-passive cellular processes that transport Se and S across cell membranes allowing them to be fixed into various tissues (Ogle et al., 1988; Riedel et al., 1991; Besser et al., 1993; Stewart et al., 2010). Algae are capable of taking up organic forms of Se approximately 1000 times more readily than inorganic forms (Amweg et al., 2003). Microorganisms (e.g. bacteria and phytoplankton) are generally very tolerant of inorganic Se and convert it into more bioavailable organic forms (mainly selenomethionine) (Bowie et al., 1996). Therefore the rapid conversion of inorganic Se by microorganisms into organic forms is the main factor mobilising Se into the food chain and leading to Se bioaccumulation (Ogle et al., 1988; Stewart et al., 2010). Biological compartments containing Se will ultimately be redeposited into sediments as part of detrital matter, leading to elevated Se concentrations in the sediment and native benthos (Saiki et al., 1993; Lemly, 1999).

The primary mode of Se uptake in fish is via the diet and its high bioavailability makes the trophic transfer of Se a threat to aquatic ecosystems. A large body of research indicates that elevated Se levels can bioaccumulate and biomagnify in aquatic food webs leading to impacts in native fish populations (Woock, 1987; Hamilton and Buhl, 1990; Besser et al., 1993; Lemly, 1997, 2003; Hamilton, 2004; Holm et al., 2005; Muscatello et al., 2006, 2008). Lemly (1997) indicated that dietary Se requirements for fish are approximately 0.1-0.5 µg/g dry weight, whereas negative effects have been reported at dietary concentrations as low as 3 µg/g dry weight (Hamilton and Buhl, 1990; Lemly, 1993a, 1997). Even at low environmental concentrations Se has a tendency to bioaccumulate as it moves to upper trophic levels of the food chain and can lead to significant population effects at higher trophic levels (e.g. fish and aquatic

birds). Chronic Se exposure in adult fish has led to increased incidences of deformities and reduced survival in fry (Woock, 1987; Hamilton and Buhl, 1990; Lemly, 1993b, 1997, 2003; Besser et al., 1993; Holm et al., 2005; Muscatello et al., 2006). Under controlled conditions high levels of dietary Se have been shown to cause reduced condition factor, liver and kidney damage and eventually death (Coughlan and Velte, 1989). Ultimately aquatic organisms can excrete Se by reducing it to hydrogen selenide and methylating it for excretion via urine, bile or through the gill (ATSDR, 2003). However, depending on the fish species the rate of excretion may not be sufficient to match Se accumulation in a contaminated system, thus leading to high body burdens of Se.

1.2.2 Selenium metabolism and the biological role of selenium

Dietary Se absorbed by the body is generally either incorporated into specific Se containing enzymes or fixed into proteins. The rapid conversion of Se into organic forms means that all bioavailable Se will be complexed, thus minimising its chemical reactivity in an organism's body. Among its primary uses, Se is important to all living organisms because it is utilised as selenocysteine at the active site of antioxidant enzymes such as glutathione peroxidase (Reddy et al., 1983; Reilly, 2006). By aiding in the reduction of reactive oxygen species, glutathione peroxidase acts as a defence mechanism against oxidative damage (Matés, 2000; Reilly, 2006). However, when Se concentrations exceed an individual's specific requirements the excess Se begins to interfere with normal protein and enzyme function and can cause oxidative stress (Palace et al., 2004). Exposure to organic Se forms (predominantly selenomethionine) via the diet is the chief cause of the production of reactive oxygen species and oxidative stress (Palace et al., 2004; Janz et al., 2010). Cellular dysfunction occurs because Se

can act as an analogue for sulphur thereby indiscriminately replacing it during protein synthesis (Besser et al., 1993). Selenium substituted proteins cannot form disulphide bonds leading to structurally unstable molecules with altered folding geometry, thus increasing the potential for dysfunctional enzymes and proteins (Alaimo et al., 1994; Simmons and Wallschlager, 2005). Adult fish can typically tolerate some cellular dysfunction without overt signs of toxicity but in developing organisms it may disrupt cellular processes leading to teratogenic effects such as deformities (skeletal, craniofacial, and fin) and edema (Reddy et al., 1983; Lemly, 1993b, 1997; Muscatello et al., 2006). Increases in teratogenic effects may lead to reduced survival and poor recruitment in subsequent generations (Muscatello et al., 2006).

The increase in bioavailable forms of Se (mainly selenomethionine) at the sediment water interface facilitates its uptake by benthic invertebrates (Lemly, 1997). These bioavailable forms of Se are incorporated into the food chain and can lead to impacts on fish populations via bioaccumulation and biomagnification (Bowie et al., 1996; Fan et al., 2002; Tsopelas et al., 2004; Hamilton, 2004; Simmons and Wallschlager, 2005). The dietary uptake of Se as selenomethionine has a significant effect on aquatic organisms because it cannot be synthesised by vertebrates and therefore any excess selenomethionine that is not metabolized will be incorporated in organs where high rates of protein synthesis occur such as the liver (Fan et al., 2002). These tissues (e.g. liver, gonads, kidneys, skeletal muscle, stomach and erythrocytes) may incorporate excess selenomethionine into proteins instead of methionine (ATSDR, 2003; Reilly, 2006).

The effect of Se enrichment at each trophic level is largely determined by the local ecology and species-specific sensitivities. Local ecology may differ between water bodies due to differences in water chemistry, retention time, sediment characteristics, or biological cycling.

Therefore, the presence of elevated Se bound to sediments is a long term Se source for fish and wildlife due to biological cycling that mobilises Se through the food chain (Lemly, 1993a, 1997). Examples of Se impacts such as Belews Lake show that elevated Se concentrations can often lead to the local extirpation of sensitive fish species and the persistence of tolerant fish species (Lemly, 1985). This means that Se concentrations in water may be relatively low (< 10 μg/L) yet tissue concentrations in higher trophic levels may reach levels that lead to complete reproductive failure of fish populations (Lemly, 1997, 2003; Muscatello et al., 2008). Selenium concentration increases of greater than 100 fold have been observed between primary producers and primary consumers, representing the largest change observed at any tropic level (Stewart et al., 2010). However, the increments by which Se concentrations increase at the higher trophic levels are much smaller.

The life cycle of benthic invertebrates requires close contact with sediments, where they build their cases, process food, and in contaminated ecosystems where they accumulate elevated levels of Se (Lemly, 1993a, 1997; Bowie et al., 1996; Sappington, 2002). Generally benthic invertebrate communities show no negative effects of Se accumulation on species abundance or general community structure yet they act as a source of high Se concentrations to predators at higher trophic levels such as fish (Lemly, 1985, 1993a, 1999; Bowie et al., 1996; Sappington, 2002, Andrahennadi et al., 2007). The dietary transfer efficiency of Se between invertebrates and fish has been shown to be nearly 100% and therefore is the most important route of Se uptake (Lemly, 1993a; Hamilton, 2004; Schlekat et al., 2005). Neither invertebrates nor vertebrates have the ability to synthesize selenomethionine for basic biological functions, which means that they must acquire organic Se from lower trophic level organisms (Alaimo et al., 1994). As a result, Se loading in an aquatic system will lead to large increases in Se uptake by

primary producers and dramatically increase Se availability for higher trophic level organisms such as fish (Lemly, 1999).

The maternal transfer of Se is another key route of accumulation in fish. Adult fish have been observed to survive chronic exposure to elevated Se without showing symptoms of toxicity (Lemly, 1997, 2003). However, a female with a high Se body burden will transfer excess Se to her eggs during their development (DeForest et al., 1999). The maternal transfer of Se therefore leads to impacts at an early developmental stage in the form of embryo mortality or teratogenic defects in the surviving offspring (Lemly, 1993b; DeForest et al., 1999; Muscatello et al., 2006). Non fatal symptoms of Se exposure in developing fish include edema, cataracts and protruding eyes (Muscatello et al., 2008). Significant developmental abnormalities in fish have been linked to whole-body and egg Se residues in adult females, including teratogenic defects such as spinal deformities (lordosis, scoliosis, and kyphosis), craniofacial deformities, and missing or deformed fins (Lemly, 1993b, 1997; Muscatello et al., 2006, 2008). In some cases, Se accumulation in an otherwise healthy population can cause complete reproductive failure leading to population collapse over a relatively short period of time (Lemly, 1997). However, once Se loading has stopped whole-body Se concentrations in fish have been shown to decrease rapidly via depuration, and population recoveries have been reported (Lemly, 1982; Garrett and Inman, 1984; Hamilton, 2004).

1.2.3 Selenium management

The management of Se in aquatic environments has been slow to develop in many jurisdictions and disagreement continues on universally protective criteria to protect the sustainability of aquatic species. Selenium has been referred to as one of the most hazardous

trace metals after mercury, although this fact has not been widely recognised. In 1987 the United States Environmental Protection Agency (USEPA) implemented a 5 µg/L water quality criterion to protect fish against chronic Se exposure. This criterion generated significant discussion on the value of a water quality based approach because it didn't take into account the contribution of dietary uptake of Se (DeForest et al., 1999; Sappington 2002; Lemly, 2003; Hamilton, 2004). A universal water standard also fails to account for the differences in climate and water chemistry between ecological regions of the United States and beyond.

As a protective metric for managing Se impacts in freshwater systems, DeForest et al. (1999) proposed a dietary threshold of 11 µg/g and a whole-body Se threshold of 6 µg/g dry weight for coldwater species. Subsequently, the USEPA proposed replacing the water criterion with a whole-body fish tissue standard of 7.91 µg/g dry weight (USEPA, 2004) based primarily on bluegill sunfish (*Lepomis macrochirus*), a warm water centrarchid species. A tissue based value for evaluating Se is generally superior because the single endpoint takes into account several factors (e.g., Se speciation, metabolism and exposure duration) which are linked to potentially adverse effects in fish (USEPA, 2004). This value is still considerably higher than the conservative values present in the Se literature which propose a dietary threshold of 3 µg/g and a whole-body Se concentration of 4 µg/g dry weight (Hamilton, 2004).

In Canada comparatively little research has been conducted on Se concentrations that are relevant for the protection of fish populations in northern environments (Kennedy et al., 2000; Holm et al., 2005; Muscatello et al., 2006, 2008). The Canadian Council of Ministers of the Environment (CCME) has established a guideline for waterborne Se of 1 µg/L to protect fish. This ambient water quality threshold appears to be the most stringent guideline in the world;

however, critics argue that it lacks regulatory authority (Outridge et al., 1999). Overall, environmental policies that have addressed Se issues have not resulted in immediate successes and as a result continued scientific research and regulatory discussion is required in order to successfully manage Se risks. Any attempts by the CCME to establish site-specific criteria for northern fish populations needs to take into account both physical and biological data from northern aquatic ecosystems. Site specific criteria would be valuable in many instances because bioaccumulation and biomagnification can vary between ecosystems due to Se accumulation rates, elimination rates, and the differences in Se compartmentalisation within a specific environment. Site specific Se criteria would also avoid the use of surrogate species from other ecosystems that most likely do not provide adequate protection for the most sensitive species in each aquatic ecosystem.

1.2.4 Uranium mining and milling operations at Key Lake, Saskatchewan

The Key Lake uranium mine is located in northern Saskatchewan, Canada (Figure 1.1).

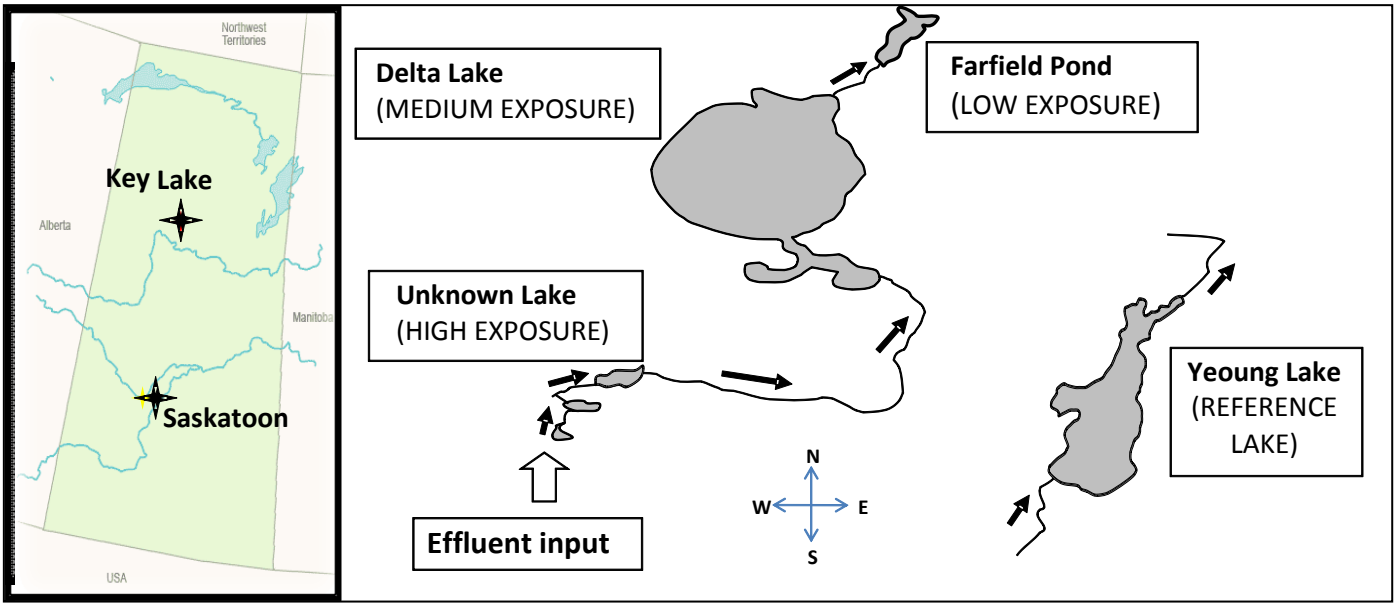

Figure 1.1: Location of the Key Lake uranium mill (Saskatchewan, Canada) and map of the study lakes located downstream of where the mill effluent joins the David Creek drainage (57˚13'N, 105˚38'W). The map shows the relative size and orientation of the exposure lakes and the reference lake in an adjacent watershed. The lakes are shown in grey and the black arrows indicate the direction of water flow.

Uranium has been milled at Key Lake since 1982, however uranium ore extraction ceased in 1997 and the mill now processes ore from a nearby deposit in McArthur River. Selenium concentrations in the aquatic environment located downstream of the Key Lake uranium mill have increased over the operating life of the mill. Selenium is a naturally occurring element found within the uranium ore processed in Key Lake. The processing of this Se rich ore for more than 25 years has resulted in a legacy of elevated Se concentrations being released into the local aquatic ecosystem. Study sites at the Key Lake uranium milling operation in northern Saskatchewan, Canada have been characterised by elevated Se concentrations in water, sediment and biota (Muscatello et al., 2008; Golder, 2008). Recent data (2007 – 2011) indicates that the average Se concentration in the effluent released by the Key Lake mill (primarily as selenate) has ranged from 15 – 48 µg/L, with an overall trend towards reduced Se concentration in the effluent (Anne Gent, Key Lake environment department, personal communication). The current administrative control for the Se reduction program is 27 µg/L (Anne Gent, Key Lake environment department, personal communication).

The presence of elevated Se in the Key Lake receiving environment is of environmental concern because even at low aqueous concentrations Se accumulates in the sediments and is incorporated into the local food chain where it can potentially lead to effects on fish populations.

Environmental sampling at Key Lake has been extensive in recent years, including Environment Canada's Environmental Effects Monitoring (EEM) program conducted by Golder Associates (2004-2007), avian studies conducted by Minnow Consultants (2006-current), and various aquatic toxicology research projects conducted by researchers from the Toxicology Centre at University of Saskatchewan. Findings from previous field investigations indicated evidence of benthic invertebrate and fish population impacts in the near field study lakes (Golder, 2008), evidence of increased condition factor of fish in the far field study lakes possibly due to ecosystem enrichment (Bennett and Janz, 2007; Golder, 2008), decreasing surface water Se concentrations with increasing distance from the effluent source (Golder, 2008), little spatial variation in aqueous Se concentration within individual study lakes (Golder, 2008; Wiramanaden et al., 2010a), and considerable variability in Se concentrations in sediment within study lakes (Golder, 2008; Wiramanaden et al., 2010a). The end result is that elevated concentrations of Se have been found in biota downstream of the Key Lake uranium milling operation (Golder, 2008; Muscatello et al., 2008; Wiramanaden et al., 2010a).

The Key Lake receiving environment offers the opportunity to address some of the unanswered questions in Se research that will lead to a greater understanding of the aquatic ecotoxicology of Se. These issues include Se accumulation rates in newly exposed naive fish, the trophic transfer of Se between aquatic compartments, and the role Se speciation plays in the bioaccumulation and toxicity of Se in aquatic environments.

1.3 Test organisms

1.3.1 Ecological role of lake chub, spottail shiner, and northern pike

Lake chub (*Couesius plumbeus*), spottail shiner (*Notropis hudsonius*) and northern pike (*Esox lucius*) are all present in the study area and are ubiquitous across the Canadian boreal forest region with ranges extending into the United States (Scott and Crossman, 1973). All three species spawn in the spring after the ice thaws with the exact date dependant on seasonal weather and the latitude of the resident water body. Lake chub and spottail shiner are both small-bodied fish averaging approximately 100 mm and 76 mm in length, respectively (Scott and Crossman, 1973). Larger spottail shiners have been reported in the range of 127 mm (Scott and Crossman, 1973). Both species have similar body shape and fin positions but differ in terms of their colouration. Lake chub are generally identified by their silvery lead appearance which may be highlighted during spawning season by a strong red or orange colour on the underbelly, around the fins, or around the mouth (Scott and Crossman, 1973). Spottail shiner can be distinguished from lake chub because of their olive coloured back and silvery sides (Scott and Crossman, 1973). During summer the diurnal activities of lake chub see them moving to deeper waters during the daytime compared to spottail shiner which typically form large schools over sandy shoals. The diet of lake chub and spottail shiner generally consists of aquatic insects, free floating zooplankton, and algae (Scott and Crossman, 1973). Both lake chub and spottail shiner are very important forage fish for larger predators such as northern pike and even for larger individuals of other small-bodied species.

Northern pike have a circumpolar distribution making adult pike one of the dominant top-level freshwater aquatic predators in the northern hemisphere (Scott and Crossman, 1973;

Stewart and Watkinson, 2004). Adult northern pike are characterised by their olive green colour, light spots and yellow to white underbelly. Females generally grow larger than males, reaching lengths greater than 1 m and weighing in excess of 20 kg. During early development pike are omnivores feeding on both zooplankton and benthic invertebrates. Adult northern pike are known as excellent ambush predators preferring less turbid water with weedy cover where they can hunt by sight (Scott and Crossman, 1973). They are also generally indiscriminate predators resorting to cannibalism as a matter of habit rather than necessity.

1.3.2 The use of *Chironomus dilutus* for feeding experiments

Chironomus dilutus (Diptera: Chironomidae), is a non-biting midge that is widely used as an experimental organism. This species is ubiquitous in northern aquatic systems and is often used as a model species for evaluating environmental impacts because it is generally tolerant of stressful conditions (Postma, 1995). The culturing and handling of *C. dilutus* is relatively easy and they are tolerant to a range of water and sediment conditions including elevated Se concentrations (Benoit et al., 1997). In natural systems a high ratio of chironomids relative to other more sensitive benthic invertebrate species (i.e. Ephemeroptera, Plecoptera and Trichoptera; EPT) is often used as an indicator of environmental impacts (Rabeni, 2001). Differences in invertebrate density and diversity between sites are often evaluated using community impact metrics such as the EPT: Chironomid ratio or benthic invertebrate diversity assessments such as the Shannon-Weaver diversity index (Krebs, 1989; Barbour et al., 1999). *Chironomus dilutus* are also rapid and efficient accumulators of many toxicants in contaminated aquatic environments (Krantzberg, 1989). The major pathways contributing to bioaccumulation in *C. dilutus* are through interactions with surface water, pore water, and sediment.

The *C. dilutus* life cycle involves three aquatic life stages (egg, larvae, and pupae) followed by an adult flying life stage. Developing larvae will pass through 4 stages called instars which each last approximately 4-7 days (Environment Canada, 1997). In preparation for their adult life stage, 4th instar *C. dilutus* will cease feeding to prepare for pupation. After less than 24 hours of pupation the adult insect emerges from its aquatic habitat as a flying midge. During the adult portion of the life cycle flying midges focus on mating and egg production. Within 24 hours of successfully mating, a female *C. dilutus* will lay a gelatinous egg mass in the water column. Typical hatching times for these eggs are approximately 2-4 days, but may require as much as 6 days (Benoit et al., 1997). Under laboratory conditions this entire life cycle requires between 23 and 30 days at approximately 23˚C (Benoit et al., 1997).

1.3.3 Caging of small-bodied fish (a field based bioassay)

As part of Environment Canada's EEM program, mining developments must conduct benthic invertebrate surveys, fish population surveys, and fish tissue surveys using both exposure and reference sites. Field based bioassays using sinking cages may be a useful tool in this process because it evaluates exposure to actual field conditions as a proxy for effluent toxicity tests (Palace et al., 2005). In contrast, laboratory studies may have difficulty replicating complex environmental mixtures or conditions that have been altered by pH, light levels, or changes in Se speciation. Therefore, the use of caging studies has a potential advantage in the complex conditions found in many ecosystems impacted by metal mining effluent. A standardized technique regarding the deployment and use of sinking cages for the evaluation of small-bodied fish exposed to industrial effluent was proposed by Palace et al. (2005). The use of this standardised technique aims to eliminate the uncertainty regarding fish exposure due to the

mobility of wild populations outside of exposure waters. The main strength of caging studies using small-bodied fish such as lake chub and spottail shiner is that concerns regarding confinement stress are minimised by both the size of the fish and the relatively small home range they require (Palace et al., 2005). In addition, restricting fish movement within cages is an effective means of controlling small-bodied fish interactions with the surrounding environment (water, sediment, and biota). The utility of caging studies in evaluating contaminant accumulation as well as typical EEM endpoints has been supported by recent research (Doebel et al., 2004; Palace et al., 2004; Klaverkamp et al., 2006).

1.3.4 Selenium speciation analysis

An understanding of Se speciation is required to fully evaluate the potential effects caused by biologically available Se concentrations in both individual fish and in fish populations (Stewart et al., 2010). Selenium accumulation and speciation is related to both the availability and spatial variability of Se in the food web. The Se concentrations and speciation in benthic invertebrates, zooplankton, or algae at a specific locale will depend on adsorption, dietary assimilation, gut biochemistry and biotransformation in these aquatic organisms (Andrahennadi et al., 2007). Selenium uptake and speciation in fish is primarily affected by prey density, Se accumulation in prey items as well as individual and interspecies differences in feeding behaviour, Se assimilation and biotransformation (Stewart et al., 2010). Andrahennadi et al. (2007) noted that Se exposure led to increased organic Se fractions and a corresponding reduction of inorganic Se at higher trophic levels. At the population level, an increase in the bioaccumulation of organic forms of Se is important because even though Se levels do not

undergo large increases in biomagnification between small-bodied fish and larger predators, the biomagnification of organic Se species may be significant.

Recent work determining Se speciation spectra using K near-edge X-ray absorption spectroscopy (XAS) has been conducted at the Stanford Synchrotron Radiation Lightsource (SSRL) in Menlo Park, CA (Andrahennadi et al., 2007) and is currently being conducted at the Canadian Light Source synchrotron in Saskatoon, SK. The benefit of XAS analysis is that it is sensitive to the local electronic environment of Se and therefore it is able to distinguish the different chemical environments in which Se is present. XAS results are fit to organic Se standards such as selenocystine (R-Se-Se-R) and selenomethionine (R-Se-R) as well as inorganic forms such as selenite (SeO_3^{2-}), selenate (SeO_4^{2-}) and solid elemental Se^0. XAS results are normalised and background corrected before being analysed according to the standard methods outlined in Pickering et al. (2000). The resulting near K-edge spectra produce the fractional sums of each form of Se compared to the total Se present.

1.3.5 Stable isotope analysis

Stable isotope analysis is a common technique used in geology and archaeology. More recently it has been adapted for the field of biology to allow researchers to trace the movement of food or contaminants within an ecosystem (Cabana and Rasmussen, 1994). During trophic transfer the relative amounts of carbon (C), nitrogen (N) and sulphur (S) isotopes generated by primary producers can be traced to the top of a food chain. The use of stable isotope analysis makes it possible to track food consumption and make inferences regarding the potential bioaccumulation of contaminants in higher trophic levels. For example, isotopic analysis of

caged mussels has been used successfully to determine connections between marine pollution levels and high tissue concentrations in test organisms (Deudero et al., 2009).

Carbon, nitrogen and sulphur isotopes are the most commonly used markers for investigating impacts to aquatic ecosystems because they have more than one isotope, are naturally abundant, can be precisely measured, and are basic requirements for growth (Lajtha and Michener, 1994; Connolly et al., 2004). One of the most important criteria for the use of stable isotopes is that each food source has a distinct isotopic signature that does not change as it transfers through successive trophic levels (Lajtha and Michener, 1994). Isotope studies utilising ratios of naturally occurring ^{12}C, ^{13}C, ^{14}N and ^{15}N stable isotopes allow researchers to make inferences about biological cycling within an ecosystem. The use of sulphur isotopes has been shown to be an effective tool in separating food sources that have similar C and N isotope signatures (Connolly et al., 2004). The evaluation of sulphur isotopes has also been shown to be an effective tool for evaluating between producer variation and within producer variation in different treatment groups (Connolly et al., 2004).

1.3.6 Research objectives and hypotheses

The goal of my research is to evaluate the uptake, speciation and trophic transfer of Se in an impacted lake system in northern Saskatchewan.

The objectives of my project have been divided into the following hypotheses:

1. Fish Survey: Evaluate the concentrations of total Se and Se speciation in resident lake chub, spottail shiner, and northern pike collected from lakes downstream of a uranium mill and from a reference lake.

Ho: There is no difference in Se bioaccumulation and speciation in resident fish collected from reference and exposure lakes.

2. Species Determination: Determine potential differences in survival and growth between lake chub and spottail shiner used in a 21 day *in situ* feeding cage study.

   Ho: *There is no difference in growth and survival between lake chub and spottail shiner used in a 21 in situ feeding cage study.*

3. Caging Study: Determine Se bioaccumulation and speciation in small-bodied fish caged in lakes located downstream of the Key Lake uranium mill.

   Ho_1: *The rate of Se accumulation and speciation in lake chub does not differ between reference and exposure lakes.*

   Ho_2: *There is no difference in Se accumulation and speciation in lake chub grazing on native benthos and those fed controlled diets of low or high Se.*

4. Evaluate the dominance of the feeding pathway using C, N, and S isotope analysis of food web components from each of the study lakes.

   Ho: Dietary exposure is the dominant pathway of Se accumulation in small-bodied fish.

CHAPTER 2

2 SELENIUM UPTAKE AND SPECIATION IN WILD AND CAGED FISH DOWNSTREAM OF A METAL MINING AND MILLING DISCHARGE

2.1 Introduction

Treated effluent from uranium milling operations in Saskatchewan, Canada typically contains elevated concentrations of certain trace metals, including dissolved inorganic forms of selenium (Se) such as selenate or selenite (Muscatello et al., 2008; Wiramanaden et al., 2010a). Even at low aqueous concentrations, Se can potentially bioaccumulate in aquatic food chains and cause effects at higher trophic levels in oviparous species such as fish and aquatic birds (Bowie et al., 1996; Fan et al., 2002; Hamilton, 2004; Tsopelas et al., 2004; Simmons and Wallschläger, 2005). Inorganic Se is taken up by primary producers directly from water; however the dietary transfer of Se, beginning at the primary producer step is the most important route of exposure in higher trophic organisms such as fish (Stewart et al., 2010). Selenium is an essential micronutrient that is vital to biological systems in small amounts, but has a narrow range between essentiality and toxicity in fish (Wilber, 1980). Dietary requirements of Se for fish are approximately 0.1 to 0.5 µg/g dry weight, whereas negative effects have been reported at dietary concentrations as low as 3 µg/g dry weight (Lemly, 1993, 1997).

At elevated concentrations the uptake, mobility and toxicity of Se are regulated by its chemical form (speciation) (Andrahennadi et al., 2007). Inorganic dissolved forms of Se (selenate and selenite) are incorporated into food webs by macrophytes, algae and microorganisms as organic Se species which bioaccumulate in benthic invertebrates, zooplankton and fish (Feldmann, 1986; Gomez-Ariza et al., 1999; Orr et al., 2006; Stewart et al., 2010). The increase in bioavailable forms of Se (e.g., organoselenides) at the sediment water

interface facilitates its uptake into the aquatic food web (Lemly, 1997). Previous studies have shown that sediment total organic carbon content is positively correlated with sediment total Se concentration, illustrating the important influence of biotic processes in Se bioavailability (Zawislanski et al., 2001; Wiramanaden et al., 2010a). Bioavailable forms of Se are incorporated into the food chain and can lead to impacts on fish populations via bioaccumulation and biomagnification (Bowie et al., 1996; Fan et al., 2002; Hamilton, 2004; Tsopelas et al., 2004; Simmons and Wallschläger, 2005). Selenite can also be reduced by microorganisms in anoxic sediments to elemental selenium (Se^0) which is generally considered to have low bioavailability (Oremland et al., 1989; Wiramanaden, et al., 2010b).

In 1987 the USEPA implemented a 5 µg/L water quality criterion to protect fish against chronic Se exposure. This generated significant discussion on the value of a water quality based approach because it did not take into account the contribution of dietary uptake of Se (DeForest et al., 1999; Sappington, 2002; Lemly, 2004; Hamilton, 2004). As a protective metric for managing freshwater systems, current management strategies agree that a tissue-based Se exposure criterion is required. DeForest et al. (1999) proposed a whole-body Se threshold of 6 µg/g dry weight for coldwater fish species whereas an even more conservative criteria of 4 µg/g dry weight was proposed by Hamilton (2004). These recommendations were based on studies looking at the effects of dietary selenium on the reproductive success of warm water fish from Belews Lake during the 1970s (Barwick and Harrell 1997). Studies of Se impacts on fish populations at Belews Lake, including the most sensitive species bluegill sunfish (*Lepomis macrochirus*), established the maternal transfer of Se between adults and their eggs and its effects in embryos and larvae causing dramatic declines in their population (Janz et al., 2010).

Subsequently, the USEPA proposed switching to a whole-body fish tissue threshold of 7.91 µg/g dry weight (USEPA, 2004).

A tissue based threshold is important to fisheries management because the maternal transfer of Se from adult females to developing eggs may cause embryo mortality or teratogenic defects in the surviving offspring (Lemly, 1993; DeForest et al., 1999; Muscatello et al., 2006). In some cases tolerant fish species have been shown to survive in environments with elevated levels of Se without showing symptoms of Se toxicity while more sensitive species may suffer complete reproductive failure and population collapse over a relatively short period of time (Lemly, 1997).

As part of Environment Canada's Environmental Effects Monitoring (EEM) program mining developments must conduct benthic invertebrate surveys, fish population surveys, and fish tissue surveys using both exposure and reference sites. Caging studies have been shown to be beneficial for measuring growth and contaminant uptake in reference and exposure lakes along a gradient of exposure (Doebel et al., 2004; Oikari, 2006; Allert et al., 2006). Field-based caging experiments may be well suited for field studies and EEM monitoring if wild fish population mobility occurs, or when lab studies require the simulation of complex environmental conditions (Palace et al., 2005). Standardised methodologies have been suggested by Palace et al. (2005) and Doebel et al. (2004), but little data are available for selecting appropriate wild species from the northern boreal forest region of Canada. The adoption of a standardized technique for caging small-bodied fish will address concerns regarding confinement stress and the control of fish interactions with the surrounding environment (water, sediment, and biota).

The main objective of this study was to evaluate the dominance of the feeding pathway with respect to Se uptake and speciation in both caged (wild naïve) and resident populations of northern small-bodied fish in areas downstream of a uranium milling operation in northern Saskatchewan, Canada. Comparisons of survival and growth during 21-day caging studies were used to determine a suitable, local small-bodied fish species for subsequent caging work. Whole-body Se concentrations and speciation were compared among study lakes, as well as to proposed tissue and ambient water quality guidelines in order to validate the use of *in situ* caging experiments as part of dietary exposure studies and future monitoring work. Overall, the results of this research will add to the understanding of the role of speciation and trophic transfer in selenium aquatic ecotoxicology.

2.2 Materials and Methods

2.2.1 Study area

Key Lake is located in north central Saskatchewan (57°11'N, 105°34'W) approximately 600 km north of Saskatoon, SK, Canada. The Key Lake uranium milling operation processes uranium ore into yellowcake (U_3O_8) and produces an effluent waste stream characterized by elevated ammonia, trace metal and metalloid concentrations, increased hardness and conductivity. The rate of treated effluent discharged to the environment is approximately 6000 m^3/d. The research sites used in this study include one reference site (Yeoung Lake) and three exposure sites, Unknown Lake (high exposure), Delta Lake (medium exposure) and Farfield Pond (low exposure), representing a gradient of Se exposure. The exposure sites are located approximately 4, 10, and 15 km downstream of the uranium mill effluent release point, respectively, while the reference site is located in a nearby unaffected watershed. Further details

regarding these study lakes can be found in Muscatello et al. (2008) and Wiramanaden et al. (2010a,b).

2.2.2 Fish collection

Previous work conducted in the Key Lake area has indicated that the most common fish species found in these study lakes were lake chub (*Couesius plumbeus*), spottail shiner (*Notropis hudsonius*), and northern pike (*Esox lucius*) (Muscatello et al., 2008), which are ubiquitous across the Canadian boreal forest region (Scott and Crossman, 1973). These species were selected to determine whole-body Se concentrations in small and large-bodied fish throughout the study lakes. All fish were collected opportunistically by angling (pike), fyke nets, beach seines and electrofishing (chub and shiner). Spottail shiner from the medium exposure lake were collected at a single location, whereas lake chub from the same lake were collected at two separate shoreline locations. Similarly, lake chub and spottail shiner from the reference lake were collected at different locations.

In early June (approximately three weeks following spring thaw), spottail shiner, lake chub and northern pike were collected (n = 5) from the medium exposure lake and the reference lake to determine whole-body Se concentrations. Additional northern pike (n = 5) were collected from the low exposure lake. No fish were collected from the high exposure lake due to low fish abundance. Fish collected during the fish survey were immediately euthanized using an overdose (0.8 g/L) of MS-222 (Tricaine, Sigma-Aldrich, Oakville, ON) and then frozen on dry ice in an airtight container. Samples remained on dry ice until they were transferred to a -80˚C freezer at the Toxicology Centre, University of Saskatchewan.

For the *in situ* caging study, lake chub (n = 90) and spottail shiner (n = 90) were collected approximately 3 weeks after spring thaw from the reference lake and anesthetised with a mild dose (0.1 g/L) of MS-222 to determine initial size measurements (weight and length). Captured fish were held in net pens for approximately 24 hours, transported to the study lakes in tanks aerated with supplied air and deployed into feeding cages immediately upon arrival at the study lakes. Mortality during this procedure was low for both lake chub (n = 1) and spottail shiner (n = 5).

2.2.3 In situ caged fish studies

Feeding cages were deployed to determine fish species suitability, Se uptake and Se speciation in fish held in lakes influenced by uranium milling discharge. The small-bodied fish species selected for the initial study were wild naive lake chub and spottail shiner collected from the reference lake. The choice for the most suitable species for future *in situ* caging work was determined based on differences in survival and growth for the two fish species.

A standardized technique regarding the construction and deployment of sinking cages for the evaluation of small-bodied fish exposed to industrial effluent was proposed by Palace et al. (2005). In the present study three 0.5 m^3 feeding cages (1.0 × 1.0 × 0.5 m; l × w × h) were placed in the reference lake (Yeoung Lake) and in the two highest exposure lakes (Unknown Lake and Delta Lake). Cage frames were constructed using ¾ inch (1.90 cm) PVC pipe and covered on five sides with ¼ inch nylon mesh. The bottom of each cage was covered with a larger $^3/_8$ inch (0.95 cm) mesh to allow fish to feed on benthic invertebrates *in situ* from the lake sediment on which the cages were placed. Cages were pressed into the lake bottom forcing sediment through the mesh and each cage was anchored into the sediment using a post that passed through a

hollow corner of the frame. This cage design also allowed fish to graze on food that accumulated on the cage netting as well as free floating food items that passed through the cage.

A previous study found aqueous total and dissolved Se concentrations in the same study lakes to show little spatial variation within lakes (Wiramanaden et al., 2010a). However, the same study noted more variability within sediments collected from Delta and Unknown Lakes, with a strong correlation noted between Se concentration and organic carbon content of the lake sediment (Wiramanaden et al., 2010a). To investigate the link between sediment organic carbon, Se concentration, and Se bioavailability, three cages were deployed in each lake along a gradient of sediment organic carbon content (low, medium and high organic carbon in sediment). Caging sites were selected based on GPS locations provided by Wiramanaden et al. (2010a).

On day 1 of the *in situ* caging study, ten fish (lake chub or spottail shiner) were placed in each cage. A total of 18 cages (9 per fish species) were deployed in the 3 study lakes (3 cages per lake), with one cage per sediment organic carbon type within each lake. Cages were deployed for 21 days and the fish were allowed to forage for food in the sediment below, on the cage netting and in the water column. To ensure adequate food availability, cages were rotated once every 7 days by approximately 120˚. After 21 days, fish were removed from the cages and euthanized, their weight and length were recorded, then they were placed in airtight containers and stored at - 80˚C. Survival was determined following the 21 day exposure period. All sampling and experimental procedures involving animals in this study were conducted in accordance with the Canadian Council on Animal Care (University of Saskatchewan Animal Care and Use Protocol 20030088).

2.2.4 Water chemistry

Triplicate water samples were collected adjacent to the caging locations and approximately 30 cm above the sediment surface (approximate mid height of cages) on day 10 of the 21-day caging experiments for analysis of dissolved Se, total hardness, ammonia and nitrate. Samples were collected using a Van Dorn horizontal acrylic beta water sampler (Wildlife Supply Company, Buffalo, NY) and passed through a 53-µm mesh sieve to remove floating debris and zooplankton. Water samples collected for dissolved Se analysis were filtered through 0.45 µm polyethersulfone filters (VWR International, Mississauga, ON) and preserved using 2% ultra-pure nitric acid (Omnitrace ultra grade, EMD chemicals, Gibbstown, NJ) in the field. All samples were collected in 250 ml acid washed high density polyethylene (HDPE) bottles and stored at 4˚C until analysis. General water chemistry (pH, conductivity, dissolved oxygen (DO), and temperature) were also assessed on-site using a multi-parameter YSI probe (6 series - YSI Inc., Yellow Springs, OH). Water hardness was measured using a HACH 16900 Digital Titrator (Hach Company, Loveland, CO). Water samples collected for nitrate were analysed using Ion Chromatography. Ammonia was measured using a VWR Symphony SB301 pH/ISE meter (VWR International, Mississauga, ON).

2.2.5 Benthic invertebrates

Triplicate benthic invertebrate samples were collected for taxonomic evaluation at each caging location using a 0.15 m^2 Eckman grab sampler (Wildlife Supply Company, Buffalo, NY) and immediately sieved through a 500 µm mesh sieve. The sieved samples were stored with no void space in pre-cleaned 1 L HDPE plastic bottles filled with 10% buffered formalin. All

samples were stored at 4ºC with minimal light exposure and were analyzed within ten days of collection. Invertebrates were sorted by taxa to family and stored in 70% ethanol. Identified taxa included mussels (Bivalvia), chironomid larvae (Insecta, Diptera), caddisfly larvae (Insecta, Trichoptera), leeches (Hirudinea), segmented worms (Annelida), and dragonfly larvae (Insecta, Odonata) according to Merritt and Cummins (1984). Differences in invertebrate density and diversity between caging sites were evaluated using the Shannon-Weaver Diversity Index (Krebs, 1989).

2.2.6 Sediment

Triplicate sediment cores were collected from each of the nine caging locations for determination of the percentage total organic carbon (% TOC), total sediment Se concentration, and particle size analysis. A hand-held corer was used to collect 4.8 cm diameter sediment cores, approximately 20 cm deep, into acrylic core tubes and stored at 4 ºC. Sediment samples were prepared from each core by removing the top 2.5 cm of sediment and homogenizing the sample in a glass beaker. Analysis of % TOC in sediment was conducted using a Leco Carbon Determinator (CR-12; Leco, St. Joseph, MI). Sediment samples were freeze dried in a Dura-Dry freeze dryer (FTS Systems, Stone Ridge, NY) and ground to a powder using a porcelain mortar and pestle. Freeze dried sediment samples were prepared for Se analysis using microwave digestion (CEM microwave MDS-2100, Matthews, NC) following a procedure outlined in Wiramanaden et al. (2010a) and analysed using inductively coupled plasma-mass spectroscopy (ICP-MS: X-Series II, Thermo Electron Corporation, Gormley, ON).

2.2.7 Selenium analysis of biological samples

All caged and wild fish samples collected for Se analysis were freeze dried for 72-96 hours and ground using a porcelain mortar and pestle for subsequent tissue digestion. The moisture content was 79 ± 0.02% for lake chub (n = 83), 77 ± 0.05% for spottail shiners (n = 5), 78 ± 0.03% for northern pike muscle tissue (n = 5), and 73 ± 0.16% for northern pike liver (n = 5). For each fish approximately 0.1 g samples were cold digested in Teflon vessels using 5 ml of ultra-pure nitric acid and 1.5 ml of hydrogen peroxide (30%, Suprapur, EMD Chemicals, Gibbstown, NJ). Digested samples were evaporated slowly in Teflon vessels at approximately 65°C and then reconstituted in 5 ml of 2% nitric acid. Prior to instrumental analysis reconstituted samples were syringe filtered using 0.45 µm polyethersulfone filters to remove any particulate matter.

Selenium concentrations in water (µg/L), sediment (µg/g dry weight) and fish (µg/g dry weight) were evaluated using ICP-MS at the Toxicology Centre (University of Saskatchewan, Saskatoon, SK). Method blanks and a standard reference material (TORT-2 lobster hepatopancreas, National Research Council of Canada, Ottawa, ON) were analyzed a minimum of every 10 samples. A method detection limit of 0.02 µg Se/g was determined using 19 method blanks. The concentration of the reference material was certified as 5.63 ± 0.67 mg/kg. Average Se recovery (>95%) was determined using 19 replicates of the standard reference material.

2.2.8 Selenium speciation determination using X-ray absorption spectroscopy

Whole fish samples were ground over liquid nitrogen as described previously (Wiramanaden et al., 2010b). A homogenised subsample was packed into a custom 2 mm

cuvette with a window constructed from sulphur and metal-free Mylar® tape, ensuring that there were no air pockets. Each cuvette was sealed with glycerol and stored in liquid nitrogen until it could be analysed using X-ray absorption spectroscopy (XAS). The cuvette was made of a polymer which contains no elemental interferences up to the bromine K-edge. Selenium speciation information was derived from Se K-edge XAS measured at the Canadian Light Source synchrotron in Saskatoon, SK, Canada. XAS is sensitive to the local electronic environment of Se and therefore is able to distinguish the different oxidation states and chemical environments in which Se is present. Selenium K near-edge spectra of prepared fish samples as well as aqueous Se standards (buffered at pH 7) were determined using the Hard X-ray Micro Analysis (HXMA) beamline at a temperature of 10 Kelvin in order to minimize speciation changes during data acquisition. Spectra of whole-fish and dilute Se standards were collected in fluorescence using a 30-element germanium detector (Canberra) equipped with arsenic filters. Further details regarding the Se K near-edge XAS methodology are outlined in Wiramanaden et al. (2010b).

Synchrotron data were normalized and background corrected according to standard methods (EXAFSPAK program suite) outlined in Pickering et al. (2000). The near-edge spectra of fish samples were analysed by least-squares fitting of each fish spectrum to the sum of the spectra of Se standards, using the EXAFSPAK program suite. A standard whose initial fractional contribution to the fit was less than 3 times its estimated standard deviation in the fit was excluded from subsequent fits. The contribution of the spectrum in the fit is equivalent to the contribution of that Se species type to the total Se. XAS identifies classes of Se species rather than specific molecules. For example, selenomethionine is used to represent Se bound to two aliphatic C groups (R-Se-R). Standards used to fit fish tissue samples included dilute aqueous solutions of organic forms of Se such as selenomethionine (R-Se-R), selenocystine (R-Se-Se-R),

seleno-bis-diglutathione (S-Se-S) and trimethyl selenonium iodide (R_3Se^+), as well as the inorganic forms selenite ($Se^{IV}O_3^{2-}$) selenate ($Se^{VI}O_4^{2-}$) and solid elemental Se^0 (Wiramanaden et al., 2010b).

2.2.9 Statistical analyses

Statistical analysis was conducted using SPSS version 17.0 (SPSS Inc., Chicago, IL) and a confidence level of 95% ($\alpha = 0.05$). Condition factor (weight/length^3 × 100) was calculated as a general health metric for each fish collected. Differences in condition factor were compared among fish from each lake using analysis of covariance (ANCOVA) with length as the covariate. The hepatosomatic index (HSI) values for pike were calculated by dividing the organ mass by the total body weight and multiplying by 100%. Data were tested for homogeneity of variance using Levene's test and normality using the Shapiro-Wilk test. The data passed the above tests and were analysed using the following statistical comparisons. A paired *t*-test was used to compare changes in condition factor for each cage between day 0 and day 21. A two-way ANOVA was used to detect significant differences in whole-body Se concentrations and benthic invertebrate densities between study lakes and *in situ* caging locations (i.e. the contribution of sediment Se concentrations and TOC on whole-body Se accumulation). If an interaction was observed, a one-way ANOVA and Tukey's test (when appropriate) were used to detect significant differences in whole-body Se among lakes or *in situ* caging locations. ANOVA and Tukey's test (when appropriate) were also used to identify differences among lakes in water chemistry (dissolved oxygen, pH, conductivity, total hardness, ammonia, nitrate, and dissolved Se), condition factor of wild fish, Shannon Weaver Diversity Index values at each caging location, northern pike HSI, and Se concentrations in northern pike liver and muscle tissue. The

relationship between the fraction of selenomethionine and whole-body Se concentration was determined using Graphpad Prism 3.0 (Graphpad Software Inc., La Jolla, CA). Due to beamtime restrictions at the Canadian Light Source synchrotron, statistical analyses were not performed on Se speciation evaluations since only one fish was analyzed from each treatment.

2.3 Results

2.3.1 Water chemistry

Conductivity ($p < 0.01$), hardness ($p < 0.01$), and ammonia ($p < 0.01$) were significantly higher, and dissolved oxygen was significantly lower ($p < 0.05$), in the exposure lakes than in the reference lake (Table 2.1). Selenium concentrations in lake water showed little variation among caging locations in the reference lake (<0.1 µg/L) and ranged from 1.48 to 1.52 µg/L at the medium exposure lake and 3.49 to 3.93 µg/L at the high exposure lake (Table 2.1). Aqueous Se concentrations in the exposure lakes were significantly greater ($p < 0.01$) compared to the reference lake, and were significantly different among all lakes, displaying a distinct Se gradient (Table 2.1).

Table 2.1 Water quality variables were recorded adjacent to the caging locations from one reference lake (Yeoung Lake) and three exposure lakes (Farfield Pond, Delta Lake, and Unknown Lake) downstream of the Key Lake uranium mill on June 24th, 2008. Data are means ± standard error of n = 3 samples.

Variable	Lake			
	Reference (Yeoung Lake)	Low Exposure (Farfield Pond)	Medium Exposure (Delta Lake)	High Exposure (Unknown Lake)
Dissolved oxygen (mg/L)	9.6 ± 0.1^{a}	8.2 ± 0.2^{b}	8.4 ± 0.2^{b}	8.5 ± 0.3^{b}
Temperature (˚C)	16.7 ± 0.9	18.1 ± 0.7	18.2 ± 1.0	18.9 ± 1.3
pH	6.6 ± 0.3^{a}	6.3 ± 0.2^{a}	7.3 ± 0.3^{b}	6.7 ± 0.1^{a}
Conductivity (µS/cm)	16.1 ± 0.5^{a}	510 ± 4.9^{b}	496 ± 9.3^{b}	641 ± 3.5^{c}
Total hardness (as $CaCO_3$) (mg/L)	7.0 ± 0.1^{a}	219 ± 2.1^{b}	396 ± 2.6^{c}	609 ± 3.2^{d}
Ammonia (as N) (mg/L)	0.1 ± 0.0^{a}	0.4 ± 0.0^{b}	0.6 ± 0.0^{c}	3.0 ± 0.1^{d}
Nitrate (mg/L)	0.3 ± 0.2^{a}	1.2 ± 0.0^{a}	1.2 ± 0.0^{a}	3.5 ± 0.2^{b}
Dissolved Selenium (µg/L)	$<0.1^{a}$	1.00 ± 0.01^{b}	1.49 ± 0.01^{c}	3.66 ± 0.05^{d}

Different letters (a-d) indicate significant differences among lakes tested using a one-way ANOVA and Tukey's test ($p < 0.05$).

2.3.2 Validation of caging protocol

Overall, the survival of caged lake chub (Table 2.2) was greater (range 80-100%) than that of spottail shiner (range 20-100%; Table 2.3). Using length as a covariate, significant decreases in weight were observed for lake chub caged at the medium (-0.21g) and high (-0.37g) organic carbon locations within the reference lake ($p < 0.05$). In contrast, there were significant increases at the low (+1.06g) and medium (+0.99g) organic carbon locations within the medium exposure lake, and the high organic carbon location (+0.59g) within the high exposure lake (all $p < 0.05$; Table 2.2). Lake chub caged in the reference lake for 21 days had reduced condition factor at each location (-0.04, -0.13, and -0.09 for low, medium and high % TOC in sediment, respectively) compared to lake chub caged in the medium (+0.16, +0.22, and +0.04) and high exposure (+0.03, +0.05, and +0.16) lakes, which had increased condition factors (Table 2.2). Within the reference lake there were significant decreases in condition factor for lake chub caged at the medium ($p < 0.01$) and high ($p < 0.01$) organic carbon locations. There were significant increases in condition factor for lake chub caged at the medium ($p < 0.05$) and high ($p < 0.001$) organic carbon locations in the high exposure lake, as well as at the low ($p < 0.001$) and medium ($p < 0.001$) organic carbon locations in the medium exposure lake.

Mean changes in body weight with length as covariate at the conclusion of the 21-day caging experiment were only positive for spottail shiners caged at the medium organic carbon location (0.42g) in the medium exposure lake, where survival was only 20% (Table 2.3). The changes in mean body weight with length as a covariate for spottail shiners caged in the reference (-0.47, -0.98, and -0.76 g), and high (-0.64, -0.72, and -0.64 g) exposure lakes were negative (Table 2.3). In the medium exposure lake negative changes in weight occurred at the low (-0.34 g) and high

(-0.07 g) organic matter locations. Significant decreases in body weight were observed for spottail shiner caged in the reference lake at the medium and high organic carbon locations and in the high exposure lake at the low and medium organic carbon locations (all $p < 0.05$; Table 2.3). There were significant decreases in condition factor for spottail shiner caged at the medium ($p < 0.01$) and high ($p < 0.001$) organic carbon locations in the reference lake, the low organic carbon location in the medium exposure lake ($p < 0.05$) and medium organic carbon location in the high exposure lake ($p < 0.001$). There were no significant differences in condition factor in wild (uncaged) lake chub or spottail shiners between the reference lake and the medium exposure lake (Tables 2.2 and 2.3).

Table 2.2 A, Total length, wet weight and condition factor for wild lake chub (*Couesius plumbeus*) and B, change (Δ) in wet weight, length, condition factor and average survival for lake chub caged for 21 days in one reference lake (Yeoung Lake) and two exposure lakes (Delta Lake and Unknown Lake) downstream of the Key Lake uranium processing mill (low, medium, and high refer to total organic carbon content of the sediment; see Table 2.4). Values for wild fish (A) represent means ± standard error of n = 6-10 fish. Values for caged fish (B) represent means ± standard error of n = 8-10 fish per cage.

A. Wild Fish		Lake Chub			
		Weight (g)	Total Length (mm)	Condition Factor (weight/length^3 × 100)	
Reference Lake (n = 10) (Yeoung Lake)		4.59 ± 0.40	77.7 ± 2.3	0.96 ± 0.02	
Medium Exposure Lake (n = 6) (Delta Lake)		1.66 ± 0.23	54.3 ± 2.1	1.00 ± 0.03	
B. Caged Fish		Weight Δ (g)	Total Length Δ (mm)	Condition Factor Δ	Survival
Reference Lake (Yeoung Lake)	Low	-0.20	0.0	-0.04	100%
	Medium	-0.21†	1.0	-0.13*	90%
	High	-0.37†	0.1	-0.09*	100%
Medium Exposure Lake (Delta Lake)	Low	1.06†	1.0	0.16*	100%
	Medium	0.99†	5.0	0.22*	100%
	High	0.55	2.0	0.04	80%
High Exposure Lake (Unknown Lake)	Low	0.49	2.0	0.03	100%
	Medium	0.73	3.0	0.05*	90%
	High	0.59†	3.0	0.16*	100%

† Significant difference in lake chub weight with length as a covariate using ANCOVA ($p < 0.05$). * Significant difference in lake chub condition factor between day 0 and day 21 for each caging location within each study lake, tested using paired *t*-tests ($p < 0.05$).

Table 2.3 A, Total length, wet weight and condition factor for wild spottail shiner (*Notropis hudsonius*) and B, change (Δ) in wet weight, length, condition factor and average survival for spottail shiner caged for 21 days in one reference lake and two lakes downstream of the Key Lake uranium processing mill (low, medium, and high refer to total organic carbon content of sediment; see Table 2.4). Values for wild fish (A) represent means ± standard error of n = 10 fish. Values for caged fish (B) represent means ± standard error of n = 2-10 fish per cage.

		Spottail Shiner			
A. Wild Fish		Weight (g)	Total Length (mm)	Condition Factor (weight/length^3 × 100)	
Reference Lake (n = 10) (Yeoung Lake)		3.66 ± 0.33	71.8 ± 2.0	0.98 ± 0.02	
Medium Exposure Lake (n = 10) (Delta Lake)		7.73 ± 0.20	91.6 ± 0.5	1.00 ± 0.01	
B. Caged Fish		Weight Δ (g)	Total Length Δ (mm)	Condition Factor Δ	Survival
Reference Lake (Yeoung Lake)	Low	-0.47	1.0	-0.09	90%
	Medium	-0.98†	-1.0	-0.20*	60%
	High	-0.76†	-1.0	-0.18*	100%
Medium Exposure Lake (Delta Lake)	Low	-0.34	0.1	-0.07*	100%
	Medium	0.42	0.0	0.13	20%
	High	-0.07	-1.0	-0.01	90%
High Exposure Lake (Unknown Lake)	Low	-0.64†	-2.0	-0.07	70%
	Medium	-0.72†	1.0	-0.16*	100%
	High	-0.64	-1.0	-0.10	70%

† Significant difference in spottail shiner weight with length as a covariate using ANCOVA (p < 0.05). * Significant difference in spottail shiner condition factor between day 0 and day 21 for each caging location within each study lake, tested using paired t-tests (p < 0.05).

2.3.3 Sediment and benthic invertebrate community characterization

Selenium concentrations in the exposure lake sediments were considerably more variable than the aqueous Se concentrations, but followed the same general trend among lakes (Table 2.1). A gradient of increasing % TOC was chosen for the caging locations within the reference lake (0.32 to 4.91 % TOC), medium exposure lake (1.27 to 11.48 % TOC), and high exposure lake (0.69 to 4.13 % TOC). In the reference lake, there was no trend of increasing Se in sediment with increasing % TOC in sediment (range 0.68 to 0.80 µg/g dry weight). However, both the medium (range 0.15 to 12.47 µg/g dry weight) and high (range 0.83 to 9.80 µg/g dry weight) exposure lakes had marked increases in total Se in sediment with increasing % TOC in sediment.

Benthic community diversity was evaluated using the Shannon Weaver Diversity Index (Table 2.4). Using this index, no significant differences in benthic community diversity between the reference and exposure lakes were detected. The dominant taxa at the majority of sites were Trichoptera and Diptera. The densities of benthic invertebrates were similar between the reference lake (733 to 2017 /m^2) and high exposure lake (1117 to 2233 /m^2). A much greater range of benthic invertebrate densities were observed in the medium exposure lake (1050 to 5783 /m^2), where the highest density was significantly greater than any of the other locations ($p < 0.05$). No other significant differences were observed in benthic invertebrate densities among exposure and reference lake caging locations.

Table 2.4 Total organic carbon (%TOC) and selenium concentration in sediment, total benthic invertebrate densities /m^2, and Shannon Weaver Diversity Index values for abiotic and biotic compartments collected at each of the in situ caging locations from one reference lake (Yeoung Lake) and two exposure lakes (Delta Lake and Unknown Lake) downstream of the Key Lake uranium processing mill. Values represent means ± standard error of $n = 3$.

Cage Location	Sediment TOC (%)		Selenium Concentration in Whole Sediment (µg/g dry weight)	Total Benthic Invertebrate Density/m^2	Shannon Weaver Diversity Index (H')
Reference Lake	Low	0.32 ± 0.02	0.80 ± 0.03	733 ± 192[a]	1.17 ± 0.06
	Medium	1.83 ± 0.21	0.68 ± 0.01	450 ± 29[a]	1.31 ± 0.09
	High	4.91 ± 0.80	0.68 ± 0.01	2017 ± 617[a]	0.70 ± 0.09
Medium Exposure Lake	Low	1.27 ± 0.08	0.15 ± 0.01	1267 ± 246[a]	1.18 ± 0.24
	Medium	7.89 ± 0.42	1.30 ± 0.50	1050 ± 200[a]	1.31 ± 0.09
	High	11.5 ± 0.47	12.5 ± 1.99	5783 ± 1871[b]	0.96 ± 0.10
High Exposure Lake	Low	0.69 ± 0.02	0.83 ± 0.03	1117 ± 347[a]	1.23 ± 0.04
	Medium	2.38 ± 0.58	2.33 ± 0.08	2233 ± 606[a]	1.03 ± 0.30
	High	4.13 ± 1.69	9.80 ± 1.87	1483 ± 338[a]	0.97 ± 0.05

Different letters (a,b) indicate significant differences in benthic invertebrate densities between all sampling sites, determined using one-way ANOVA and Tukey's Test ($p < 0.05$).

2.3.4 Whole-body selenium in caged fish

Whole-body Se concentrations in caged fish revealed a significant interaction between lake and substrate type. Whole-body Se concentrations in caged lake chub from the low, medium and high organic carbon locations in the reference lake (1.79 ± 0.09, 1.63 ± 0.06 and 1.66 ± 0.08 µg/g, respectively) were not significantly different (Figure 2.1). Whole-body Se concentration in caged lake chub from the medium organic carbon location (5.46 ± 0.19 µg/g) in the medium exposure lake was significantly greater than the low (4.36 ± 0.17 µg/g) and high (4.21 ± 0.33 µg/g) organic carbon locations ($p < 0.05$; Figure 2.1). In the high exposure lake, whole-body Se concentrations in caged lake chub from the high organic carbon location (6.06 ± 0.33 µg/g dry weight) was significantly greater than the low (3.50 ± 0.22 µg/g) and medium (4.32 ± 0.21 µg/g) organic carbon locations ($p < 0.001$; Figure 2.1). Overall, mean whole-body Se concentrations increased with increasing Se in sediment and increasing percentage of total organic carbon in sediment in the high exposure lake, whereas in the medium exposure lake the highest mean whole-body Se concentration was observed in lake chub caged at the medium organic carbon location (Figure 2.1).

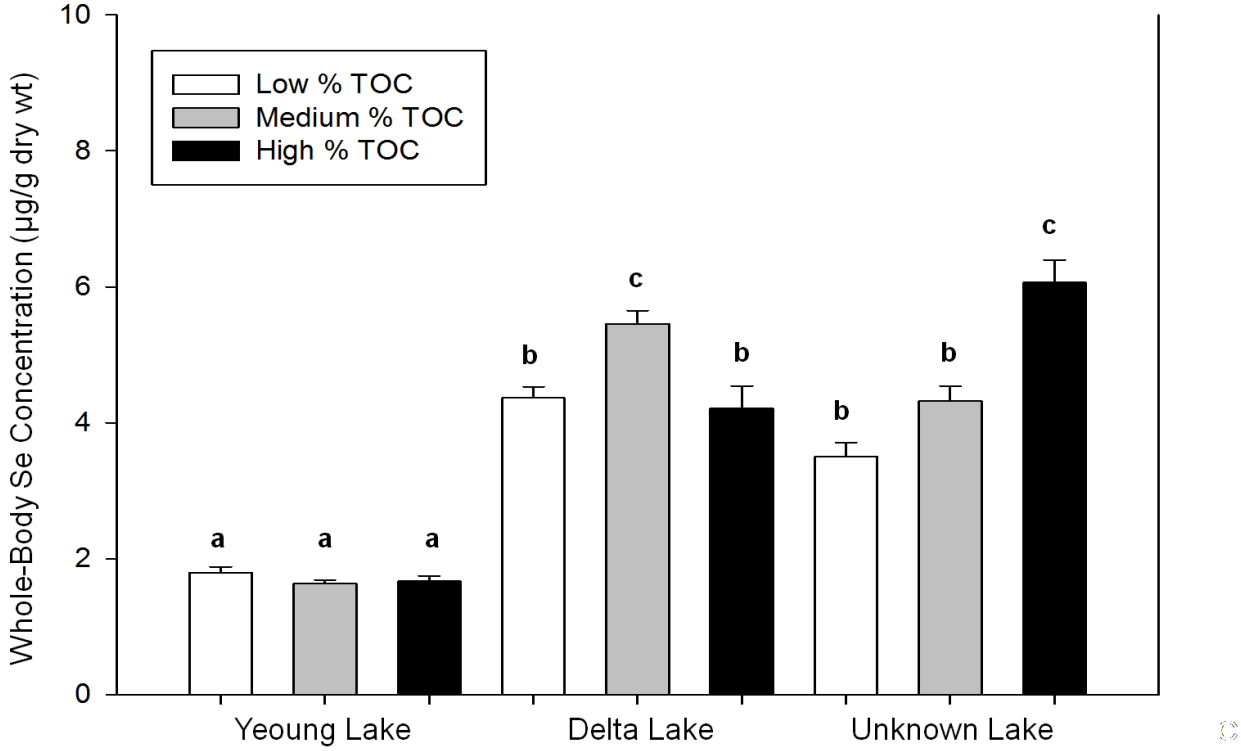

Figure 2.1 Whole-body Se concentrations of lake chub caged at locations with varying percentage total organic carbon (% TOC) content at the reference (Yeoung Lake) and exposure lakes (Delta Lake and Unknown Lake). Values represent means ± standard error of n = 8-10 fish. Different letters (a-c) indicate significant differences in Se concentration among caged lake chub using a one-way ANOVA and Tukey's test ($p < 0.05$).

Whole-body Se concentrations in caged lake chub combined from all three locations in the medium (4.63 ± 0.18 µg/g) and high (4.73 ± 0.28 µg/g) exposure lakes were significantly greater than fish caged in the reference lake (1.70 ± 0.05 µg/g) ($p < 0.05$; Figure 2.2A). However, there was no significant difference between the whole-body Se concentrations in lake chub caged in the medium and high exposure lakes (Figure 2.2A).

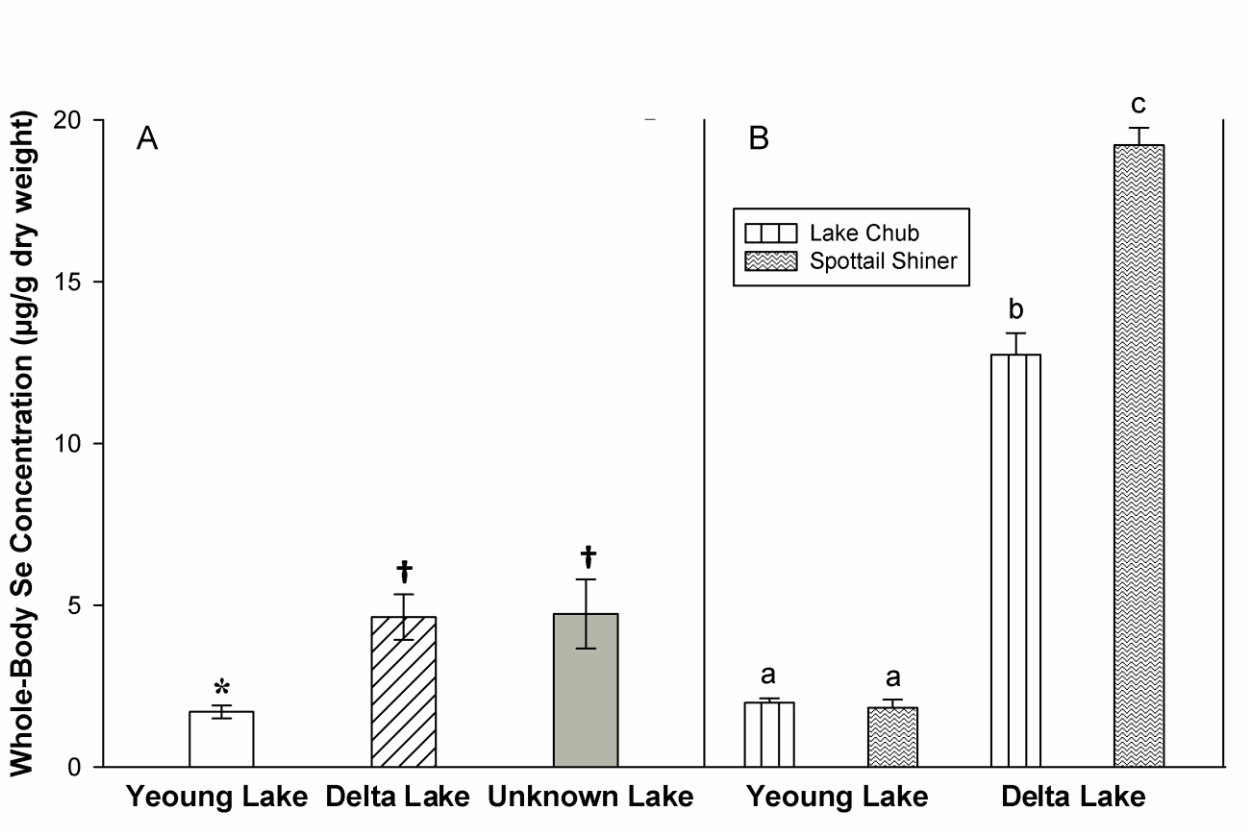

Figure 2.2 (A) Whole-body Se concentrations in caged lake chub from the reference (Yeoung Lake), medium exposure (Delta Lake) and high exposure (Unknown Lake) lakes and (B) whole-body Se concentrations in wild lake chub and spottail shiner collected from the reference and medium exposure lakes. Values represent means ± standard error for n = 5-10 fish. Different symbols (*,†) indicate significant differences between lakes for caged lake chub tested using a one-way ANOVA and Tukey's Test ($p < 0.05$). Different letters (a-c) indicate significant differences in wild lake chub and spottail shiner between the reference and medium exposure lakes using a one-way ANOVA and Tukey's test ($p < 0.05$).

2.3.5 Whole-body selenium in wild fish

No wild fish were collected from the high exposure lake (Unknown Lake). Whole-body Se concentrations in wild (uncaged) spottail shiner (19.22 ± 0.54 µg/g) and lake chub (12.74 ± 0.67 µg/g) from the medium exposure lake (Delta Lake) were significantly greater than those collected from the reference lake (1.83 ± 0.28 µg/g and 1.98 ± 0.14 µg/g, respectively) ($p < 0.001$; Figure 2.2B). Wild spottail shiner collected from the medium exposure lake also had significantly greater whole-body Se concentrations than wild lake chub ($p < 0.001$; Figure 2.2B).

Although body lengths and weights of northern pike varied among study lakes, there were no differences in condition factor for northern pike among the lakes (Table 2.5). The hepatosomatic index for northern pike was significantly lower in fish from the exposure lakes compared to fish from the reference lake ($p < 0.05$). Selenium concentrations in muscle and liver increased significantly along a gradient of increasing Se exposure ($p < 0.05$; Table 2.5). Selenium concentration in muscle was significantly greater in northern pike collected from the medium exposure lake (26.8 ± 2.6 µg/g) than in the low exposure lake (11.9 ± 0.3 µg/g; $p < 0.01$) and the reference lake (1.5 ± 0.2 µg/g; $p < 0.001$). Similarly, Se concentration in northern pike liver collected from the medium (22.7 ± 2.23 µg/g; $p < 0.001$) and low (16.2 ± 2.90 µg/g; $p < 0.05$) exposure lakes were also significantly greater than in the reference lake (4.98 ± 0.27 µg/g) (Table 2.5).

Table 2.5 Total length, wet weight, condition factor, hepatosomatic index (HSI), and Se concentrations in muscle and liver tissues of wild adult northern pike (Esox lucius) collected from the reference lake (Yeoung Lake), low exposure lake (Farfield Pond) and medium exposure lake (Delta Lake) downstream of the uranium processing mill at Key Lake.

Location	Length (cm)	Weight (g)	Condition Factor (weight/length^3 × 100)	HSI	Muscle [Se] (µg/g dry weight)	Liver [Se] (µg/g dry weight)
Reference Lake (Yeoung Lake)	61.8 ± 5.3	1553 ± 336	0.61 ± 0.02	1.10 ± 0.12^a	1.45 ± 0.18^a	4.98 ± 0.27^a
Low Exposure Lake (Farfield Pond)	53.6 ± 10.0	1023 ± 266	0.61 ± 0.01	0.65 ± 0.06^b	11.9 ± 0.27^b	16.2 ± 2.90^b
Medium Exposure Lake (Delta Lake)	55.4 ± 3.2	1101 ± 91	0.65 ± 0.03	0.70 ± 0.09^b	26.8 ± 2.56^c	22.7 ± 2.23^c

Values represent means ± standard error of $n = 5$ fish. Different letters indicate significant differences determined using one-way ANOVA and Tukey's Test ($p < 0.05$).

2.3.6 X-ray absorption spectroscopy

The chemical forms of Se in fish tissue samples were determined using Se K near-edge X-ray absorption spectroscopy (XAS). Overall, the Se spectra indicated differences in the Se species present among experimental groups (Figure 2.3A-D). The spectra analysed from wild and caged lake chub showed a decreasing fraction of inorganic Se with increasing Se exposure as well as increasing fraction of organic Se forms modeled as selenocystine and selenomethionine with increasing Se exposure (between exposure lakes). However, the organic form of Se (R-Se-Se-R) modeled as selenocystine was observed to be the most abundant form of Se in the reference lake (Figure 2.3A). In contrast, the organic form of Se (R-Se-R) modeled as selenomethionine was found to be the most abundant in fish from the medium and high Se exposure lakes (Figures 2.3B and 2.3D). Small fractions of inorganic Se species were found in two of the samples analysed, although Se^0 and SeO_3^{2-} were not found to make up a large proportion of the Se analysed in the fish tissue (Figure 2.3D).

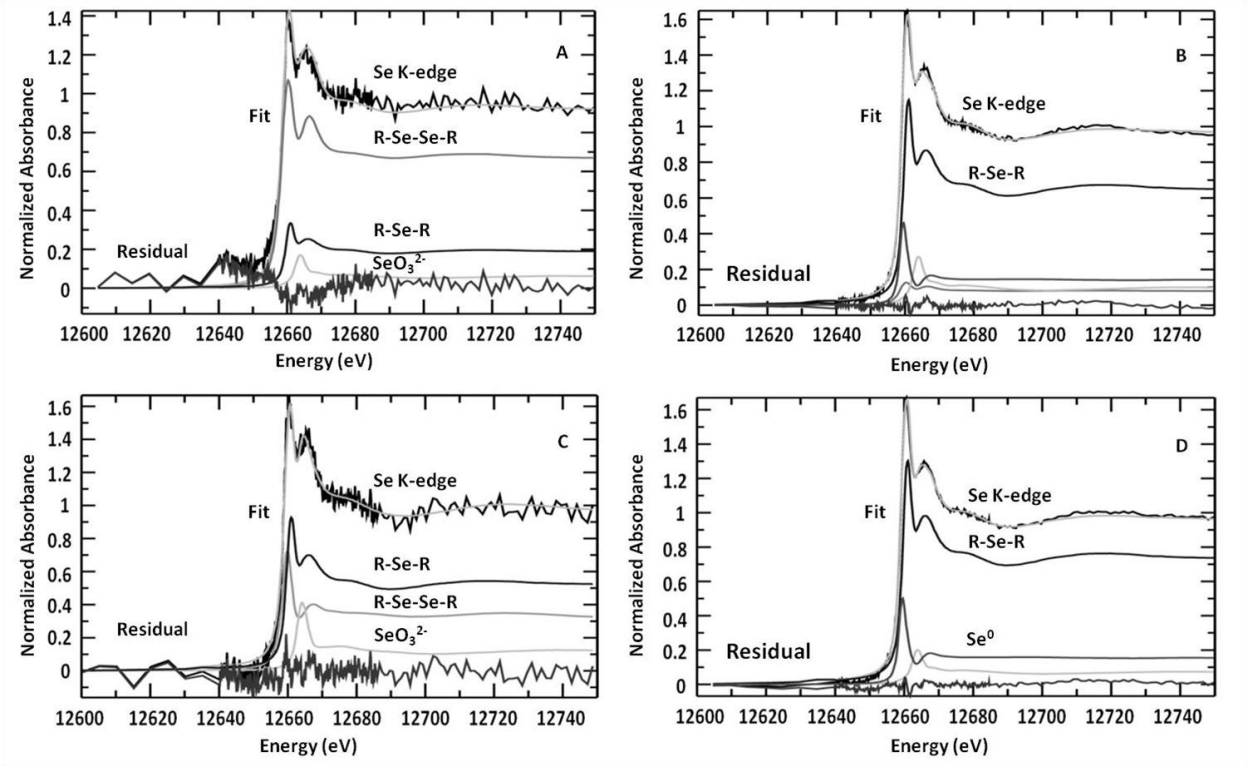

Figure 2.3 Selenium speciation spectra determined using K near-edge X-ray absorption spectroscopy for wild lake chub from (A) the reference lake (Yeoung Lake) and (B) medium exposure lake (Delta Lake), and for lake chub caged at the high sediment organic carbon location in (C) the reference lake (Yeoung Lake) and (D) high exposure lake (Unknown Lake). The Se K near-edge for each representative fish, the fit, the individual components scaled to represent their approximate contributions to the fit and the residual are shown (see text for details). Selenium standards included R-Se-Se-R (modeled as selenocystine), R-Se-R (modeled as selenomethionine), elemental Se (Se^0) and selenite (SeO_3^{2-}).

Combining all caged lake chub as well as wild lake chub and spottail shiner (representing whole lake averages), the relationship between whole-body Se concentrations and the proportion of Se modeled as selenomethionine (R-Se-R) indicated that selenomethionine was directly associated with increasing Se exposure ($r^2 = 0.649$; $p < 0.05$; Figure 2.4). Since whole-body Se concentration increased in both caged and wild lake chub, the fraction of total Se that was modeled as selenomethionine increased and appeared to reach a plateau between 0.6-0.8 (Figure 2.4).

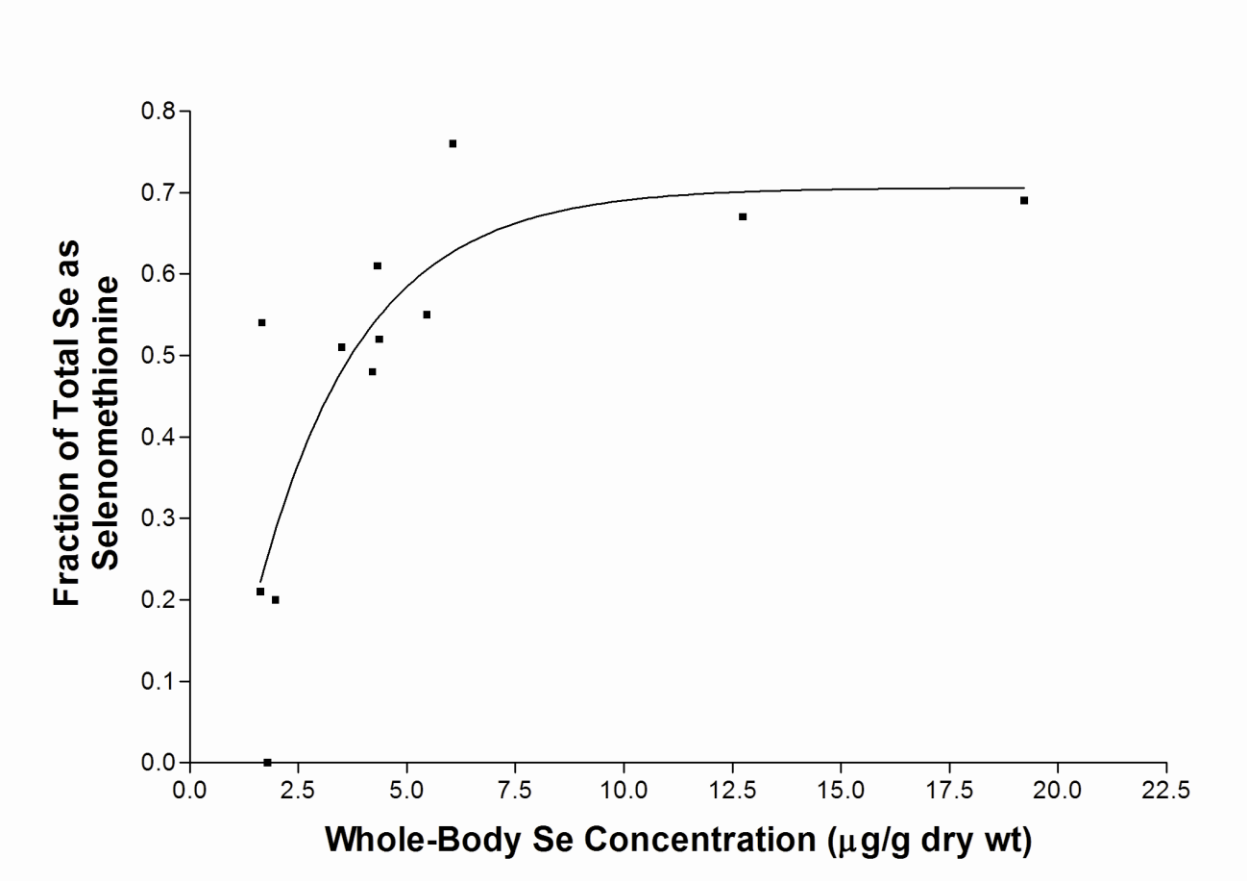

Figure 2.4 The fraction of selenomethionine in relation to whole-body Se concentrations in lake chub and spottail shiner from the reference and exposure lakes. Data points represent both caged fish and wild fish ($r^2 = 0.649$; $p < 0.05$).

2.4 Discussion

2.4.1 Abiotic and biotic environment – exposure characterization

The release of uranium milling effluent at the Key Lake operation has led to elevated trace metal and metalloid concentrations, increased hardness and conductivity in downstream lakes compared to nearby reference lakes (Muscatello et al., 2006, 2008; Golder 2008). Water quality measurements collected in this study were confirmatory samples for comparison with the more detailed studies previously conducted at these study lakes. A full spectrum of trace metals analyses was not conducted for water in the present study; however elevated levels of uranium, nickel, arsenic, and molybdenum in water were detected in 2007 and reported by Golder (2008). Increased nitrogen compounds in the form of ammonia and nitrate has contributed to elevated nutrient levels in the exposure lakes (Kelly and Janz, 2008). Muscatello et al. (2008) identified differences in Se concentrations in algae, biofilm, and free floating debris that were available to fish inhabiting the exposure lakes. In the present study, sediment Se concentrations were higher in the exposure lakes than in the reference lake and a trend of increasing total Se in sediment with increasing %TOC in sediment was observed in the exposure lakes, similar to that reported previously at these same study sites (Wiramanaden et al., 2010a).

The benthic invertebrate community diversity differed somewhat among study lakes, however the same core species were present across all study sites (Odonata, Plecoptera, Trichoptera, Diptera, Crustacea, Annelida, and Mollusca). Although not statistically significant, there was a trend for higher total numbers of benthic invertebrates/m^2 at caging sites in the exposure lakes compared to the reference lake. Similarly, Golder (2005) reported elevated, though not significantly greater, densities of benthic invertebrates in the same exposure lakes

compared to a reference lake. Both wild and caged fish species inhabiting the exposure lakes were subject to different water chemistry and may have had greater food availability when compared to fish inhabiting the reference lake. Greater ecosystem productivity and food availability may also allow mature fish in the exposure lakes to approach maximum size estimates (observed in wild spottail shiner from the medium exposure lake) as reported by Scott and Crossman (1973).

2.4.2 Species suitability for caging

Of the two small-bodied fish species investigated, lake chub proved to be the most suitable species for caging studies in lakes downstream of the Key Lake uranium mill based on higher survival, an increase in condition factor and better tolerance to capture and handling stress. This may indicate a higher level of stress tolerance and/or a greater ability to access available food (grazing on netting, suspended food particles and native benthos in sediment). The major sources of stress on caged lake chub and spottail shiner included handling during collection, transportation, and confinement. Handling stress in spottail shiner was evident by scale loss, potentially contributing to mortality. This phenomenon was not observed in lake chub used in this study. It is possible that the visibility of predators outside of the cages may have contributed to fish stress during the caging trials, however no evidence of cage or net damage caused by aquatic predators was observed.

Differences in growth and condition factor of lake chub during the 21-day feeding study may have been confounded by the time of spawning of the lake chub and spottail shiner used in the *in situ* caging trials. Fish collections were carried out near the end of spawning in these species (Scott and Crossman, 1973), and may have included some spawning individuals. Since

whole-body Se concentrations were determined, gonad masses were not recorded. However, at the conclusion of the caging trials approximately 20% of the lake chub and spottail shiner were observed to be readily expressing eggs or milt during handling. As a result, minor weight loss and a corresponding reduction in condition factor measured in caged spottail shiner and lake chub may not have been solely the result of reduced feeding or limited food availability, but may have also been influenced by egg or milt release.

2.4.3 Selenium in wild fish

Whole-body Se concentrations in wild (uncaged) lake chub and spottail shiner from the medium exposure lake were significantly greater than in fish collected from the reference lake and 1.5 to 2.5 times greater than the proposed USEPA criterion to protect aquatic life of 7.91 µg/g (USEPA, 2004). Accumulation of Se in wild small-bodied fish living in the medium exposure lake occurred despite a relatively low aqueous Se concentration that was only slightly higher than the CCME guideline for the protection of fish of 1µg/L (CCME, 2003) and below the USEPA ambient water criterion of 5 µg/L (USEPA, 1987). The bioaccumulation of Se in the food web of the exposure lakes is the result of historical Se loading from the Key Lake uranium processing mill, sediment water interactions, and geochemical processes affecting mobility and bioavailability, and is therefore not reflected in Se concentrations of lake water. However, the results presented here compare well with earlier studies by Lemly (1985, 1993) and Hamilton (2004) which suggested that freshwater ecosystems with Se concentrations ≤1µg/L may lead to Se body burdens capable of causing deleterious effects at higher trophic levels. Incorporation of the dietary component of Se exposure may account for increased body burdens in fish resulting from low aqueous Se exposure (Luoma and Presser, 2009). The Se concentrations observed in

wild spottail shiner from the medium exposure lake and reference lake in the present study were similar to those reported by Muscatello et al. (2008). However, the whole-body Se concentrations in spottail shiner were approximately 1.5 times greater than in lake chub collected from the same lake in the present study, which may reflect differences in diet between the two species. Overall, the results for wild fish were comparable to Muscatello et al. (2008), who reported 1.5 to 6 fold biomagnification of Se between plankton and small-bodied fish in these study lakes and little evidence of biomagnification between small-bodied fish (shiners and chub) and larger predators such as northern pike.

The muscle and liver Se concentrations in northern pike collected from the medium exposure lake in the present study were approximately two-fold higher than the whole-body Se of lake chub collected from the same lake, but only slightly greater than the whole-body Se concentrations of spottail shiner. Muscle and liver Se concentrations in northern pike were similar to muscle and liver concentrations from the same exposure lakes reported by Muscatello et al. (2006). Elevated egg and muscle tissue Se concentrations have been correlated with an increase in deformities in pike fry (Muscatello et al., 2006). The results for northern pike were based on a small sample size and variable results might therefore be expected based on the spatial variation of Se in the sediment and biota within the medium exposure lake. No evidence of deformities was observed in adult lake chub or spottail shiner collected from the exposure lakes in the present study or by Golder (2008). In addition, differences in whole-body Se concentrations among fish species collected from the medium exposure lake were likely the result of interspecies differences in diet, resulting in differences in Se uptake, metabolism, and excretion, as well as the spatial variation of Se in sediment and benthos within the lake. Within

population variation in whole-body Se concentrations have been shown not to differ significantly based on fish length, weight, and age (Janz et al., 2010).

Data collected for wild lake chub and spottail shiner from the medium exposure lake (Delta Lake) suggested that steady-state Se concentrations in small-bodied fish will occur at approximately
12 µg/g for lake chub and 18 µg/g for spottail shiner from this lake. These concentrations are greater than the draft USEPA criterion for the protection of fish populations, which was based on warm water fish species. Comparable tissue concentrations were observed in cutthroat trout (*Oncorhynchus clarkii*) fed diets high in selenomethionine that exhibited no toxicological effects or reproductive impairment (Hardy et al., 2009). Active Se excretion was suggested as the primary means of regulating Se below toxic levels, although specific kinetic mechanism(s) involved in Se depuration in fish are not known (Hardy et al., 2009).

2.4.4 Differences in whole-body selenium in caged fish

Significant increases in whole-body Se in caged lake chub were measured in the exposure lakes, showing a 2-3 fold increase compared to Se concentrations in caged lake chub from the reference lake. Whole-body Se concentrations in fish caged in the exposure lakes for 21 days were below the 7.91µg/g criterion proposed by the USEPA (2004). However, whole-body Se concentrations were above the 4 µg/g dry weight threshold for toxicity proposed by Lemly (1993) in all exposure lake locations except the low organic carbon location in the high exposure lake. The duration of the present caging experiments was inadequate to achieve steady-state whole-body Se concentrations as suggested by the greater Se observed in the wild fish of the same species collected from the medium exposure lake. Therefore, considerably longer trials,

such as the 87-day caging experiment conducted by Allert et al. (2006) may be necessary to achieve steady state Se. Depuration of accumulated Se can also be expected to require up to 3 months, as was estimated for june suckers (*Chasmistes liorus*) by Allert et al. (2006).

The trend of increasing whole-body Se in caged lake chub with increasing Se in sediment and percentage total organic carbon in sediment was more evident in the high exposure lake than in the medium exposure lake. However, the correlation of Se in the sediment and percentage total organic carbon was not as strong as predicted based on previous work conducted by Wiramanaden et al. (2010a). Possible reasons for this whole-body Se trend include the feeding habits of the caged fish and the Se content of the available food (primarily benthos). Differences in the Se concentration of available benthos was dependant on the type of organic carbon covering the lake bottom and differences in the rate of Se cycling throughout each lake. Both of these factors can affect food availability and Se bioavailability in each of the treatment cages and therefore the potential for Se accumulation and bioavailability (Lemly, 1993, 1997; Simmons and Wallschläger, 2005). These factors may account for the greatest whole-body Se increase in lake chub caged at the medium organic carbon caging location in the medium exposure lake. The lack of a strong relationship between total Se in sediment and whole-body Se concentration in caged lake chub is likely because this does not account for the influence of the entire food chain (excludes benthic invertebrates) between the sediment and caged fish. As the primary prey items for the caged lake chub, benthic invertebrates likely represented the main dietary source of Se. The strongest possible link between sediment Se and whole-body Se in caged small-bodied fish would have to include a more detailed evaluation of the Se concentration in food sources.

The application of an *in situ* caging technique was useful in measuring Se accumulation in exposure lakes using naive wild fish collected from a reference lake. Due to the inability to quantify caging mortality in real time, the use of lakes downstream of the milling effluent release point may be more desirable than exposing caged fish at the point of effluent discharge where higher mortalities have been observed (Pyle et al., 2001). Such natural water bodies are realistic models for studying contaminant exposures and bioaccumulation, and reduce the likelihood of having confounding toxicological responses caused by high concentrations of effluent components at the point of discharge such as ammonia and other trace metals. However, the similarity in sediment Se concentrations and whole-body Se caging results in the medium and high exposure lakes suggests that exposure scenarios for Se rely on many factors and natural systems may not have a strong enough gradient to significantly alter exposure. Nevertheless, the results of the present study suggest that *in situ* caging of small-bodied fish can be applied to studies investigating bioaccumulation of Se in aquatic ecosystems receiving complex industrial effluents.

2.4.5 Selenium speciation determination using X-ray absorption spectroscopy

In this study the fraction of whole-body (R-Se-R) modelled as selenomethionine increased with increasing Se exposure in both wild and caged fish. These results also suggested an apparent maximum of selenomethionine between 60 and 80%. This suggests a biological limit to the metabolism, storage and use of selenomethionine in lake chub. At the population level, an increase in the bioaccumulation of organic forms of Se is important because even though Se does not undergo a large degree of biomagnification between small-bodied fish and larger predatory fish, the biomagnification of organic Se species may be significant. As a result, a better

understanding of Se speciation in all relevant food chain components is required to fully understand the potential effects caused by biologically available Se concentrations in both individual fish and in fish populations.

Selenium accumulation and speciation in individual fish is related to the spatial variability of Se and the availability of Se in the food web. The Se concentrations and speciation in the available benthic invertebrates, zooplankton, or algae/periphyton/biofilm present in each cage will also depend on adsorption, dietary assimilation, gut biochemistry and biotransformation in these aquatic organisms (Andrahennadi et al., 2007). Depending on food availability, the feeding habits of caged fish likely differed in the proportion of food obtained from the water column, the cage netting, or benthic invertebrates. Selenium uptake and speciation in caged lake chub was likely influenced by prey density, prey type and prey Se concentrations, as well as by individual differences in feeding behaviour, Se assimilation and biotransformation, all of which require further investigation. Andrahennadi et al. (2007) reported that Se exposure led to increased organic Se fractions and a corresponding decrease in inorganic Se fractions at higher trophic levels, which was also observed in the present study. There was also a trend within study lakes of increasing organic Se modeled as selenomethionine with increasing percentage organic carbon in the lake sediment (data not shown). Overall, the combination of total Se concentrations and Se speciation in lake chub caged in the exposure lakes strongly suggests that dietary exposure routes dominated the bioaccumulation of this trace element, although specific food sources (e.g., benthic macroinvertebrates, zooplankton, algae/biofilm) could not be identified. Our ongoing work using similar caging experiments and combining Se speciation with stable isotope analyses of biotic and abiotic components of the aquatic system will begin to address these data gaps.

In the present study, a small fraction of elemental selenium was observed in a few fish samples with greater whole-body Se concentrations. Elemental Se is not a form of Se predicted to exist in appreciable amounts in fish because it is not believed to be a bioavailable form that contributes to cellular processes (Burk, 1986). Therefore, the presence of small portions of elemental Se in fish from the present study may simply be the result of inorganic materials such as sediment consumed during benthos foraging that were present in the gut of fish during sample collection. However, it is also possible that elemental Se is a metabolite produced in response to higher levels of Se. In support of this, Misra et al. (2010) have observed elemental Se to be the primary metabolite when isolated hepatocytes of rainbow trout (*Oncorhynchus mykiss*) were exposed to 100 µM of selenate or selenite. In addition, since gut contents were not removed prior to whole-body Se determinations, it is also possible that gut contents contained other Se forms, including organoselenides such as selenomethionine, and that this may have influenced total Se and Se speciation results. Further work is needed investigating tissue-specific Se speciation in fish, including elemental Se.

2.4.6 Selenium speciation and whole-body selenium comparison

The presence of predominantly selenomethionine like compounds in both wild fish and fish caged in the exposure lakes indicates that it may be a suitable marker of Se exposure, and that selenomethionine accumulation occurs relatively quickly. The sharp rise in and subsequent plateau of selenomethionine levels identified in this study (between 60 and 80% of total Se) was correlated to increasing whole-body Se concentration. This correlation included both wild and caged fish and thus represented both short-term and long-term exposure periods. Dietary uptake of Se is the dominant pathway of Se accumulation in fish (Fan et al., 2002; Hamilton, 2004;

Stewart et al., 2010) and this relationship illustrates that Se uptake can occur over a relatively short period of time. The presence of an apparent maximum regarding the fraction of total Se as selenomethionine in exposed fish with increasing whole-body Se concentrations represents an interesting physiological response. It may illustrate a limit on the proportion of selenomethionine that a fish can tolerate and suggest that steady-state Se concentration may be maintained by excretion. While the sample size was small, the steady-state whole-body Se concentration in the wild fish collected from the exposure lakes in the current study correspond with the levelling off of selenomethionine at 60-80% of whole-body Se.

2.5 Conclusions

This study indicated that compared to spottail shiner, lake chub may be better suited for fish caging studies in northern coldwater ecosystems. The results of the *in situ* caging studies in these lakes show that short-term (21 day) exposures can lead to significant Se uptake, but were not sufficient to reach steady state Se concentrations. Such short term exposures may also exceed conservative whole-body Se guidelines designed to protect fish species. The Se speciation results indicated that the predominant form of Se in both wild fish and fish caged in the exposure lakes was selenomethionine, suggesting that dietary exposure was the primary route of bioaccumulation. Importantly, the presence of selenomethionine appears to be a marker of elevated Se exposure. To adequately define the risks of Se to fish and other aquatic organisms, knowledge of the Se species present in exposed fish may be extremely important. Overall, this study did not attempt to link the percentage of organic Se in exposed fish to specific toxicological effects. However, in the future it may be possible to link the observed selenomethionine maximum to the onset of potential population level effects (increased

frequencies of larval fish deformities) from chronic Se exposure such as those previously identified for northern pike in these same study lakes (Muscatello et al., 2006).

CHAPTER 3

3 EVALUATING THE TROPHIC TRANSFER OF SELENIUM IN AQUATIC ECOSYSTEMS USING CAGED FISH, X-RAY ABSORPTION SPECTROSCOPY AND STABLE ISOTOPE ANALYSIS

3.1 Introduction

Previous characterisation of lakes downstream of the Key Lake uranium milling operation in northern Saskatchewan, Canada has indicated elevated Se concentrations in water, sediment and biota (Muscatello et al., 2008; Golder, 2008; Wiramanaden et al., 2010a). Using synchrotron-based x-ray absorption spectroscopy, selenomethionine-like compounds have been shown to be the dominant form (species) of Se accumulated in fish following both short-term and long-term Se exposure (Phibbs et al. 2011). The presence of predominantly selenomethionine-like compounds in fish exposed to elevated Se indicates it may be a suitable marker of elevated Se exposure (Phibbs et al., 2011).

Caging of small-bodied fish in contaminated aquatic systems has been shown to be an effective method of evaluating bioavailability of trace elements in certain fish species (Pyle et al., 2001; Doebel et al., 2004; Palace et al., 2005; Allert et al., 2006; Oikari, 2006; Phibbs et al., 2011). Field-based caging studies are most suitable for evaluating impacts on fish species with high mobility or where the simulation of complex environmental conditions would be too difficult to conduct in laboratory studies (Palace et al., 2005). A standardized technique for caging small-bodied fish used by Palace et al. (2005) and adapted by Phibbs et al. (2011) has shown promising results in controlling fish interactions with the surrounding environment (water, sediment, and biota) and minimising stress caused by confinement. A species comparison between lake chub (*Couesius plumbeus*) and spottail shiner (*Notropis hudsonius*),

two common small-bodied fish species inhabiting northern Canadian ecosystems, has shown lake chub are well suited for measuring contaminant uptake in caging experiments (Phibbs et al., 2011).

As an essential ultra trace micronutrient, Se is required by fish in small amounts, but shows a narrow range between essentiality and toxicity (Janz et al., 2010). In aquatic environments Se is converted to organic Se species (organoselenides) by algae, macrophytes, and bacteria, which then bioaccumulate in higher food chain organisms (consumers) via dietary pathways (Feldmann, 1986; Gomez-Ariza et al., 1999; Orr et al., 2006; Stewart et al., 2010). Selenite can also be reduced by microorganisms in anoxic sediments to elemental selenium (Se^0) which is generally considered to be less bioavailable (Oremland et al., 1989; Wiramanaden et al., 2010b). Even at relatively low aqueous concentrations, bioavailable forms of Se bound to sediment are taken up by benthic invertebrates and bioaccumulate in aquatic food webs (Bowie et al., 1996; Lemly, 1997; Fan et al., 2002; Hamilton, 2004; Tsopelas et al., 2004; Simmons and Wallschläger, 2005). The mechanisms of Se biotransformation into organic forms by primary producers are not completely understood at this time, however, the metabolism of Se species into more toxic organic forms increases at higher trophic levels (Andrahennadi et al., 2007). The chemical species of Se may also be an important factor affecting uptake, mobility and trophic transfer through the aquatic food web (Andrahennadi et al., 2007; Wiramanaden et al., 2010b). The trophic transfer of Se in aquatic food webs can lead to potential population impacts at higher trophic levels, such as fish and aquatic birds (Bowie et al., 1996; Lemly, 1997; Fan et al., 2002; Hamilton, 2004; Tsopelas et al., 2004; Simmons and Wallschlager, 2005; Janz et al., 2010).

Stable isotope analysis has been adapted to the field of aquatic toxicology, allowing researchers to track the movements of food and contaminants within an ecosystem (Cabana and Rasmussen, 1994). For example, stable isotope analysis of caged mussels has been successfully used to determine connections between marine pollution levels and high tissue concentrations (Deudero et al., 2009). By tracing the relative amounts of naturally-produced carbon (C), nitrogen (N), and sulphur (S) isotopes from primary producers to the top of a food chain, it is possible to track food consumption and make inferences regarding biological cycling within an ecosystem, including the bioaccumulation of contaminants in higher trophic levels. Carbon (^{12}C and ^{13}C), nitrogen (^{14}N and ^{15}N) and sulphur (^{32}S and ^{34}S) isotopes are the most commonly used markers in aquatic ecotoxicology, because they have more than one isotope, are naturally abundant and can be precisely measured (Lajtha and Michener, 1994; Connolly et al., 2004). The C isotope signature ($\delta^{13}C$) of an ecosystem is established during the assimilation of ^{13}C by primary producers and differs between terrestrial, freshwater, and marine environments based on the available carbon in each ecosystem (DeNiro and Epstein, 1978). The N isotope signature ($\delta^{15}N$) of an organism reflects its diet and the trophic position of the species analysed because it tends to increase during trophic transfer (DeNiro and Epstein, 1981; Cabana and Rasmussen, 1994). The S isotope signature ($\delta^{32}S$) of an ecosystem will be very narrow in a system that has not been involved in the sedimentary process and have a wider range of values in weathered or disturbed systems which have undergone various degrees of biological sulphur cycling. A common example of this is through the reduction of sulphate to sulphide by bacteria in anaerobic environments resulting in increased ^{34}S of the remaining sulphate. Since $\delta^{32}S$ have the ability to vary over much smaller distances than can be expected with $\delta^{13}C$ and $\delta^{15}N$ it can be used as an effective tool in separating food sources with similar $\delta^{13}C$ and $\delta^{15}N$ signatures (Connolly et al.,

2004; Croisetiere et al., 2009). In addition, $\delta^{32}S$ can aid in distinguishing between producers for different treatments (Connolly et al., 2004).

The main objective of this study was to evaluate the dominant exposure pathways with respect to the bioaccumulation and trophic transfer of Se using caged lake chub. Lake chub collected from a reference lake were caged in both a reference lake and in a lake downstream of a uranium milling operation in Saskatchewan, Canada. Comparisons of dietary Se uptake were made by feeding caged lake chub a diet spiked with Se or a basal (normal Se) diet in both study lakes. Whole-body Se concentrations were compared between exposure and reference lakes, as well as between diets. Whole-body Se concentrations were evaluated in conjunction with Se speciation in fish and the stable isotope signatures of fish, experimental diets, and water. The overall goal of this research was to enhance our understanding of the dietary transfer of Se and the role of Se speciation in the aquatic ecotoxicology of this trace element.

3.2 Materials and Methods

3.2.1 Study area

This research was conducted in the receiving waters downstream of the Key Lake uranium processing mill, approximately 600 km north of Saskatoon in north central Saskatchewan, Canada (57˚11'N, 105˚34'W). The Key Lake uranium milling operation processes high grade uranium ore into yellowcake (U_3O_8) and releases approximately 6000 m^3/d of effluent characterised by elevated trace element concentrations (Muscatello et al., 2008). The research sites used in the present study included a reference lake (Yeoung Lake) and an exposure lake (Unknown Lake). The exposure lake is located in a small watershed approximately 4 km downstream of the uranium effluent release and the reference lake is located in a nearby

unaffected watershed. For comparison, wild lake chub were collected and analysed from both the reference lake and the closest available exposure lake where lake chub were present (Delta Lake), located approximately 6 km downstream of Unknown Lake. The exposure lakes used in this study have been influenced by approximately 30 years of uranium milling effluent release and are characterised by elevated trace elements, hardness, conductivity, ammonia and nitrate compared to nearby reference lakes (Muscatello et al., 2006, 2008; Golder, 2008). Further details regarding these study lakes can be found in Muscatello et al. (2008), Wiramanaden et al. (2010a), and Phibbs et al. (2011).

3.2.2 Fish collection

Lake chub were selected for this feeding experiment because of their ubiquity across the Canadian boreal forest region and the availability of a reference population in the study area (Scott and Crossman, 1973; Muscatello et al., 2008). Lake chub were also chosen because of their demonstrated suitability for use in this type of caging experiment (Phibbs et al., 2011). Adult lake chub were captured from the reference lake using a combination of fyke nets and beach seine, and held in net pens for approximately 24 hours. On day 1, lake chub (n = 120) were transported to the caging sites in aerated tanks. Upon arrival at the study lakes, fish were lightly anesthetised using 0.1 g/L MS-222 (Tricaine, Sigma-Aldrich, Oakville, ON) before collecting initial size measurements (weight and fork length), and then deploying them (n = 10/cage) into the study lakes. At the conclusion of the 21-day feeding trials, the surviving fish were collected and immediately euthanized for future analysis (Se concentration or speciation) by administering an overdose (0.8 g/L) of MS-222. Body weight, and fork length were recorded before whole fish samples were frozen individually on dry ice in airtight containers. Samples

were held on dry ice until they were transferred to a -80˚C freezer at the University of Saskatchewan. Condition factor (a general measure of fish health) was calculated using a length to weight relationship (weight/length^3 × 100). All sampling and experimental procedures involving animals in this study were conducted in accordance with the Canadian Council on Animal Care (University of Saskatchewan Animal Care and Use Protocol 20030088).

3.2.3 Feeding cage study

Feeding cages were deployed to determine short-term Se uptake rates and Se speciation in fish fed controlled diets and caged in either the reference or exposure lake. Six 0.5 m^3 feeding cages (1.0 × 1.0 × 0.5 m; l × w × h) covered with ¼ inch (0.64 cm) nylon mesh were placed in the exposure lake (Unknown Lake) and in the reference lake (Yeoung Lake). Cage construction and design was adapted from Phibbs et al. (2011) and Palace et al. (2005). Each PVC cage was deployed approximately 1.5 m below the water surface. Previous work in these study lakes by Wiramanaden et al. (2010a) indicated that aqueous Se concentrations (total and dissolved) show little spatial variation within lakes, and the dissolved Se fraction was determined to be approximately equal to the total amount of Se in the water column. To attempt to restrict Se exposure in caged fish to only aqueous and controlled dietary sources (basal and Se-spiked diets) the cage bottom and 30 cm of each side were covered with a plastic tarpaulin and the bottom was also covered with a 10 cm layer of clean silica sand (particle size 250 – 425 µm) (Brock White Canada Inc, Saskatoon, SK). For all cages deployed the cage bottom and 30 cm of each side were covered with a plastic tarpaulin and the bottom of each cage was covered by a 10-cm layer of clean silica sand (particle size 250 – 425 µm; Brock White Canada Inc., Saskatoon, SK) to attempt to restrict Se exposure in caged fish to only aqueous and controlled dietary sources. This

barrier was implemented to eliminate the possibility of caged fish feeding on native benthic invertebrates in the sediment below. Ten lake chub were placed in each of the six cages in the reference lake and exposure lake. A total of 12 cages (3 cages per feeding treatment per lake) were deployed in the two study lakes. Cages were deployed for 21 days and the fish were limited to a daily ration of the controlled dietary treatment in addition to naturally available food that developed on the cage netting or was suspended in the water column.

Each morning, 5 g (wet weight) of the appropriate food source was thawed and administered to each feeding cage. To ensure adequate food availability each cage was administered a ration of 5 g/cage/day (approximately 10% body weight/fish/day) of commercially prepared *Chironomus* spp. larvae (Hagen Inc, Edmonton, AB) or Se-spiked diet (laboratory reared *Chironomus dilutus* spiked with a 4 µg/L solution of sodium selenite during larval development; see section 1.3.2). The Se-spiked diet was produced with a target Se concentration of 6 µg/g dry weight. The basal food source was chosen because it represents a diet with sufficient quantities of Se (1.5 µg/g dry weight) for normal metabolic function. During the first half of the experiment, local forest fire activity prevented access to the study lakes for feeding activities on two mornings (day 4 and day 6). On these days feeding was skipped and a double food ration was administered the next morning.

3.2.4 Abiotic environment

Triplicate water samples from each study lake were collected approximately 30 cm above the sediment surface (approximate mid height of cages) for analysis of dissolved Se. In order to measure the potential effects of variable effluent (and thus variable Se) output from the uranium mill during the caging trials, water samples were collected on days 1, 10 and 21. Water samples

were collected using a Wildco® Van Dorn horizontal acrylic beta water sampler (Wildlife Supply Company, Buffalo, NY) and then passed through a 53 μm mesh sieve to remove floating debris and zooplankton. Water samples collected for dissolved Se analysis were filtered through 0.45 μm acetate filters (VWR International, Mississauga, ON) and then preserved using 2% ultra-pure nitric acid (Omnitrace ultra grade, EMD Chemicals, Gibbstown, NJ). Triplicate water samples were also collected for total hardness and ammonia determinations. All samples were stored in 250 ml acid washed high density polyethylene (HDPE) bottles and kept at 4˚C until analysed. General water quality parameters (pH, conductivity, dissolved oxygen (DO), and temperature) were assessed on-site using a multi-parameter YSI probe (6 series - YSI Inc., Yellow Springs, OH). Water hardness was measured using a HACH 16900 Digital Titrator (Hach Company, Loveland, CO). Total ammonia was measured using a VWR Symphony SB301 pH/ISE meter (VWR International, Mississauga, ON).

On day 21, triplicate samples of silica sand were collected from three cages in each of the study lakes to quantify the amount of Se that adsorbed to the sand particles during the study. Sand samples were freeze dried and ground to a powder using an acetone-cleaned porcelain mortar and pestle. Sand samples were prepared for Se analysis using microwave digestion (CEM microwave MDS-2100, Matthews, NC) and analysed using inductively coupled plasma-mass spectroscopy (ICP-MS; X-Series II, Thermo Electron Corporation, Gormley, ON) following a procedure outlined previously (Wiramanaden et al., 2010a). The standard reference material (PACS-2 marine sediment certified as 0.92±0.22 mg/kg; National Research Council Canada, Ottawa, ON) was used to determine Se recovery (range 70–90%).

3.2.5 Procedure for culturing spiked chironomid diet

Breeding *C. dilutus* adults were collected from stock populations at the University of Saskatchewan using an air suction device attached to a 250 ml flask. Breeding adults were left to breed overnight in a 500 ml mason jar equipped with suitable breeding habitat. Deposited egg masses were used to seed 18 L aquaria (4-5 egg masses/aquarium) filled with 14 L of dechlorinated tap water and a 2 cm layer of silica sand (250 – 425 µm; Brock White Canada Inc, Saskatoon, SK), and kept in an environmental chamber at a constant temperature of 23˚C. Each tank was monitored daily to ensure a stable temperature and adequate dissolved oxygen levels were present (23.1˚C and 6.84 mg/L, respectively). Water quality was maintained using static water renewals every three days. Using a light regime of 16 hours light: 8 hours dark, the *C. dilutus* breeding tanks reached 4th instar (harvestable age) after approximately 25 days. During the final 10 days of larval development, water renewals were conducted using a 4 µg/L solution of sodium selenite (Na_2SeO_3; Sigma-Aldrich, Oakville, ON).

After 10 days of Se exposure, each tank was drained and the 4th instar *C. dilutus* larvae were removed and separated from the sediment using a #30 (0.6 mm) U.S.A. Standard Testing Sieve (Fisher Scientific Canada, Toronto, ON). Any pupating larvae were discarded and the larvae were rinsed with tap water to remove any remaining sediment. Based on the yield in each tank, *C. dilutus* were weighed out into 1, 2, or 5 g increments and placed in airtight vials for storage at -80˚C. A total *C. dilutus* biomass of 500 g wet weight was cultured for the Se-spiked food feeding experiment. Small subsets of the prepared diet and the commercially available diet were stored separately for Se analysis and stable isotope analysis. The Se concentration of the cultured chironomids was determined to be 5.5±0.72 µg/g dry weight using ICP-MS.

3.2.6 Selenium analysis

Frozen whole fish samples were freeze dried using a Dura Dry freeze dryer (FTS Systems, Stone Ridge, NY) for 72-96 hours. The moisture content of lake chub was 77.0 ± 0.1%. Freeze dried samples were ground to a fine powder using an acetone-cleaned porcelain mortar and pestle. Teflon vessels were used to cold digest 0.1g samples using 5 ml of nitric acid and 1.5 ml of hydrogen peroxide (30%, Suprapur, EMD Chemicals, Gibbstown, NJ). Digested samples were evaporated slowly in the Teflon vessels at approximately 65˚C before being reconstituted in 5 ml of 2% nitric acid. To remove any particulate matter, reconstituted samples were syringe filtered using disposable 0.45 µm acetate filters. Digested tissue samples were analysed for Se using ICP-MS. A method detection limit of 0.03 µg Se/g was determined using 15 method blanks. A total of 14 replicates of certified reference material (TORT-2, Lobster hepatopancreas) obtained from the National Research Council of Canada (Ottawa, ON) were used to determine Se recovery (range 80–95%). Method blanks and the standard reference material (certified as 5.63 ± 0.67 mg/kg) were analyzed a minimum of every 10 samples.

3.2.7 Selenium speciation determination by X-ray absorption spectroscopy (XAS)

Fish samples were analysed for Se speciation using XAS following the same procedure as Phibbs et al. (2011). Briefly, homogenised subsamples of whole lake chub were packed with no head space in 2 mm custom cuvettes, sealed with glycerol and stored in liquid nitrogen. Each cuvette was constructed of a polymer which contains no elemental interferences up to the bromine K-edge. Selenium speciation was determined at the Stanford Synchrotron Radiation Light source (SSRL) in Menlo Park, CA. Selenium K near-edge spectra were recorded for

prepared fish samples as well as aqueous Se standards (buffered at pH 7) using the Structural Molecular Biology XAS beamline 9-3. Further information regarding details of the K near-edge XAS methodology is outlined in Wiramanaden et al. (2010b).

The Se K near-edge spectra of the lake chub tissues were normalised and background corrected before being analysed according to standard methods (EXAFSPAK program suite) outlined previously (Pickering et al., 2000). Each lake chub spectrum was analyzed by least squares fitting to a linear combination of spectra of Se standards. A standard spectrum was excluded from subsequent fits if the initial fractional contribution to the fit was less than 3 times its estimated standard deviation. The fractional contribution of the standard spectrum to the fit is equivalent to the contribution of that Se species type to the total Se. XAS is sensitive to the local environment of Se, and this analysis identifies classes of Se species rather than specific molecules. For example, selenomethionine is used to represent Se bound to two aliphatic C groups (R-Se-R). Thus, for each sample analysed, the compounds modeled as selenomethionine will be referred to as selenomethionine-like compounds. Standards used to fit to fish tissue samples included dilute aqueous solutions of organic forms of Se such as selenomethionine (R-Se-R), selenocystine (R-Se-Se-R), seleno-bis-diglutathione (S-Se-S) and trimethyl selenonium iodide (R_3Se^+), as well as the inorganic forms selenite (SeO_3^{2-}) selenate (SeO_4^{2-}) and solid elemental Se^0 (Wiramanaden et al., 2010b).

3.2.8 Stable isotope analysis

To infer the trophic transfer of Se and pathways of Se accumulation in the aquatic environment at the study lakes, the isotopic signatures of C, N and S were analysed in homogenised whole-body samples of caged fish, wild fish, controlled diet samples, and lake

water. Wild lake chub analysed for stable isotopes were collected from the exposure lake (Delta Lake) during a previous study (data collected in 2008; Phibbs et al., 2011) and from the reference lake (Yeoung Lake) during the current study. Triplicate unfiltered water samples (2L) were collected from the reference lake (Yeoung Lake) and the exposure lakes (Delta Lake and Unknown Lake) and immediately frozen on dry ice. Wild (from Yeoung Lake and Delta Lake) and caged (from Yeoung Lake and Unknown Lake) lake chub, water samples (from Yeoung Lake, Delta Lake, and Unknown Lake) and controlled diet samples (Se-spiked and basal diets) were freeze dried for 72-96 hours prior to stable isotope analysis. Freeze dried samples (fish, water, and controlled diets) were ground to a fine powder using an acetone-cleaned porcelain mortar and pestle. The pulverised samples were prepared for lipid extraction by Isoprep Laboratory Services (Regina, SK). Lipid extraction was conducted to normalise carbon stable isotope ratios because lipids are 13C-depleted relative to proteins and considerable variability exists both within and among tissues (Sweeting et al., 2006). Samples prepared for C and N isotope analysis were soaked overnight in a 2:1 mixture of methanol and chloroform (EMD Chemicals, Gibbstown, NJ). After the samples were rinsed, decanted with a fresh 2:1 mixture of methanol and chloroform, and dried, 5 mg subsamples were stored in scintillation vials for stable isotope analysis. Samples prepared for sulphur analysis did not undergo lipid extraction and were immediately weighed out in 5 mg subsamples and then stored for analysis. Stable isotope analysis was conducted using elemental analyzer combustion to CO_2 and N_2 and measured by continuous-flow isotope ratio mass spectrometry at the Environment Canada Stable Isotope Hydrology and Ecology Laboratory in Saskatoon, SK, Canada.

3.2.9 Statistical analyses

SPSS version 17.0 (SPSS Inc., Chicago, IL) was used to conduct statistical analyses at a confidence level of 95% ($\alpha = 0.05$). Data were tested for homogeneity of variance using Levene's test and normality using the Shapiro-Wilk test. The data passed the above tests and were analysed using the following statistical comparisons. Significant differences in the condition factor of lake chub between day 0 and day 21 were determined using a paired t-test (Table 3.1). Differences in fish body weights were compared between lakes using analysis of covariance (ANCOVA) using length as a covariate. Significant differences in whole-body Se using lake and diet as experimental factors were determined using a two-way analysis of variance (ANOVA) and Tukey's test. One-way ANOVA and Tukey's test (when appropriate) were used to determine differences in water chemistry, including dissolved aqueous Se concentrations and sand Se concentrations between lakes. One-way ANOVA and Tukey's test (when appropriate) were also used to determine differences in stable isotope signatures between lake water, controlled diet types, caged lake chub and wild lake chub. Due to limits in available synchrotron beamtime, only one fish from each treatment was analysed for Se speciation.

3.3 Results

3.3.1 Abiotic environment

Temperature ($p < 0.01$), total hardness ($p < 0.01$), conductivity ($p < 0.01$), and total ammonia ($p < 0.01$) were significantly greater at the caging locations in the exposure lake (Unknown Lake) compared to the reference lake (Yeoung Lake), while pH ($p < 0.05$) and dissolved oxygen ($p < 0.05$) were significantly greater in the reference lake compared to the

exposure lake (Table 3.1). Dissolved Se concentrations in the exposure lake were greater than in the reference lake (Table 3.2). Following a period of shutdown at the uranium processing mill, temporal variations in aqueous Se concentrations in the exposure lake were detected (Table 3.2). During the 21-day caging experiment Se concentrations increased significantly by approximately 20% between day 0 (2.32 µg Se/L) and day 10 (2.88 µg Se/L; $p < 0.005$) and then stayed relatively constant between day 10 and day 21 (2.89 µg Se/L; Table 3.2). A full spectrum analysis of trace elements was not conducted on water, sediment or fish samples; however elevated concentrations of other trace elements in the exposure lake water such as uranium, nickel, arsenic, and molybdenum have been reported recently (Golder, 2008).

In this study, clean silica sand was used to line the bottom of each feeding cage. Analytical results of samples collected from the sand layer at the bottom of the feeding cages indicated that it did not accumulate measurable Se and was neither a source nor sink of Se in this study.

Table 3.1. Water quality parameters from a reference lake (Yeoung Lake) and an exposure lake (Unknown Lake) located downstream of the Key Lake (SK, Canada) uranium mill (mean ± SE of n = 3 samples).

Variable	Reference Lake	Exposure Lake
Dissolved Oxygen (mg/L)	9.2 ± 0.1	8.1 ± 0.1*
Temperature (˚C)	16.9 ± 0.3	21.4 ± 1.5**
pH	7.2 ± 0.1	6.4 ± 0.3*
Conductivity (µS/cm)	12 ± 1.1	496 ± 2.8**
Total hardness (mg $CaCO_3$/L)	6.8 ± 0.4	623 ± 3.5**
Ammonia (mg N/L)	0.1 ± 0.0	3.0 ±0.1**

Significantly different compared to the reference lake using *t*-test: * $p < 0.05$; ** $p < 0.01$.

Table 3.2. Total aqueous selenium (Se) concentrations from a reference lake (Yeoung Lake) and an exposure lake (Unknown Lake) downstream of the Key Lake (SK, Canada) uranium mill (mean ± SE of n=3 samples).

	Reference Lake			Exposure Lake		
	Day 0	Day 10	Day 21	Day 0	Day 10	Day 21
Total Se (µg/L)	<0.1	<0.1	<0.1	2.32 ± 0.02^{a}	2.88 ± 0.03^{b}	2.89 ± 0.00^{b}

Different letters (a,b) indicate significant differences in Se concentration of water samples collected on days 0, 10 and 21 of the 21-day caging study and compared using one-way ANOVA and Tukey's test ($p < 0.005$).

3.3.2 Caging results

For fish caged in the reference lake (Yeoung Lake) and the exposure lake (Unknown Lake), the percent survival of caged lake chub did not differ significantly between lake or diet type (Table 3.3). Changes in body weight after 21 days for lake chub caged in the reference lake increased slightly (+0.04 g) for the Se-spiked diet treatment and decreased significantly (-0.27 g) for the basal diet treatment ($p < 0.05$; Table 3.3). Body weight increased significantly for the basal and Se-spiked diet treatments (+0.69 g and +0.44 g, respectively) in the exposure lake ($p < 0.05$; Table 3.3). Between day 0 and day 21, lake chub caged in the reference lake and fed a Se-spiked diet had a larger change in mean length (0.5 mm) than for the basal diet (0.2 mm), although these differences were not significant (Table 3.3). Similarly, lake chub caged in the

exposure lake and fed a Se-spiked diet also had larger changes in mean length (1.0 mm) than for the basal diet (0.8 mm), but these differences were not significant (Table 3.3). The condition factor of caged fish decreased slightly (-0.05 and -0.01 for the basal and Se-spiked diets, respectively) in the reference lake. In the exposure lake the condition factor of caged fish appeared to increase (0.07) for the Se-spiked diet and increased significantly (0.12) for the basal diet ($p < 0.005$; Table 3.3).

Table 3.3. Survival and mean change (Δ) in weight, total length, and condition factor for lake chub caged for 21 days in a reference lake (Yeoung Lake) and an exposure lake (Unknown Lake) downstream of the uranium processing mill at Key Lake. Basal and Se-spiked diet refer to controlled Se rations administered to caged fish (see text for explanation). Values represent mean ± SE of *n* = 6-10 fish per cage.

Caged Lake Chub		Weight Δ (g)	Fork Length Δ (mm)	Condition Factor Δ	Survival
Reference Lake (Yeoung Lake)	Basal Se Diet	-0.27†	0.2	-0.05	87%
	Se-Spiked Diet	0.04	0.5	-0.01	93%
Exposure Lake (Unknown Lake)	Basal Se Diet	0.69†	0.8	0.12*	77%
	Se-Spiked Diet	0.44†	1.0	0.07	80%

† Significant differences in caged lake chub weight with length as a covariate using ANCOVA within each lake between day 0 and 21 ($p < 0.05$). * Significant differences in condition factor between day 0 and day 21 for each treatment evaluated using a paired *t*-test ($p < 0.005$).

3.3.3 Whole-body selenium in caged fish

Significant lake ($p < 0.001$) and diet ($p < 0.001$) effects were detected for whole-body Se concentrations in caged lake chub using two-way ANOVA, and no significant interaction was detected between lake and diet type. Whole-body Se concentrations in caged lake chub from the basal (5.01 ± 0.28 µg/g) and Se-spiked (5.90 ± 0.24 µg/g) diet treatments in the exposure lake were significantly greater ($p < 0.001$) than the basal (2.38 ± 0.12 µg/g) and Se-spiked (3.39 ± 0.17 µg/g) diet treatments in the reference lake (Figure 3.1). Within the exposure lake, the mean whole-body Se concentration in lake chub fed the Se-spiked diet was not significantly different than the lake chub fed the basal diet, whereas in the reference lake the mean whole-body Se concentration in lake chub fed the Se-spiked diet was significantly greater than those fed the basal diet ($p < 0.01$; Figure 3.1).

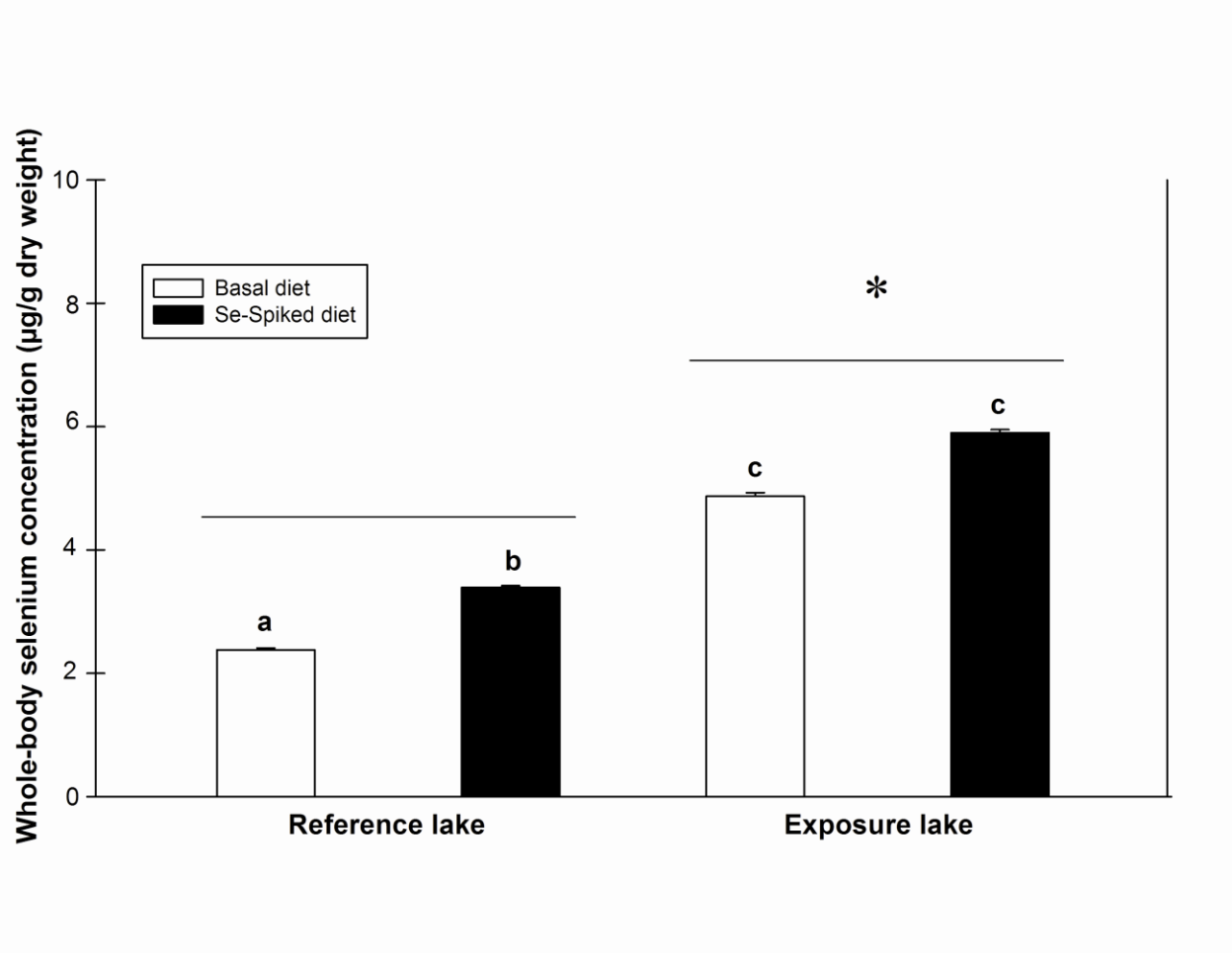

Figure 3.1 Whole-body Se concentrations (µg/g dry weight) in lake chub caged for 21 days in a reference lake (Yeoung Lake) and an exposure lake (Unknown Lake) and fed basal and Se-spiked diets. Values represent means ± SE of *n* = 6-10 fish per cage (*n* = 3 cages per treatment). Different symbols indicate significant differences between lakes (*) and food type (a-c) for caged lake chub determined using a two-way ANOVA and Tukey's test (both $p < 0.001$).

3.3.4 X-ray absorption spectroscopy (XAS)

The chemical forms (species) of Se in whole fish were determined from a single sample of each treatment using K near-edge x-ray absorption spectroscopy. The spectra from all caged lake chub analysed were dominated by organic forms of Se characterised by an increased proportion of selenomethionine-like compounds and a corresponding decrease in selenocystine-like compounds with increasing Se exposure (Figure 3.2A-D). The most abundant form of Se in fish caged in the reference lake and fed a basal diet was the organic form of Se modelled as selenocystine, in which the Se structural environment is R-Se-Se-R (Figure 3.2A). In contrast, selenomethionine-like compounds with the Se structural environment of R-Se-R were found to be the most abundant organic form of Se from the spiked diet treatment in the reference lake and both of the caging treatments in the exposure lake (Figure 3.2B to 3.2D).

Overall, the fraction of selenomethionine-like compounds increased sharply (0.39, 0.45, 0.63, 0.68 in Figures 3.2A to 3.2D, respectively) while the fraction of selenocystine dropped (0.54, 0.45, 0.33, 0.27 in Figures 3.2A to 3.2D, respectively). Small fractions of inorganic Se species were observed, but the presence of selenite (range 5-7%) was not found to make up a large proportion of the Se analysed. Taking into consideration the total Se content from Figure 1 in conjunction with the estimated species fractions (from the XAS represented in Figure 2, raw data not shown) indicates that the actual amount of selenocystine (R-Se-Se-R) remained relatively constant (1.28, 1.54, 1.54, 1.58) while selenomethionine (R-Se-R) content increased sharply (0.92, 1.54, 3.2, 4.0).

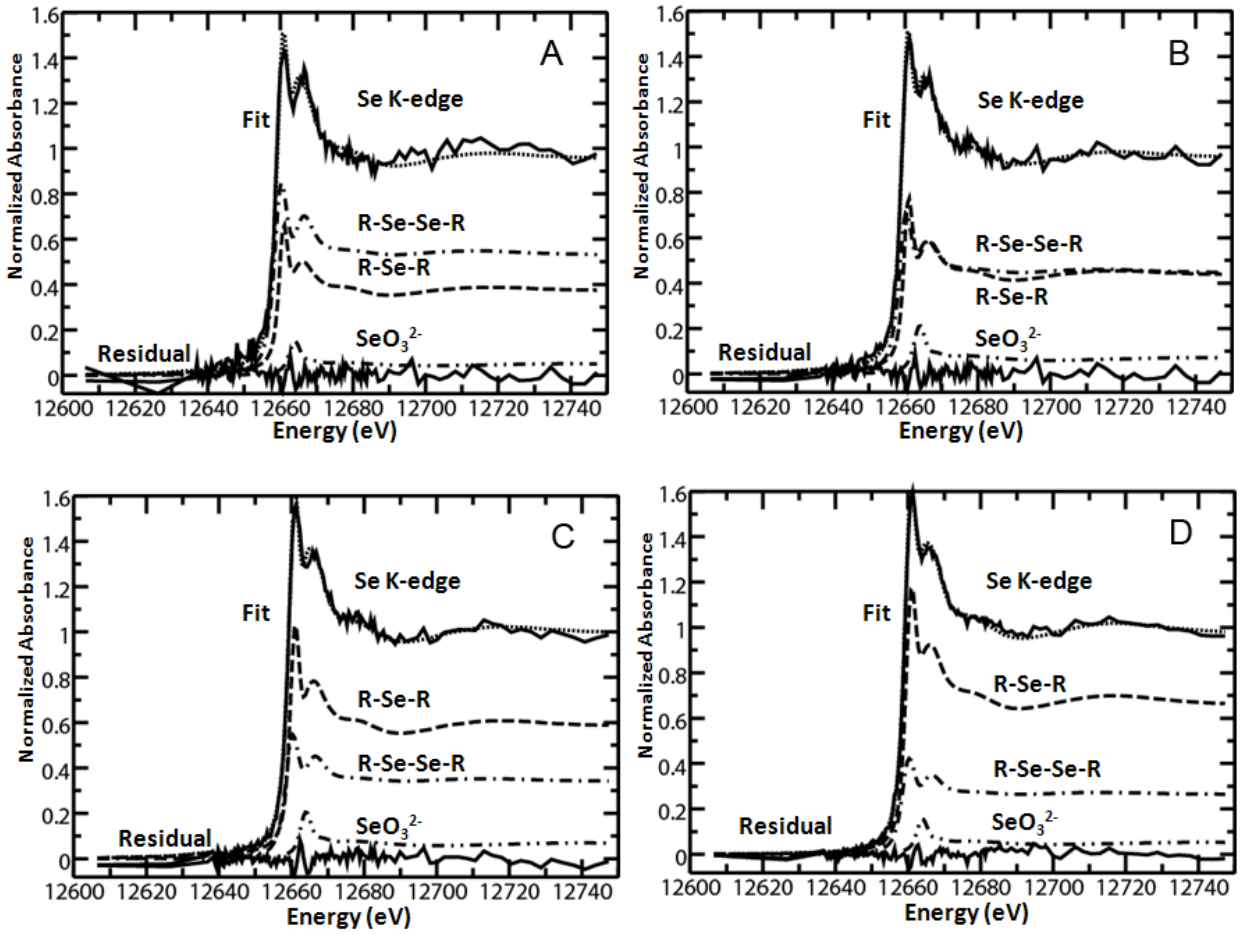

Figure 3.2 Selenium speciation spectra determined using K near-edge x-ray absorption spectroscopy (XAS) for wild lake chub collected from a reference lake (Yeoung Lake) and caged in (A) Yeoung Lake and fed a basal diet, (B) Yeoung Lake and fed a Se-spiked diet, (C) Unknown Lake (exposure lake) and fed a basal diet and (D) Unknown Lake (exposure lake) and fed a Se-spiked diet. This figure shows the Se K near-edge for each representative fish, the fit, the individual components representing their approximate contributions to the fit and the residual (see text for details). Selenium standards represented include R-Se-Se-R (modeled as selenocystine), R-Se-R (modeled as selenomethionine), and selenite (SeO_3^{2-}). See text for details regarding the proportions of Se species present.

3.3.5 Stable isotope analysis

The mean $\delta^{13}C$ signature of lake chub caged in the reference lake and fed a basal diet (-20.15 ± 0.24) was not significantly different than caged lake chub fed a Se-spiked diet in the reference lake (-18.75 ± 0.16) or lake chub caged in the exposure lake and fed a basal diet (-19.43 ± 0.47) but differed significantly compared to lake chub caged in the exposure lake and fed a Se-spiked diet (-18.20 ± 0.34) ($p < 0.05$; Figure 3.3A). Analysis of the $\delta^{15}N$ signature in caged lake chub did not distinguish between caging treatments with similar $\delta^{13}C$ signatures (Figure 3.3B). However, significant differences were detected between the $\delta^{15}N$ signatures of the lake chub caged in the reference lake and fed a basal diet (8.04 ± 0.14) and those fed a Se-spiked diet (7.22 ± 0.11; $p < 0.05$) (Figure 3.3B). Significant differences in $\delta^{15}N$ signatures were also detected between lake chub caged in the reference lake and fed a basal diet compared to fish caged in the exposure lake and fed a Se-spiked diet (7.32 ± 0.03; $p < 0.05$), but not those fed a basal diet (7.57 ± 0.17; Figure 3.3B). No significant differences were detected in the $\delta^{32}S$ signatures among lake chub caged in the reference lake and fed basal and Se-spiked diets (4.98 ± 0.30 and 5.18 ± 0.20, respectively) and those caged in the exposure lake fed basal and Se-spiked diets (4.38 ± 0.18 and 4.45 ± 0.17, respectively) (Figure 3.3C).

A significant difference was detected between the $\delta^{13}C$ signatures of the basal (-32.24 ± 0.13) and Se-spiked (-22.87 ± 0.10) food sources ($p < 0.001$; Figure 3.3A). No significant differences were detected in the $\delta^{15}N$ and $\delta^{32}S$ signatures of the basal (12.49 ± 0.09 and 6.98 ± 0.18, respectively) and Se-spiked food sources (6.78 ± 0.71 and 8.03 ± 0.65, respectively; Figure 3.3B and 3.3C).

Significant differences were detected in the $\delta^{13}C$ (-23.2 ± 0.4, -26.5 ± 0.1, and -27.3 ± 0.0) signatures of the water collected from the reference lake, Unknown Lake and Delta Lake (an exposure lake downstream of Unknown Lake), respectively ($p < 0.001$; Figure 3.3A). Significant differences were also detected in the $\delta^{15}N$ (0.2 ± 0.6, 4.0 ± 0.4, and 7.5 ± 0.5) signatures of the water collected from the reference lake, Unknown Lake and Delta Lake, respectively ($p < 0.001$; Figure 3.3B). No significant differences were detected in the $\delta^{32}S$ signatures of the water samples collected from Delta Lake and Unknown Lake (6.5 ± 0.2, and 6.5 ± 0.1, respectively; Figure 3.3C). Due to naturally low dissolved anion concentrations in the reference lake, no $\delta^{32}S$ signature was detected during isotopic analysis of this element.

Wild (uncaged) lake chub could be separated between the reference lake and Delta Lake based on $\delta^{32}S$ (Figure 3.3C) and $\delta^{15}N$ (Figure 3.3B) signatures, but were not distinguishable based on $\delta^{13}C$ signatures (Figure 3.3A). Mean $\delta^{15}N$ and $\delta^{32}S$ signatures of wild lake chub from the reference lake (8.31 ± 0.60 and 4.57 ± 0.19, respectively) differed significantly from Delta Lake (4.28 ± 0.16 and -0.12 ± 0.25, respectively) ($p < 0.005$ and $p < 0.001$, respectively). The $\delta^{13}C$ signature did not differ significantly between wild fish collected from the reference lake (-17.69 ± 0.86) and Delta Lake (-19.87 ± 2.24).

Comparisons of $\delta^{13}C$ signature and whole-body Se concentration for wild and caged lake chub did not isolate any trends in $\delta^{13}C$ signatures (Figure 3.3A). Compared to the low variability in $\delta^{13}C$ signatures of caged fish, wild lake chub from the reference lake and Delta Lake had a higher range of $\delta^{15}N$ signatures (Figures 3.3A and 3.3B). Visual comparisons of $\delta^{15}N$ signatures and Se accumulation for wild and caged lake chub did not separate caging treatments or lake treatments with similar $\delta^{13}C$ signatures (Figure 3.3B). The basal and Se-spiked food source had

visibly different $\delta^{13}C$ and $\delta^{15}N$ signatures, but very little difference in $\delta^{32}S$ signatures. More specifically, the Se-spiked food source had a much larger variation in $\delta^{15}N$ signature than $\delta^{13}C$ signature (Figures 3.3A and 3.3B). The small spread in the $\delta^{13}C$ signature visible after 21 days of caging between the dietary treatments in both the reference and exposure lakes was reflective of the differences in the $\delta^{13}C$ signatures of the diets (Figure 3.3A). Similarly, the small spread in $\delta^{15}N$ signatures in the reference lake caged fish reflected the difference in the $\delta^{15}N$ signatures of the two diets but this trend was less noticeable in the exposure lake (Figure 3.3B).

In general there was a relationship between $\delta^{32}S$ signatures of Se-exposed fish and whole-body Se concentrations. Comparisons of $\delta^{32}S$ signatures and whole-body Se concentrations in wild and caged lake chub showed significant divergence between wild lake chub collected from the reference lake and Delta Lake (Figure 3.3C). Figure 3.3C shows the divergence of $\delta^{32}S$ signatures between lake chub caged in the reference and exposure lakes. Comparisons of the $\delta^{15}N$ and $\delta^{32}S$ signatures indicated that wild lake chub from Delta Lake had much lower $\delta^{15}N$ and $\delta^{32}S$ signatures than the caged lake chub originally collected from the reference lake (Figures 3.3B and 3.3C). Caged lake chub from the exposure lake had lower $\delta^{32}S$ signatures than the wild and caged lake chub from the reference lake (Figure 3.3C).

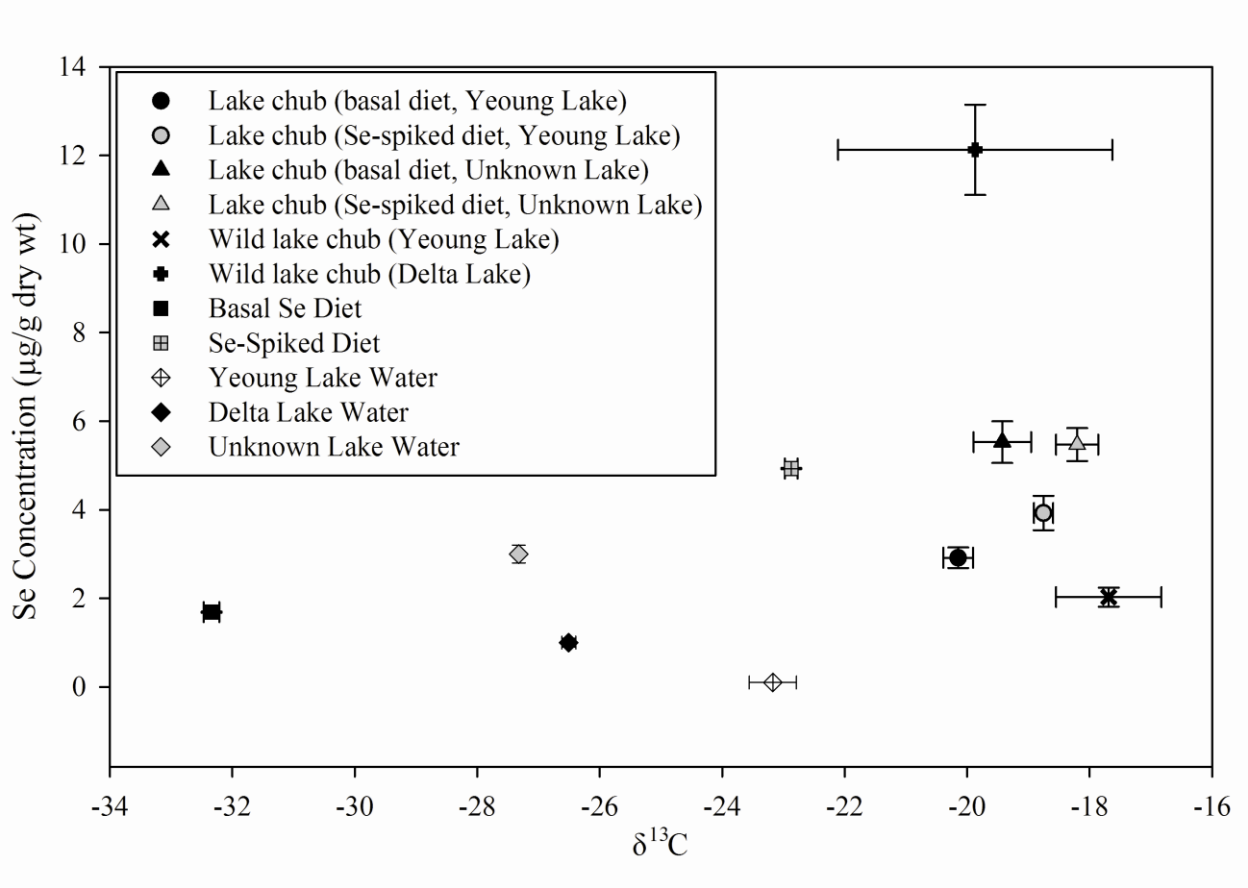

Figure 3.3**(**A) The relationship between whole-body Se concentration and carbon ($\delta^{13}C$) stable isotope signature in lake chub caged in the reference lake (Yeoung Lake) and fed basal and Se-spiked diets, lake chub caged in an exposure lake (Unknown Lake) and fed basal and Se-spiked diets, wild lake chub collected from the reference lake and an exposure lake (Delta Lake) located approximately 6 km downstream of Unknown Lake, water samples collected from the reference lake, Unknown Lake, and Delta Lake, and samples of the basal Se diet and the Se-spiked diet. Values for the isotopic signatures of caged fish, lake water, and administered diets represent mean ± SE of $n = 3$.

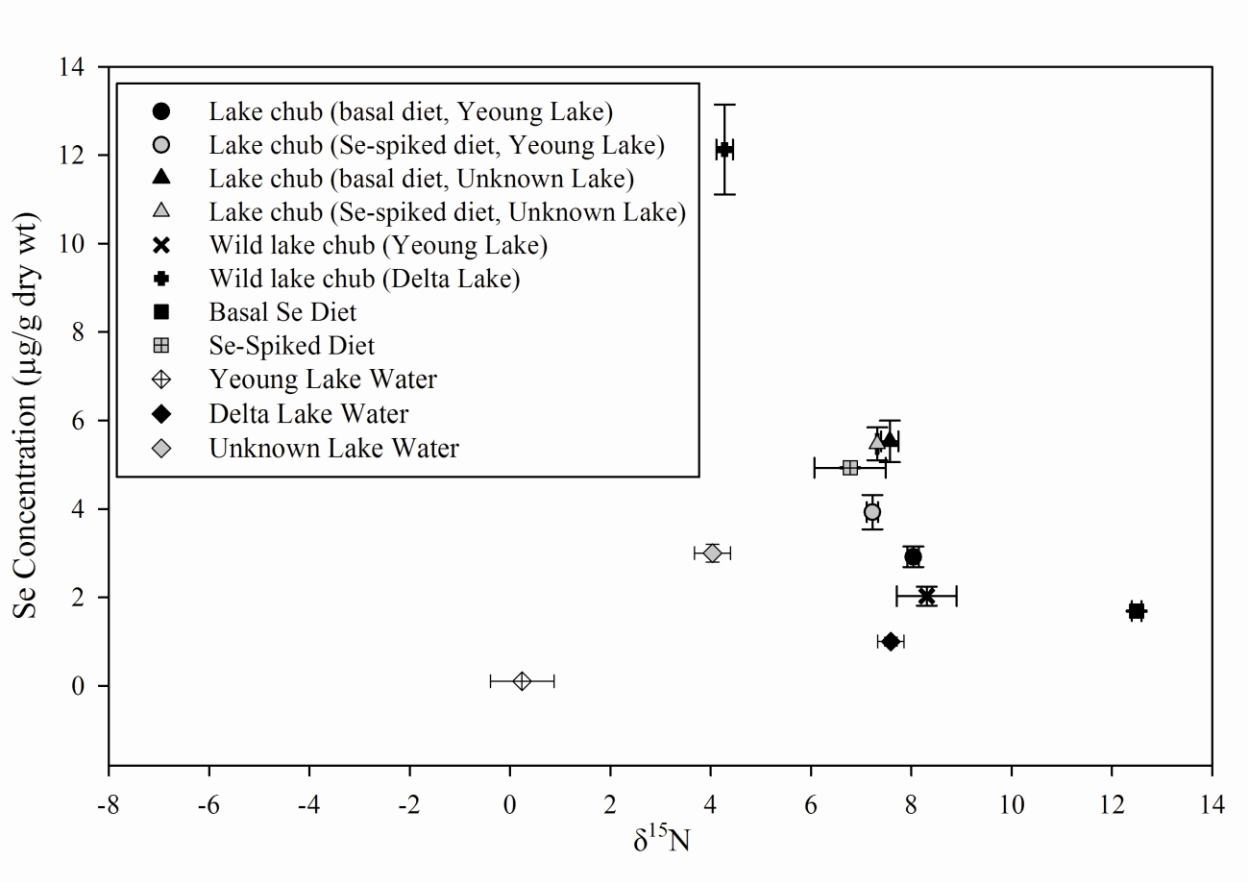

Figure 3.3(B) The relationship between whole-body Se concentration and nitrogen ($\delta^{15}N$) stable isotope signature in lake chub caged in the reference lake (Yeoung Lake) and fed basal and Se-spiked diets, lake chub caged in an exposure lake (Unknown Lake) and fed basal and Se-spiked diets, wild lake chub collected from the reference lake and an exposure lake (Delta Lake) located approximately 6 km downstream of Unknown Lake, water samples collected from the reference lake, Unknown Lake, and Delta Lake, and samples of the basal Se diet and the Se-spiked diet. Values for the isotopic signatures of caged fish, lake water, and administered diets represent mean ± SE of $n = 3$.

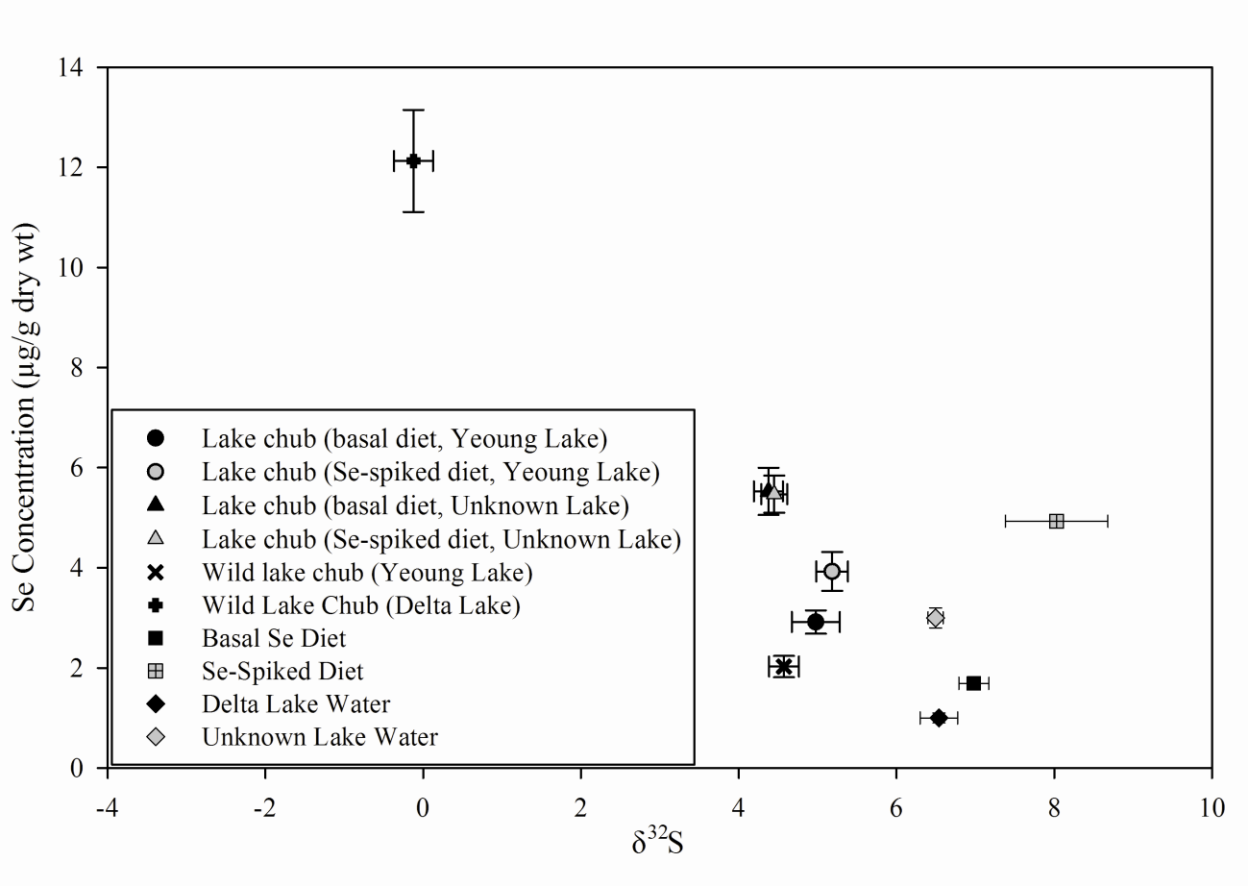

Figure 3.3(C) The relationship between whole-body Se concentration and sulphur ($\delta^{32}S$) stable isotope signature in lake chub caged in the reference lake (Yeoung Lake) and fed basal and Se-spiked diets, lake chub caged in an exposure lake (Unknown Lake) and fed basal and Se-spiked diets, wild lake chub collected from the reference lake and an exposure lake (Delta Lake) located approximately 6 km downstream of Unknown Lake, water samples collected from the reference lake, Unknown Lake, and Delta Lake, and samples of the basal Se diet and the Se-spiked diet. Values for the isotopic signatures of caged fish, lake water, and administered diets represent mean ± SE of $n = 3$.

3.4 Discussion

To our knowledge this is the first study combining metalloid speciation and stable isotope analyses to investigate Se bioaccumulation pathways in wild fish. Overall, the results of this caging study indicated that whole-body Se concentrations increased with increasing exposure to dietary Se. Whole-body Se concentrations in lake chub caged in the reference lake for 21 days were 1.5 times higher in the Se-spiked diet than the basal diet, and whole-body Se concentrations for both diet types were 2-3 times higher in the fish caged at the exposure lake compared to the basal diet in the reference lake, which was not as large as the 4-fold difference in Se concentration between the two diets. The predicted result for the basal diet treatment in the exposure lake was to approximate the basal diet treatment in the reference lake, since the barrier fitted on the cage bottoms was designed to exclude feeding on native benthic invertebrates. Instead, the whole-body Se concentrations for the two treatment groups in the exposure lake were significantly greater than the reference lake treatments but not significantly different from each other. No significant differences were detected in the whole-body Se concentrations of the basal diet treatment in the reference lake after 21 days compared to wild uncaged lake chub collected from the same lake by Phibbs et al. (2011).

Observations made during this experiment indicated that the controls established to isolate chub in the feeding cages from natural prey were insufficient to exclude benthic invertebrate activity present in the study lakes. In the exposure lake several species of benthic invertebrates (Trichoptera and Odonata) were observed using the cages and stake markers as aids to help them emerge from their aquatic life stage. As a result, caged lake chub in the exposure lake were likely able to forage on native benthos during the experiment, resulting in greater Se

bioaccumulation than was contributed from either the basal or Se-spiked diets alone. Thus, the Se exposure of caged lake chub in the exposure lake was likely due to both the controlled diets and natural prey. Limited results for benthic invertebrates from the exposure lake indicate 2-4 fold higher Se concentrations than the Se-spiked diet (Muscatello et al., 2008). In the reference lake basal diet treatment, the limited growth results may be the result of limited access to native benthos during caging.

Comparing whole-body Se concentrations from lake chub caged in the exposure lake in the present study with those observed from an *in situ* cage study carried out in the same study lake by Phibbs et al. (2011), where lake chub were allowed to forage on native benthic invertebrates freely (i.e., no barrier on cage bottoms was used), indicated that Se bioaccumulation in caged fish was similar, approximately 2-3 times above reference concentrations. Statistical comparisons between the current study and data from Phibbs et al. (2011) demonstrated that selenium concentrations of the lake chub caged in the exposure lake from all three dietary exposure regimes (basal, Se-spiked, or *in situ*) were not significantly different from one another, which suggests that the *in situ* diet likely contributed to all exposures regimes. However, while significant increases in whole-body Se concentrations could be attained in 21 days, this limited time period was inadequate to achieve whole-body Se concentrations in equilibrium with the surrounding environment, as indicated by higher Se concentrations in wild lake chub previously collected from Delta Lake (12.74±0.67 µg Se/g; Phibbs et al., 2011). Steady-state whole-body Se concentrations that are in equilibrium with the surrounding environment have been reported to require much longer exposure periods, such as in the 87-day cage experiment conducted by Allert et al. (2006).

Overall, selenomethionine-like compounds were found to be the most abundant form of Se at higher Se exposures. The fraction of total Se represented by selenomethionine-like compounds from lake chub caged in the reference lake and fed a basal or Se-spiked diet increased with increasing Se exposure, and the highest fraction of selenomethionine-like compounds was observed in fish fed the Se-spiked diet and caged in the exposure lake. The fraction of selenomethionine-like compounds from the exposure lake basal diet treatment were consistent with the results from the *in situ* feeding study conducted in the same study lake by Phibbs et al. (2011). The unexpected increase in whole-body Se concentrations for the basal diet treatment in the exposure lake and the stable isotope results further supports the likelihood that caged lake chub in the exposure lake had access to natural prey items (benthic invertebrates, zooplankton, and algae/biofilm) despite attempts to isolate caged fish from the native benthos.

The highest fractions of selenomethionine-like compounds observed in the exposure lake fish in the present study fall within the apparent biological maximum (approximately 0.60–0.80) suggested for lake chub by Phibbs et al. (2011). A previous study conducted by Phibbs et al. (2011) suggested that the maintenance of a biological maximum for the metabolism and storage of organic forms of Se in prey such as lake chub may be the result of efficient Se metabolism and excretion. The importance of large increases in organic forms of Se (selenomethionine-like compounds) is that large increases in bioavailable forms of Se in prey would be passed on to higher trophic levels. The percentage of organic Se compounds (predominantly selenomethionine) is likely to biomagnify at higher trophic levels even though the trophic transfer of Se has been observed to be relatively low at higher trophic levels (Fan et al., 2002; Andrahennadi et al., 2007; Muscatello et al., 2008; Stewart et al., 2010).

Stable isotope analysis indicates that the fish caged in the exposure lake were able to deviate from administered diet by consuming native benthos. In the present study there was an inverse relationship between $\delta^{32}S$ signature and Se exposure for the whole-body fish samples analysed. For the exposure lake treatments the $\delta^{32}S$ signatures of the lake water and the administered diets were not consistent with the $\delta^{32}S$ signature of the whole-fish samples analysed in this study. This relationship supports the greater differences in $\delta^{32}S$ signature observed in lake chub caged in the reference lake compared to those caged in the exposure lake, the latter of which had access to both a controlled diet and presumably more native benthos. Therefore the heterogeneous diet of the fish caged in the exposure lake led to similar $\delta^{32}S$ signatures for each caging treatment.

These results may also suggest a dose-dependent substitution of Se for S in methionine as a consequence of dietary Se exposure, a shift likely caused by the substitution of Se for S in primary producers due to the elevated aqueous Se concentration in their environment. This relationship is also supported by the trend of increasing selenomethionine-like compounds with increasing Se exposure observed in the present study and by Phibbs et al (2011). Proulx and Hare (2008) and Croisetiere et al. (2009) noted that benthos feeding on particles in anoxic sediment tend to have lower $\delta^{32}S$ signatures than benthos feeding in more oxic environments, likely leading to lower $\delta^{32}S$ signature in foraging fish. Anoxic sediments have been identified in past research conducted at these exposure lakes at Key Lake (Wiramanaden et al., 2010a), but these specific lake locations were not targeted for fish caging in the present study.

In the present study there was a relationship between $\delta^{15}N$ and the whole-body Se concentrations in caged fish-fed controlled diets. The $\delta^{15}N$ isotopic signatures of wild lake chub

collected from Delta Lake were isotopically lighter than each of the caged lake chub treatments as well as the wild (uncaged) lake chub collected from the reference lake. When comparing just the $\delta^{15}N$ signatures of the caged lake chub in the reference lake, the differences between those fed basal and Se-spiked diets diverged from each other relative to the signatures of their respective diets. Overall, the $\delta^{15}N$ signatures of the caged lake chub that were exposed to Se were isotopically lighter than the lake chub caged in the reference lake and fed a basal diet. This trend was much less obvious between the two diet treatments in the exposure lake indicating their $\delta^{15}N$ signatures were not singly influenced by the administered diet, which appears to be the case for fish caged in the reference lake. This $\delta^{15}N$ signature difference further suggests that the diet of lake chub caged in the exposure lake was influenced by prey items other than the experimental diets.

No relationships were observed between $\delta^{13}C$ signatures and Se accumulation for both wild and caged lake chub. $\delta^{13}C$ signatures are representative of the primary producers in the aquatic food web and generally do not change significantly between trophic levels, whereas $\delta^{15}N$ signatures reflect dietary differences and trophic status, and tend to increase during trophic transfer (DeNiro and Epstein, 1978, 1981). In this study no apparent influences of the $\delta^{13}C$ and $\delta^{15}N$ signatures in water were detected in caged or wild lake chub. However, the large differences in $\delta^{13}C$ and $\delta^{15}N$ signatures between the basal and Se-spiked food sources can be attributed to differences in the ecosystems in which the food originated. The Se-spiked *C. dilutus* produced in Saskatoon, SK had much different $\delta^{13}C$ and $\delta^{15}N$ signatures than the *Chironomus* spp. produced commercially in San Francisco, CA.

Due to the relatively short duration of this caging experiment, only small changes in isotopic signatures were produced in experimental fish. We believe that based on the trends in the data and evidence in the literature, a longer feeding study would have yielded isotopic signatures in the caged fish that were more representative of their diets. In the future, isotopic signatures may be produced from short-term caging studies by using an isotopic tracer in the controlled diets. Isotopic tracers can be monitored in aquatic ecosystems to help track the movement of the tracer through the food chain (Peterson et al., 1994). The use of a Se-isotope tracer would likely link Se accumulation with specific food sources within an aquatic system. In this way the use of a Se isotope tracer would likely yield more accurate results in terms of linking Se accumulation with specific food sources within an aquatic system. In the present study, there was a relationship between $\delta^{32}S$ signatures of Se-exposed fish and contaminant concentrations. The use of a tracer could help identify links between isotopic signatures and contaminant biomagnifications, similar to the work conducted in northern Canadian lakes by Cabana and Rasmussen (1994) and Kidd et al. (1995).

Over the course of the 21-day feeding study, the aqueous Se concentration in the exposure lake increased significantly by approximately 20%. Temporal variations in aqueous Se concentrations over the 21-day feeding study were anticipated due to activities related to the uranium milling effluent management. Prior to the initiation of this research project the uranium processing mill at Key Lake was offline for approximately one month of scheduled maintenance on its effluent management system. The feeding trial began approximately 10 days after the uranium mill had begun regularly discharging effluent. The significance of the shutdown on the caging trials and their results is expected to be low because the primary mode of Se bioaccumulation in fish is via the diet, and the influence of aqueous Se concentrations on

sediment Se concentrations occurs over relatively longer time periods. Therefore, the assimilation of aqueous Se by primary producers would be expected to be the first biotic or abiotic component of the ecosystem to respond to changes in Se inputs (Riedel et al., 1991; Besser et al., 1993). The dietary significance of Se-enriched primary producers to caged lake chub is relatively low, but they would be expected to have some influence on the dietary uptake of Se. Therefore, we do not expect that small temporal changes in aqueous Se concentrations to alter the significance of the dietary pathways for Se accumulation.

3.5 Conclusions

The results of this study support the hypothesis that dietary uptake of Se is the dominant form of Se uptake in fish. This study also indicated that dietary uptake of Se by caged lake chub can lead to significant increases in whole-body Se in a relatively short period of time (21 days). Controlled feeding in this study was successful in the reference lake, but through the use of stable isotope analysis it appeared likely that caged fish in the exposure lake were able to significantly increase their Se concentrations by foraging to a greater extent on native benthic invertebrates in addition to the controlled basal diet, leading to similar $\delta^{32}S$ signatures for each of the exposure lake treatments**.** The predominant form of Se in exposed fish was selenomethionine-like compounds, which further supports the findings of our previous study (Phibbs et al., 2011) and others (Fan et al., 2002; Andrahennadi et al., 2007) that the presence of selenomethionine-like compounds appears to be a marker of elevated Se exposure. Therefore, the combination of total Se, Se speciation and stable isotope use (in particular $\delta^{15}N$ and $\delta^{32}S$ isotopes) were useful in this field study, because it allowed us to track Se uptake from both native and prepared diets into caged lake chub. Furthermore, the combination of stable isotope

analysis and Se speciation indicated that non-specific substitution of Se for $\delta^{32}S$ in methionine may be a consequence of elevated Se fixed by primary producers in aquatic systems.

CHAPTER 4

4 GENERAL DISCUSSION

4.1 Introduction

Overall the goal of my thesis research was to increase the understanding of Se speciation and trophic transfer with regards to the aquatic ecotoxicology of Se. This study was conducted in a chain of lakes in northern Saskatchewan subjected to more than 25 years of uranium milling effluent discharge with elevated Se levels. The main approaches used in this research project included: Evaluation of Se concentrations and Se speciation in large and small-bodied fish inhabiting lakes receiving partially treated effluent and fish from a nearby reference lake; the validation of a caging methodology using wild naïve lake chub and spottail shiner to determine the best potential species for further caging work; feeding studies using caged lake chub aimed at evaluating Se accumulation via dietary uptake from an *in situ* food source, a Se-spiked food source and a basal (normal Se) food source; and the use of stable isotope (C, N, S) analysis to detect trends in the dietary uptake and trophic transfer of Se and possible links to Se speciation.

4.2 Selenium in Northern Saskatchewan

4.2.1 The effects of dietary uptake on whole-body selenium concentrations and selenium speciation in fish from the Key Lake area using *in situ* feeding cages

Chapter 2 described a field study conducted in early summer 2008 investigating Se uptake and speciation in both wild and caged small-bodied fish. Fish used in this caging study were collected from a reference lake and caged in the reference lake (Yeoung Lake), a medium exposure lake (Delta Lake) and a high exposure lake (Unknown Lake) downstream of the Key Lake uranium milling operation. Caged fish were left for 21 days to feed *in situ* on available native benthos and algae at their caging location. The use of three different caging locations per

lake based on total organic carbon in sediment (one cage per location; n = 1) means that fish were used as the experimental unit for this experiment. The use of an experimental design that includes pseudoreplication exaggerates statistical significance by treating the data as independent observations when they are in fact interdependent. In this study it was not feasible (permitting and logistics) to catch 270 lake chub in order to be able to place 3 cages at each sediment type location in each of the three lakes. However, the overall lake averages which represent three cages from each lake are also presented and they reflect n = 3.

The fish species selected for this experiment included lake chub, spottail shiner and northern pike because of their widespread distribution across the boreal forest region. Selenium uptake was evaluated using whole-body Se concentrations measured using ICP-MS. Subsamples of fish from each treatment group were also analysed for Se speciation using K near-edge XAS at the Canadian Light Source synchrotron in Saskatoon, SK and the Stanford Synchrotron Radiation Lightsource in Menlo Park, CA. Aqueous Se concentrations in the exposure lakes from this study are elevated, exceeding the 1μg/L water criteria established by the CCME (CCME, 2003), but are relatively low compared to the lakes immediately downstream of the effluent release point.

Increased whole-body Se concentrations were expected based on trophic transfer work conducted in the same study lakes by Muscatello et al. (2008). Muscatello et al. (2008) found that despite relatively low Se concentrations in water, Se concentrations in lake sediment increased up to 200-4000 times and that significant increases in Se were observed in all biotic compartments compared to an upstream reference lake. After 21 days in the same exposure lakes, significant increases (2-3 fold) in the whole-body Se concentration occurred in lake chub caged *in situ*. These concentrations were below the proposed U.S. Environmental Protection

Agency (USEPA) whole-body Se threshold of 7.91 µg/g and much less than the steady-state whole-body Se concentrations observed in lake chub and spottail shiner collected from Delta Lake during the summer of 2008 (approximately 12 µg/g and 18 µg/g dry weight, respectively).

Validation of the caging methodology indicated that small-bodied fish were suitable for measuring dietary uptake of Se and that lake chub were the most suitable species for Se caging studies in this region. Lake chub suitability was based on higher survival, a positive increase in condition factor and relative tolerance to capture and handling stresses. These factors all indicated that lake chub have a higher level of stress tolerance and/or a greater ability to access available food (grazing on netting, suspended food particles and native benthos in sediment) during this type of experiment.

Selenium speciation results from wild fish and fish caged in the reference and exposure lakes indicated that selenomethionine was the predominant form of Se in exposed fish and appears to be a marker of high Se exposure. In addition, correlation of Se uptake with the percentage of whole-body Se present as selenomethionine indicated that rising whole-body Se concentrations lead to a large increase in the fraction of selenomethionine, reaching an observed maximum of approximately 60-80%. The importance of large increases in organic forms of Se including the marker of Se exposure (selenomethionine) is that it may lead to large increases in bioavailable forms of Se in prey. Therefore at higher trophic levels the percentage of organic Se compounds (predominantly selenomethionine) is likely to biomagnify even though the trophic transfer of Se has been observed to be relatively low at higher trophic levels (Fan et al., 2002; Andrahennadi et al., 2007; Muscatello et al., 2008).

4.2.2 The uptake, speciation and trophic transfer of selenium in caged fish fed controlled selenium diets

Chapter 3 described a field study conducted in June 2009 investigating Se uptake and speciation in caged lake chub from the reference lake (Yeoung Lake) fed basal and Se-spiked diets of 1.5 and 5.5 µg Se/g dry weight, respectively, in the reference lake and an exposure lake (Unknown Lake). In addition to whole-body Se and Se speciation analyses, the trophic transfer of Se was also investigated using C, N, and S stable isotope analysis. This project aimed to repeat the methodology and analysis outlined in Chapter 2 with a change in the food source to examine the uptake of dietary Se from food sources with known Se concentrations. The use of controlled food sources was also intended to clarify the role of aquatic Se concentrations with regard to Se uptake and speciation. Since fish do not appreciably take up Se via aqueous exposure from aquatic systems, whole-body Se concentrations in caged fish were predicted to be linked to food treatment and not lake treatment (Fan et al., 2002; Hamilton, 2004; Stewart et al., 2010).

At the end of the 3 week caging experiment, whole-body Se concentrations measured in caged lake chub from the reference lake were approximately 1.5 times higher in lake chub fed a Se-spiked diet compared to a basal diet. Whole-body Se concentrations for both diet types were 2-3 times higher in the exposure lake compared to the basal diet in reference lake. In the high exposure lake there were no significant differences in whole-body Se concentrations between fish fed the Se-spiked and basal diet. This result deviates from the predicted result for the basal diet and water only exposure. However, field observations indicated that the measures taken to isolate lake chub from the available *in situ* food sources were not sufficient to eliminate the availability of free swimming benthic prey items in active and mobile pre-emergence stages.

In this study there was a correlation between both $\delta^{15}N$ and $\delta^{32}S$ and the whole-body Se concentrations in caged fish fed controlled diets. With respect to N, wild lake chub collected from the medium exposure lake were isotopically lighter than each of the caged lake chub treatments as well as the wild (non caged) lake chub collected from the reference lake. The difference in $\delta^{15}N$ signature suggests that the diet of caged lake chub was likely the result of something other than the supplied food source. Sulphur had the strongest correlation between isotopic signature and whole-body Se concentration. In the present study there was an inverse relationship between $\delta^{32}S$ signature and Se exposure for the whole-body fish samples analysed. The high $\delta^{32}S$ may also point to a non-specific substitution of Se for S in methionine as a consequence of Se exposure. Therefore a decreasing trend between $\delta^{32}S$ signature and increasing Se exposure also suggests that Se substituted for S in methionine (i.e. selenomethionine) may be responsible for the $\delta^{32}S$ change.

4.2.3 Integration of *in situ* and controlled diet caging results at Key Lake

In Chapter 2 it was shown that elevated whole-body Se concentrations in wild fish species collected downstream of the Key Lake uranium mill were similar to those reported in previous investigations (Golder, 2005, 2008; Muscatello et al., 2006, 2008). The caging experiments conducted in Chapters 2 and 3 indicated that caged lake chub exposed to elevated dietary Se (*in situ* or Se-spiked diet) had 2-3 fold increases in whole-body Se compared to Se concentrations in wild or caged lake chub from the reference lake. The results from both studies suggest that naive small-bodied fish caged in Se impacted systems may accumulate significant amounts of Se, thereby increasing their whole-body concentration over a relatively short period of time. In 21 days fish accumulated Se in excess of conservative whole-body Se guidelines despite water

concentrations of Se within the range of the protective thresholds set by the CCME and the USEPA (1 µg/L and 5 µg/L respectively). However, caging trials of such short duration are inadequate to achieve whole-body Se concentrations in equilibrium with the surrounding environment such as those from wild fish collected from Delta Lake (medium exposure lake). To achieve steady state whole-body Se concentrations that are in equilibrium with the surrounding environment much longer exposure periods are required, such as the 87 day caging experiment conducted by Allert et al. (2006).

The use of *in situ* caging to evaluate contaminant uptake via aqueous or dietary exposure is a relatively new approach (Pyle et al., 2001; Doebel et al., 2004; Palace et al., 2005; Allert et al., 2006; Oikari, 2006). The strength of this caging approach is that it yields a time integrated measurement of Se accumulation that can be compared to reference and chronically exposed wild fish populations. A secondary goal of this thesis was to help validate the use of fish caging for measuring contaminant uptake. The results of this research were successful in validating this technique as a tool to investigate Se bioaccumulation and speciation in cold water ecosystems. The final motivation for the *in situ* caging trial conducted in Chapter 2 was to compare the use of two of the dominant small-bodied fish species available in northern coldwater ecosystems. Results indicated that lake chub may be better suited for biomonitoring purposes than spottail shiner due to their higher stress tolerance (handling and confinement). The value of this research is that it identifies a suitable small-bodied fish species that may be valuable for evaluating EEM endpoints and metals accumulation from mining effluent that is increasingly common in northern environments.

In both Chapters 2 and 3 the fraction of selenomethionine-like compounds increased with increasing Se exposure and reached an apparent biological maximum of approximately 60-80%. For this reason the presence of selenomethionine-like compounds acts as a good marker of Se exposure. This strength of this marker as a metric for Se exposure is that the results from the exposure scenarios used in Chapter 3 increased the r^2 value from 0.649 (Figure 2.4) to 0.681 when incorporated into the correlation (Figure 4.1).

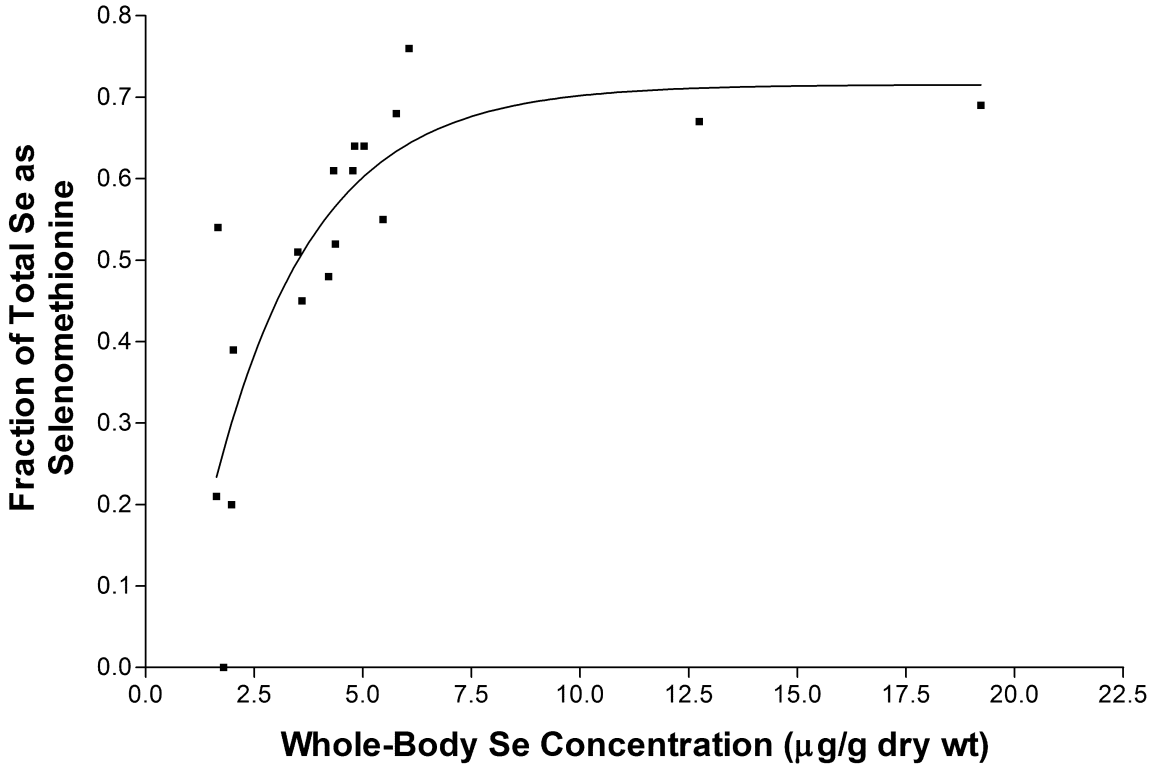

Figure 4.1. The fraction of selenomethionine in relation to whole-body Se concentrations in lake chub and spottail shiner from the reference and exposure lakes. Data points represent both caged and wild fish collected in 2008 and 2009, $r^2 = 0.681$ ($p < 0.05$).

The maintenance of this apparent maximum during the metabolism and storage of organic forms of Se in fish was likely the result of efficient Se metabolism, the biomagnification of organic Se species and active Se excretion. Maintenance of high organic forms of Se in prey species is important because it may lead to large increases in bioavailable Se at higher trophic levels despite low bioconcentration factors of Se between prey and predators. It is also interesting to note that the inflection point of the fraction of selenomethionine in Figure 4.1 corresponds with the USEPA proposed guideline for whole body Se concentration.

4.2.4 Comparison between study lakes

The lakes downstream of the Key Lake mill are characterised by elevated Se levels in water and aquatic biota. This area is ideal for this type of Se uptake study because its long history of effluent release has resulted in a legacy of Se enrichment and subsequent cycling of Se from sediments to the aquatic food chain and back again. The differences in Se concentrations in the study lakes followed a gradient of Se accumulation from the high exposure lake (Unknown Lake) through the medium exposure lake (Delta Lake) to the low exposure lake (Farfield Pond). Interspecies variation in the whole-body Se increases measured within the exposure lakes was considerable. There were significantly greater whole-body Se concentrations in wild spottail shiner from medium exposure lake compared to lake chub collected from the same lake. Both of these fish species had significantly higher whole-body Se concentrations compared to spottail shiner and lake chub collected from the reference lake (approximately 6 and 9 times higher for lake chub and spottail shiner, respectively).

Biota in the exposure lakes have been shown to bioaccumulate Se despite relatively low Se concentrations in the lake water (Muscatello et al., 2008). This may be because the majority

of Se found in contaminated environments occurs in the top layer of sediments and detrital matter (Bowie et al., 1996; Lemly, 1999; Simmons and Wallschläger, 2005; Orr et al., 2006). This layer is the active zone from which Se is cycled back into the aquatic food chain by benthic organisms (Simmons and Wallschläger, 2005). This is particularity true in the Key Lake receiving environment because mill effluent is released into a chain of shallow slow moving lakes which facilitates high Se recycling and sedimentation rates as well as accumulation of Se in the food chain (Orr et al., 2006).

4.2.5 Use of stable isotope analysis as a tool for assessing trophic transfer of contaminants

In Chapter 3 stable isotope analysis was utilised to determine if differences in C, N, and S isotopes could detect trends in the movement of Se in the environment. By comparing stable isotope results with contaminant concentrations, Se uptake was correlated with reductions in $\delta^{15}N$ and $\delta^{32}S$. In addition, the use of $\delta^{32}S$ was a valuable method for determining differences in samples with similar $\delta^{15}N$ signatures and identifying trends in contaminated systems with elevated sulphur signatures. The use of stable isotope analysis in biology is a growing practice and this study showed some promising results despite a short exposure time and the lack of a tracer isotope. Incorporating a stable Se isotope (e.g. ^{74}Se) into the food produced for this study may have allowed us to pinpoint the movement of the tracer directly from the food source to the caged fish. In this study, stable isotope analysis yielded quantifiable information regarding dietary uptake which would not have been possible using techniques such as gut content analysis. Increased use of stable isotope analysis in ecological research should yield new opportunities for studying the movement of contaminants, such as Se, through trophic transfer

and improve the evaluation of environmental risks of specific contaminants to fish and other aquatic organisms.

4.3 Conclusion

The accumulation of Se in effluent enriched environments occurs relatively quickly once Se enters the food chain and becomes bioavailable. Both Chapters 2 and 3 showed that the uptake of dietary Se by caged lake chub may exceed conservative Se thresholds and approach the USEPA regulatory threshold (7.91 µg/g dry weight) designed to protect fish species in as little as 21 days (USEPA, 2004). The whole-body Se concentrations after 21 days of caging were between 33 and 50% of the steady-state concentrations found in chronically exposed fish in Delta Lake. Fish species collected from the exposure lakes in Chapter 2 and by Muscatello et al. (2008) have also shown differences in steady-state whole-body Se levels between species, indicating interspecific differences in Se accumulation, excretion and tolerance. The tolerance of different cold water fish species has been a matter of debate in recent literature (Chapman, 2007). In Chapter 2 my research compared three of the dominant fish species found in the study lakes (lake chub, spottail shiner, and northern pike) for steady-state Se concentrations as well as comparing the suitability of lake chub and spottail shiner for caging studies evaluating Se accumulation from dietary sources. Chapter 3 attempted to refine this approach using controlled food with mixed success. However, the results from Chapter 3 did help to validate the Se uptake and speciation results from Chapter 2. Focusing on caging methodology, the results of this study show the value of *in situ* caging in evaluating the uptake of dietary Se. For example this study yielded a time integrated measurement of Se accumulation that can be compared to reference and chronically exposed wild populations. It also showed the suitability of using lake chub over

spottail shiner for contaminant exposures dominated by dietary uptake, or other EEM type assessments and research.

The second main focus of this thesis was the analysis of Se speciation which confirmed the presence of selenomethionine as a biomarker of Se exposure (Fan et al., 2002; Andrahennadi et al., 2007). However, the mechanisms of toxicity caused by the bioaccumulation of this form of Se is largely unstudied and would seem to be a critical data gap in understanding Se toxicity in the future. The final focus of this thesis was the use of stable isotope analysis in Chapter 3. Using this approach I set out to increase the understanding of the trophic transfer of Se in the aquatic ecosystem. The use of $\delta^{32}S$ and $\delta^{15}N$ isotopes helped to identify possibility of external food sources (native benthos) in the Unknown Lake cages during the controlled food experiment, which was vitally important because caging coincided with the emergence of a variety of insects into the terrestrial life stage. Stable isotope analysis also yielded quantifiable results for dietary consumption which would not have been possible based using single time point observations such as gut content analysis. In comparison with whole-body Se concentrations, the use of stable isotopes indicated that the bioaccumulation of Se was associated with a reduction in $\delta^{15}N$ and $\delta^{32}S$, as well as confirming the value of analysing S isotopes when studying contaminated systems with elevated sulphur signatures. Overall the use of speciation and stable isotope analysis in ecological experiments may provide new opportunities for understanding the trophic transfer and the ecotoxicology of Se. I feel the results of my research have made a unique contribution to the discussions of Se ecotoxicology in aquatic environments. In particular this research will be helpful in understanding the potential impacts of Se in northern ecosystems, aid in the establishment of regulatory thresholds to protect cold water fish species, and site specific guidelines for the protection of fish in the future.

4.4 Future Research Needs and Recommendations

4.4.1 Research needs

The USEPA has proposed a chronic whole-body Se threshold in fish of 7.91 µg/g dry weight which is based on the warm water centrarchid species, bluegill sunfish (USEPA, 2004). This whole-body Se criterion is only based on a few studies and may not be applicable to cold-water fish species. Chapman (2007) makes the case that cold-water fish species can tolerate higher Se tissue concentrations than warm-water fish. Research on the effects of Se on fish in northern environments has focused on Se uptake in reproducing fish and its potential for causing Se-induced deformities (Kennedy et al., 2000; Holm et al., 2005; Muscatello et al., 2006). Chapman (2007) hypothesizes that these studies indicate the USEPA threshold may provide a conservative level of protection for northern fish species. However, a realistic protective guideline requires more site specific studies. Muscatello et al. (2009) echoed the need for more studies on the environmental fate of Se and the effects of Se on the aquatic environments in north temperate regions such as the boreal forest region in Canada.

The results from Chapter 2 of this thesis concurs with Muscatello et al. (2008) that wild small-bodied fish species in the exposure lakes can accumulate as much as 2-3 times more Se than the USEPA threshold of 7.91 µg/g dry weight. The study outlined in Chapter 2 indicated that in these lakes wholebody fish concentrations could approach the USEPA threshold of 7.91 µg/g dry weight in as little as 21 days. Both Chapters 2 and 3 indicated that the Se species selenomethionine can be used as a marker of Se exposure. Selenium uptake and the build up of organic forms of Se have been shown to be very rapid and there appears to be a theoretical maximum for selenomethionine in exposed fish. As these were not reproductive studies, no Se

induced deformities were observed during these experiments. However, the rapid uptake of Se in a short exposure period gives some cause to question the effectiveness of the USEPA threshold for protecting small-bodied fish. The use of guidelines that utilise regional or site specific standards rather than the universal application of one standard across an entire country may be more appropriate. Achieving a better, more protective whole-body Se threshold for northern fish populations will require (1) a better understanding of the mechanisms of Se toxicity and (2) more research focusing on the application of site specific solutions. The research suggestions that have arisen from this project include:

- *Understanding Se speciation and its effects on Se toxicity in aquatic environments.*

This study used XAS technology to determine the different Se species present in fish exposed to a gradient of Se. The results suggested that selenomethionine can act as a marker of Se exposure. This biomarker of exposure could be an important tool going forward to determine if fish are accumulating excess Se from the environment. Research in this area could be furthered by looking at the toxicological mechanism(s) that causes selenomethionine-like compounds to cause toxicity in fish, which may relate to the apparent correspondence of the inflection point of the fraction of whole body selenomethionine to the USEPA proposed guideline for whole body Se concentration. Therefore future development of environmental criteria for the protection of aquatic life may need to incorporate information on whole-body Se speciation of exposed fish.

- *The need for increased understanding of Se geochemistry.*

In field based experiments such as the ones conducted in this thesis there is little control over the water and sediment geochemistry in the study environment. In some environments other contaminants which are known to influence Se toxicity are available concurrently in the

environment, such as pH, arsenic or mercury (Lemly, 1999; ATSDR, 2003). In such cases these factors should be evaluated as well. It was beyond the scope of this project to collect other water and sediment chemistry data from the research lakes or to relate whole-body Se results with other contaminants. However, a better understanding of the potential effect these factors have on Se bioaccumulation and toxicity may be beneficial to understanding complex ecosystems such as those affected by mining effluent.

- *Analysis of differential Se accumulation in small-bodied fish tissues.*

In Chapter 2 the analysis of Se speciation in northern pike was conducted on different fish tissues (liver and muscle). This was done as a proxy for whole-body analysis (Se concentration and speciation) in a larger species that is more difficult to analyse using this method. The analysis of different fish tissues will also help to determine if different tissues have different rates of Se accumulation and differences in Se speciation. The application of this technique to multiple tissues in other fish such as the small-bodied fish species used in Chapter 2 may yield valuable new information regarding the uptake, metabolism, and excretion of Se species in specific tissues.

- *Analysis of chronic lifetime Se accumulation patterns using Laser Ablation Inductively Coupled Plasma Mass Spectrometry (LA-ICP-MS) analysis of selenium in otoliths.*

The study of fish chronically exposed to Se in enriched ecosystems can be evaluated using LA-ICP-MS analysis of Se in the calcified structure of a fish (otolith). Research conducted by Palace et al. (2007) has established methods for analysing Se accumulated in otoliths. By analysing the incremental uptake of Se in fish from a Se enriched lake such as Delta Lake it may be possible to determine the pattern of Se uptake over a fish's lifetime in that environment. It

may also yield valuable information to help link Se uptake to toxicity by identifying the life stage(s) with the highest rate of Se uptake or help identify lifetime exposures complicated by fish migration.

- *Investigation of Se-induced reproductive effects (Se-induced deformities) in small-bodied fish.*

Following up on the discussion of uptake and speciation of Se in small-bodied fish species (lake chub and spottail shiner) it may be worthwhile to look into the sensitivity of each species to Se-induced reproductive effects. These fish are common to the study lakes used in the larval deformity research conducted by Muscatello et al. (2006) and therefore the same experimental design could be used to investigate potential Se-induced deformities resulting from Se exposed adults. Results could be compared between wild naive lake chub and spottail shiner as well as to results from predators such as northern pike. The wild and caged adult lake chub and spottail shiner studied in Chapters 2 and 3 did not show high sensitivity to Se accumulation. Taking into consideration the significant increases in Se-induced deformities identified by Muscatello et al. (2006) it may be helpful to evaluate deformities in small-bodied fish and its potential effects on their population. The link between Se-induced deformities and population level impacts of multiple species in an ecosystem will yield better information for predicting food web level effects caused by Se contamination as well as aid in the development of a more appropriate Se criterion for the protection of fish populations.

- *Further testing of Se-spiked diets on the apparent selenomethionine maximum of 60-80%.*

The results from Chapters 2 and 3 show that the presence of selenomethionine is a marker of Se exposure. Both studies also found that there was an apparent maximum (60-80%) of selenomethionine-like compounds in the exposed fish. This apparent maximum that was

observed is likely a combination of an individual fish's uptake, metabolism and excretion of Se. Future work could be conducted to see if this maximum is common (occurrence and range) for other species and if the maximum is linked to the sensitivity or tolerance of fish species to Se. For example Holm et al. (2005) indicated that different fish species have different sensitivities to Se. This has been supported by the different results observed for warm water species (i.e. bluegill) and cold water species (i.e. northern pike) (Kennedy et al., 2000; USEPA, 2004; Muscatello et al., 2006). This idea could be built upon with the inclusion of Se speciation data. It could also be combined with the investigation of Se-induced deformities to investigate if a correlation exists between Se speciation and the occurrence of Se-induced deformities. The exploration of this research focus should increase our understanding of Se speciation and its links to Se toxicity/tolerance. This information will also aid decision makers in choosing more protective Se standards whether it is between cold and warm water species or small-bodied fish and large predators.

4.4.2 Recommendations

During the course of this research project a number of recommendations were highlighted. They are briefly discussed below:

- *Sample drying for Se analysis (freeze dry vs. oven dry)*

The volatilisation of Se occurs at 80°C and Se evaluations can be underestimated if losses occur during sample preparation. Drying samples in a 60°C oven has been a standard practice at many research facilities including the Toxicology Centre at the University of Saskatchewan. However for this study the availability of a high efficiency freeze dryer allowed all the samples analysed for Se in this experiment to be dried at high vacuum pressures and low temperatures. Results

from the freeze dryer showed similar moisture content reductions (lake chub 75%, spottail shiner 76%, northern pike 74%) to previous samples analysed at the Toxicology Centre (Muscatello et al., 2008). However the benefit of this method is that the potential for Se losses due to volatilisation at high temperatures was eliminated. Therefore, the preparation of samples for analysis of volatile trace metals such as Se by freeze drying is the most prudent method because it reduces the uncertainty related to volatilisation losses from other methods.

- *Two strengths of field based studies were outlined in this thesis:*

1. The application of *in situ* caging techniques was useful in measuring Se accumulation in exposure lakes using naive wild fish collected from a reference lake. This approach is relatively new (Pyle et al., 2001; Doebel et al., 2004; Palace et al., 2005; Allert et al., 2006; Oikari, 2006) and this research successfully used this technique to investigate Se bioaccumulation and speciation in cold water ecosystems. Overall the strength of this approach is that it yields a time integrated measurement of Se accumulation which can be compared to both reference and chronically exposed wild fish populations.

2. Using lakes downstream of the milling effluent release point may be more desirable than exposing caged fish at the point of effluent discharge. The selection of exposure locations at the effluent release point would expose fish to other factors besides the contaminant of concern such as higher temperatures, low dissolved oxygen, high ammonia and other trace metals. Therefore the use of a natural water body is also a more realistic model of field exposures to contaminants and reduces the likelihood of confounding toxicological endpoints with other effects caused by effluent components such as those listed above.

- *The identification of a suitable small-bodied fish species for caging experiments*

Caged lake chub proved to be the most suitable species for Se uptake studies in lakes downstream of the Key Lake uranium mill based on higher survival, a positive increase in condition factor and their relative tolerance to capture and handling stress. This indicates a higher level of stress tolerance and/or a greater ability to access available food (grazing on netting, suspended food particles and native benthos in sediment).

- *Timing of caging studies*

Many factors are involved in the timing and execution of field experiments and they often vary from year to year. Start date issues such as the timing of ice break-up and seasonal ease of collecting each species were the important constraints for these experiments. The timing of the *in situ* caging study may have led to differences in growth and condition factor of lake chub and spottail shiner because caging was conducted near the end of their respective spawning periods (Scott and Crossman, 1973). As a result the selection of fish for the caging experiments may have included some spawning individuals. Therefore any weight loss and corresponding reduction in condition factor measured in caged lake chub and spottail shiner may not have been solely the result of feeding habits or limited food availability but actually the result of decreased gonad size or egg release over the 21 day caging trail.

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