SELENIUM BIOACCUMULATION AND SPECIATION IN THE BENTHIC INVERTEBRATE *CHIRONOMUS DILUTUS*: AN ASSESSMENT OF EXPOSURE PATHWAYS AND BIOAVAILABILITY

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ABSTRACT

Uranium mining and milling operations at Key Lake, Saskatchewan, Canada, have been releasing effluent since 1983, resulting in the accumulation of selenium in surface water, sediment, and biota in downstream lakes relative to pre-development and reference lake conditions. Elevated selenium can pose a risk to fish and bird populations in aquatic ecosystems as a result of the trophic transfer of selenium from the base of the aquatic food web. This research program was designed to address specific knowledge gaps related to the bioaccumulation of selenium at the benthic-detrital link of aquatic food webs. To fulfill this objective, laboratory and in situ field experiments were conducted using the benthic invertebrate *Chironomus dilutus* to identify the exposure pathway(s) and selenium species associated with the bioaccumulation of selenium by benthic invertebrates downstream from the Key Lake operation.

Laboratory bioaccumulation tests that exposed midge larvae to 4.3 µg/L as dissolved selenate for 10 d resulted in negligible accumulation of selenium. However, larvae rapidly accumulated selenium over 10 d of exposure to 3.8 and 1.8 µg/L selenite and seleno-DL-methionine (Se-met), respectively. Furthermore, once selenium was accumulated by the larvae from the selenite and Se-met treatments, the majority of it was retained after a 10 d depuration period in clean water. When additional midge larvae were exposed to selenium until emergence, selenium accumulated during the larval stage was passed onto the adults following metamorphosis, with only a small percentage of the selenium (< 5%) transferred to the exuvia. Selenium speciation analysis using X-ray absorption spectroscopy showed that increases in total selenium concentrations corresponded to increased fraction of organic selenides, modeled as selenomethionine, in *C. dilutus* larvae and adults.

Results from the 2008 in situ caging study confirmed that surface water is not a significant selenium exposure pathway for benthic invertebrates at Key Lake. *Chironomus*

dilutus larvae accumulated between 20- and 90-fold more selenium from exposure to sediment compared to exposure to surface water in the high exposure lake. In response to these findings, a second in situ experiment was conducted in 2009 to investigate the importance of dietary selenium (biofilm or detritus) vs. whole-sediment as an exposure pathway. Larvae exposed to sediment detritus (top 2 – 3 mm of sediment) from the exposure site had the highest selenium concentrations after 10 d of exposure (15.6 ± 1.9 µg/g) compared to larvae exposed to whole-sediment (12.9 ± 1.7 µg/g) or biofilm (9.9 ± 1.6 µg/g). Biofilm had lower total selenium concentrations than the detritus and whole-sediment fractions, but nearly 80% of the selenium was present as organic selenides similar to selenomethionine. Biofilms appear to be an enriched source of organic selenium and are important food items for many benthic consumers. Integrating the separate lines of evidence that were generated during laboratory and in situ bioaccumulation tests helped strengthen the understanding of selenium accumulation in the benthic-detrital food web and subsequent trophic transfer of selenium to benthic invertebrates.

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ΔC	change in concentration
Δt	change in time
µg/g	micrograms per gram
µg/L	micrograms per litre
µm	micrometer
AGTMF	Above Ground Tailing Management Facility
ANOVA	analysis of variance
ATSDR	Agency for Toxic Substances and Disease Registry
BCF	bioconcentration factor
BN	bulk neutralization
$C_5H_{11}NO_2Se$	seleno-DL-methionine
CCME	Canadian Council of Ministers of the Environment
CF	concentration factor
CLS	Canadian Light Source
CNSC	Canadian Nuclear Safety Commission
COPC	contaminant of potential concern
CRM	certified reference material
d	day/days
DL	David Lake
DO	dissolved oxygen
DOC	dissolved organic carbon
dry wt	dry weight
DTMF	Deilmann Tailings Management Facility
EEM	Environmental Effect Monitoring
EMP	Environmental Monitoring Program
ERA	Environmental Risk Assessment
FL	Fox Lake
HXMA	Hard X-ray Micro Analysis
ICP-MS	inductively-coupled plasma mass spectrometry
K_e	elimination rate constant
K_u	uptake rate constant
LoD	limit of detection
ml	millilitre
MMER	Metal Mining Effluent Regulations
NH_3	unionized ammonia
RO	reverse osmosis

R-Se-H	selenocysteine
R-Se-R	selenomethionine
R-Se-Se-R	selenocystine
R-S-Se-S-R	seleno-*bis*-glutathione
Se	selenium
Se^0	elemental selenium
Se-met	seleno-DL-methionine
SeO_3^{2-}	selenite
SeO_4^{2-}	selenate
SMOE	Saskatchewan Ministry of Environment
SRM	standard reference material
SSRL	Stanford Synchrotron Radiation Light Source
TOC	total organic carbon
UL	Unknown Lake
U_3O_8	yellow cake
USDOI	United States Department of the Interior
USEPA	United States Environmental Protection Agency
XAS	X-ray absorption spectroscopy
YL	Yeoung Lake

PREFACE

This thesis is organized as manuscripts for publication; as such, there is some repetition of the Introduction and Materials and Methods sections in this thesis. Chapter 2 was accepted for publication by *Environmental Toxicology and Chemistry* on June 3rd, 2011. Supplementary data that was not published from Chapter 2 has been included in the Appendix. Chapter 3 will be submitted to *Environmental Toxicology and Chemistry* in June 2012. The full citations are as follows:

Franz ED, Wiramanaden CIE, Janz DM, Pickering IJ, Liber K. 2011. Selenium bioaccumulation and speciation in *Chironomus dilutus* exposed to water-borne selenate, selenite, or seleno-DL-methionine. *Environmental Toxicology and Chemistry* 30:2292-2299.

Franz ED, Wiramanaden CIE, Gallego-Gallegos M, Tse JJ, Phibbs JR, Janz DM, Pickering IJ, Liber K. 2012. An in situ assessment of selenium bioaccumulation from surface water, sediment, and dietary exposure pathways using caged *Chironomus dilutus* larvae. *Environmental Toxicology and Chemistry* (in preparation).

CHAPTER 1

INTRODUCTION

1.1 Selenium: chemical properties and geographic distribution

Selenium is classified as a metalloid, exhibiting properties of both metals and non-metals. Within the periodic table, selenium occurs in Group 16 of elements (old style designation VI.A) with an atomic number 34 and an atomic mass of 78.96 (ASTDR, 2003). Five stable isotopes of selenium occur naturally in the environment. Isotope ^{80}Se is the most abundant at 49.61%, followed by ^{78}Se (23.78%), ^{76}Se (9.36%), ^{77}Se (7.63%), and ^{74}Se (0.87%) (Ralston et al., 2008). ^{82}Se is a naturally occurring unstable isotope with a half-life of 1,020 years. Other unstable isotopes include ^{79}Se, which is a by-product of ^{235}U fission reactions, and ^{75}Se that is used as a radioactive tracer in environmental studies (Ralston et al., 2008). Selenium shares fundamental aspects of its chemical behavior with other elements in this group including oxygen, sulfur, tellurium, and polonium. The fundamental similarity between selenium and sulfur accounts for many analogous chemical and biological interactions between these two elements (Reilly, 2006). In the natural environment, selenium is commonly found in four oxidation states: -II (selenides), 0 (elemental selenium), IV (selenite), and VI (selenate). Reduced forms of selenium include volatile methylated selenides, seleno-amino acids (selenomethionine and selenocysteine) and their associated proteins, and inorganic selenides. Selenium is an essential micronutrient for many organisms, and species-specific biotransformation pathways contribute to the complex speciation and biogeochemical cycling patterns observed in aquatic environments (Maher et al., 2010).

The average estimated concentrations of selenium in the earth's crust are between 0.05 and 0.09 mg/kg (ASTDR, 2003). The distribution of selenium in soil and sediment is highly variable and dependent on the bedrock geology of the region. This is especially evident in certain regions in China and Brazil where both selenium deficient (< 0.1 mg/kg) and seleniferous (> 0.5 mg/kg) soils are present, sometimes separated by only a few kilometers (Lenz and Lens, 2009). Periods of increased volcanic activity are often associated with higher concentrations of selenium in rock formations because selenium, along with sulfur, is vaporized and released in gaseous form under high temperatures. After cooling, selenium condenses and precipitates with rainfall. This phenomenon contributes to higher concentrations of selenium in rock formations formed during periods of increased volcanic activity. Large areas of central North America are characteristically seleniferous because the soils were formed during the highly volcanic Carboniferous, Triassic, Jurassic, Cretacous, and Tertiary Ages (Ralston et al., 2008).

There are currently no economically significant selenium ore deposits (Lenz and Lens, 2009); however, selenium can be enriched in black shale, phosphate rocks, crude oil, and coal deposits (Maher et al., 2010). Selenium is also an important elemental component of the mineral matrix of silver, copper, and nickel ore deposits (ATSDR, 2003), as well as sandstone-type uranium ore deposits (CCME, 2007). Low concentrations of selenium are typical of mineral ores and fossil fuels, but in some circumstances high levels may be present depending on the geological origin of the deposit. Concentrations in excess of 1,000 mg/kg have been reported in the vicinity of some sandstone-type uranium deposits in the western United States; although concentrations typically range between 0.05 and 1.0 mg Se/kg (Shamberger, 1981). Similarly, coal can exceed 100 mg Se/kg depending on the source of the deposit (Ralston et al., 2008); however, the typical range of concentrations is between 0.4 and 24 mg/kg (Lemly, 2004).

1.2 Selenium in the aquatic environment

1.2.1 Sources of selenium contamination

Mobilization and distribution of selenium in the environment occurs during natural processes and anthropogenic activities. Concentrations of selenium in aquatic environments are highly variable, depending on the geological and biological characteristics of the particular region, and the extent of anthropogenic activity (CCME, 2007). In the absence of direct input of selenium, background concentrations typically range between 0.1 and 0.4 μg/L in surface water and 0.2 and 2.0 mg/kg dry weight (dry wt) in sediment (USDOI, 1998). Natural weathering of rock and soil can contribute to elevated background concentrations in some aquatic and terrestrial environments; however, human activity is primarily responsible for the mobilization of selenium to aquatic environments (Lemly, 2004). Several industrial, municipal, and agricultural practices can mobilize and distribute selenium, but the major contributors of selenium to aquatic environments are oil refineries, coal-fired power plants, agricultural irrigation projects, and metal mining and milling operations (Maher et al., 2010).

Revelations that selenium is a contaminant of potential concern (COPC) in some aquatic ecosystems emerged in the late 1970s and early 1980s with two well-publicized cases of adverse effects on fish and wildlife populations (Hamilton, 2004). In Belews Lake, North Carolina, the disappearance of 19 of the 20 endemic fish species was linked to the discharge of effluent from nearby ash ponds of a coal-fired power plant (Lemly, 1985). Surface water selenium concentrations ranged between 5 and 10 μg/L, and while other elements were also found to be elevated in the lake, selenium was implicated in the fish population collapse because of its propensity to bioaccumulate in aquatic food webs. The processing and combustion of coal is one of the primary human activities that mobilize selenium in the environment. With increased

global reliance on coal for generating electricity, safe storage of coal combustion waste presents a serious environmental safety concern for environmental managers (Lemly, 2004).

Perhaps the most well documented case of selenium contamination occurred in the Kesterson National Wildlife Refuge in the San Joaquin Valley, California. A reservoir in this area served as a collection and storage point for agricultural drainage water originating from large irrigation projects in the western San Joaquin Valley (Schuler et al., 1990). Irrigation practices in the San Joaquin Valley used subsurface drains to keep salt from accumulating near the root zone in order to increase crop productivity. A consequence of this irrigation practice was that trace elements were leached from the soil along with the salts. The seleniferous soils characteristic of the region produced selenium concentrations up to 1,400 μg/L in subsurface drainage water flowing into the Kesterson Reservoir (Ohlendorf, 1987). In 1985, mass mortalities were reported for several fish and aquatic bird species. Elevated selenium was documented at every level of the food web, and congenital deformities and reproductive impairment were severe for several species of fish and aquatic birds. This combination of geological, biogeochemical, and anthropogenic factors contributing to biological effects was termed the "Kesterson Effect," and in 1987 the Kesterson National Wildlife Refuge was classified a toxic waste dump (Presser, 1994).

1.2.2 Partitioning of selenium in the abiotic environment

Surface water and sediment contain the majority of selenium in contaminated environments, but predicting the risk of selenium exposure to biological communities requires knowledge of not only the concentration of selenium in each compartment, but also of the various selenium forms (species) that are present (Andrahennadi et al., 2007). Several different inorganic and organic forms of selenium occur in aquatic environments: inorganic selenate

(SeO_4^{2-}) and selenite (SeO_3^{2-}), elemental selenium (Se^0), volatile methylated species, seleno-amino acids and associated proteins, and inorganic selenides (Se^{2-}) (Simmons and Wallschläger, 2005). Each selenium species exhibits unique chemical properties that determine its bioavailability and toxicity, while site-specific biogeochemical factors dictate the partitioning and transfer of different selenium species between abiotic and biotic environmental compartments (Lemly, 2002). Among the most important biogeochemical processes influencing the distribution of different selenium species are the hydrology of the area (e.g., lentic versus lotic systems), rates of biotic and abiotic transformations, redox conditions in the sediments, the structure of the food web, and species-specific differences in uptake and depuration kinetics (Sappington, 2002). Ultimately, knowledge of the biogeochemical processes that govern the partitioning and transfer of selenium is critical for understanding and predicting the risk of exposure and toxicity to aquatic organisms (Maher et al., 2010).

1.2.2.1 Selenium in surface water

Selenate and selenite are the most common inorganic forms of selenium dissolved in surface waters (Besser et al., 1993; Cutter, 1991). Selenate dominates well-aerated surface waters where alkaline conditions prevail due to its kinetic stability and the slow reduction of selenate to selenite (USEPA, 2004). As such, selenate has been characterized as the most mobile selenium species present in aquatic environments. Under moderate oxidizing conditions found in some surface waters, selenium can occur predominately as inorganic selenite. Both inorganic species are considered stable in natural waters, but several processes can effectively remove selenium from the water column. Phytoplankton/bacterioplankton communities present in the water column have been shown to absorb both inorganic species of selenium (Riedel et al., 1996; Simmons and Wallshläger, 2011). Riedel et al. (1991) observed that uptake of selenite by some

algal species can be largely explained by non-biological, rapid adsorption to cell surfaces. This is due to the polar nature of selenite, which confers greater reactivity than selenate. Greater reactivity causes selenite to preferentially adsorb to particulates and phytoplankton suspended in the water column. Selenite also forms stable adsorption complexes with ferric oxides, and in the case of ferric hydroxide, selenite can be almost completely adsorbed, which is a property not shared by selenate (Maher et al., 2010). Selenate can be substantially and rapidly removed from the water column and transferred into the sediment by microbial reduction to elemental selenium, as well as by binding to organic matter (Zhang and Moore, 1996).

Organic forms of selenium in the water column are, in general, poorly characterized (Fan et al., 2002; Lemly, 2002; Hamilton, 2004). Most of the organic selenium found in aquatic environments likely occurs as selenoamino acids (e.g., selenomethionine and selenocysteine) incorporated into proteins and peptides, and not as free amino acids in water (Fan et al., 2002). Microbial decomposition of biological material is cited as one potential mechanism for the release of organic selenium back into the water column (Lemly, 2002). Dissolved organic selenium occurs at concentrations significantly less than both selenate and selenite. However, trace concentrations of organic selenium in surface water (< 1 µg/L), may contribute disproportionately to selenium accumulation by some species due to its increased bioavailability compared to selenate and selenite (Orr et al., 2006).

1.2.2.2 Selenium accumulation in sediment

Selenium enters aquatic ecosystems mainly as inorganic selenate and selenite in the water column, but sediments are the dominant sink in most aquatic environments impacted by anthropogenic activity (Maher et al., 2010). Partitioning of selenium from the water column to the sediments happens mainly via two processes: 1) absorption of inorganic and organic

selenium by aquatic organisms, followed by death and decomposition, and 2) by binding/complexation and precipitation with particulate matter (Canton and Van Derveer, 1997). Selenium shows a greater tendency to adsorb to fine particulate material, and a positive relationship has been shown to exist between sediment organic carbon and selenium concentrations in the sediment of both lotic (Van Derveer and Canton, 1997) and lentic (Wiramanaden et al., 2010a) aquatic environments. Lentic environments are more conducive to the accumulation of selenium in sediments than lotic systems due mainly to longer hydraulic retention times and larger deposits of organic material in the sediments (Canton and Van Derveer, 1997).

Once selenium partitions to the sediment, it is transformed and cycled between particulate (sediment) and dissolved phases (overlying water and pore water) depending on redox potential, pH, biological activity, and the presence of complexing ligands (Siddique et al., 2006). Reducing environments, common in the sediments and pore waters of lentic systems, favor the microbial reduction of selenate to elemental selenium and other reduced forms of selenium (Zhang and Moore, 1996; Belzile et al., 2000; Oram et al., 2010; Martin et al., 2011). Elemental selenium, unlike inorganic selenate and selenite, is not measurably soluble in water (USEPA, 2004), and is considerably less bioavailable than other selenium species (Lenz and Lens, 2009). Once formed, elemental selenium is stable over a wide range of pH values, (ASTDR, 2003) making the sediments a potentially large pool for elemental selenium in aquatic ecosystems. Sediment microbes may further reduce elemental selenium to organic selenides, thereby contributing to the pool of organic of selenium in the sediments (Bowie et al., 1996). Organic selenium can also accumulate in sediments via the deposition and decay of primary producers that have absorbed and transformed inorganic selenium to organic forms in the water column

(Fan et al., 2002; Orr et al., 2006). Combined, these two processes can contribute to the accumulation of highly bioavailable organic selenides in sediment, which can comprise a significant portion of the total selenium in contaminated systems (USEPA, 1998).

Partitioning of selenium species among overlying water, sediment, and pore water is strongly related to the vertical gradient of redox conditions in the sediments and overlying water. Selenium in pore water undergoes reduction from selenate to selenite, elemental selenium, or selenides as sediments become more reducing, partially explaining the inverse relationship between selenium sequestration in sediments and pore water selenium concentrations (Oram et al., 2010). Furthermore, a recent study contrasting two lentic environments found that more reducing redox conditions near the sediment-water interface favored the recycling of dissolved selenite and organic selenium into overlying water and surficial pore water (Martin et al., 2011). The authors concluded that microbial communities residing at specific redox zones were primarily responsible for determining the depth at which selenium diffused into pore water or overlying water (Martin et al., 2011). Desorption of selenium from iron and manganese oxyhydroxides has also been demonstrated to be an important process leading to the diffusion of selenium (i.e., selenite) into pore waters under reducing conditions (Belzile et al., 2000). Because of the complex cycling of different selenium species between overlying water, sediment, and pore water, sediment-dwelling aquatic invertebrates can be exposed to bioavailable selenium via multiple pathways.

As mentioned earlier, sediments are the main repository for selenium in contaminated systems, with the top layer of sediment and organic material (detritus) containing as much as 90% of the selenium in contaminated systems (Lemly, 2002). This tendency for selenium to partition and cycle within the top layer of sediments is partly responsible for the slow rates of

ecosystem recovery for some lentic systems. Furthermore, the same characteristics that enhance the accumulation of selenium in lentic systems (e.g., long hydraulic retention times and low sedimentation rates) also slow the rate recovery (Lemly, 1997a). This reality was demonstrated in an experiment where artificial stream mesocosms were dosed with sodium selenite, and sediment selenium concentrations were monitored after dosing ceased. In the artificial stream receiving 10 µg/L as selenite, concentrations in the sediment were still as high as 5 to 10 µg/g two years after dosing ceased (Swift, 2002). According to Lemly, relatively little reduction in the toxic impact of selenium is observed until sediment selenium concentrations drop to approximately 1 µg/g due to the propensity for selenium to bioaccumulate in the benthic-detrital food web (Lemly, 1997a). This implies that once selenium is introduced into an aquatic environment, the legacy of contamination and ecotoxicological effects can continue long after the input has ceased.

1.2.3 Bioaccumulation and trophic transfer of selenium

Ecological risks associated with elevated selenium in aquatic environments are related to the propensity for selenium to bioaccumulate in aquatic food webs. An extensive amount of research has been undertaken on this topic, and two paradigms form the fundamental basis for understanding selenium bioaccumulation and trophic transfer in aquatic ecosystems. The first is that uptake of selenium at the base of aquatic food webs by primary producers (algae and macrophytes) and microorganisms (bacteria and fungi) constitutes the single largest bioconcentration step in aquatic ecosystems (Luoma and Presser, 2009; Presser and Luoma, 2010). The second fundamental paradigm is that diet constitutes the principal pathway of bioaccumulation, toxicity, and transfer through aquatic food webs (Stewart et al., 2010). Bioaccumulation, as defined by the USEPA (2000a), is “the accumulation of chemicals in the

tissues of organisms through any route, including respiration, ingestion, or direct contact with contaminated water, sediment, and pore water in the sediment." Uptake of selenium can also occur from water-borne pathways (bioconcentration) for most aquatic consumers; however, compared to the dietary pathway, water-borne exposure does not pose a significant risk to most aquatic species (Janz et al., 2010).

The importance of dietary versus water-borne exposure is evident when comparing the acute and chronic toxicity criteria. In the case of water-borne exposure, acute toxicity criteria established by the USEPA for selenite and selenate in freshwater environments are set at 258 µg/L and 417 µg/L, respectively (www.epa.gov), which is directly related to the tolerance of aquatic organisms to short-term exposure to high concentrations of inorganic selenium. Available chronic freshwater criteria, however, are considerably lower to account for bioaccumulation and the potential for selenium to cause sublethal effects in sensitive species after long-term exposure. In Canada, the freshwater guideline for protection of aquatic life is 1 µg/L measured as total selenium (CCME, 2012). The chronic freshwater criterion in the United States was set at 5 µg/L (USEPA, 1987) until the USEPA recommended the water-based criterion be replaced with a fish tissue criterion of 7.91 µg/g (dry wt) to more accurately address the potential risk to sensitive species (USEPA, 2004). The draft criterion is still in the process of revision, which is a direct reflection of the difficulty in establishing a comprehensive criterion that is protective of aquatic life on a site-specific basis.

1.2.3.1 Selenium uptake by primary producers

Selenium is an essential nutrient for algal growth, and uptake of selenate and selenomethionine occurs mainly via active transport by membrane proteins, while uptake of selenite is occurs primarily by passive absorption (Riedel et al., 1991). The single largest

enrichment of selenium occurs between water and primary producers, although bioaccumulation varies as a function of species-specific uptake rates, the concentration and speciation of selenium in the water, and the water chemistry (Bowie et al., 1996). In a laboratory study comparing the bioavailability and toxicity of inorganic and organic selenium in a simulated laboratory food chain, the algal species *Chlamydomonas reinhardtii* accumulated 34.5 µg/g (dry wt) in 24 h of exposure to 10 µg/L selenium as selenomethionine. Similar dissolved concentrations of selenite and selenate resulted in lower algal selenium concentrations of 12.0 µg/g and 4.1 µg/g, respectively (Besser et al., 1993). This difference in bioavailability between organic and inorganic selenium results in varied degrees of selenium enrichment in aquatic ecosystems. In water bodies dominated by selenate, primary producers can accumulate 100 to 500 times more selenium compared to the ambient surface water concentration. However, when selenite or organic selenium dominate, this ratio can increase from 1,000 to 10,000 (Luoma and Presser, 2009). In freshwater environments, selenium enrichment from surface water to primary producers is generally within the range of 300 to 1,900 (Conely et al., 2011). Downstream from Key Lake, Muscatello et al. (2008) reported accumulation factors for periphyton of 240 in Unknown Lake (2.7 ± 0.1 µg/L) and 245 in Delta Lake (0.7 ± 0.0 µg/L). The comparatively low accumulation factors for periphyton at these locations reflect that selenate is the dominant form of selenium present in surface water downstream from the Key Lake operation.

Besides being efficient at accumulating dissolved selenium, primary producers are also largely responsible for introducing highly bioavailable forms of selenium into aquatic food webs. Selenomethionine is the main form of selenium linked to cases of toxicity and teratogencity in fish and birds, and primary producers and microorganisms are the only classes of organisms capable of synthesizing selenomethionine (refer to section 1.2.4.2). The base of the benthic-

detrital food web is comprised of primary producers and microorganisms living on the sediment surface, as well as primary producers that have died and precipitated from the water column. Combined, these two processes contribute to elevated concentrations of selenium in the upper few centimeters of sediment and detritus in contaminated systems (Orr et al., 2006). Ultimately, the enrichment of selenium by primary producers and microorganisms, combined with the biotransformation of inorganic selenium to organic selenium, largely determines the magnitude of selenium bioaccumulation in successive trophic levels (Luoma and Presser, 2009).

1.2.3.2 Invertebrates: the link in the trophic transfer of selenium

As mentioned previously, the majority of the selenium in contaminated systems is found in the upper few centimeters of sediment and detritus. This is significant because benthic invertebrates inhabiting this layer of sediment form the critical link between selenium bioconcentration by primary producers and microorganisms and the trophic transfer of selenium to fish and wildlife (Alaimo et al., 1994). Many invertebrate species are important prey for fish and birds, and are therefore the principal conduits of selenium accumulation (i.e., organic selenium) for top-level consumer organisms in selenium-impacted aquatic systems. Uptake of selenium by invertebrates is determined largely by the concentration and bioavailability (and hence speciation) of selenium in the sediment/detritus and surface water. In addition, species-specific habitat preferences, feeding patterns, and physiological responses to accumulated selenium (i.e., uptake and elimination rates) account for much of the difference in bioaccumulation among invertebrate taxa (Presser and Luoma, 2010). These differences are propagated through successive trophic levels and often result in varied concentrations of selenium in different top-level predator species within the same environment (Stewart et al.,

2010). Knowledge of the site-specific food web interactions is therefore critical when assessing the risk selenium poses to top predator species (Luoma and Presser, 2009).

Fundamental to the understanding of selenium bioaccumulation in aquatic invertebrates is the importance of dietary accumulation compared to direct uptake via water-borne exposure. Water-borne exposure may contribute to selenium concentrations in some cases, especially when biogeochemical cycling processes enhance the formation and release of bioavailable organic selenium and selenite into pore water and overlying water (Bowie et al., 1996; Amweg et al., 2003; Orr et al., 2006). Notwithstanding potential uptake from water-borne exposure, the majority of research published on this topic implicates dietary uptake as the principal cause of elevated concentrations of selenium in aquatic invertebrates from selenium-impacted systems (Alaimo et al., 1994; Malchow et al., 1995; Conley et al., 2009). Beyond this basic assertion, there is only a limited understanding of the processes of selenium bioaccumulation in freshwater invertebrates. Much of the uncertainty relates to poor characterization of the concentration and speciation of selenium in the inorganic and organic particulate material that forms the diet of aquatic invertebrates (Andrahennadi et al., 2007). The uncertainty is further compounded by the fact that the dietary preferences of many invertebrate species are not well established (Stewart et al., 2010). The microbial biomass component of sediment detritus is thought to be a significant source of dietary selenium for many benthic invertebrates (Alaimo et al., 1994, Fan et al., 2002; Orr et al., 2006), but estimates of the selenium in invertebrate diets are generally inexact due to the inherent difficulty in separating the biotic and abiotic fractions of detrital material (Stewart et al., 2010). More research is needed to clarify the importance of food preference and its relationship to selenium bioaccumulation in benthic-detrital food webs.

1.2.4 Physiological role of selenium

1.2.4.1 Selenoproteins

Selenium is as an essential micronutrient required for growth and development in virtually all species of bacteria, plants, and animals. The metabolism of selenium is highly specific, and involves several inorganic and organic selenium-containing compounds and intermediate metabolites during the formation of selenium-containing proteins. There are three groups of selenium-containing proteins: 1) selenoproteins containing the 21st amino acid selenocysteine, 2) proteins containing non-specifically incorporated selenium, and 3) proteins that bind selenium (Janz et al., 2010). All known physiological functions of selenium are mediated through selenoproteins that specifically incorporate selenocysteine (R-Se-H) at the active site during protein synthesis (Lobanov et al., 2007). In contrast to the other 20 amino acids found in proteins, selenocysteine is utilized only when it is required for protein function, specifically as a key functional (and almost always catalytic) group in enzymes involved in antioxidant functions, thyroid hormone metabolism, and redox balance (Reilly, 2006; Gladyshev, 2012). The highly specific function of these enzymes is related to selenocysteine being fully ionized (R-Se^{-}) at physiological pH, which makes it a very efficient redox catalyst (Reilly, 2006). In comparison to homologous proteins with cysteine at the active site, selenocysteine has been shown to be 1,000 times more effective in catalytic redox reactions (Lobanov et al., 2009).

Physiological functions of several selenoprotein families are well known in prokaryotic and eukaryotic organisms. The most well-defined selenoproteins are the glutathione peroxidases (antioxidant defence), thioredoxin reductases (support cell proliferation, antioxidant defence, and redox regulation of protein function and signalling), thyroid hormone deiodinases (thyroid hormone regulation), selenophosphate synthetase (formation of an intermediate compound involved in selenocysteine synthesis), and selenoprotein P (selenium transport to various organs

and tissues) (Zwolak and Zaporowska, 2012). All of these proteins have a single selenocysteine molecule inserted at the active site, with the exception of selenoprotein P that contains 10 to 18 individual selenocysteine molecules (Shchedrina et al., 2007). Recently, another family of selenoproteins, designated SeIL, were identified that contain a natural diselenide bond between two selenocysteine residues (Shchedrina et al., 2007). The function of these proteins is currently unknown, but structural comparisons of SeIL to thioredoxin selenoproteins suggest that they may function in redox regulation (Shchedrina et al., 2007). Interestingly, the selenoproteins with a diselenide bond have so far only been identified in aquatic organisms, such as fish, invertebrates, and marine bacteria (Lobanov et al., 2009). With the exception of vertebrates, aquatic organisms are known to have larger sets of selenoproteins (selenoproteomes) compared to terrestrial organisms (Gladyshev, 2012). Fish, for example, are among the organisms with the largest selenoproteomes with between 30 and 37 individual selenoproteins (Lobanov et al., 2009); whereas some higher level plants, fungi, and insects (beetles and silkworms) have completely lost the expression of selenoprotein genes and selenocysteine biosynthetic/insertion machinery during evolution (Lobanov et al., 2007). Environmental factor(s) clearly play an important role in the evolution of selenium utilization, but as yet the cause of differential expression of selenoproteins between terrestrial and aquatic organisms remains largely unknown.

1.2.4.2 Metabolism and biotransformation of selenium

The most important biosynthetic pathways involve in the metabolism and biotransformation of selenium into the amino acids selenocysteine and selenomethionine (Janz et al., 2010). These amino acids are homologous to the amino acids cysteine and methionine, with selenium substituting for sulfur during amino acid synthesis. In the case of selenocysteine synthesis, the process is tightly regulated for each selenoprotein, and governed by a UGA codon

on mRNA that is specific to selenocysteinyl-tRNA (Daniels, 1996). This tRNA utilizes selenophosphate generated from inorganic selenide as the precursor for the synthesis of selenocysteine by the enzyme selenocysteine synthetase (Xu et al., 2012). Selenocysteine metabolism is unique compared to other amino acids in that it must be synthesized during each cycle of protein synthesis. Free selenocysteine is not found within the cell because its highly reactive properties would result in cellular damage (Daniels, 1996). Instead, the enzyme selenocysteine β-lyase catalyzes the decomposition of selenocysteine to alanine and hydrogen selenide, the latter being the key metabolite in the synthesis of selenocysteine and other selenium-containing proteins, as well as methylated volatile selenium species (Daniels, 1996; Schrauzer, 2000; Spallholz and Hoffman, 2002). Hydrogen selenide is also formed from the sequential reduction of inorganic selenite to selenide mediated by glutathione and the intermediate metabolite seleno-*bis*-glutathione (R-S-Se-S-R) (ASTDR, 2003). For bacteria, the subsequent reduction of seleno-*bis*-glutathione results in the formation of elemental selenium within the cell. Microbially-derived elemental selenium is characteristically orange-red in color, and is frequently associated with the cell wall and/or cell membrane (Turner et al., 1998). Microbial reduction of inorganic selenate and selenite to elemental selenium is particularly important in selenium-impacted aquatic ecosystems because elemental selenium is considerably less bioavailable to aquatic organisms than selenite and organic selenium (Wiramanaden et al., 2010b).

All organisms that express selenoproteins are capable of the *de novo* synthesis of selenocysteine, but in the case of selenomethionine, the biosynthetic pathway is exclusive to primary producers, bacteria, and yeast (Schrauzer, 2000). In aquatic ecosystems, algae, macrophytes, and bacteria are responsible for the introduction of selenomethionine into the food

web, which is propagated to higher trophic level organisms primarily via the dietary pathway. Unlike selenocysteine, selenomethionine is not required for any specific physiological function; rather, it is synthesized analogously to methionine based on the relative abundance of selenium versus sulfur within the cell (Turner et al., 1998; Schrauzer, 2000). At the level of the consumer organism, exogenous dietary selenomethionine is incorporated non-specifically into proteins in place of methionine because the tRNA for methionine does not discriminate between the two amino acids (Schrauzer, 2000). The non-specific incorporation of selenomethionine into body proteins allows organisms to effectively store excess selenium in a comparatively non-toxic form, while also providing a readily-available source of selenium for the synthesis of selenoproteins (Daniels, 1996). Although animals are incapable of synthesizing selenomethionine, they are capable of metabolizing selenomethionine to selenide that can then be utilized for the synthesis of biologically active selenocysteine (Schrauzer, 2000). Selenomethionine has gained special attention in assessments of selenium-impacted aquatic ecosystems because it is highly bioavailable and has been implicated in toxic effects to fish and bird populations (Spallholz and Hoffman, 2002; Hamilton, 2004).

1.2.4.3 Toxicity of selenium to aquatic organisms

Selenium is unique in comparison to other micronutrients in animal nutrition in that it shows a very narrow range between essentiality and toxicity. There are three levels of physiological activity for selenium that are applicable to animals: 1) the metabolism of selenium (primarily selenomethionine) for use in the synthesis of seleoproteins, 2) storage of excess selenium by substituting selenomethionine for methionine during protein synthesis, and 3) toxic effects (Hamilton, 2004). In aquatic environments impacted by elevated selenium, toxicity is most commonly observed among populations of egg-laying vertebrates during early

developmental life stages. Effects manifest when developing embryos are exposed to selenium as a result of the maternal transfer of excess dietary selenium accumulated by the parent organism (Simmons and Wallschläger, 2005). The hallmark effects (most sensitive endpoints) for fish are teratogenic development of fry, edema, and/or larval mortality (Janz et al., 2010), while effects in bird populations are most often associated with reduced hatching success and deformities of embryos and chicks (Ohlendorf et al., 1988). Increased incidence of mortality and teratogenic effects during these early life stages can result in significant population declines for sensitive species if the effects are observed in successive generations (Ohlendorf et al., 2011). Furthermore, effects can begin to manifest at dietary concentrations less than an order of magnitude above dietary requirements (Simmons and Wallschläger, 2005). For fish, dietary selenium concentrations less than 3 µg/g dry wt are considered optimal while only slight increases (7 to 30 times) may increase the incidence of teratogenic effects in the embryo (Janz et al., 2010). The dietary threshold for effects to birds is also quite narrow, with egg selenium concentration greater than 5 µg/g dry wt associated with deformities in the developing embryo (Ohlendorf et al., 2011).

The transition from essentiality to toxicity is ultimately related not only to the concentration of selenium in the diet, but also to the form of selenium and its subsequent metabolism during development (Spallholz and Hoffman, 2002). Selenomethionine is the suspected cause of embryonic mortality and teratogenic effects fish and birds because it is the primary form of selenium deposited in eggs during reproduction (Janz et al., 2010). In a reproductive study on mallards by Heinz et al. (1987), birds that were fed a diet containing 10 µg/g dry wt selenomethionine had a 40 – 44 % reduction in the number of eggs that hatched compared to the controls, along with increased incidence of malformations in the hatchlings.

The proposed mechanisms of selenium-induced toxicity were thought to be related to improper protein folding and/or altered enzyme function due to the substitution of selenomethionine for methionine during embryonic development (Lemly, 1997b). This proposed mechanism of toxicity has recently been questioned as protein structure has been shown to be largely unaffected by the substitution of selenomethionine for methionine (Schrauzer, 2000). Altered enzyme activity, however, has been documented for *E. coli* when selenomethionine replaces methionine near the active site of some enzymes (Schrauzer, 2000).

Recently, oxidative stress caused by the metabolism of selenomethionine by the developing embryo has garnered attention as a mechanism of toxicity in birds (Spallholz and Hoffman, 2002) and fish (Palace et al., 2004). While selenomethionine has not been directly linked to the formation of reactive oxygen species, the metabolism of selenomethionine to other reactive selenium metabolites could generate free radicals causing DNA damage and(or) altered integrity/function of proteins involved in DNA repair (Lenz and Lens, 2009). Furthermore, ongoing research in this area has recently linked dietary selenomethionine to increased stress responses (Wiseman et al., 2011) and altered swim performance and energetics (Thomas and Janz, 2011) in fish. These added stressors, when coupled with the potential for embryonic mortality and teratogensis, have the potential to cause significant population-level effects in aquatic ecosystems impacted by elevated concentrations of selenium.

Invertebrate populations have traditionally been considered less at risk to the effects of selenium at environmentally relevant concentrations compared to fish and bird populations (May et al., 2007; Malchow et al., 1995). This notion has been based largely on an assessment of field studies where benthic invertebrates and some forage fishes were able to accumulate 20 to 370 μg/g selenium and still maintain stable populations (Lemly, 1993). By comparison, the

estimated dietary threshold concentration for effects in fish and birds is between 3 and 11 µg/g dry wt (deBruyn and Chapman, 2007). In the Kesterson National Wildlife Refuge, several field investigations reported concentrations of selenium in excess of 100 µg/g dry wt for several aquatic invertebrate taxa including Zygoptera, Anisoptera, and Diptera (Schuler et al., 1990). Midge larvae from this area had particularly high selenium concentrations, ranging from 76 to 180 µg/g dry wt (Schuler et al., 1990). These findings, among others, ultimately led to the pervading assumption that the most important aspect of selenium accumulation by invertebrates is not direct toxicity to the organisms themselves, but rather the dietary source of selenium (i.e., organic selenium) they provide to fish and wildlife (Lemly, 1993). This premise has merit insofar as aquatic invertebrates are considered as a single class of organisms serving as dietary food items for higher trophic levels. However, it does little to identify specific species that may be at risk within the larger aquatic invertebrate community.

Assumptions about the tolerance of aquatic invertebrates to selenium are partly related to the high water-borne concentrations of selenium required to elicit effects. A review by deBruyn and Chapman (2007) found a wide range of sensitivity among aquatic invertebrate taxa exposed to dissolved forms of selenium, with lethal and sublethal effects observed at dissolved selenium concentrations one to two orders of magnitude above concentrations that typically occur in contaminated aquatic systems. When whole-body invertebrate selenium concentrations were evaluated, sublethal effects to aquatic invertebrates were observed between 1 and 30 µg/g dry wt, suggesting that water-borne exposure consistently underestimates the potential for effects (deBruyn and Chapman, 2007). In a laboratory study with *Chironomus decorous,* significant reductions in larval growth were observed when larvae accumulated more than 2.55 µg/g dry wt selenium from a diet of seleniferous algae containing 2.11 µg/g dry wt selenium (Malchow et al.,

1995). Recent studies with the mayfly *Centroptilum triangulifer* demonstrated significant reductions in survival and growth when larvae were fed periphyton containing 11.9 µg/g dry wt selenium (Conely et al., 2011). Delays in development (time to first emergence) were reported at a dietary concentration of 27.2 µg/g, while reduced fecundity (number of eggs) occurred at dietary concentrations as low as 4.2 µg/g (Conley et al., 2011). Subtle changes in growth, development, and reproduction can have dramatic effects on invertebrate density, thereby limiting the abundance of prey for successive trophic levels. In this respect, the effects of selenium on higher trophic levels may be two-fold: first, via elevated concentrations of selenium in the food, and second, by reducing the abundance of invertebrate prey. Although comparatively few studies have examined the risk specifically to aquatic invertebrates, there is evidence to suggest that some sensitive aquatic invertebrate taxa may be at risk from effects of elevated selenium at concentrations previously considered protective of fish and birds.

1.3 Uranium operations at Key Lake, Saskatchewan

1.3.1 History

Northern Saskatchewan is home to the largest high-grade uranium deposits in the world. Cameco Corporation (Cameco) is the principal owner (87%) and sole operator of the high-grade uranium milling facility at Key Lake (57°13' N, 105°38’ W) in northern Saskatchewan, Canada. The operation is located approximately 600 km north of Saskatoon on the southeastern edge of the Athabasca Basin (Figure 1.1). Uranium ore extraction at Key Lake began in 1982 with the open pit mining of two ore zones. The Gaertner ore body was mined until 1987, followed by the mining of the Deilmann ore body from 1986 until 1997. Stockpiled uranium ore from Key Lake was milled until 1999, at which point the facility began processing high-grade ore from the McArthur River Operation located approximately 80 km northwest of the Key Lake operation.

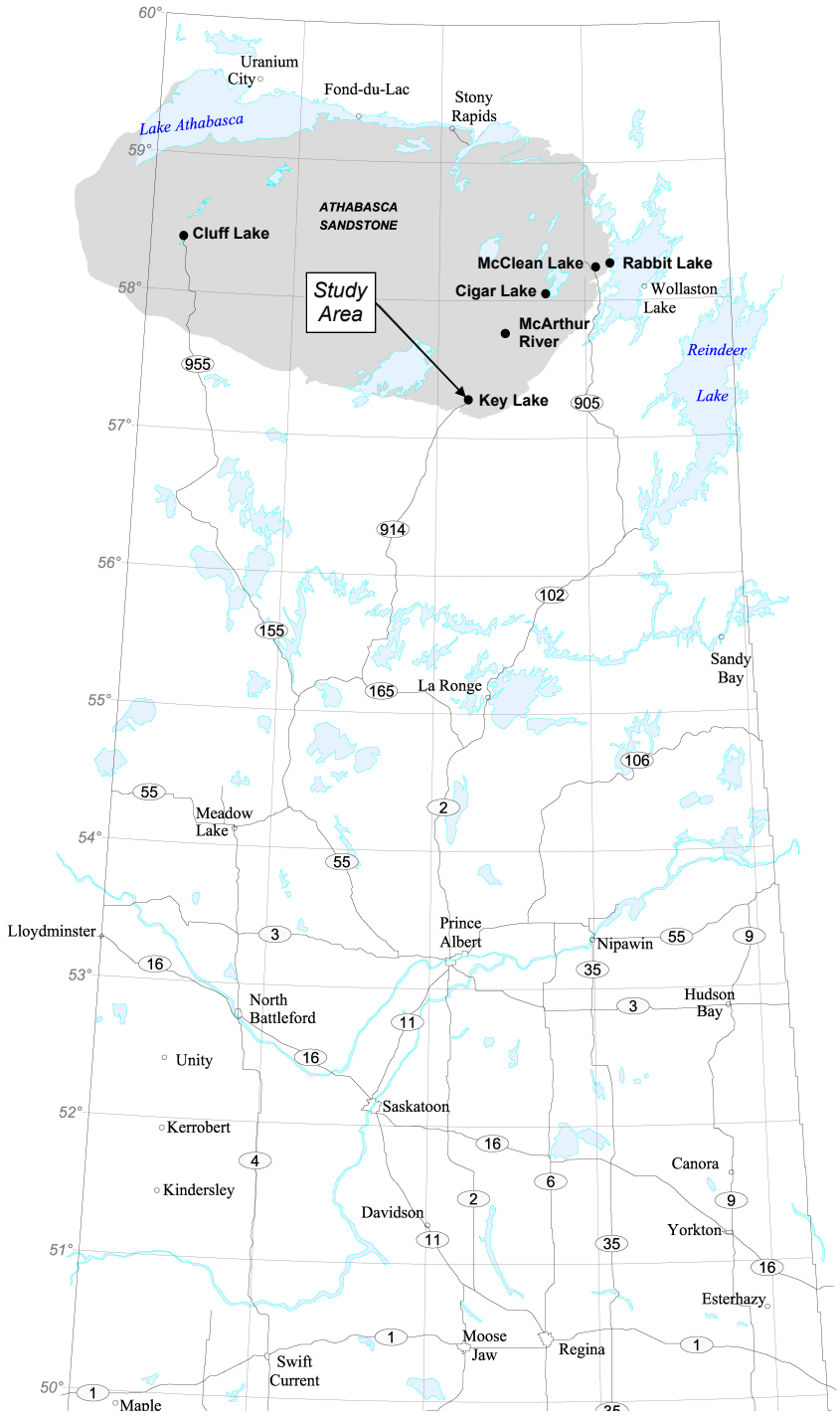

Figure 1.1: Location of the Key Lake uranium operation, northern Saskatchewan, Canada. Map provided by Canada North Environmental Services, Saskatoon, SK.

McArthur River is the largest high-grade uranium mine in the world with an average grade of 15.24% U_3O_8. As of 2010, Key Lake is licensed to produce 18.7 million lbs of U_3O_8 annually (Cameco, 2010a).

1.3.2 Drainage basin and study area

Key Lake is located in the Wheeler River watershed, which flows northeast and ultimately drains into Wollaston Lake. There are two separate discharge points for all treated water at Key Lake. Water from dewatering operations at the Deilmann Tailings Management Facility (DTMF) is treated by reverse osmosis and released into the McDonald Creek drainage system. Treated effluent from the bulk neutralization circuit is batch discharged into the David Creek drainage basin at Wolf Lake once the final effluent quality meets regulatory and operational requirement for release. Effluent flows downstream from Wolf Lake through Fox Lake and Yak Creek before meeting up with David Creek (Figure 1.2). Water flowing from David Lake (located upstream from the milling operation) into David Creek dilutes the effluent from Yak Creek prior to entering Unknown Lake. Downstream from Unknown Lake water flows from Delta Lake through Far-field Pond before entering the Wheeler River. In general, the lakes downstream from the effluent discharge point are characterized as shallow with sparsely vegetated littoral zones, limiting both the abundance and diversity of aquatic vegetation (Minnow, 2010).

A large volume of effluent is generated during the extraction of U_3O_8 during the milling process. A total of 1,205,392 m^3 of treated effluent was released into the David Creek drainage basin downstream from the mill in 2010 (Cameco, 2010a). Conductivity measurements of the surface water downstream from the point of discharge estimate that approximately 72% of the water flowing out of Wolf Lake is treated effluent (Golder, 2003). The estimated percent

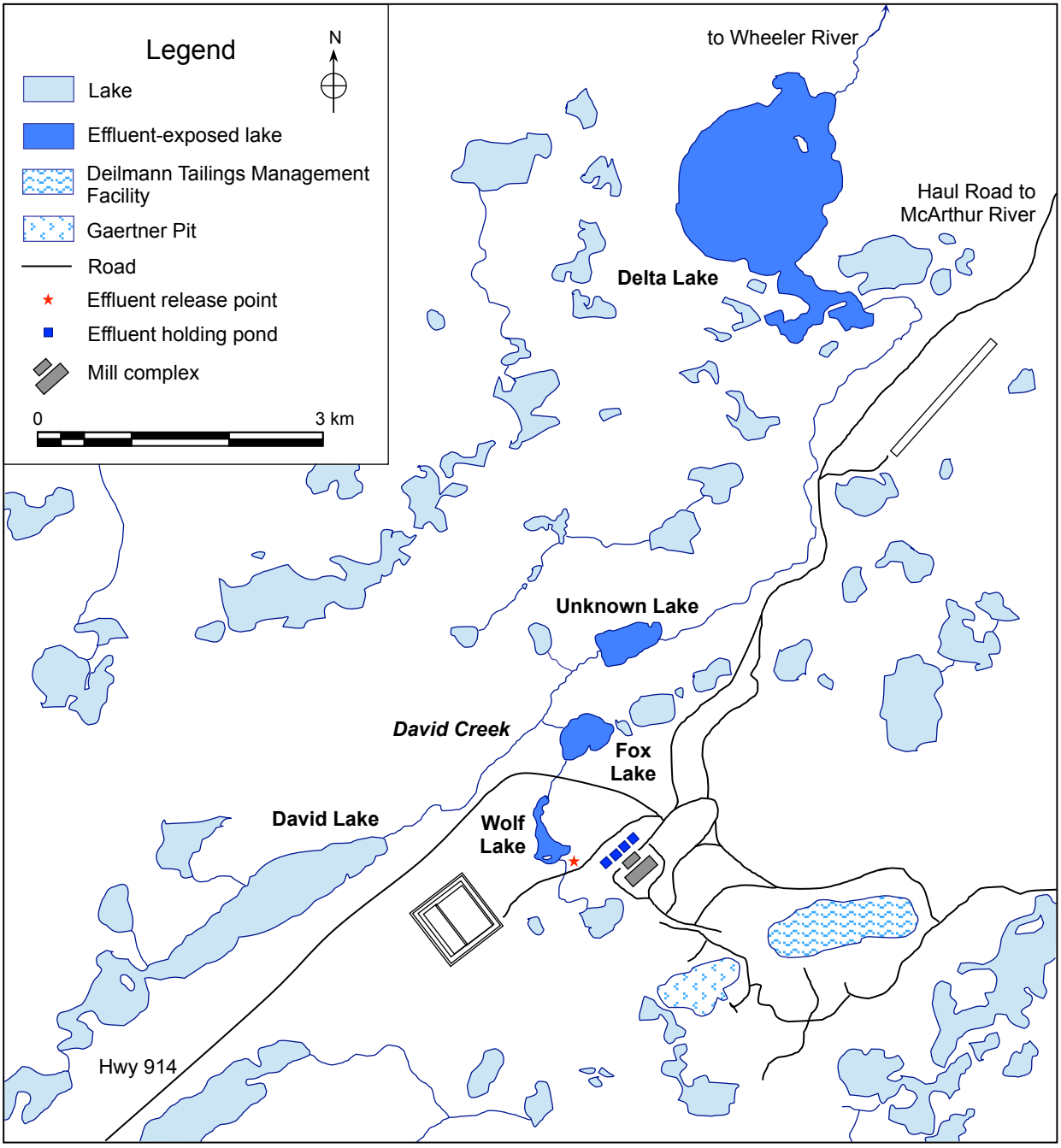

Figure 1.2: Map of the Key Lake uranium operation and treated effluent discharge area. Adapted from Cameco Corporation (2010a).

effluent at the outflow of Fox Lake (Yak Creek) is 69%. Past the confluence of Yak and David Creeks, the percentage of effluent drops to approximately 37% due to the dilution effect of water originating from upstream at David Lake. Once the flow reaches the Wheeler River, the effluent contribution is indistinguishable from background constituent levels in the area (Golder, 2003).

1.3.3 Effluent treatment and release

Under the Metal Mining Effluent Regulations (MMER) of the *Fisheries Act*, metal mines operating in Canada are prohibited from discharging effluent that is acutely lethal to fish (Government of Canada, 2002, 2006). These regulations also impose limits on the release of contaminants such as metals, cyanide, radium-226, and suspended solids into aquatic ecosystems. In the process of extracting, purifying and concentrating uranium into yellowcake, many naturally occurring heavy metals and radionuclides are also extracted from the ore. The majority of these constituents leach into the wastewater stream produced during solvent extraction (Cameco, 2005). Contaminated water requiring treatment also originates from multiple sources around the mine site not associated with milling. Dewatering well water for environmental containment, water level management at the Deilmann Tailings Management Facility (DTMF), and seepage and runoff from waste rock and the Above Ground Tailings Management Facility (AGTMF) can all contain levels of contaminants exceeding mill requirements for release (SENES, 2005).

Water treatment at Key Lake is accomplished mainly through bulk neutralization (BN), although reverse osmosis (RO) is used to treat groundwater pumped from dewatering wells (Cameco, 2005). The BN process at Key Lake involves neutralization of the mill effluent with the addition of lime, solids thickening, and pH adjustment to remove metals from the wastewater stream. Increasing the pH of the water to 10.5 precipitates many of the metals, including

cadmium, chromium, cobalt, copper, iron, lead, manganese, nickel, vanadium, uranium, and zinc with removal efficiencies as high as 99% (SENES, 2005). In the case of radium-226, barium chloride is added to the water resulting in the precipitation of radium as a barium-radium salt (Cameco, 2005). The precipitated solids are subsequently thickened and deposited with other waste solids in the DTMF. Effluent is treated with acid to lower the pH to between 6 and 7 in order to meet mill requirements for release. Lastly, the effluent is pumped into one of four holding ponds where contaminant concentrations are monitored. Once analysis has confirmed that the effluent meets mill requirements for release, the effluent is released into the receiving environment at Wolf Lake. Effluent that does not meet the requirements for release is cycled back to the BN circuit and treated again until all contaminant levels meet mill requirements (Cameco, 2005).

The wastewater treatment process at Key Lake underwent a significant upgrade in 2007 with the construction of a new circuit for the targeted removal of molybdenum and selenium. Selenium and molybdenum have both been identified as COPCs at Key Lake, but prior to 2007 the BN circuit could not effectively remove these metals from the final treated effluent. The new treatment circuit separates solids containing selenium and molybdenum from the wastewater stream, resulting in lower concentrations of selenium and molybdenum in the mill effluent. The precipitated solids are pumped to the DTMF, while the effluent is diverted back to the BN circuit to continue the water treatment process described above. With optimization of the new molybdenum/selenium removal process in 2009, the selenium concentration in final effluent is now consistently below the administrative level of 0.028 mg/L, and well below the maximum allowable monthly mean concentration of 0.6 mg/L set by the Saskatchewan Ministry of Environment (SMOE, 2009). Average yearly selenium concentrations in the effluent have also

decreased since 2006, as mass loadings of selenium to Wolf Lake have been reduced from 51.2 kg in 2007 to 20.1 kg in 2010 (Cameco 2010a)

1.3.4 Selenium-related research at Key Lake

Several research initiatives have been undertaken at Key Lake in recent years to investigate the scale and extent of potential effects on biological communities related to current and/or historical mining and milling activities. The first study specifically designed to investigate the potential for treated effluent-related effects downstream from the mine was conducted in the late 1990's by Pyle et al. (2001). In the study, caged fathead minnow larvae (*Pimephales promelas*) were placed in Fox Lake and Unknown Lake downstream from the effluent release point, as well as in two lakes receiving mine-watering effluent with elevated levels of nickel, and one reference lake (David Lake). After 7 d of in situ exposure, fathead minnow larvae from Fox and Unknown Lakes had higher rates of mortality than fish that were caged in David Lake, or the two lakes receiving mine-dewatering effluent (Pyle et al., 2001). Concentrations of aluminum, arsenic, cadmium, copper, molybdenum, and selenium correlated strongly with mortality, but when the surface water concentrations were screened against available toxicity threshold data, only selenium (via dietary accumulation) was identified as the likely cause of increased mortality in fathead minnow larvae caged in Fox and Unknown Lakes (Pyle et al., 2001).

In 2003 the CNSC put forth a formal request for an evaluation of selenium in the David Creek drainage basin, marking the beginning of an extensive period of selenium-related research at the Key Lake operation. The environmental risk assessment (ERA) that followed in 2005 identified potential risks associated with elevated concentrations of selenium, along with arsenic, molybdenum, uranium, and polonium-210 (EcoMetrix, 2005). Potential effects were limited to

near-field locations (Wolf Lake and Fox Lake) in the case of arsenic, uranium, and polonium-210; however, selenium was predicted to exceed the proposed United States Environmental Protection Agency (USEPA, 2004) tissue-based benchmark of 7.91µg/g dry wt in fish downstream to Delta Lake (EcoMetrix, 2010). Conclusions from the risk assessment pertaining to selenium were strengthened by a study on selenium toxicosis in larval northern pike (*Esox lucius*) (Muscatello et al., 2006). The main objective of this study was to identify whether developmental effects in larval northern pike related to maternal transfer of selenium or to exposure of the embryo to selenium in surface water collected from three locations downstream from Key Lake. Results strongly implicated maternal transfer of selenium as the primary source of selenium exposure resulting in increased deformities and edema in developing fry, with effects detected as far downstream as Delta Lake (Muscatello et al., 2006). Furthermore, the observed relationship between deformities in northern pike fry and tissue selenium concentrations suggested that northern pike show similar sensitivity to selenium as other warm water and cold water fish species. Subsequent to research conducted by Muscatello et al. (2006, 2008), a multi-year collaborative research program, involving several researchers from the University of Saskatchewan, was initiated to investigate selenium in the David Creek drainage basin. The main goals of the program were to investigate the distribution, fate, and transfer of selenium through environmental compartments and up the food web, and to determine whether adverse effects are occurring in biological communities due to elevated selenium exposure in the receiving environment.

1.4 *Chironomus dilutus:* a model test organism

Chironomus dilutus (formerly *C. tentans*; Diptera: Chironomidae) is a widely distributed (holarctic) non-biting midge often used in both field and laboratory tests (Liber et al., 1996;

Leppänen et al., 2006). There are three aquatic life stages which *C. dilutus* progresses through: egg, larvae, and pupae, followed by a terrestrial adult stage. Eggs, laid in gelatinous egg masses, typically hatch within 2 to 4 days of oviposition, although eggs can take up to 6 days to complete the hatch (Benoit et al., 1997). Larvae go through four instars, each lasting between four and seven days (Environment Canada, 1997). At the end of the 4th instar, larvae cease feeding and enter pupation. The pupal stage generally lasts no longer than 1 day. Pupation is followed by emergence of an adult insect. Emergence typically follows a bimodal distribution with peak male emergence occurring prior to female emergence with metamorphosis taking approximately 23 to 30 days at 23˚C under laboratory conditions. Females typically produce a single egg mass within 24 h of mating (Benoit et al., 1997).

Several factors have led to the use of *C. dilutus* as a test species: they are relatively straightforward to culture and handle, larvae inhabit and feed on the sediment surface, they are tolerant to a wide range of physical and chemical conditions in both sediment and water, they complete their life history in a short period of time, a number of developmental and reproductive endpoints can be monitored, and they are representative of a ubiquitous and ecologically important taxon in many aquatic environments (Benoit et al., 1997; Environment Canada, 1997; USEPA, 2000b). *Chironomus* spp. are also well suited for in situ toxicity and bioaccumulation tests (Castro et al., 2003; Sibley et al., 1999). Midge larvae have been used in situ to assess water and sediment by measuring survival (Chappie and Burton, 1997; Crane et al., 2000), growth (Sibley et al., 1999), and head capsule width (Faria et al., 2007). Larvae burrow and feed on the sediment surface of aquatic environments, and are exposed to contaminants that accumulate in the sediment. Ingested organic and inorganic material is expected to contribute to the bioaccumulation and toxicity of metals for chironomid larvae (Simpson and Bately, 2007;

Stewart et al., 2010); however, chironomid larvae also accumulate contaminants from water (Stuijfzand et al., 2000), making this species ideal for investigating potential pathways of contaminant exposure (Faria et al., 2007).

1.5 In situ toxicity and bioaccumulation testing

1.5.1 Advantages of in situ-based test methods

Toxicity testing is widely recognized as a key line of evidence in the development and administration of environmental regulations (Wharfe et al., 2007). Simple laboratory tests are most often employed to assess the bioavailability, bioaccumulation, and toxicity of contaminants on aquatic organisms, but the results are often difficult to extrapolate to field conditions (Graney et al., 1995). This is largely related to the complexity of abiotic and biotic interactions that affect contaminant mobility, partitioning, transformation, and accumulation in aquatic food webs. The challenges of conducting environmentally realistic laboratory toxicity tests using aquatic invertebrates has led to the development of test methods designed to measure biological and chemical responses using caged organisms (Chappie and Burton, 1997; Sibley et al., 1999; Castro et al., 2003; Burton et al., 2005; Robertson and Liber, 2007). The main advantage of in situ testing is that they integrate site-specific abiotic and biotic environmental conditions that influence the bioaccumulation and toxicity of contaminants while allowing the experimenter some control over the exposure scenario (Liber et al., 2007).

In situ test methods using caged benthic invertebrates are particularly well suited for assessing metal bioaccumulation pathways because sediment-dwelling organisms can accumulate metals from contaminants in pore water, sediment, and overlying water (Ingersoll, 1995; Simpson and Bately, 2007). In the case of sediment exposures, in situ toxicity testing incorporates site-specific sediment characteristics into the exposure regime, such as organic

carbon, pH, redox potential, sediment particle size, acid volatile sulfide, and sediment mineral constituents (oxides of iron, manganese, and aluminum), as well as biotic factors such as microbial activity. These sediment properties confer a high degree of spatial and temporal variability in sediment composition and chemistry, and are difficult, if not impossible, to mimic in the laboratory (Burton, 1991; Di Toro et al., 1991). Similar to sediment-associated contaminants, the bioavailability and toxicity of complex metal mining effluents can also be altered substantially by site-specific abiotic and biotic characteristics of the receiving environment. Laboratory tests using reconstituted water can mimic some water quality parameters, but many environmental conditions are difficult to replicate. Dissolved oxygen and temperature, for example, are not easily replicated under controlled conditions because both fluctuate diurnally, as well as temporally. Water temperature fluctuations can influence the metabolic rate in aquatic organisms, which in turn can influence the uptake rates of environmental contaminants (Armstead and Yeager, 2007). Small-scale temporal changes in water quality are seldom replicated in laboratory bioaccumulation and toxicity testing; whereas in situ methods account for subtle changes in water quality that can influence the response by the test organisms. Where the objective is to define site-specific effects of exposure, relying on data generated from traditional laboratory bioassays may not accurately characterize the risk of exposure for aquatic organisms (Cairns, 1993).

1.5.2 Invertebrate caging techniques

Caging methods using freshwater invertebrates to assess sediment toxicity were first proposed by Nebeker et al. (1984). Since their inception, in situ testing methods have been adapted for multiple uses within ecological risk and hazard exposure assessments, including site investigations, monitoring, establishing causal linkages, investigating exposure pathways, and

identifying stressor classes (Crane et al., 2007). The basic premise of all caging studies involves designing a chamber that minimizes the disruption of the site-specific biotic and abiotic factors that may influence the response being measured in the caged organisms. One of the more common chamber designs, depicted in Figure 1.3, uses clear tubes designed for sediment coring with polyethylene caps to enclose each end and mesh-covered openings to allow for water exchange. Clear core tubes allow for light to penetrate inside the chamber, while the mesh screen allows water to circulate between the chamber and the surrounding surface water. In general, the mesh opening should be large enough to allow water to flow freely without allowing the caged organisms to escape the chamber, or indigenous invertebrates to enter. In a study by Chappie and Burton (1997), two mesh sizes were evaluated for their ability to keep indigenous organisms out of the test chamber. The authors observed that reducing the mesh size from 149 µm to 74 µm reduced the number of indigenous zooplankton, oligochaetes, and chironomids inside the chamber, which made sorting and collecting the test organisms more efficient.

Several effective options have been developed for discriminating between the environmental compartments from which stressors may dominate as an exposure pathway (Burton et al., 2005; Crane et al., 2000; Faria et al., 2007). Exposure pathways can be compartmentalized into water column, surficial sediments, sediment-water interface, and pore water treatments by altering the placement of the chambers within the surface water and sediment (Greenberg et al., 2002; Burton et al., 2005). This enables biological and chemical effects endpoints to be evaluated for individual exposure pathways, which can help identify the source and nature of stressors to benthic organisms. Robertson and Liber (2007) used this approach to investigate surface water and sediment as potential pathways/causes of benthic invertebrate community impairment downstream from a northern Saskatchewan uranium operation. Results from the in situ study

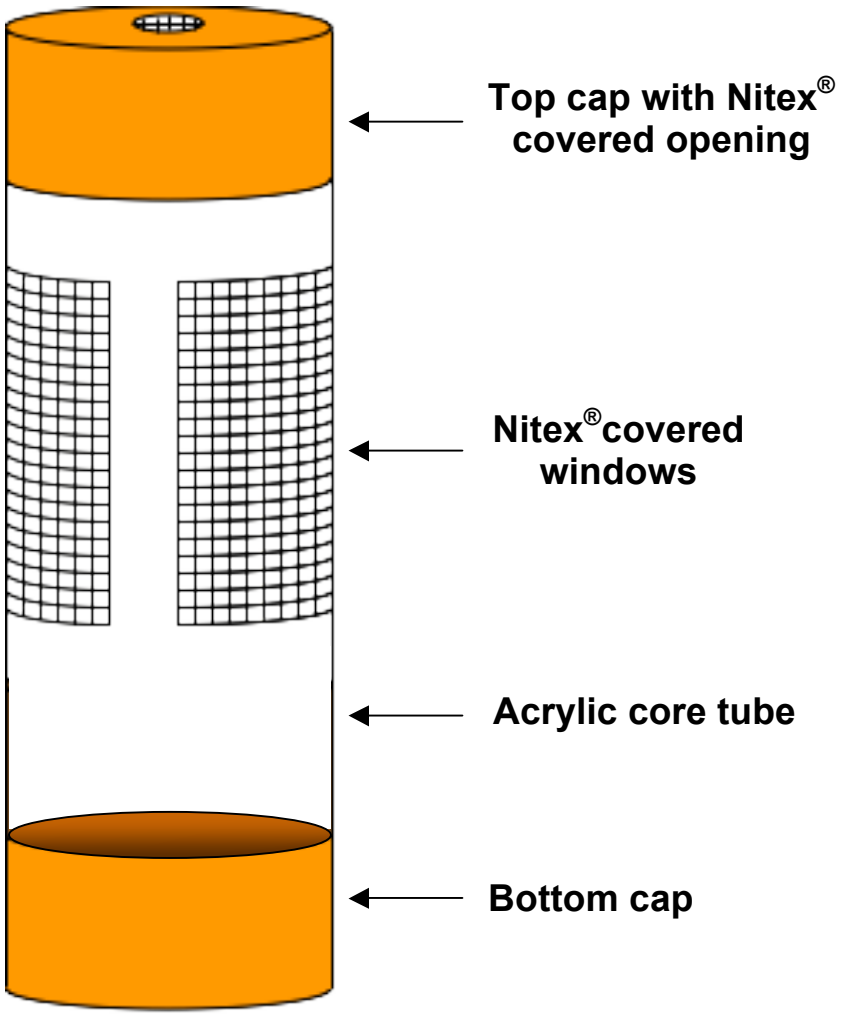

Figure 1.3: Chamber design for in situ bioaccumulation and toxicity testing using benthic invertebrates (Nitex®, Wildlife Supply Company, Buffalo, NY, USA).

contributed to a weight-of-evidence approach that helped to identify the dominant exposure pathway and COPCs that were likely contributing to benthic community effects downstream from the mining operation.

Caging studies have been successfully conducted using a variety of macroinvertebrates, such as cladocerans (Ireland et al., 1996; Pereira et al., 1999), amphipods (DeWitt et al., 1999; Schulz and Liess, 1999; Robertson and Liber, 2007), chironomids (Sibley et al., 1999; Crane et al., 2000; Castro et al., 2003), oligochaetes (Sibley et al., 1999), and bivalves (Soucek et al., 2000). However, there are currently no standardized methods for conducting in situ toxicity or bioaccumulation testing using freshwater invertebrates. Selection of a suitable invertebrate species for in situ toxicity will depend on the specific objectives of each study, but each study should take into account factors such as sensitivity to contaminant exposure, the organisms feeding habits, ecological relevance, taxonomic relation to indigenous aquatic invertebrates, and amenability to caging (Ingersoll, 1995). In the case of sediment toxicity and bioaccumulation evaluations, the chosen species should be either epifaunal or infaunal as the ecology of these species involves close contact with sediment. Macroinvertebrate species, such as midge larvae, amphipods, oligochaetes, and mayflies are often chosen because established protocols exist for their culturing and use in laboratory bioassays. Extensive databases are also available for many of these species, which facilitates comparisons of field and laboratory results (DeWitt et al., 1999). Test organisms are typically obtained from a laboratory culture where organisms of the same age can be preferentially selected for experimentation. Indigenous organisms have been successfully used for in situ bioassays, but collecting an adequate number of indigenous organisms of the same age can be difficult (Chappie and Burton, 2000). For this reason, laboratory-reared species are more often chosen for in situ toxicity and bioaccumulation testing.

1.6 Selenium speciation analysis using X-ray absorption spectroscopy

Knowledge of the chemical forms of selenium present in the environment is essential for accurately assessing the risk selenium poses in aquatic ecosystems (Maher et al., 2010; Ohlendorf et al., 2011). One of the more refined techniques for identifying the chemical forms of selenium in solid samples is X-ray absorption spectroscopy (XAS). The major advantage of XAS is its ability to detect different selenium species in complex samples without the need for sample pre-treatment (Pickering et al., 1999). Speciation data for this thesis were generated at two synchrotron facilities: beamline 9-3 at the Stanford Synchrotron Radiation Laboratory (SSRL; Menlo Park, CA), and the Hard X-ray Micro Analysis (HXMA) beamline at the Canadian Light Source (CLS; Saskatoon, Saskatchewan).

The experimental approach is fundamentally the same at both facilities. Electrons travelling in a circular orbit at close to the speed of light emit electromagnetic radiation that contains nearly the full electromagnetic spectrum ranging from infrared to hard X-rays. A monochromator converts the polychromatic synchrotron beam into a monochromatic beam (in this case hard X-rays) that is subsequently focused and directed toward the sample using mirrors (Andrahennadi, 2008). X-ray absorption spectra are generated when incident photons (X-rays) interact with electrons in bound atoms (i.e., selenium). At high enough energies, the incident photon results in ionization of core electrons. The core hole caused by emission of the core electron is filled by the decay of a higher-level electron with emission of an Auger electron or a fluorescent X-ray photon. Consequently, X-ray absorption can be monitored by directly measuring the transmission of X-rays through the sample, or by measuring the X-ray fluorescence, or electron yield (George and Pickering, 2007). X-ray fluorescence is the preferred detection method for analyzing environmental samples because of increased sensitivity for detecting low concentrations of selenium.

Absorption spectra are operationally separated into two regions that reflect different physical phenomena: the near-edge region and the extended X-ray absorption fine structure (EXAFS). The near-edge region measures the binding energy of the core electrons. This region of the absorption spectra is sensitive to the oxidation state of selenium in the sample, with higher oxidation states requiring more energy to ionize core electrons. Consequently, each selenium near-edge spectrum is highly distinctive and allows for characterization of the local electronic and atomic environment of an element (Vickerman et al., 2004).

Analysis of the absorption spectra is carried out using computer programs such as the EXAFSPAK software (George and Pickering, 1995). Initial data analysis involves isolating the absorption edge for the element of interest from background fluorescence of other elements in the sample. Principal component analysis (PCA) is then used to generate a statistical framework for choosing the number of standards to include when fitting the sample spectrum. In general terms, PCA identifies the number of different forms of the element present in the sample. Once the number of components is determined, target transformation is used to determine the most likely chemical standards to include when fitting the data. Several selenium standards are available, including inorganic selenate (SeO_4^{2-}) and selenite (SeO_3^{2-}), organic selenomethionine, selenocystine (the dimer of the seleno-amino acid selenocysteine), seleno-*bis*-glutathione, and red elemental selenium (Se^0). Linear combinations of near-edge spectra for a set of environmentally relevant selenium standards are then fit to the sample spectra to determine the relative percent contribution of each standard species in the sample (Figure 1.4). A standard is excluded from the fit if its fractional contribution is less than three times its estimated standard deviation. The residual, as depicted in Figure 1.4, is a visual representation of the goodness-of-fit of the various selenium standards to the data.

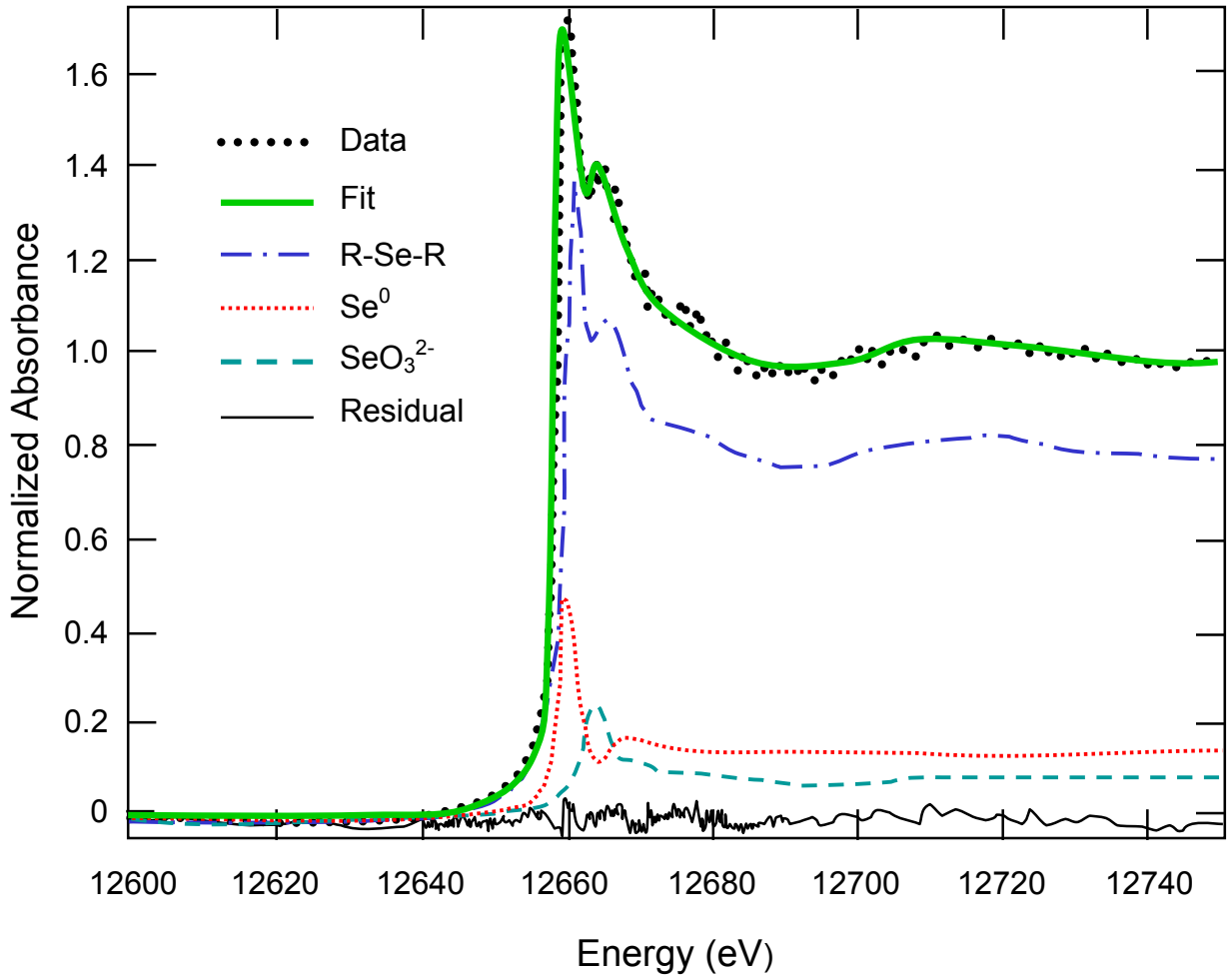

Figure 1.4: X-ray absorption near-edge spectra of *Chironomus dilutus* larvae exposed to dissolved seleno-DL-methionine for 10 days (described in Chapter 2). The near-edge spectrum (Data) was fit to the sum of the spectra of relevant selenium standards to determine the percent contribution of each selenium species. Standards included in the fit are organic selenides (R-Se-R; i.e., selenomethionine), elemental selenium (Se^0), and inorganic selenite (SeO_3^{2-})

1.7 Research goals and objectives

The goal of this work was to identify the dominant exposure pathway(s) and selenium species involved in the bioaccumulation of selenium by chironomid larvae inhabiting lakes downstream from a uranium milling operation. To attain this goal, the research was divided into two specific objectives:

1. Describe the selenium uptake and elimination kinetics, selenium speciation, and retention of selenium in *C. dilutus* larvae and adults based on exposure to different forms of dissolved selenium (selenate, selenite, or seleno-DL-methionine) under laboratory conditions.

   H_0: The rates of selenium uptake and depuration and the degree of selenium bioaccumulation in C. dilutus larvae are not dependent on the speciation of selenium (inorganic vs. organic) in the exposure water.

   H_0: There is no difference in the transfer of selenium from C. dilutus larval to adult stages during development based on exposure to either dissolved inorganic or organic selenium.

   H_0: Selenium speciation profiles in C. dilutus larvae or adult insects are not dependent on the chemical form of selenium (inorganic vs. organic) in the exposure water.

2. Conduct in situ bioaccumulation experiments to determine the significance of exposure route (water vs. sediment/diet) and sediment selenium speciation on bioaccumulation in *C. dilutus* larvae.

   H_0: There is no difference in C. dilutus accumulation of selenium, on a site-specific basis, regardless of whether larvae are exposed to selenium contaminated surface water and sediment, or surface water only.

H_0: Bioaccumulation and speciation of selenium in C. dilutus larvae is not dependent on whether larvae are exposed to whole sediment, biofilm, or detritus as a source of selenium.

CHAPTER 2

SELENIUM BIOACCUMULATION AND SPECIATION IN *CHIRONOMUS DILUTUS* EXPOSED TO WATER-BORNE SELENATE, SELENITE, OR SELENO-DL-METHIONINE

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2.1 Introduction

Selenium bioaccumulation within aquatic food chains has emerged as an important ecotoxicological issue in recent years because of well-documented cases of selenium toxicity to fish and birds. In the Kesterson National Wildlife Refuge in the San Joaquin Valley, California, USA, sub-surface irrigation drainage water, containing elevated levels of selenium, accumulated in a number of wetlands and resulted in reproductive impairment and deformities in several species of fish and wildlife (Ohlendorf et al., 1986; Saiki and Ogle, 1995). Since this event, and heightened public awareness of selenium pollution issues, a significant volume of research has been published on the bioaccumulation and toxicity of selenium in aquatic environments, with the consensus being that diet is the dominant pathway of selenium accumulation in fish and birds (Hamilton, 2004; Lemly, 2004).

Accumulation of selenium within the benthic food web is an important step in the dietary transfer of selenium to higher trophic level organisms living in or around aquatic environments.

Invertebrates are important in the trophic transfer of selenium because they are the dietary source of selenium for many species of fish and wildlife (Lemly, 1993). Dietary exposure is likely the dominant route of selenium accumulation in chironomids, with evidence suggesting that the microbial biomass component of sediment detritus is an important source of dietary selenium (Alaimo et al., 1994). However, due to their habitat preference and feeding patterns, accumulation of selenium from water can contribute to elevated body-burdens of selenium in invertebrates (Stewart et al., 2010). Although selenium oxyanions, selenate (SeO_4^{2-}) and selenite (SeO_3^{2-}), are the predominant forms of selenium in surface waters, it has been suggested that trace amounts of dissolved organo-selenium compounds may contribute disproportionately to selenium bioaccumulation in aquatic food webs because of their increased bioavailability (Besser et al., 1993; Rosetta and Knight, 1995).

Knowledge of chemical speciation and biotransformation processes that lead to selenium accumulation at higher trophic levels is essential to understanding the risks associated with selenium exposure for aquatic organisms (Unrine et al., 2007). Each selenium species in the abiotic environment and biota of aquatic ecosystems behaves differently with respect to mobility and bioaccumulative potential. Biotransformation of selenium and the incorporation of organo-selenium compounds into proteins are key steps in the trophic transfer of selenium (Fan et al., 2002). Aquatic insects can biotransform inorganic selenium into organic forms (Vickerman et al., 2004), which are the predominant forms of selenium ingested by birds and fish (Spallholz and Hoffman, 2002). In particular, selenomethionine has been shown to cause embryonic deformities, other teratogenic effects, and mortality of bird embryos at dietary concentrations as low as 8 µg/g wet-weight (Heinz et al., 1989).

In the present study, we examined the bioavailability and bioaccumulation of selenium in larvae of the midge *Chironomus dilutus*, and documented the uptake and elimination kinetics for three different forms of dissolved selenium. We also used X-ray absorption spectroscopy (XAS) to describe selenium speciation in *C. dilutus* following exposure to the three different forms of selenium. X-ray absorption spectroscopy is a powerful tool in the assessment of selenium bioavailability, bioaccumulation, and biotransformation because it can provide unique insights into the chemical forms of selenium present in tissues without extensive pretreatment such as digestion. Furthermore, selenium K-edge XAS has been optimized for the detection of relatively low concentrations of selenium, making this technique particularly useful in ecotoxicological assessments of selenium-impacted environments. The objectives of the present study were to determine whether the selenium species present in *C. dilutus* at various life stages are dependent on the chemical form of selenium to which the organism is exposed (selenate, selenite, or seleno-DL-methionine), and to assess the efficiency of selenium transfer from larvae to adult insects.

2.2 Materials and methods

2.2.1 Test organism

Chironomus dilutus (Diptera: Chironomidae) was chosen as the test species to assess selenium bioaccumulation and speciation in both the larval and adult insect stages. Formerly classified as *C. tentans*, this widely distributed non-biting midge is commonly used in laboratory tests because it is relatively easy to culture and it completes its life cycle in a relatively short period of time (Benoit et al., 1997). Ecologically, *Chironomus* spp. can be one of the more abundant benthic invertebrate taxa found in aquatic environments because they can tolerate many different types of habitats and a wide range of environmental conditions. Their ubiquity in aquatic ecosystems makes them important prey items for a large number of invertebrate, fish,

and bird species (Pinder, 1986). Midge larvae were obtained from an in-house culture maintained at the Toxicology Centre, University of Saskatchewan, Saskatoon, SK, Canada.

2.2.2 Experimental design

All experiments were conducted in an environmental chamber at the Toxicology Centre, University of Saskatchewan. Tests were performed as modified static-renewal assays at a temperature of 25 ± 1ºC and under a 16:8 h light:dark cycle (illumination intensity ranging from 600 to 1100 lux during the light cycle). Sodium selenate (Na_2SeO_4), sodium selenite (Na_2SeO_3), and seleno-DL-methionine (Se-met) ($C_5H_{11}NO_2Se$) were the three selenium species evaluated in the present study (Sigma-Aldrich Canada). Replicate 300-ml tall-form glass beakers containing a thin layer of silica sand (particle size 250 – 425 µm) served as the experimental units. Larvae were 7-9 d old (2^{nd} instar) at the start of each test, with a total of 29 replicate beakers used per treatment (10 larvae per beaker). Stock concentrations (5.0 mg/ml) for each selenium species were prepared using ultrapure water (Barnstead® NANOpure) and then diluted using carbon-filtered municipal water to achieve a nominal test concentration of 4 µg/L. Water changes were performed every 3 d to ensure adequate water quality and consistent selenium concentrations. Triplicate water samples were collected from each treatment immediately before and after each water change during the uptake phase to verify the aqueous selenium exposure concentration. Water samples were also collected on day 3 of the elimination phase to confirm that the selenium concentration in all treatments was similar to the control water selenium concentration. Dissolved oxygen and temperature were monitored during the water changes, and separate water samples were collected at each water change to measure pH, alkalinity, hardness, conductivity, and ammonia. Larvae were fed 1 ml of fish food slurry (6 mg/ml dry weight (dry wt), Bio Flakes®, Sera, Heinsburg, Germany) that was low in total selenium (0.60 µg/g), on a daily basis.

To evaluate selenium uptake, elimination, and tissue speciation in *C. dilutus*, larvae were exposed to test solutions for an uptake period of 10 d followed by an elimination period of 10 d in clean water. To evaluate the selenium uptake, chironomid larvae from three randomly selected beakers per treatment were removed on days 1, 3, 6, 9, and 10 and analyzed for whole-body selenium. Following the initial 10 d uptake period, larvae from the remaining replicates were transferred to clean test beakers containing clean culture water and a clean sand substrate with no added selenium. Larvae from three replicate beakers from each treatment were collected and analyzed for whole-body selenium concentrations on days 11, 14, 17 and 20 to assess selenium elimination. One additional replicate was collected from each treatment at the end of the uptake phase (day 10) and at the end of the elimination phase (day 20) to determine selenium speciation in the larvae. Larvae from the control treatment were sampled only on days 10 and 20, as uptake and elimination of selenium was expected to be negligible.

An additional study was conducted to evaluate selenium retention and tissue speciation in emerged *C. dilutus* adults following exposure of the entire larval and pupal stage to selenate, selenite, or Se-met. This experiment was performed according to the procedures and conditions described above for the uptake/elimination tests, but with a longer duration of exposure (9 to 65 d post-hatch). Second instar larvae were exposed to approximately 4 µg/L of each selenium species until adult emergence was complete. A total of five replicates per treatment were used for this test with four of the replicates analyzed for whole-body selenium concentration and the remaining replicate analyzed for bulk tissue-selenium speciation. An emergence trap was placed on top of each beaker to contain the *C. dilutus* adults as they emerged. The beakers were checked on a daily basis, and newly emerged insects were gently aspirated and transferred into a vial prior to analysis for whole-body selenium concentration and bulk tissue-selenium speciation.

2.2.3 Selenium concentration analysis

Total selenium concentrations in test water and *C. dilutus* tissue samples were determined using inductively coupled plasma-mass spectrometry (ICP-MS; X Series II Thermo Electron Corporation). Analysis involved monitoring ^{80}Se and the use of collision cell technology as described previously (Wiramanaden et al., 2010a). Water samples collected for dissolved selenium analyses were membrane filtered (0.45 µm polyethersulfone (PES), Pall Corporation) and acidified to 2% nitric acid (omnitrace ultra, EM Science). All samples were kept at 4ºC prior to analysis. The ICP-MS limit of detection was 0.07 µg/L.

Chironomus dilutus larvae designated for total selenium analysis were rinsed three times with deionized water and dried at 60 ºC for a minimum of 48 h prior to digestion. Adult insects avoid full contact with water during emergence; consequently, these samples were dried without being rinsed with deionized water. Dried samples were cold digested using nitric acid (5 ml) and hydrogen peroxide (1.4 ml; 30% Omnipure, VWR International), followed by selenium concentration determination using ICP-MS as previously described. The tissue digestion method was verified using a lobster hepato-pancreas standard reference material (Tort-2 National Research Council Canada, Ottawa, ON, Canada). Standard reference material (SRM) and method blanks were analyzed every ten samples using ICP-MS; all replicate SRM samples were within 10% of the certified value of 5.63 ± 0.67 µg/g. The method limit of quantification determined from the SRM was 0.15 µg/g.

2.2.4 Selenium speciation analysis

Processing and analysis of midge larvae, adults, and exuvia for selenium speciation was carried out according to methods outlined by Wiramanaden et al. (2010b). Briefly, samples designated for speciation analysis were stored at -80 ºC prior to processing. Sample preparation

involved homogenizing the tissue while keeping the samples frozen using liquid nitrogen. Samples with low mass were not homogenized to minimize sample loss. Each sample was then packed in an XAS cuvette and stored in liquid nitrogen until analysis. Selenium K-edge spectra were generated at beamline 9-3 at SSRL and at the HXMA beamline at the CLS. The K-edge is the energy at which a non-binding core electron is ejected from the inner electron orbital. Spectra of dilute aqueous selenium standards were also collected, which included inorganic SeO_4^{2-}, and SeO_3^{2-}, organic selenomethionine (R-Se-R), selenocystine (R-Se-Se-R; the dimer of the seleno-amino acid selenocysteine), seleno-*bis*-glutathione (R-S-Se-S-R), and red elemental selenium (Se^0). The near-edge spectra for each sample were then quantitatively fit to the sum of the spectra of standards using edge-fitting software (EXAFSPAK). For a selenium standard to be included in the fit, its fractional contribution had to exceed three times its estimated standard deviation. The final data analysis shows the percent contribution of the individual selenium standards to the total selenium present in a given sample.

2.2.5 Uptake and elimination kinetics

Uptake and elimination rate constants were determined using the graphical method assuming a one-compartment model of bioconcentration (Rand, 1995). This method of calculating the elimination rate constant K_e (/d) does not require steady-state concentrations in the test organisms prior to initiating the elimination phase, which was the case for larvae from the selenite and Se-met treatments in the present study. Briefly, K_e was calculated from the slope of the decline in whole-body selenium during the first 7 d of elimination, on a logarithmic scale, against the elimination time according to the equation.

$$\ln C = \ln C_0 - K_e t \tag{2.1}$$

where C_0 is the whole-body selenium concentration at the beginning of the 10 d elimination phase and t is time (days). The uptake coefficient K_u (L/g • d) was calculated from the linear plot of the increase in selenium concentration over the 10 d uptake phase according to the equation:

$$K_u = \left(\frac{\frac{\Delta C}{\Delta t} + K_e C_{D10}}{C_w} \right) \tag{2.2}$$

where ΔC is the change in whole-body selenium concentration during the uptake phase, C_{D10} is the larval concentration on day 10, and C_w is the average dissolved selenium concentration in the test solution. Rate constants were not determined for the control and selenate treatments due to the negligible accumulation and elimination of selenium observed during the experiment. Concentration factors (CFs) for each experimental selenium treatment were calculated using the following equation:

$$CF_{larvae} = \frac{C_{D10}}{C_w} \tag{2.3}$$

Given the short life span of *C. dilutus* under favorable laboratory conditions and the need for an elimination phase, steady-state was likely not achieved during the uptake phase in the selenite and Se-met treatments. Consequently, CFs for the midge larvae is based on whole-body selenium concentrations after 10 d of exposure.

2.2.6 Statistical analysis

Data analyses were performed using SigmaPlot® version 11 (Systat Software) with a 95% ($\alpha = 0.05$) confidence level. Data are presented as the mean ± one standard deviation. Significant differences in larval, adult, and exuvia selenium concentrations across treatments were determined using a one-way ANOVA. Data that failed testing for normality or equal variance were log-transformed prior to statistical analysis. Tukey's post-hoc test, when appropriate, was used to compare selenium concentrations among treatment groups for larvae, adults, and exuvia. A one-way ANOVA was also used to compare time to emergence and mean emergence among treatments. Mean emergence time was defined as the average number of days (post-hatch) for 50% of the larvae to emerge from each replicate.

2.3 Results and discussion

2.3.1 Selenium uptake and elimination by *C. dilutus* larvae

Measured selenium concentrations in both the selenate and selenite treatments were in good agreement with the nominal concentration (4 µg/L) during the uptake phase (Table 2.1). However, the dissolved selenium concentration for the Se-met treatment was lower than the nominal concentration and had greater variability throughout the 10 d of exposure. The selenium concentration measured in the new test water on days 0, 1, 3, 6, and 9 was 3.3 ± 0.9 µg/L, but after 3 d the concentration dropped below 1µg/L, with a mean concentration of 1.8 ± 1.0 µg/L during the 10-d uptake period. These results were consistent with a previous study in which Se-met in the test water decreased by 50 to 60% over a 96-h period (Besser et al., 1993). Mass balance estimations indicated that approximately 10% of the Se-met added to the beakers was accumulated by the larvae during the uptake phase (data not shown). No speciation analysis was

Table 2.1: Uptake and elimination kinetics, bioaccumulation, and times to emergence for *Chironomus dilutus* larvae exposed to three forms of selenium (Se).

Measurement	Aqueous Selenium Treatment			
	Control	Selenate	Selenite	Seleno-DL-methionine
Test water [Se][a]	0.5 ± 0.1	4.3 ± 0.3	3.8 ± 0.8	1.8 ± 1.0
K_u[b]	–[f]	–[f]	0.49	3.51
K_e[b]	–[f]	–[f]	0.03	0.16
CF_{larvae}[c]	2120	480	3830	17900
CF_{adults}[d]	3060	360	3550	24460
Emergence[e]				
First adult	29	29	33	27
Median	40	39	42	37

[a] Dissolved Se concentration (geometric mean ± 1 SD in µg/L) during the 10-d uptake phase.
[b] Uptake rate constant (K_u, L/g · d) and elimination rate constants (K_e, /d).
[c] Larval concentration factors (CF) were derived from mean larval whole-body Se concentration on day 10.
[d] Adult CF values are based on the mean whole-body Se concentrations for emerged insects.
[e] Time in days (post-hatch) until emergence of the first adult insect and time until median (50%) emergence.
[f] No uptake or elimination rate constants calculated due to negligible Se accumulation in these treatments.

done on the control water; however, the low whole-body selenium concentrations in control midges suggest that selenate was the dominant dissolved selenium species (Figure 2.1).

Chironomus dilutus larval survival was > 85% across all treatments. Growth rates in the selenate and selenite treatments were similar to the control treatment during the experiment. Mean larval dry wt at day 10 was 61% of the day 20 weight in the control treatment, 10 larval dry weights in the selenate and selenite treatments were 63% and 69% of the day 20 weight, respectively (data not shown). This suggests that *C. dilutus* larvae are tolerant of whole-body selenium concentration up to 14.5 µg/g dry weight. Larvae from the Se-met treatment exhibited a reduction in growth rate during the uptake phase, with the day 10 mean larval dry weight accounting for only 48% of the larval dry weight at day 20. Growth resumed when the larvae were transferred to clean water to assess elimination, with no significant difference in the day 20 dry weight observed among treatments ($p = 0.178$, data not shown).

Results from the uptake phase of the experiment showed that *C. dilutus* larvae rapidly accumulated selenium from the selenite and Se-met treatments (14.5 and 32.2 µg/g dry wt, respectively) compared to the control and selenate treatments (1.1 and 2.1 µg/g dry wt, respectively) (Figure 2.1a). Larvae from the selenite and Se-met treatments had significantly elevated ($p < 0.05$) selenium concentrations following 10 d of exposure compared to the control larvae (Figure 2.1a). Exposure of *C. dilutus* larvae to a mean Se-met concentration of 1.8 µg/L resulted in a whole-body selenium concentration of 32.2 ± 8.2 µg/g after 10 d of uptake, with a corresponding CF of 17,900 (Table 2.1). Steady-state was likely not achieved during the 10 d exposure period (Figure 1a,) but continuing the exposure phase past 10 d to document steady-state was not feasible without either shortening the duration of the elimination phase, or altering,

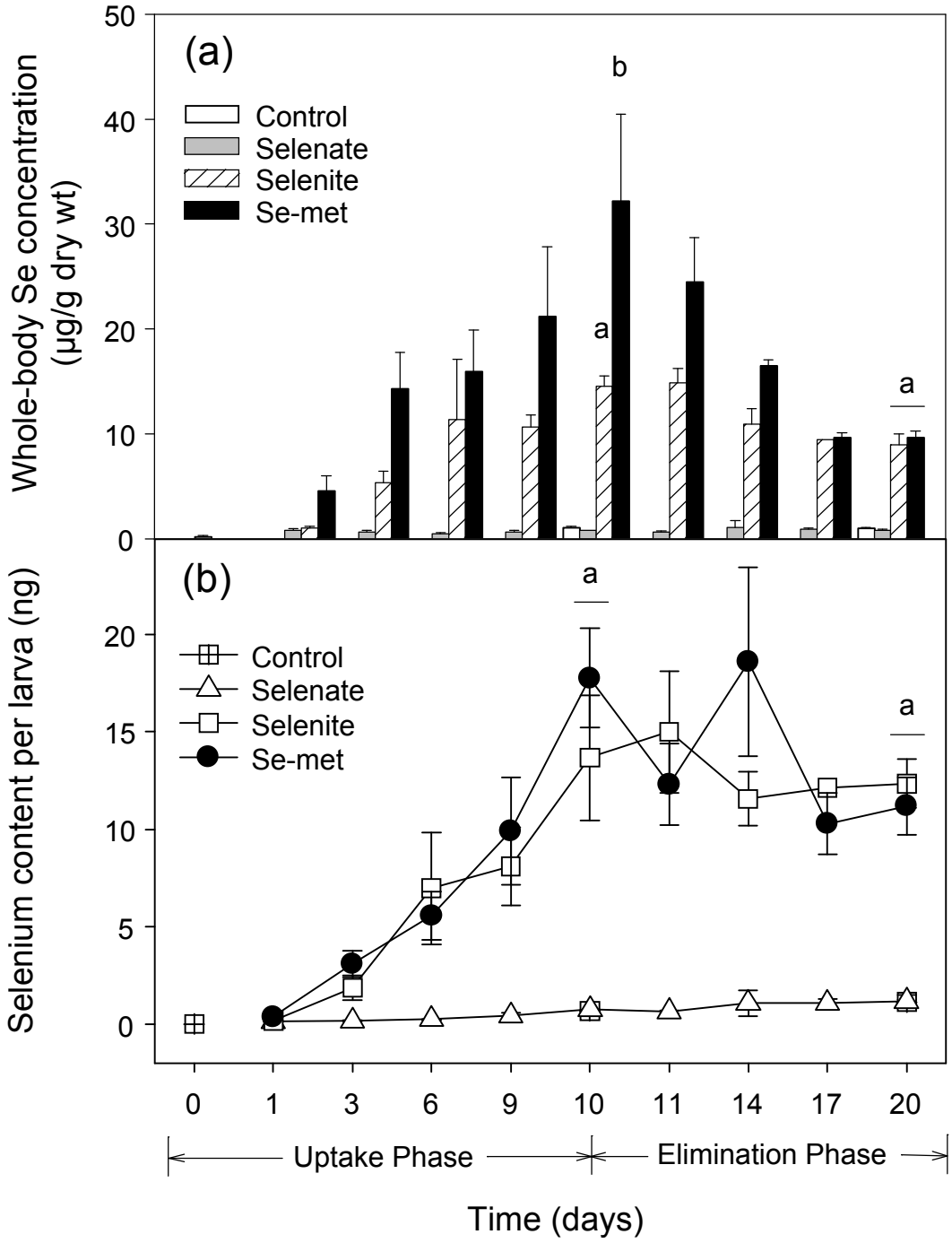

Figure 2.1: Uptake and elimination of selenium (Se) by *Chironomus dilutus* larvae exposed to aqueous selenium as selenate, selenite, or seleno-DL-methionine (Se-met). (a) Larval whole-body selenium concentration (µg/g dry wt). (b) Total selenium content per larva (ng). Letters indicate a significant difference ($p < 0.05$) among treatments on days 10 and 20. Data are the mean ± 1 SD ($n = 3$). Values for selenate day 10 and selenite day 17 are the means of 2 samples.

the test conditions to slow the rate of larval growth and prolong the time to adult emergence. However the 10 d concentration in larvae from the Se-met treatment represented approximately 75% of the whole-body selenium concentration in adult insects (44.0 ± 4.8 μg/g) following continuous aqueous selenium exposure of the larvae until insect emergence (Figure 2.2). Larvae from the selenite treatment approached steady-state following 10 d of exposure, with whole-body selenium concentrations in the larvae similar to that of the adult insects (14.5 vs. 13.5 μg/g). Although some elimination of selenium may occur as larvae undergo metamorphosis, the adults retained the bulk of accumulated selenium, with only approximately 2% of the selenium contained in the exuvia (data not shown). These data suggest that the selenium concentration in adult insects should closely approximate the steady-state concentration in larvae.

The relatively high CF values for midge larvae and adults from the control treatment are consistent with the inverse relationship observed between dissolved selenium concentrations and bioaccumulation metrics (Besser et al., 1993; DeForest et al., 2007). Despite an order of magnitude greater concentration of selenate in the test water, the CF for larvae was approximately four-fold lower compared to the control treatment (Table 2.1). These data demonstrate that uptake from the dissolved phase is not an important route of selenium bioaccumulation for lower trophic level consumers when selenium is present as selenate.

Selenium was largely retained by larvae in the selenite and Se-met treatments following a 10 d elimination period in clean water (Figure 2.1b). Larvae exposed to selenite for 10 d retained 89% of the selenium after 10 d of elimination, compared with 62% retention by larvae exposed to Se-met (data not shown). Although rapid elimination of selenium apparently takes place once the larvae are removed from the Se-met spiked water (Figure 2.1a), much of the decrease in concentration appears to be the result of growth dilution. As shown in Figure 2.1b,

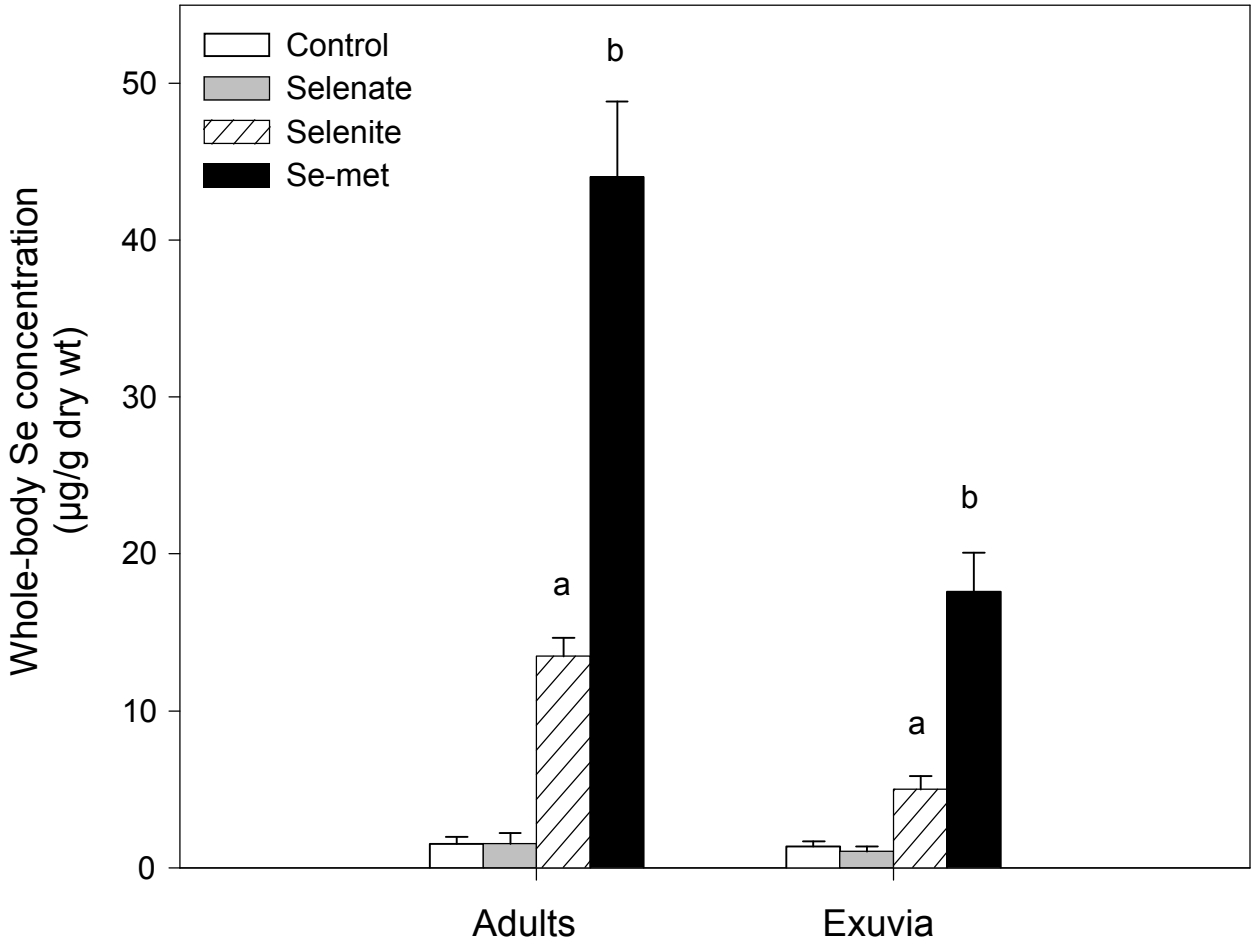

Figure 2.2: Selenium (Se) concentration in *Chironomus dilutus* adults and exuvia after larval exposure to selenate, selenite, or seleno-DL-methionine (Se-met). Data are the mean ± 1 SD (n = 4). Different letters (a, b) indicate a significant difference ($p < 0.05$) among treatment groups for the adult insects and exuvia.

the majority of selenium accumulated by the larvae from the selenite and Se-met treatments was retained during the elimination phase. It is hypothesized that larvae from these two treatments incorporated Se-met non-specifically into protein in place of methionine during larval growth. When the animals were subsequently transferred to clean water, the non-proteinaceous pool of selenium was quickly eliminated, with the steady-state concentration at day 17 indicative of the concentration of selenium incorporated into protein. The greater percentage of selenium eliminated by larvae from the Se-met treatment compared to the selenite treatment suggests that the larvae exposed to Se-met accumulated selenium faster than selenium could be incorporated into protein, resulting in a greater concentration of free selenium readily available for elimination. The observation that larvae exposed to selenite depurated selenium to a similar whole-body concentration as the larvae exposed to Se-met is consistent with a two-compartment elimination model where rapid selenium elimination is dependent on the concentration of free selenium in the organism and slower elimination rates are related to the concentration of selenium incorporated into tissue proteins. We were unable to calculate an elimination rate constant from the slow compartment for the selenite and Se-met treatments because only two sampling times (days 17 and 20) were representative of the slow elimination phase. This need for several data points during the latter stages of elimination is a practical drawback of the two-compartment model and was not possible in the present study given that the larvae were approaching metamorphosis after 10 d of elimination. Once selenium is incorporated into protein, it is likely that the rate of elimination would likely be negligible, because proteins would need to be catabolized in order for selenium to be excreted. Consequently, for this species of aquatic invertebrate, a one-compartment model may best represent the process of selenium elimination.

Based on 10 d whole-body selenium concentrations, seleno-DL-methionine was the most bioavailable form of dissolved selenium, followed by selenite. Selenate was not significantly bioaccumulated during the uptake phase (Figure 2.1). These results are in good agreement with other studies that have compared the bioavailability of these selenium species to aquatic invertebrates (Maier and Knight, 1993; Rosetta and Knight, 1995). While diet is likely the dominant route of selenium accumulation by benthic detritivores such as *C. dilutus* in situ, our results suggest that if selenite or organic selenium is present in surface water or sediment pore water at low concentrations, the aqueous exposure route can contribute to elevated whole-body selenium concentrations. Given that benthic invertebrates can be exposed to contaminants via surface water and pore water, and not solely their diet, uptake of bioavailable forms of selenium from the dissolved phase cannot be discounted as a potential exposure pathway for selenium accumulation in the field. Other authors have noted that aqueous selenium exposure can, under some conditions, be an important route of uptake for a number of species occupying lower trophic levels (Stewart et al., 2010). Although we found selenate to have negligible bioavailability compared to selenite at approximately 4 µg/L, microbial-mediated reduction of dissolved selenate has been shown to increase the concentration of reduced selenium species at the sediment-water interface and in pore water (Martin et al., 2011). Furthermore, highly bioavailable dissolved organo-selenium can be found at the sediment-water interface as a result of decomposition of organic matter in surficial sediments (Belzile et al., 2000). For example, at two sites in the Elk River Valley, BC, Canada, sub-oxic conditions near the sediment-water interface resulted in increased selenite and organo-selenium in the surface water and pore water, demonstrating that these selenium species can be released back into the water column (Martin et al., 2011). At one site, selenite and organo-selenium were present at concentrations above 3

µg/L at the sediment-water interface, concentrations similar to those used in the present study. Given the habitat preference of benthic detritivores, the accumulation of more bioavailable species of selenium from pore water and surface water could contribute, in addition to diet, to elevated selenium concentrations in benthic-dwelling invertebrate (Orr et al., 2006).

2.3.2 Retention of selenium by adult insects

Adult insects retained the majority of selenium accumulated during the larval stage. Adult insects and exuvia from the Se-met and selenite treatments contained significantly greater concentrations of total selenium than the respective controls ($p < 0.05$; Figure 2.2). In the Se-met treatment, the mean total selenium concentration in adults was 44.0 ± 4.8 µg/g dry weight compared to 32.2 ± 8.2 µg/g dry weight for larvae after 10 d of exposure. Based on the adult selenium concentration, it is reasonable to assume that the steady-state whole-body selenium concentration in the larvae from this treatment would have been greater than or equal to 44 µg/g. Nonetheless, the data clearly demonstrate that selenium accumulated during the larval stage is largely retained by the pupa and transferred to the adult during metamorphosis. Some selenium is deposited in the exuvia once the adult insect completes metamorphosis, but this represents less than 5% of the total selenium, on a mass basis, in the adults. The relationship between selenium concentration in the adults and exuvia exhibited a positive linear relationship when all four treatments were plotted together ($r^2 = 0.987$, $p < 0.001$) (Figure 2.3). From these data, it appears that selenium incorporation into the exuvia is not a mechanism of detoxification or excretion of excess selenium, but rather a function of normal protein synthesis when the organisms are exposed to dissolved selenium concentrations near 4 µg/L.

There were no significant differences in adult emergence among treatments, presented as time until first emergence or median (50%) emergence (Table 2.1). Adult emergence in the

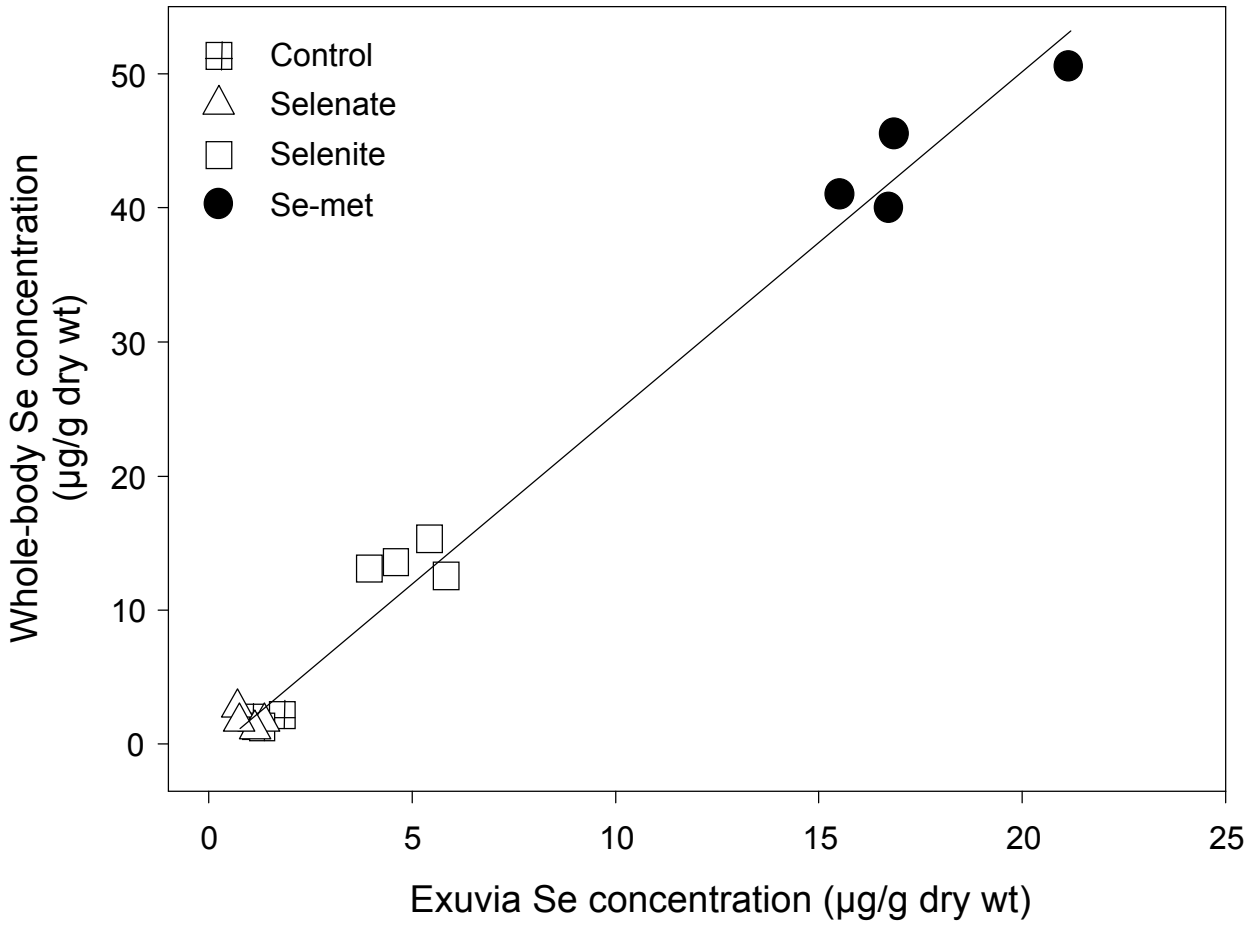

Figure 2.3: Relationship between selenium (Se) concentrations in emerged *Chironomus dilutus* adults and exuvia after larval exposure to selenate, selenite, or seleno-DL-methionine (Se-met). ($y = 2.54x - 0.80$; $r^2 = 0.987$).

control treatment was 82%, which exceeds the 70% criterion found in standard test methods (USEPA, 2000b). Furthermore, cumulative emergence met the 70% criterion in the selenate (70%) and selenite (70%) treatments, but was only 56% in the Se-met treatment (Figure 2.4). These data suggest that a mean dissolved Se-met concentration of 1.8 µg/L can decrease midge emergence. Low microgram per liter concentrations of some forms of selenium in surface water may therefore affect *C. dilutus* populations in aquatic environments and possibly other aquatic insects as well. There were no significant differences in emerged adult insect dry weight across all treatments. However, the gender ratio (male:female) differed among treatments, with the controls having the highest ratio (1.41), followed by selenate (1.11), Se-met (0.87), and selenite (0.35) treatments. Individual weights of male and female adults were not collected, so the effect of selenium exposure on gender-specific weight was not evaluated.

Conventional understanding of selenium toxicity to aquatic organisms has viewed insects simply as a conduit of selenium transfer to higher trophic level organisms; however, recent research has suggested that some invertebrate species may also be sensitive to selenium exposure at environmentally relevant concentrations (deBruyn and Chapman, 2007; Conley and Funk, 2009). A study investigating selenium bioaccumulation and maternal transfer in mayflies has shown that a significant percentage of selenium can be transferred to the insect eggs (Conley and Funk, 2009). In our study, *C. dilutus* adults were collected prior to oviposition, and male and female insects were grouped together for analysis. This method of sampling allowed us to determine the highest possible whole-body selenium concentration in the adult insects, as oviposition of egg masses would likely have resulted in the maternal transfer of selenium to eggs, thus reducing the total body-burden in female midges post-partum.

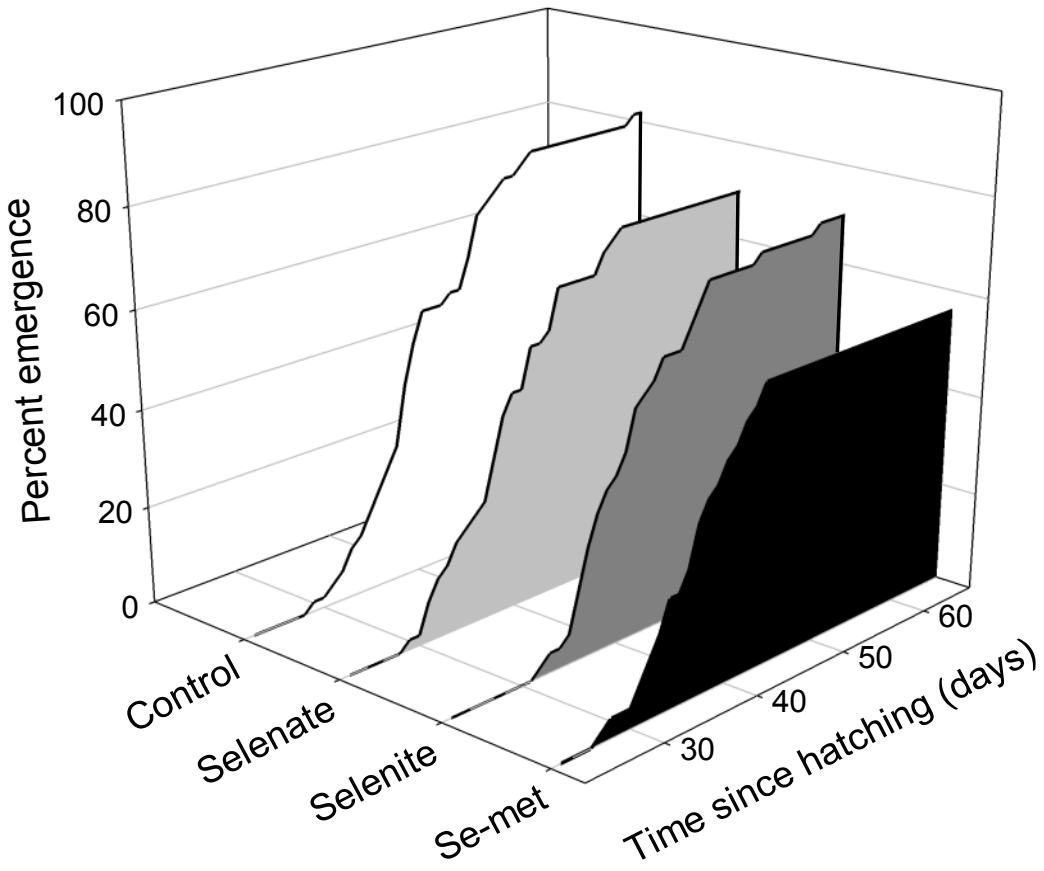

Figure 2.4: Percent emergence of *Chironomus dilutus* adults following larval exposure to selenate, selenite, or seleno-DL-methionine (Se-met).

2.3.3 Selenium speciation in larvae, adults, and exuvia

Selenium K-edge X-ray absorption spectroscopy was used to obtain spectra for *C. dilutus* larvae, adults, and exuvia following exposure to different water-borne forms of selenium. Each selenium K-edge spectrum is a unique fingerprint that provides insight into the local electronic environment of selenium present in the sample (Andrahennadi et al., 2007). Individual spectra were quantitatively fit to the sum of spectra of known selenium standards. Organo-selenium standard compounds included selenomethionine, selenocystine, and seleno-*bis*-glutathione used to represent the selenium environments R-Se-R, R-Se-Se-R, and R-S-Se-S-R, respectively. Inorganic selenium standards included selenate (SeO_4^{2-}), selenite (SeO_3^{2-}), and red elemental selenium (Se^0). Our objective was to evaluate whether chironomids exposed to different chemical forms of selenium express different whole-body selenium speciation, and whether the selenium speciation profile changes between chironomid larval and adult stages. Given the importance of organic forms of selenium in trophic transfer of selenium in aquatic environments, it is important to understand not only the comparative bioavailability of different selenium species, but also how different chemical forms of selenium are biotransformed, in particular, by lower trophic-level organisms that can readily bioaccumulate selenium. Because of a low whole-body selenium concentration (1.06 ± 0.08 µg/g), selenium standards could not be fit to the spectrum for larvae from the control treatment on day 10. Similarly, no selenium K-edge was generated for exuvia from the control and selenate treatments due to low selenium concentrations and insufficient sample mass.

The organic selenides R-Se-R (modeled as selenomethionine) and R-Se-Se-R (modeled as selenocystine) were the predominant chemical forms of selenium detected in most of the *C. dilutus* larvae, adults, and exuvia in the present study. Following exposure to dissolved selenite or Se-met, larvae had 76% and 79%, respectively, of the total selenium occurring as R-Se-R.

Animals exposed to selenate had 64% of their whole-body selenium present as organic diselenides with no detectable amount of R-Se-R (Figure 2.5, Table 2.2). Organic diselenides were modeled as selenocystine, which is a dimer of selenocysteine, the 21st amino acid. Selenocysteine synthesis is genetically regulated and is required for proper function of several selenoproteins. Selenocysteine is highly reactive at physiological pH, which makes detecting this compound difficult. Consequently, the dimer selenocystine served as a proxy for estimating the abundance of selenocysteine in *C. dilutus* larvae and adults in the present study. Concentrations of organic diselenides ranged from 0.35-1.54 μg/g (Table 2.2), which suggests that selenocystine levels remain relatively constant, regardless of the selenium speciation in the test water or the whole-body selenium concentration in the organisms. Diselenides were not detected in day 10 larvae and adult insects from the selenite and Se-met treatments; however, this is likely related to the detection limit of XAS and the high concentrations of R-Se-R compounds masking the presence of R-Se-Se-R in the samples.

The predominance of organic selenides (R-Se-R; modeled as selenomethionine) in larvae from the selenite treatment was surprising. Animals have no mechanism for the synthesis of the amino acid methionine, and consequently are unable to synthesize Se-met; instead, animals obtain methionine and Se-met primarily from their diet (Spallholz and Hoffman, 2002). Bacterial reduction of selenite to organic selenides is postulated as the most likely mechanism whereby larvae from the selenite treatment accumulated elevated concentrations of organic selenides. Bacteria have been shown to rapidly accumulate and metabolize selenite into amino acids and proteins, whereas selenate is less efficiently metabolized (Burton et al., 1987). This is supported by speciation data from our selenate treatment, where selenate-exposed larvae had no detectable levels of organic selenides (R-Se-R) after 10 d of exposure, and only low levels of

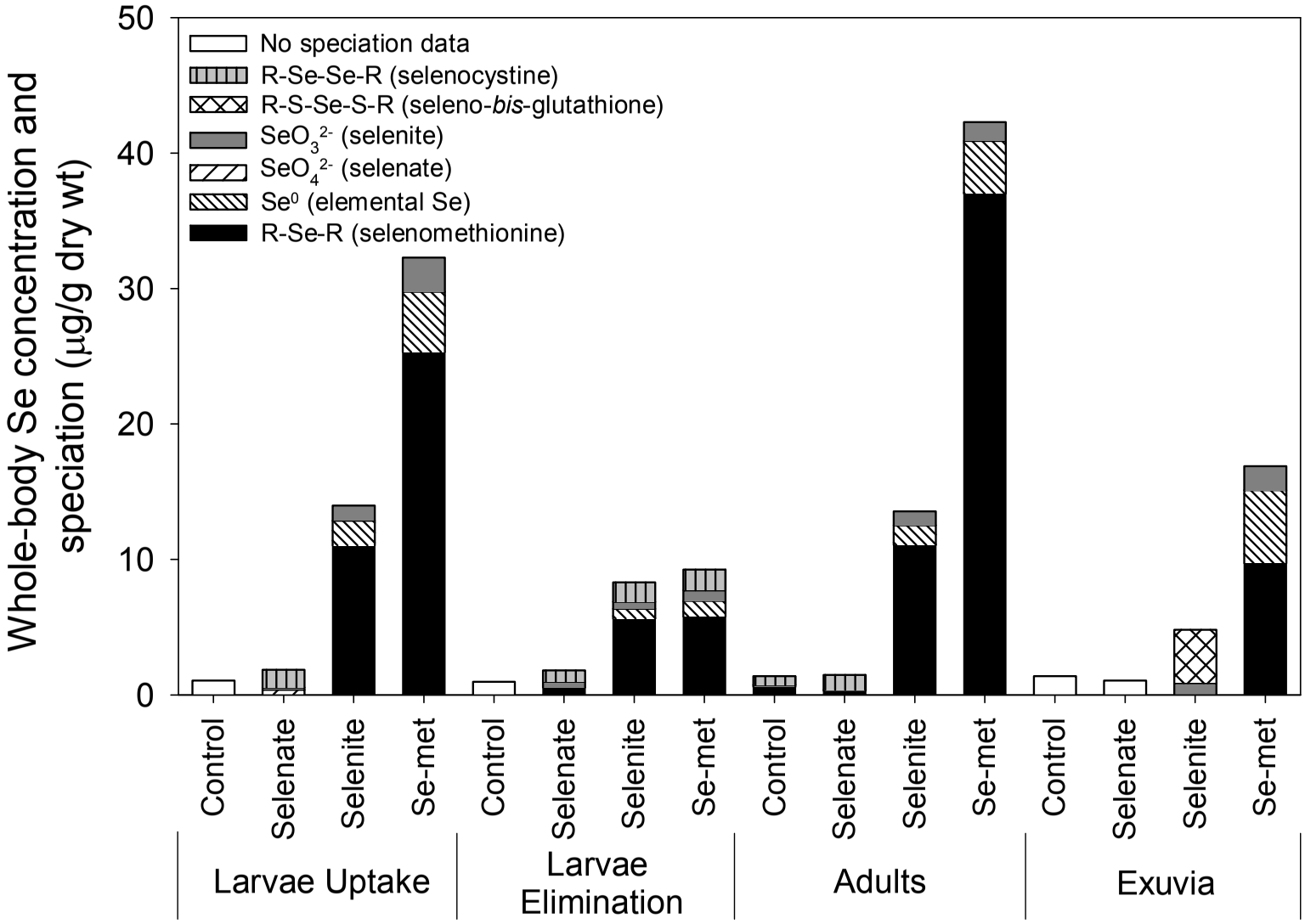

Figure 2.5: Whole-body selenium (Se) concentration and Se speciation in *Chironomus dilutus larvae*, adults, and exuvia after exposure of larvae to three different aqueous forms of Se (selenate, selenite, and seleno-DL-methionine (Se-met).

Table 2.2: Selenium (Se) speciation in *Chironomus dilutus* larvae, adults, and exuvia. Data are expressed as µg/g dry wt with the percent contribution of each Se standard listed in parentheses. The selenium standards used to derive the best fit for the selenium K-edge were SeO_4^{2-} (selenate), SeO_3^{2-} (selenite), Se^0 (red elemental selenium), R-Se-R (selenomethionine), R-Se-Se-R (selenocystine), and R-S-Se-S-R (seleno-bis-glutathione).

Exposure Phase	Treatment	SeO_4^{2-}	SeO_3^{2-}	Se^0	R-Se-R	R-Se-Se-R	R-S-Se-S-R
Larvae Uptake[a]	Selenate	0.37 (8)	0.17 (18)			1.32 (64)	
	Selenite		1.13 (8)	1.81 (12)	11.04 (76)		
	Se-met		2.52 (8)	4.44 (14)	25.32 (79)		
Larvae Elimination[a]	Selenate		0.18 (22)		0.22 (27)	0.35 (43)	
	Selenite		0.51 (6)	0.73 (8)	5.63 (63)	1.43 (18)	
	Se-met		0.77 (7)	1.16 (11)	5.78 (61)	1.54 (16)	
Adults	Control		0.13 (9)		0.60 (39)	0.65 (42)	
	Selenate		0.08 (5)		0.30 (20)	1.09 (70)	
	Selenite		1.04 (8)	1.46 (11)	11.05 (82)		
	Se-met		1.40 (3)	3.87 (9)	37.02 (84)		
Exuvia[a]	Selenite		0.87 (17)				3.94 (79)
	Se-met		1.76 (10)	5.39 (31)	9.73 (55)		

[a] No speciation data available for the control treatment from the larval uptake and elimination phases, as well as for control and selenate exuvia.

R-Se-R in larvae following 10 d of elimination (0.22 µg/g) and adult emergence (0.30 µg/g). Whether bacterial reduction of selenium occurred in the test media, in the guts of the chironomid larvae, or both is unknown. Gut bacteria in some mammalian species have demonstrated the ability to accumulate selenite and biotransform it into seleno-amino acids, making ingested selenite more bioavailable to the host organism (Whanger et al., 1978; Hudman and Glenn, 1984). Microbial transformation in the chironomid gut is a possible explanation for the presence of selenomethionine-like compounds in the chironomid larvae exposed to selenite; however, another explanation is that microbes present in the test media, or biofilm microbial communities formed on the sand substrate, absorbed selenite from the water and converted it into organic selenides. Larvae feeding at the sediment-water interface could have then accumulated Se-met through ingestion of these microbes.

In both the selenite and Se-met treatments, the fraction of organic selenides decreased in larvae during the period of elimination compared to larvae at the end of the uptake phase (Figure 2.5, Table 2.2). Organic selenides in larvae from the selenite treatment dropped from 76 to 63% during elimination, while the fraction of R-Se-R in larvae from the Se-met treatment dropped from 79 to 61%. The decrease in the fraction of organic selenides in both of these treatments is likely related to the elimination of selenium as observed in Figure 2.1b, as well as the catabolism of R-Se-R compounds (selenomethionine) which serves as a reserve supply of selenium for the synthesis of selenocysteine (Whanger et al., 1978). A similar study demonstrated that chironomid larvae can rapidly eliminate uranium once removed from the source of exposure (Muscatello and Liber, 2010). However, in such cases, rapid elimination is expected to occur when metals are in a free state in the organisms and not associated with tissues.

Adults from the selenite and Se-met treatments expressed R-Se-R as the dominant form of selenium in their tissues. In both treatments, the adults had more than 80% of the selenium present as organic selenides, with the remainder of the selenium present as elemental selenium and selenite. Similar fractions of organic selenide compounds were observed in the larvae from the selenite and Se-met treatments at the end of the uptake and elimination phases, indicating that the majority of selenium accumulated during the larval stage is retained in the adult insect without further biotransformation. The similarity between larval and adult speciation profiles was also observed in the selenate treatment, with R-Se-Se-R as the predominant selenium species. This suggests that little biotransformation of selenium occurs during metamorphosis, with the bulk of the selenium being retained by adult midges as organic selenides.

Selenium speciation data generated for *C. dilutus* exposed to selenite or Se-met in the present study are similar to spectra derived for aquatic invertebrates collected from downstream of industrial mining activities (Andrahennadi et al., 2007; Wiramanaden et al., 2010a). Aquatic invertebrates collected downstream of a coal-mining impacted site (Andrahennadi et al., 2007), with dissolved selenium concentrations ranging from 1.3 to 9.2 µg/L, had organic selenides as the greatest fraction of total selenium. Speciation analysis was not done on the surface water in that study; however, selenate was likely the dominant chemical form of selenium, as it tends to be the predominant selenium species under aerobic conditions. When comparing selenium speciation results, the spectra for invertebrates collected downstream of industrial mining activities most closely resemble the spectra for larvae exposed to selenite and Se-met in the present study. These speciation results further suggest that surface water exposure to selenate does not contribute directly to elevated whole-body concentrations of organic selenides (R-Se-R) in benthic invertebrates.

In another study, chironomid larvae collected from two lakes downstream of a uranium mining operation in northern Saskatchewan (Wiramanaden et al., 2010a) also displayed selenium speciation profiles similar to the spectra obtained for the selenite and Se-met treatments in the present study. The chironomid larvae samples from these lakes had 84% and 85% of the selenium present as the organic selenium species R-Se-R, modeled as selenomethionine, with the remaining fraction modeled as selenocystine. These results are similar to the selenite and Se-met treatments presented here, where organic selenides accounted for 76% and 79% of the whole-body selenium, respectively (Figure 2.5, Table 2.2). The dominant selenium species in the surface water of the two lakes was selenate (Wiramanaden et al., 2010b), yet the speciation profile for *C. dilutus* larvae exposed to selenium as selenate for 10 d in the present study showed no detectable R-Se-R. This observation is consistent with the understanding that dissolved selenate is not directly bioavailable to aquatic organisms, and that the dietary/sediment route of exposure is likely the dominant pathway of selenium accumulation into the benthic-detrital food chain in situ.

Overall, the use of standard bioaccumulation tests, along with synchrotron-based XAS methods, demonstrated the relationship between the bioavailability of different chemical forms of selenium in water and subsequent bioaccumulation and biotransformation in chironomids. At each stage of *C. dilutus* development, whole-body selenium speciation was dominated by organic selenides and diselenides, regardless of the speciation of selenium in the exposure water. However, the proportions of organic selenides to diselenides increased when the organisms were exposed to the more bioavailable forms of selenium, selenite and Se-met. Understanding the link between exposure to different chemical forms of selenium and selenium biotransformation in

invertebrates provides a better understanding of the exposure risks associated with fish and birds preying on these lower trophic level organisms in selenium-contaminated environments.

CHAPTER 3

AN IN SITU ASSESSMENT OF SELENIUM BIOACCUMULATION FROM WATER, SEDIMENT, AND DIETARY EXPOSURE PATHWAYS USING CAGED *CHIRONOMUS DILUTUS* LARVAE

This chapter will be submitted (with minor modification) to the journal Environmental Toxicology and Chemistry. The full citation is: Franz ED, Wiramanaden CIE, Gallego-Gallegos M, Tse JJ, Phibbs JR, Janz DM, Pickering IJ, Liber K. 2012. An in situ assessment of selenium bioaccumulation from surface water, sediment, and dietary exposure pathways using caged Chironomus dilutus larvae. Environmental Toxicology and Chemistry (in preparation).

3.1 Introduction

The potential adverse effects of elevated selenium concentrations in aquatic ecosystems have been well documented in several cases involving industrial, agricultural, and mining activities (Lemly, 2004). In northern Saskatchewan, Canada, uranium mining and milling operations have contributed to increased downstream concentrations of selenium in water, sediment and biota (Muscatello and Janz, 2009; Wiramanaden et al., 2010a). Selenium is an element of concern because of its propensity to bioaccumulate and cause reproductive and teratogenic effects for some sensitive species of fish and birds (Lemly, 1997b). Accumulation of selenium occurs primarily via the dietary pathway for top predators inhabiting freshwater systems (Presser and Luoma, 2010), and aquatic invertebrates are key to this trophic transfer of selenium since they serve as the primary food source for many fish and bird species (Lemly,

1993). A study investigating selenium accumulation in northern pike (*Esox lucius*) found a significant increase in deformities and edema in fry originating from female fish collected from two sites downstream of a uranium milling operation despite surface water selenium concentrations being less than 5 µg/L (Muscatello et al., 2006; Muscatello et al., 2008).

Characterizing selenium bioaccumulation in benthic organisms is challenging, given the diversity of site-specific physical, chemical, and biological processes that govern the distribution, mobility, and exposure pathways in benthic food webs. Selenium efficiently accumulates into aquatic food webs in freshwater environments characterized by long hydraulic retention times, high rates of primary productivity, and sediments rich in organic matter (Simmons and Wallschläger, 2005). Partitioning of selenium to sediment represents an important step in the accumulation of selenium within aquatic food webs because benthic invertebrates are known to accumulate selenium from sediment, with evidence suggesting that the microbial biomass fraction of sediment detritus is an important source of selenium for some species (Alaimo et al., 1994; Conley et al., 2009). While diet is considered the dominant route of selenium bioaccumulation for many freshwater invertebrates (deBruyn and Chapman, 2007; Luoma and Presser, 2009), aqueous exposure can contribute to selenium accumulation in some cases (Stewart et al., 2010; Franz et al., 2011). Reduced forms of selenium have been shown to accumulate within surface water and sediment pore water of lentic environments (Martin et al., 2011), and the release of organic selenium into surface water from decaying biological material has been postulated as a factor contributing to elevated selenium concentrations in benthic organisms inhabiting lentic environments (Orr et al., 2006).

The diversity of inorganic and organic forms (species) of selenium that can be present in the environment adds another level of complexity when assessing the risk selenium poses to

aquatic organisms. Each form of selenium differs in its bioavailability and potential to cause toxicity; therefore, knowledge of the selenium species present in the environment is essential for accurately defining and predicting the risk selenium poses to aquatic organisms (Unrine et al., 2007). Advances in spectroscopy techniques utilizing synchrotron-generated X-rays have proven successful for detecting organic and inorganic selenium species in environmental samples, making this technique a powerful tool for investigating the distribution, bioavailability, and bioaccumulation of selenium in aquatic systems (Wiramanaden et al., 2010b; Andrahennadi et al., 2007).

The principal research objectives of this study were to identify the dominant exposure pathways and selenium species contributing to selenium bioaccumulation in benthic macroinvertebrates downstream from the Key Lake uranium milling operation in northern Saskatchewan, Canada. Using a modified in situ caging method with larvae of the midge *Chironomus dilutus*, we assessed selenium accumulation from surface water and sediment/dietary exposure pathways at several locations within the drainage basin. X-ray absorption spectroscopy (XAS) analysis was used to assess selenium speciation in caged *C. dilutus* larvae exposed to selenium from surface water, biofilm, and sediment pathways.

3.2 Materials and methods

3.2.1 Study site

The study was conducted during the summers of 2008 and 2009 downstream of the Key Lake uranium milling operation in northern Saskatchewan, Canada (57°11'N, 105°34'W; see Figure 1.1). Treated metal mining effluent is discharged into Wolf Lake which flows into Fox Lake (FL, high exposure site). Downstream of Fox Lake is Unknown Lake (UL, medium exposure site), which receives drainage from both Fox Lake and David Lake. David Lake (DL)

served as the reference location for the 2008 study because of its upstream location from the effluent discharge point. Six sites, located in David, Fox, and Unknown Lakes were selected as the study sites in 2008 to compare sediment vs. surface water exposure pathways of selenium bioaccumulation by *C. dilutus* larvae (Figure 3.1). Sediment chemistry data from previous investigations were used to select sites that capture the heterogeneity of sediment composition and selenium concentrations within Fox and Unknown Lakes (Wiramanaden et al., 2010a). Consideration was also given to the depth of the water at each site, because of the logistical challenges of deploying the in situ chambers in water depths greater than 2 m. Unknown Lake site 1 (UL1) was chosen in 2009 to evaluate in greater detail Se bioaccumulation from the sediment/dietary pathway in caged *C. dilutus* larvae. Reference sediment/detritus for this experiment was collected from a location in Yeoung Lake (YL).

3.2.2 Test organisms

The chosen test organism for this study was *C. dilutus*, obtained from an in-house culture at the Toxicology Centre. Larvae were cultured from egg masses deposited within 48 h by adult midges held in a breeding jar. Egg masses were then transferred to aquaria (3 to 4 egg masses per aquarium) containing a thin layer of silica sand (particle size 425 – 850 μm) to allow the larvae to hatch. Aquaria were kept in an environmental chamber with a photoperiod of 16:8 h light:dark and a temperature of 25 ± 1ºC. Carbon-filtered, bio-filtered municipal water was used for culturing the larvae, and one water change was performed during the culturing period to maintain good water quality. Larvae were cultured for 8 to 10 d post-hatch, prior to beginning the in situ exposure experiment in 2008. For the 2009 study, larvae were cultured until 12 to 14 d post-hatch to allow more growth (greater body mass) prior to beginning the experiment.

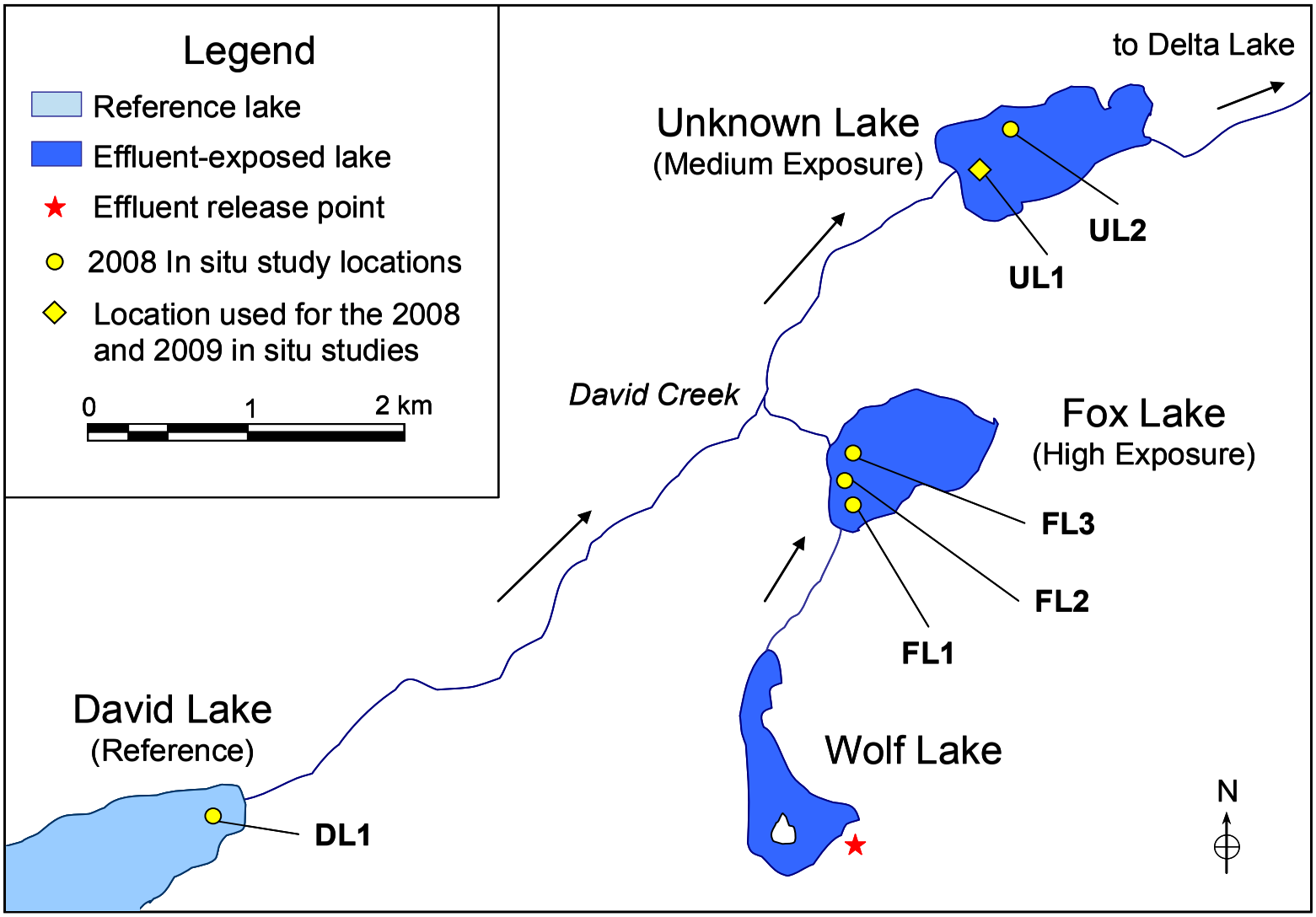

Figure 3.1: Location of the sampling sites within the David Creek drainage basin used for the 2008 and 2009 in situ selenium bioaccumulation studies (Key Lake, SK, Canada). FL = Fox Lake (high exposure). UL = Unknown Lake (medium exposure). DL = David Lake (reference). Arrows indicate direction of water flow.

During transportation to the Key Lake facility, water holding the test organisms was aerated to ensure adequate dissolved oxygen levels in the transport vessels.

3.2.3 In situ chamber design and deployment

Test chambers were designed to assess the dominant site-specific pathways (water vs. sediment) of selenium bioaccumulation in *C. dilutus*. Each chamber was constructed out of clear acrylic tubing (Figure 3.2) measuring 5 cm in diameter and 15 cm in length, similar to designs utilized by Robertson and Liber (2007). Three rectangular openings (3 x 5 cm) were cut in the chamber and covered with 300 µm Nitex® nylon screen (Wildlife Supply Company, Buffalo, NY, USA) to allow water exchange between the chamber and the surrounding surface water. The type of sediment substrate in the chambers, which served as the habitat and food source for the test organisms, was chosen based on the objective of the specific in situ exposure treatment. Once the sediment substrate was in place with a bottom core cap secured (see details below), the test organisms were gently transferred to the chamber and another core cap placed over the top of the chamber. This cap had a small opening covered with Nitex® mesh to allow air bubbles to escape during deployment of the chamber. Each chamber was fastened to a wooden stake using plastic zip ties, and inserted into the sediment so that the sediment surface inside the chamber aligned with the surrounding sediment-water interface (Figure 3.2). No food was artificially added to the test chambers throughout the duration of the test.

3.2.4 Abiotic sample collection and exposure characterization

Surface water, pore water, and sediment were collected from each site in 2008 and 2009. Samples were analyzed for water quality, sediment composition, and selenium to support

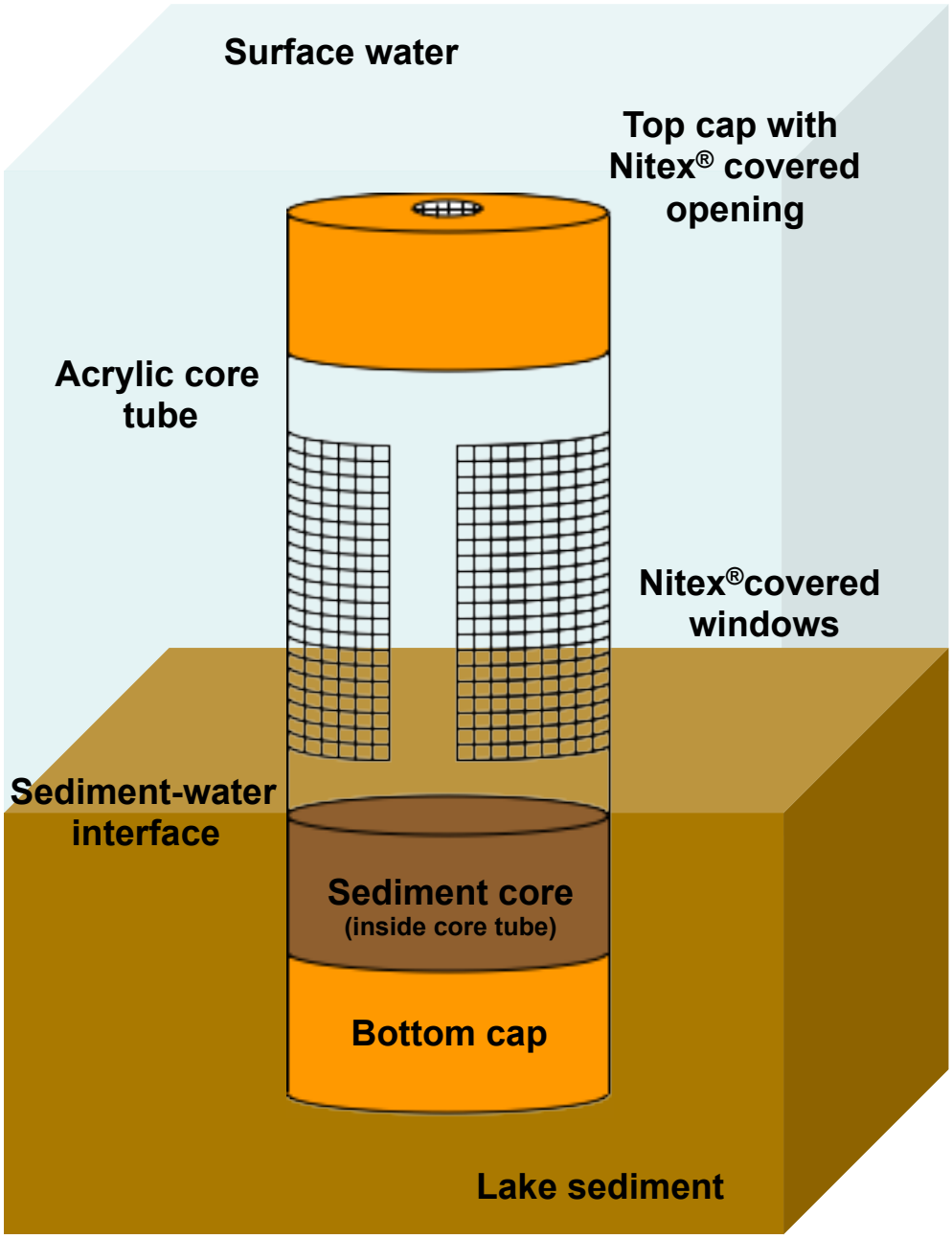

Figure 3.2: Illustration showing the design and deployment of an in situ chamber for assessing surface water and sediment exposure pathways using caged *Chironomus dilutus* larvae (Nitex®, Wildlife Supply Company, Buffalo, NY, USA).

findings from the in situ experiments; as well as to help identify possible relationships between selenium partitioning in the abiotic environment and selenium bioaccumulation in *C. dilutus* larvae. In 2008, on-site water measurement of DO and temperature were taken with a Thermo Orion® dissolved oxygen meter (model 835; Thermo Scientific, Beverly, MA, USA). For the 2009 study, a multi-meter probe (6-Series; YSI Incorporated, Yellow Springs, OH, USA) was used to measure DO, temperature, conductivity, and pH on-site at the beginning of the in situ experiment. Triplicate surface water samples for chemistry and water quality were collected both years at a depth of approximately 30 cm above the sediment surface using a Van Dorn horizontal water sampler (Wildlife Supply Company, Buffalo, NY, USA). The samples were stored in acid-washed high density polyethylene (HDPE) bottles that were rinsed three times with site water sieved through a 53 µm screen. At the on-site laboratory, subsamples were removed and stored in HDPE bottles for analysis of dissolved selenium concentration, dissolved organic carbon (DOC) content, general water quality, and major ions. Samples for dissolved selenium and DOC analysis were filtered through a 0.45 µm membrane (Supor® polyethersulfone (PES); Pall Corporation, Ann Arbor, MI, USA). The water sample for dissolved selenium analysis was acidified to 2% final concentration of double distilled nitric acid (omnitrace ultra; EM Science, Gardena, CA, USA) prior to analysis. Surface water samples collected for general water quality were stored in HDPE bottles with no headspace and analyzed within one week of returning to the Toxicology Centre for conductivity (ORION ATI® conductivity meter, model 170; Thermo Scientific, Beverly, MA, USA), pH (ORION PerpHecT® LogR® meter, model 370; Thermo Scientific, Beverly, MA, USA), alkalinity and hardness (Hach 16900 Digital Titrator; Hach Company, Loveland, CO, USA) ammonia (VWR Symphony SB301 pH/ISE meter; VWR International, Mississauga, ON), and organic carbon (Shimadzu

TOC-5050A organic carbon analyzer; Mandel Scientific, Guelph, ON). Major ion analyses were conducted on one surface water sampler for each study site in 2008 (ALS Laboratories, Saskatoon, SK).

Sediment pore water was collected at all study sites using dialysis sampling devices (peepers). These peepers were constructed of acrylic as described previously (Robertson and Liber, 2009), and designed to vertically sample pore water in the sediment at 2 cm depth intervals. Prior to deployment, the peeper chambers were filled with ultrapure water (Barnstead® NANOpure), covered with a 0.45 µm PES membrane, placed in a clean 20 L bucket containing NANOpure water, and aerated with nitrogen gas for 24 hrs. Peepers (n = 3 in 2008, n = 4 in 2009) were inserted vertically into the sediment at each site with one chamber exposed to the overlying water immediately above the sediment-water interface. The devices were left to equilibrate with the pore water for 10 d, after which time they were retrieved, and water samples from each chamber were collected for dissolved selenium and DOC analysis. Surface water within the chamber above the sediment-water interface was defined as the overlying water, while pore water measurements were taken from the first chamber completely in the sediment. Water samples for dissolved selenium and DOC were analyzed according to the procedures described for surface water.

Whole-sediment and detritus samples were collected in acrylic core tubes using a hand-held corer (constructed in-house). Three cores (5 cm outside diameter) were collected from each site. Each core tube was filled with site water to ensure there was no head space prior to securing the top core cap. Sediment cores were transported and stored at 4°C and were processed at the Toxicology Centre within 10 d of collection by extruding and homogenizing the top 2.5 cm of sediment. In 2009, sediment detritus was collected from UL1 and Yeoung Lake

(YL; reference area) by extruding and homogenizing the top 0.5 cm of the sediment core. Subsamples were then taken and oven dried at 60°C for total selenium and TOC analysis. In addition, one replicate per site was used for sediment selenium speciation and kept at -80°C until further processing. Particle size was determined for whole-sediment based on a composite of the three replicates from each site (ALS Laboratories, Saskatoon, SK). Particle size was not determined for detritus from UL1 and YL in 2009 because of insufficient sample mass. Sediment and detritus selenium concentrations were determined using a microwave digestion method optimized at the Toxicology Centre followed by analysis using collision cell inductively coupled plasma-mass spectrometry (ICP-MS). Total organic carbon in the sediment was analyzed using a Leco Carbon Determinator (CR-12; Leco, St. Joseph, MI).

Sediment biofilm was collected from ten artificial substrates that were deployed 21 d prior to the experiment near site UL1 in 2009. The substrates were constructed from acrylic plastic sheets (30 x 23 cm), with six holes (1 cm diameter) to allow primary producers to colonize the substrate surface from the sediment. After the 21 d culture period, the biofilm substrates were retrieved and gently rinsed with surface water from the site to remove any particulate inorganic material that may have settled on the substrate during the colonization period. Biofilm from five of the substrates were used as the food source in one of the treatments during the in situ bioaccumulation study (Section 3.2.5.2), while biofilm from the remaining artificial substrates were processed for selenium (total and speciation) and TOC analyses. These substrates were kept in coolers containing site water during transport back to the Toxicology Centre where they were processed by lightly scraping the biofilm into individual sample bottles (HDPE). Due to the small amount of biofilm, subsamples from each of the five replicates were pooled to obtain one sample each for TOC and selenium speciation analysis. Biofilm samples

for total selenium analysis were oven dried (60°C) for several days, subsequently digested using nitric acid (5 ml) and hydrogen peroxide (1.4 ml; 30% Omnipure, VWR International, Mississauga, ON, Canada), and analyzed for selenium (Section 3.2.6.1). The sample used for speciation analysis was stored at 4°C in site water for 3 d prior to processing and analysis (Section 3.2.6.2). No taxonomic identification was undertaken on the biofilm sample due to the limited amount of sample available for analysis.

3.2.5 In situ selenium bioaccumulation testing

3.2.5.1 Surface water vs. sediment exposure

The 2008 study focused on assessing the relative importance of sediment vs. surface water exposure routes on selenium bioaccumulation by *C. dilutus* larvae. Three exposure treatments were compared in the 2008 study: selenium accumulation from surface water only (SW), selenium accumulation from sediment only (SED), and selenium accumulation from surface water and sediment combined (SW+SED). Site-specific sediment was used as the substrate in the SW+SED treatments, while the SW treatments were carried out using reference sediment from David Lake (DL1) as the substrate. Sediment was collected to a depth of approximately 2.5 cm using a hand-held corer (constructed in-house) with the actual in situ chamber serving as the core tube. Each sediment core was measured to ensure a consistent amount of sediment was collected for each chamber. Once a suitable core was collected, a core cap was placed on the bottom of the chamber to prevent the sediment core from falling out. This method of collection minimized sediment manipulation and preserved the sediment profile. With the bottom cap in place, 10 *C. dilutus* larvae were transferred to the chamber, and another core cap fit over the top opening. For the surface water exposures, reference sediment cores from DL1 were collected in each in situ chamber and transported in DL surface water to each

test site where they were deployed within 5 m of the sediment exposure chambers. The SED treatment involved collecting sediment from FL2 into test chambers and transferring the chambers to David Lake (reference site), which had a very low surface water selenium concentration. Four replicate chambers were deployed for each treatment at each site.

Following a 10-d exposure period, the wooden stakes were retrieved and in situ chambers removed prior to being transported back to the field lab in site-specific water for processing. The contents of each chamber were sieved to retrieve the *C. dilutus* larvae, after which they were transferred to a clean test beaker and rinsed three times with de-ionized water to remove any sediment particles. Test larvae were then placed in a clean aluminum weigh pan, sealed in a petri dish, and kept at -20°C until arrival back at the Toxicology Centre, University of Saskatchewan. *Chironomus dilutus* test larvae were dried at 60°C for a minimum of 48 hrs, and digested using nitric acid and hydrogen peroxide as previously described for the biofilm samples.

3.2.5.2 Sediment/dietary accumulation patterns

The follow-up study in 2009 investigated, in greater detail, the importance of selenium bioaccumulation from the sediment/dietary route of exposure. Four in situ exposure treatments were compared at one location in Unknown Lake (UL1; Figure 3.1): surface water only, biofilm, sediment detritus, and whole-sediment. Six in situ chambers (10 larvae per chamber) were deployed for each treatment and retrieved after 10 d as described for the 2008 study. Three individual replicates were prepared for whole-body selenium analysis according to the method outlined for the 2008 experiment. Larvae from the remaining three replicates per treatment were pooled to obtain enough sample for selenium speciation analysis using XAS.

The experimental method was similar to the 2008 experiment, with a few modifications adapted specifically for testing different fractions of the sediment at UL1. For the surface water

only treatment, approximately 2.5 cm of clean silica sand (particle size 250 – 425 µm) was used as the substrate, with 2 to 3 mm of detritus from the reference location in YL. A similar procedure was used in the sediment detritus treatment where clean sand was overlaid with detritus from UL1 to provide a source of dietary selenium exposure. Selenium bioaccumulation from whole-sediment was evaluated using the procedure described for the 2008 study. Sediment biofilm used in the biofilm treatment was collected from five artificial substrates after the 21 d culture period (Section 3.2.4). Upon retrieval of the substrates, the adhered biofilm was carefully scrapped from the surface of the substrate that faced the water column and transferred to clean sample bottles. Subsequently, 2 to 3 mm of the cultured biofilm were laid overtop of 2.5 cm of sand in the in situ exposure chambers to provide a source of food for the test organisms.

Resident chironomid larvae from UL1 were also collected in 2009 for comparison to selenium concentration and speciation results in the in situ treatments. Benthic invertebrates were collected using a standard Ekman grab sampler (6" x 6"; Wildco, Buffalo, NY, USA) and a sieve bucket (500 µm). Chironomid larvae were sorted from the rest of the benthic invertebrates at the field-laboratory at Key Lake. Only three replicate samples were collected due to low overall invertebrate density at this site. Two replicates (n = l2 larvae/replicate) were kept for total selenium analysis and processed according to the same method as the in situ *C. dilutus* larvae. The remaining replicate was used to determine selenium speciation in resident chironomid larvae.

3.2.6 Selenium analyses

3.2.6.1 Total selenium

Analysis of total selenium in the surface water, sediment, pore water, and chironomid larvae was carried out using an ICP-MS operated in collision cell mode (ICP-MS; X Series II

Thermo Electron Corporation, Waltham, MA, USA). Certified reference material (CRM) and method blanks were analyzed every ten samples to validate the analytical method. Average selenium recovery for all analyses was greater than 90% based on analysis of a freshwater CRM (1643e; National Institute of Standards and Technology, Gaithersburg, MD, USA). Tort-2 CRM was used to verify the tissue digestion method, and PACS-2 marine sediment was used to verify the sediment digestion method (National Research Council Canada, Ottawa, ON, Canada). Method limits of detection (LoD) for surface water and pore water ranged from 0.11 µg/L to 0.65 µg/L. The LoD for selenium in tissue was 0.11 µg/g in 2008 and 0.04 µg/g in 2009. Limits of detection for sediments were 0.02 mg/kg in 2008 and 1.02 mg/kg in 2009. Yeoung Lake detritus collected in 2009 measured below the LoD, so half the LoD was used as an estimate for the total selenium concentration.

3.2.6.2 Selenium speciation

X-ray absorption spectroscopy was used to provide insight into the chemical speciation of selenium in the sediment from sites sampled in 2008. In addition to speciation analysis of the sediment, detritus, and biofilm, the 2009 study was expanded to include speciation analysis of the test larvae exposed in situ and resident chironomids from UL1. Sample handling, preparation, and analysis was carried out according to procedures described elsewhere (Wiramanaden et al., 2010b). Sediment, detritus, and larval samples were kept frozen and ground using an agate mortar and pestle cooled in liquid nitrogen. Ground sediment was then packed into a custom XAS cuvette (2 mm), sealed with a drop of liquid glycerol, and stored frozen in liquid nitrogen. Biofilm from UL1 was processed by transferring a sample directly from the acrylic substrate, which was submerged in site water, to the XAS cuvette. This method

minimized the time between removal of the biofilm from the site water and freezing the sample in liquid nitrogen.

Speciation data for the 2008 sediment samples were generated at the Canadian Light Source (CLS) on the Hard X-ray Micro-Analysis (HXMA) beamline. Data from the 2009 study were generated on the structural molecular biology beamline 9-3 at the Stanford Synchrotron Radiation Lightsource (SSRL). X-ray absorption spectroscopy uses synchrotron generated X-ray photons to excite a core selenium electron resulting in the emission of a fluorescent X-ray. Absorption spectra for selenium are generated by measuring the X-ray fluorescence given off after the decay of a higher-level electron. The near-edge region of the spectra provides information about the electronic environment around selenium present in a given sample. In this way, each spectrum is a fingerprint of the chemical types of selenium present, indicating the oxidation state and local coordination, though not the explicit molecule. Standards of known selenium species were then fit to the spectra to determine the percent contribution of the individual selenium standards using EXAFSPAK software (George and Pickering, 1995). Specific details concerning the generation of selenium speciation spectra have been published elsewhere (Andrahennadi et al., 2007; Wiramanaden et al., 2010b).

3.2.7 Statisical analysis

Data analyses were performed using SigmaPlot® version 11 (Systat Software, Inc.) with a 95% (α=0.05) confidence level. Significant differences in larval survival between surface water and sediment exposures at each site (2008 study) were evaluated using a Student's t-test. This test was also used to determine differences between surface water and overlying water selenium concentrations at each site. Significant differences in larval whole-body selenium at each study site were determined using a one-way ANOVA. Data that failed testing for normality or equal

variance were $\log_{10}$-transformed prior to statistical analysis. Dunnett's post-hoc test was used in the 2009 study to compare selenium concentrations of larvae from each exposure treatment against the surface water exposure group. Linear regression, with a confidence level of $\alpha = 0.05$, was used to determine the relationships between sediment selenium and TOC, *C. dilutus* whole-body selenium and sediment selenium, and *C. dilutus* whole-body selenium and pore water selenium

3.3 Results

3.3.1 Surface water quality

Water bodies in the near-field area downstream of the Key Lake uranium milling operation are characterized by elevated surface water concentrations of major ions, hardness, conductivity, ammonia, and other constituents, as a result of the release of treated metal mining and milling effluent (Table 3.1 and Table 3.2). Fox Lake had the highest concentration of selenium for the 2008 experiment, with average surface water selenium concentrations collected from the three locations (FL1, FL2, and FL3) ranging from 11.99 to 12.37 µg/L at a depth of 30 cm above the sediment surface (Table 3.3). Surface water flowing out of Fox Lake is diluted with water from David Creek prior to entering Unknown Lake, which results in lower surface water concentrations of selenium in Unknown Lake (Table 3.3). Surface water collected from Unknown Lake site 1 (UL1) in 2008 had a concentration of 3.93 ± 0.03 µg/L; in 2009, the concentration measured 3.67 ± 0.10 µg/L. Water samples collected approximately 1 to 2 cm above the sediment surface were higher in dissolved selenium than surface water samples collected 30 cm above the sediment for both the 2008 and 2009 experiments. Unknown Lake also had higher concentrations of selenium in the overlying water compared to the surface water, but only UL2 in 2008 was significantly higher ($p \leq 0.001$; Table 3.3).

Table 3.1: Water quality parameters measured at the locations in David Lake (DL, reference), Fox Lake (FL, high exposure) and Unknown Lake (UL, medium exposure) chosen for the in situ selenium bioaccumulation experiment in 2008.

Parameter[a]	Units	David Lake	Fox Lake			Unknown Lake	
		DL1[c]	FL1	FL2	FL3	UL1	UL2
Water Quality							
Temperature[b]	ºC	14.6	18.6	18.9	18.8	18.9	18.3
DO[b]	mg/L	8.9	8.3	8.4	8.3	8.3	8.1
pH	units	6.1	6.0 ± 0.0	6.1 ± 0.0	6.2 ± 0.1	5.3 ± 0.0	5.4 ± 0.0
Conductivity	µS/cm	13	1809 ± 8	1796 ± 15.3	1803 ± 14	762 ± 2	705 ± 1
Hardness	mg/L	5	930 ± 10	896 ± 12	887 ± 6	327 ± 6	313 ± 25
Alkalinity	mg/L	8.0	7.3 ± 0.6	7.7 ± 0.6	7.3 ± 0.6	6.7 ± 1.2	7.7 ± 1.2
Ammonia (N)	mg/L	0.1	11.3	11.2	11.2	11.2	2.7
Major Ions							
Bromide	mg/L	n/a	0.11	0.11	0.11	0.11	n/a
Calcium	mg/L	0.90	282	280	279	279	100
Chloride	mg/L	1.15	35.2	35.2	35.2	35.2	12.9
Fluoride	mg/L	0.02	0.60	0.64	0.65	0.65	0.30
Lithium	mg/L	0.01	0.47	0.46	0.47	0.47	0.25
Magnesium	mg/L	0.32	13.8	13.7	13.7	13.7	5.0
Nitrate	mg/L	0.28	8.3	8.0	8.5	8.5	4.0
Potassium	mg/L	0.33	25.4	25.2	25.3	25.3	9.0
Sodium	mg/L	0.76	37.0	36.8	36.8	36.8	13.4
Sulfate	mg/L	0.47	996	993	991	991	353

[a] One sample collected for analysis of major ions. Triplicate water samples collected for water quality.
[b] Surface water temperature and dissolved oxygen (DO) measured directly at each location.
[c] Only one water sample collected for analysis of water quality and major ions from David Lake.

Table 3.2: Water quality parameters for the 2009 in situ bioaccumulation study measured on-site at Unknown Lake (UL1) and at the laboratory. Laboratory data are presented as the mean ± 1 SD (n = 3).

Water Quality Parameter	Units	Unknown Lake (UL1)	
		On-site[a]	Laboratory[b]
Temperature	ºC	16.7	
DO[c]	mg/L	9.16	
pH	units	4.84	4.95 ± 0.11
Conductivity	µS/cm	655	703 ± 11
Hardness	mg/L		297 ± 4
Alkalinity	mg/L		12.6 ± 1.2
Ammonia (N)	mg/L		1.44 ± 0.04

[a] Measured on-site using an YSI multi-meter probe.
[b] Analyses conducted at the Toxicology Centre, University of Saskatchewan.
[c] Dissolved oxygen.

Table 3.3: Concentrations of selenium (µg/L) in surface water, pore water, and sediment from the sites chosen for the 2008 and 2009 in situ bioaccumulation studies in David Lake (DL, reference), Fox Lake (FL, high exposure), and Unknown Lake (UL, medium exposure). Data are presented as the mean ± 1 SD (n = 3).

Parameter[a]	David Lake	Fox Lake			Unknown Lake	
	DL1	FL1	FL2	FL3	UL1	UL2
2008 Bioaccumulation Study						
Surface water[a]	< LoD[c]	12.37 ± 0.09	11.99 ± 0.02	12.15 ± 0.04	3.93 ± 0.03	3.51 ± 0.11
Overlying water[b]	0.16[d]	17.89 ± 0.39[†]	14.95 ± 0.40[†]	12.84 ± 0.24[‡]	4.19 ± 0.32	4.73 ± 0.07[†]
Pore water	0.11[d]	16.69 ± 0.26	7.14 ± 1.02	12.04 ± 1.33	3.71 ± 1.91	4.10 ± 0.69
2009 Bioaccumulation Study						
Surface water					3.67 ± 0.10	
Overlying water					4.75 ± 1.04	
Pore water					6.63 ± 1.16	

[a] Collected 30 cm above the sediment surface (grab sample).
[b] Collected from the top chamber of the sediment pore water sampler (approximately 1 to 2 cm above the sediment surface).
[c] Limit of detection (LoD) = 0.65µg/L for the surface water sample.
[d] The mean of two samples. No SD calculated.
Significantly different from the surface water selenium concentration at each site ([†] $p < 0.001$; [‡] $p < 0.01$).

3.3.2 Sediment and pore water

Sediment selenium concentrations were generally lowest for lakes furthest from the final discharge point of the effluent at Key Lake; however, the pattern of selenium distribution in sediment is more complex than in overlying surface water. There is a high degree of spatial variability in sediment selenium concentrations downstream of the milling operation, and this variability occurs both among lakes, and among sites within the same lake. In 2008 the highest selenium concentration was found in sediment collected from FL1, near the inflow of the lake, which was nearly 5-fold greater than the selenium concentration in sediment from FL2 and FL3 (Table 3.3). Sediment selenium concentrations were similar among FL2, FL3, and UL1 at 5.10 ± 0.91, 5.33 ± 1.23, and 5.50 ± 1.49 mg/kg, respectively (Table 3.4).

Selenium concentrations in sediment pore water were higher at all sites in Fox Lake compared to Unknown Lake in 2008, a direct result of being further away from the final discharge point at the Key Lake operation (Table 3.3). The variability of pore water selenium concentration within each study site was low at all sites during the 2008 study, except FL2. Whole-sediment selenium was not a strong predictor of pore water selenium concentration (r^2 = 0.65, $p > 0.05$) (data not shown).

For the 2009 study, UL1 was chosen to further investigate the uptake pathways for selenium accumulation, and resulting whole-body selenium speciation, in *C. dilutus* larvae exposed to different components of lake sediment in situ. Mean selenium concentrations in the whole-sediment were 13.89 ± 7.43 mg/kg in 2009 compared to 5.50 ± 1.49 mg/kg 2008. These data indicated there is considerable spatial variability in sediment selenium concentration in this lake, even within a relatively small area (Table 3.5). The detrital fraction of the whole-sediment sample collected from UL1 was highest in selenium concentration (23.71 ± 3.99 mg/kg),

Table 3.4: Sediment composition and selenium concentrations from the 2008 in situ bioaccumulation study site locations in David Lake (DL), Fox Lake, (FL, high exposure) and Unknown Lake (UL, medium exposure). Data are presented as the mean ± 1 SD (n = 3).

Parameter	Units	David Lake	Fox Lake			Unknown Lake	
		DL1	FL1	FL2	FL3	UL1	UL2
Moisture	%	89.4	71.5	28.9	33.2	62.2	52.1
Sand	%	38	65	99	99	86	84
Silt	%	59	30	1	1	10	13
Clay	%	3	5	<1	<1	4	3
TOC[a]	%	31.2 ± 0.9	5.6 ± 1.4	0.7 ± 0.1	0.8 ± 0.1	5.0 ± 2.4	2.4 ± 1.0
Selenium	mg/kg	0.86 ± 0.09	24.13 ± 6.73	5.33 ± 1.23	5.10 ± 0.91	5.50 ± 1.49	2.33 ± 0.13

[a] Total organic carbon.

Table 3.5: Sediment composition and selenium concentrations from the 2009 in situ bioaccumulation study downstream from the Key Lake uranium operation in Unknown Lake. Data are presented as the mean ± 1 SD (n = 3).

Parameter	Units	Yeoung Lake	Unknown Lake		
		Reference Detritus	Whole Sediment	Detritus	Biofilm
TOC[a]	%	11.02 ± 2.98	11.22 ± 4.75	15.80 ± 0.47	9.62[b]
Selenium	(mg/kg)	<LoD	13.89 ± 7.43	23.71 ± 3.99	12.29 ± 4.41

[a] Total organic carbon.
[b] Composite of five samples due to insufficient amounts of biofilm for TOC analysis.

followed by whole-sediment (13.89 ± 7.43 mg/kg), and sediment biofilm (12.29 ± 4.41 mg/kg) (Table 3.5). Detritus collected from a nearby reference lake (Yeoung Lake) served as the low selenium (< LoD, Table 3.5) food source for the water-only exposure. Pore water selenium content at this site was slightly higher in 2009 compared to 2008, again related to the spatial heterogeneity in sediment selenium concentrations at this site.

Organic carbon in the sediment was at or below 5% for all downstream study locations in 2008 (Table 3.3). Previous work at these locations demonstrated a strong positive correlation between TOC and sediment selenium when TOC content was above 10%; however, this relationship became weaker at sites where TOC content was below 10% (Wiramanaden et al., 2010a). In 2008, selenium concentrations in whole-sediment correlated well with TOC content at both Fox Lake ($r^2 = 0.95$, $p < 0.001$) and Unknown Lake ($r^2 = 0.72$, $p < 0.05$) (Figure 3.3a). The regression line slope was less steep for Unknown Lake compared to Fox Lake, which is likely a reflection of lower concentration of selenium in Unknown Lake (Figure 3.3a). Figure 3.3b shows that a positive correlation exists between selenium concentration and TOC content in whole-sediment, detritus, and biofilm samples collected from UL1 during the 2009 study.

3.3.3 Speciation analysis of sediment-associated selenium

Whole-sediment collected from each site in 2008 was analyzed for selenium speciation in order to provide a more detailed understanding of selenium uptake by chironomid larvae from sediment exposure. Selenium K near-edge spectra were generated for one sediment sample from each study site using XAS. Near-edge spectra of environmentally relevant selenium standards were then fit to each sediment spectra to identify the fractional selenium species composition in the sediment at each site. Figure 3.4a shows the contribution of each selenium standard to the total selenium content in the sediment at each in situ study site in 2008. Elemental selenium was

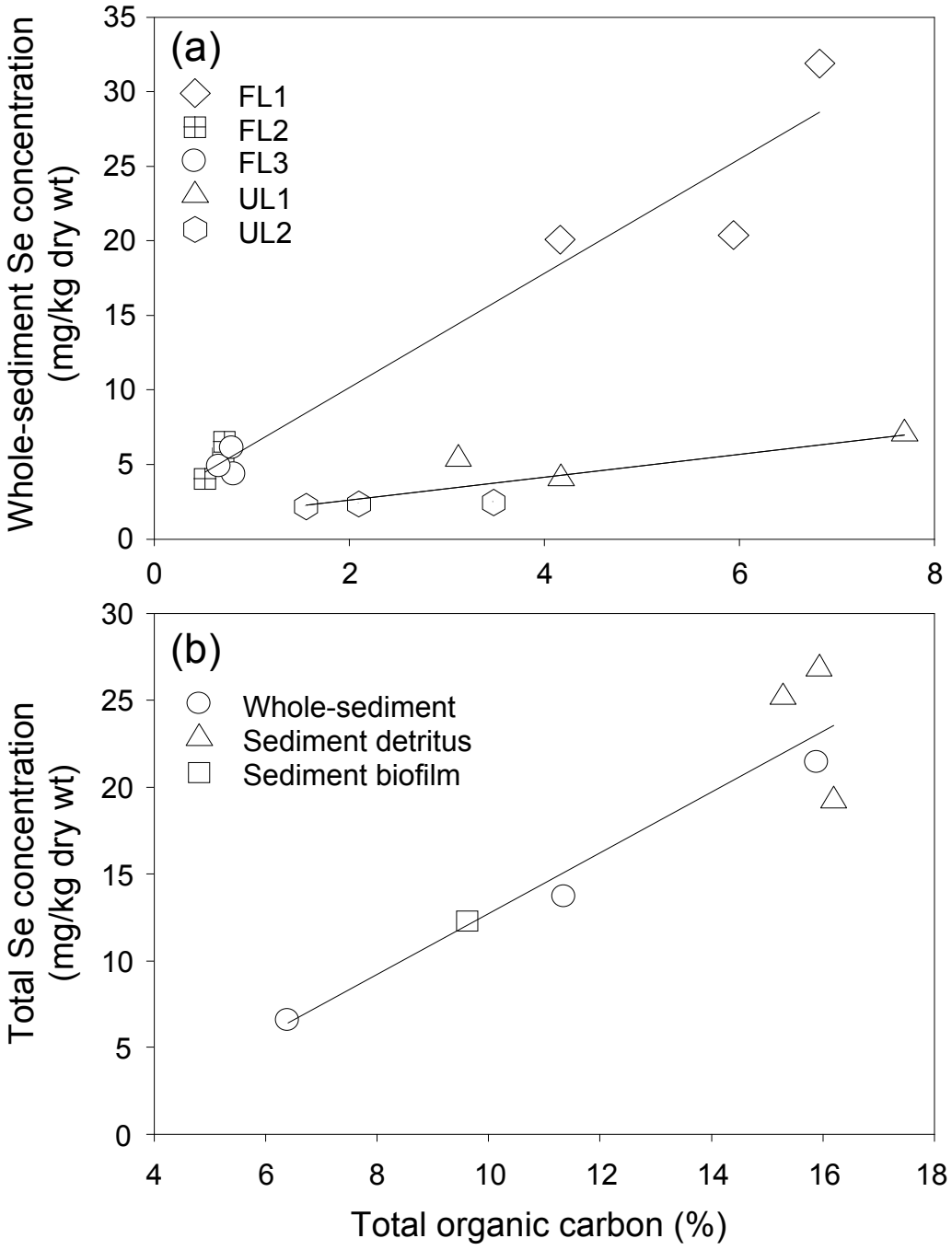

Figure 3.3: The relationship between sediment selenium (Se) concentrations and total organic carbon content (TOC) for study sites downstream of the Key Lake uranium operation, SK, Canada. (a) Whole-sediment Se concentration as a function of TOC for the study sites in Fox Lake (FL) (r^2 = 0.95, p < 0.001) and Unknown Lake (UL) (r^2 = 0.72, p < 0.05) in 2008. (b) Total Se concentrations as a function of TOC in whole-sediment, sediment detritus, or sediment biofilm at Unknown Lake site 1 in 2009 (r^2 = 0.85, p < 0.01).

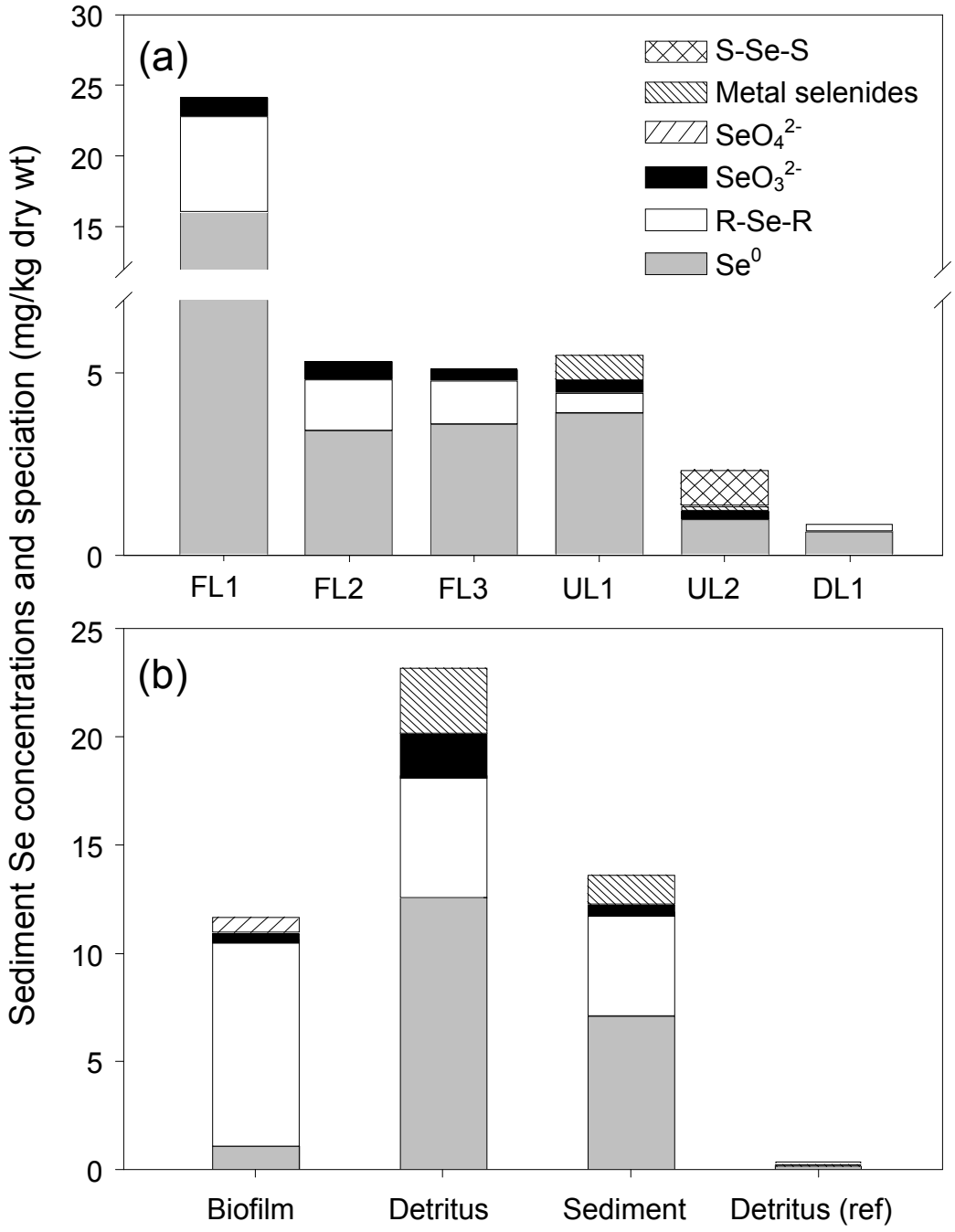

Figure 3.4: Mean concentration of different selenium (Se) species in (a) whole-sediment collected from sites within Fox Lake (FL), Unknown Lake (UL), and David Lake (DL) in 2008, and in (b) biofilm, detritus, and sediment from Unknown Lake site 1 (UL1) and reference (ref) detritus from Yeoung Lake in 2009. Selenium standards included in the fit are seleno-bis-glutathione (S-Se-S), selenate (SeO_4^{2-}), selenite (SeO_3^{2-}), selenomethionine (R-Se-R), and elemental Se (Se^0).

the most abundant selenium species in the sediment at each site, ranging from 42 to 76% of the total selenium concentration. The highest proportion of elemental selenium was found in sediment collected from the reference site in David Lake (76%). Organic selenides, modeled as selenomethionine (R-Se-R), were detected at each site with the exception of UL2 (Figure 3.4a). The highest proportion of organic selenides (28%) occurred at FL1, which was also the site downstream from the milling operation with the highest percentage of organic carbon in the sediment (5.64 ± 1.35%). Selenite was present at all the downstream study locations, but at fractions less than 10% of the total sediment selenium.

Selenium speciation analyses for the 2009 in situ experiment were conducted on the different fractions of the whole-sediment profile that served as the source of selenium exposure to caged *C. dilutus* larvae. Figure 3.4b illustrates the fractions of key environmentally relevant selenium species in whole-sediment, biofilm, and sediment detritus from Unknown Lake, as well as in sediment detritus from a reference site. Similar fractions of selenium species were observed between a homogenized sediment sample (0 to 2.5 cm) horizon and a detritus sample from Unknown Lake. The percentage of elemental selenium in whole-sediment and detritus were 51% and 53%, respectively. Organic selenides were found in each fraction at UL1 and in the detritus from the reference location (Figure 3.4b); however, substantially more organic selenium was detected in the biofilm sample (78%).

3.3.4 *Chironomus dilutus* growth and survival

Survival and growth of *C. dilutus* were poor and highly variable for the in situ experiment carried out in 2008. This variability occurred among in situ exposure treatments, as well as within treatments at many sites. Mean survival in the surface water only exposures was lower compared to sediment exposures at each site within Fox Lake and Unknown Lake, although most

sites were not significantly different (Table 3.6). Decreased survival in the surface water exposure treatments at sites FL1 and FL2 and at UL1 required pooling of experimental replicates in order to obtain sufficient biomass for ICP-MS analysis. We suspect that the suitability of the reference site sediment chosen as a substrate for the surface water only exposure chambers was a contributing factor to the low survival in these exposure groups. This sediment was high in organic carbon and lacked a consolidated sediment layer for larvae to construct cases. Handling stress, coupled with the young age of the larvae at the start of the test (2^{nd} instar) may have also contributed to decreased survival. For the 2009 experiment, the method was modified in order to address these problems. First, *C. dilutus* larvae were older (12 to 14 d post-hatch) at the beginning of the 10-d exposure period. Second, the substrate used in surface water exposure at Unknown Lake was a more consolidated surficial layer of reference sediment from Yeoung Lake. Third, larvae were transferred directly from the pre-exposure culture tanks to the in situ chambers on site at Unknown Lake to minimize handling of the larvae prior to deploying the chambers. Refining the test method resulted in greater than 80% survival across all treatment groups at the study location in Unknown Lake in 2009. However, larvae exposed to sediment detritus had lower dry weights following 10 d of exposure compared to larvae exposed to either whole-sediment or biofilm ($p < 0.05$) (Table 3.7).

3.3.5 Selenium bioaccumulation in caged *C. dilutus*

Statistical comparisons of selenium concentrations in *C. dilutus* larvae from the 2008 SW and SED treatments could not be achieved due to low survival and the need to pool larvae from replicate chambers to ensure there was an adequate sample mass for ICP-MS analysis. Nonetheless, sediment was clearly the dominant pathway of selenium accumulation for chironomid larvae during the 10 d experiment (Figure 3.5). Where statistical analyses could be

Table 3.6: Survival and weight of *Chironomus dilutus* larvae following 10 d of exposure to site-specific surface water (SW) or sediment (SED) at the study locations in David Lake (DL), Fox Lake (FL) and Unknown Lake (UL) in 2008. Data presented the mean ± 1 SD (n = 4).

Study Location	Survival (%)		Weight (mg/larva dry wt)	
	SW	SED	SW	SED
DL1	75 ± 31[†]	25 ± 13	0.18 ± 0.07	0.41[a]
FL1	13 ± 10[†]	53 ± 17	0.30[a]	0.20 ± 0.08
FL2	40 ± 35	50 ± 29	0.16[a]	0.33 ± 0.02
FL3	63 ± 26	75 ± 24	0.13 ± 0.03	0.20 ± 0.10
UL1	15 ± 12[†]	70 ± 18	0.20[a]	0.29 ± 0.05
UL2	58 ± 29	65 ± 47	0.17 ± 0.05	0.25 ± 0.06

[a] Standard deviation not calculated due to pooling of replicates for selenium analysis.
[†] Significant difference in larval survival compared to sediment exposure at each site ($p < 0.05$).

Table 3.7: *Chironomus dilutus* survival and weight for the 2009 in situ selenium bioaccumulation experiment at the study location (UL1) in Unknown Lake. Data presented as the mean ± 1 SD (n = 6).

Exposure Treatment	Survival (%)	Weight (mg/larva dry wt)
Reference Detritus	80 ± 25	0.19 ± 0.03[a]
Whole-sediment	80 ± 19	0.33 ± 0.05[b]
Detritus	83 ± 26	0.19 ± 0.01[a]
Biofilm	82 ± 17	0.56 ± 0.08[c]

Different letters indicate a significant difference among treatments in larval dry weight after 10 d of exposure ($p < 0.05$).

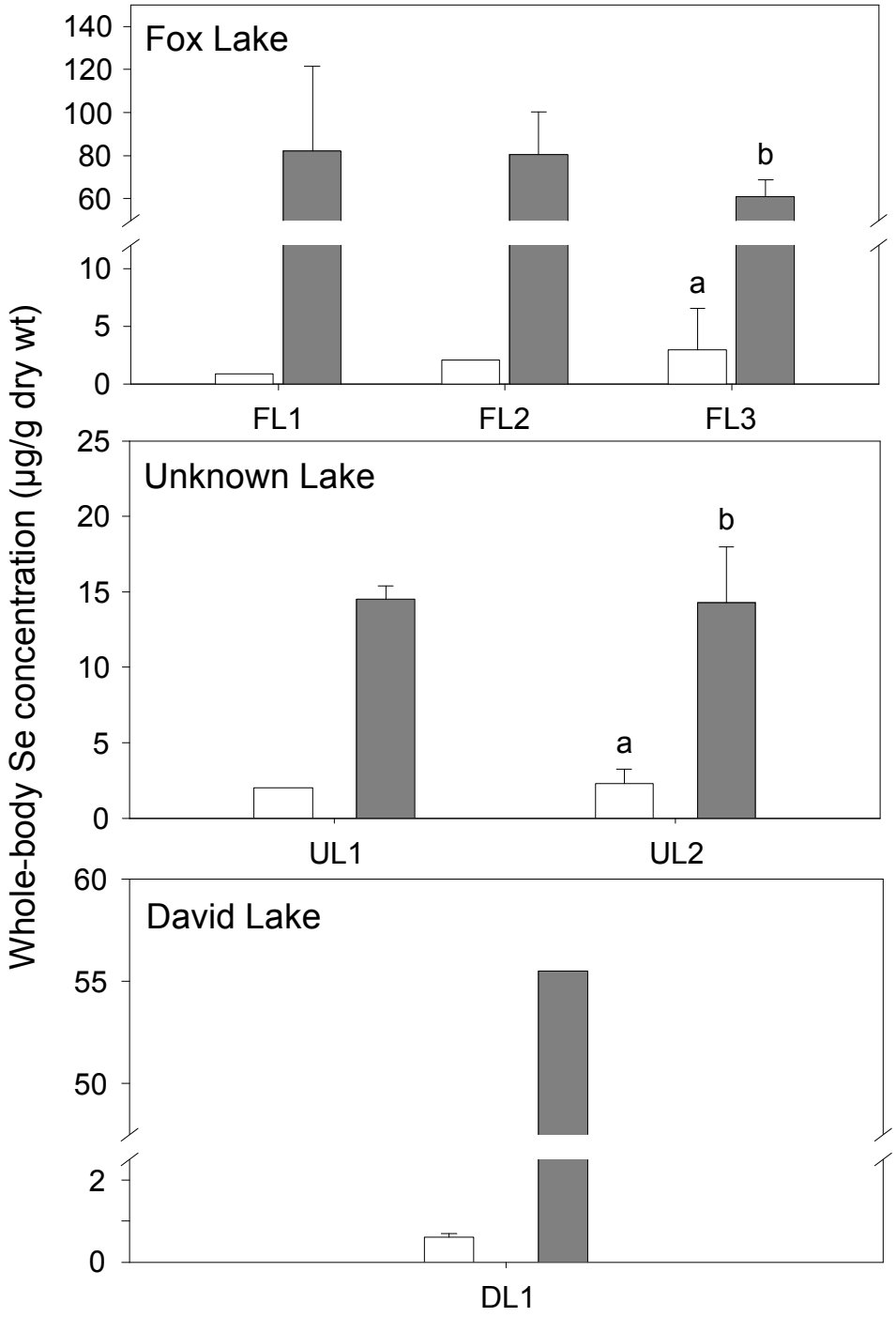

Figure 3.5: Whole-body selenium (Se) concentrations in *Chironomus dilutus* larvae exposed in situ to sediment and surface water (▬) or surface water only (▭) at study locations within the David Creek drainage basin in 2008. Data are the mean ± 1 SD. Different letters (a,b) indicated a significant difference in larval whole-body Se between the sediment and surface water treatments at each site ($p < 0.05$).

made, exposure to sediment resulted in significantly elevated whole-body selenium concentrations compared to larvae exposed to selenium in the surface water (UL2, $p < 0.01$; FL3, $p \leq 0.001$). Whole-body selenium concentrations in larvae exposed to sediment from different sites within Fox Lake and within Unknown Lake were not significantly different ($p > 0.57$ and $p > 0.63$, respectively) (Figure 3.5). At the reference location in DL, test organisms that were exposed to sediment from FL2 within the chambers accumulated 27-fold more selenium than larvae exposed to surface water at FL2 in the absence of sediment exposure. Meanwhile, larvae exposed to sediment from FL2 at the reference location accumulated 55.5 µg Se/g, compared to 80.4 ± 20.15 µg Se/g in larvae exposed to similar sediment and selenium in the surface water at FL2. Selenium concentrations in the caged larvae were strongly correlated with both pore water Se ($r^2 = 0.86$, $p < 0.01$), and whole-sediment selenium normalized to sediment organic carbon content ($r^2 = 0.80$, $p < 0.05$) (Figure 3.6), consistent with results observed for resident chironomids in this lake system (Wiramanaden et al., 2010a).

Total selenium concentration and selenium speciation data for caged *C. dilutus* larvae and resident chironomids from the 2009 study are shown in Figure 3.7. Whole-body selenium concentrations for 2008 and 2009 compare well for caged larvae exposed to whole-sediment at UL1. In 2008, the mean (± 1 SD) selenium concentration in the caged larvae was 14.5 ± 0.9 µg/g dry wt compared to 12.9 ± 1.7 µg/g dry wt in 2009. For comparison, resident chironomid larvae collected from UL1 had a total selenium concentration of 16.6 µg/g dry wt (mean of two samples). Exposure to detritus from Unknown Lake resulted in the highest selenium content in *C. dilutus* larvae, and significantly more selenium was accumulated by test larvae from each of the exposure treatments compared to the reference detritus treatment ($p < 0.05$, Figure 3.7). Organic forms of selenium were the most prevalent chemical species of selenium in both caged

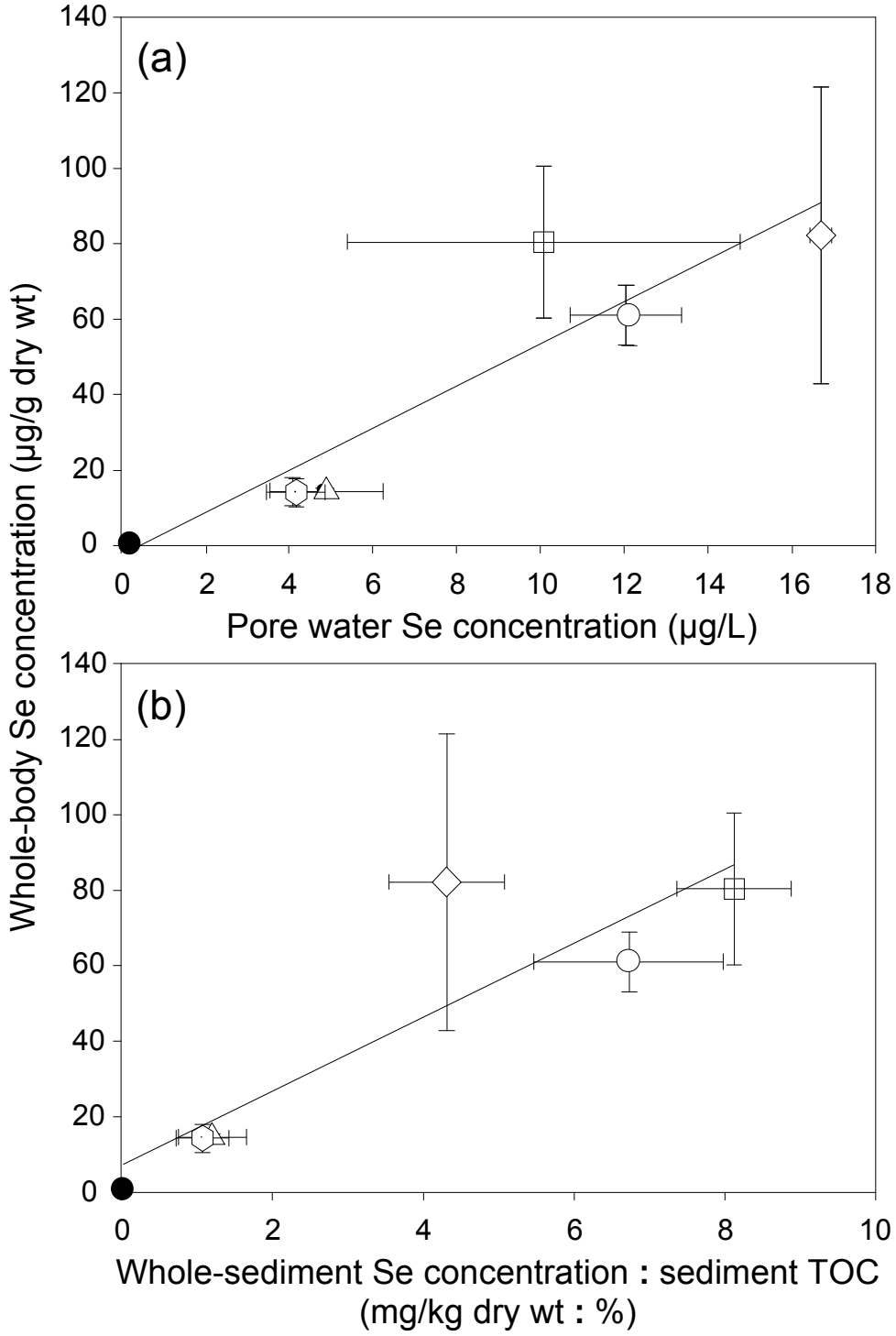

Figure 3.6: Relationship between *Chironomus dilutus* larvae selenium (Se) concentration and (a) pore water Se ($r^2 = 0.86$, $p < 0.01$) and (b) sediment Se normalized to total organic carbon ($r^2 = 0.80$, $p < 0.05$) at the in situ study locations in Fox Lake (FL1 ◇; FL2 ⊞; FL3 ○), Unknown Lake (UL1 △; UL2 ⬡) and David Lake (DL1 ●) during the 2008 experiment.

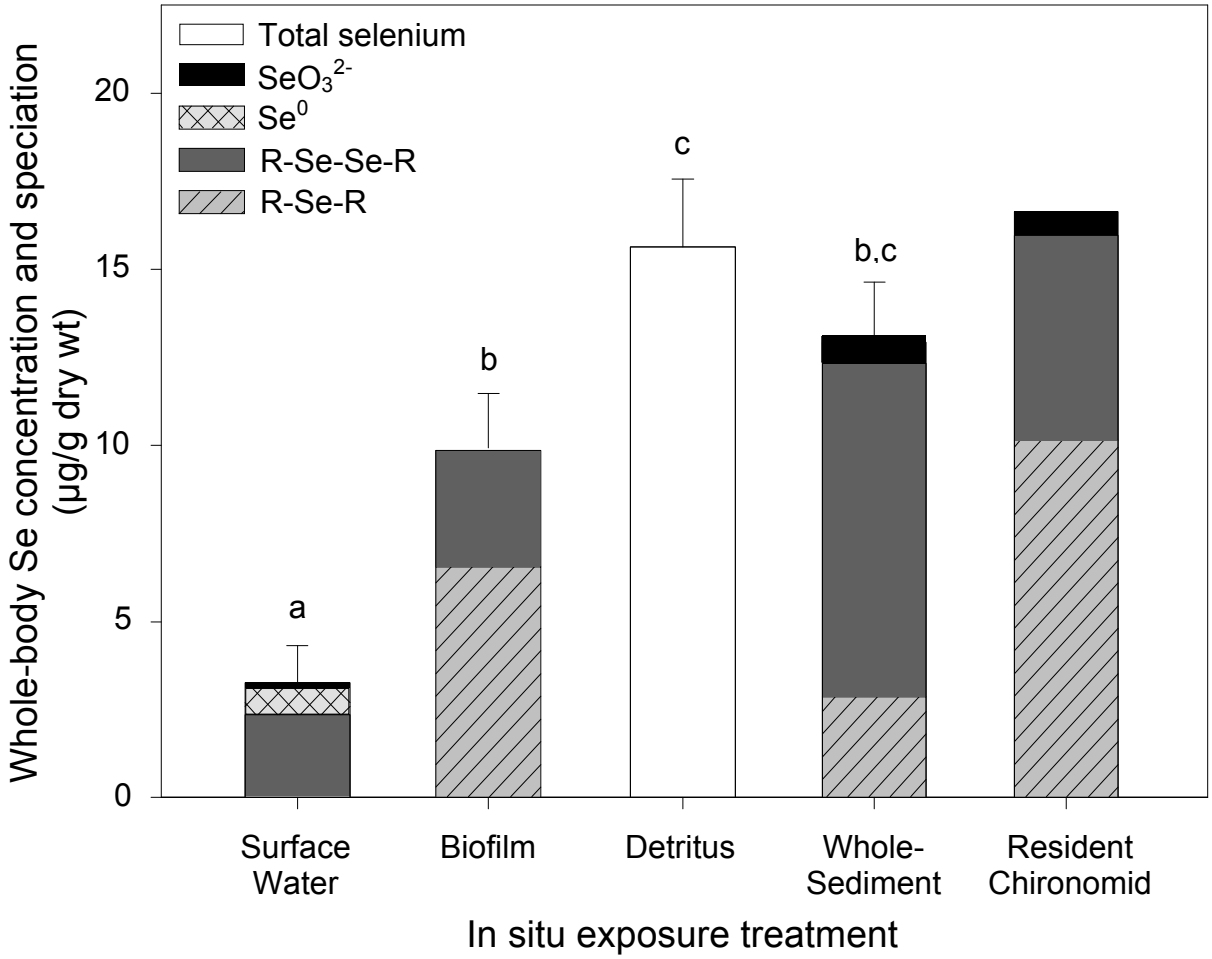

Figure 3.7: Whole-body selenium (Se) concentrations and speciation for resident chironomids and caged *Chironomus dilutus* following 10 d of in situ exposure at Unknown Lake (UL1) to biofilm, whole-sediment, detritus or reference detritus (from Yeoung Lake) in 2009. Data are presented as the mean ± 1 SD (n = 3) of the larval whole-body Se concentrations. Different letters (a,b,c) indicate a significant difference in whole-body Se concentrations among treatments ($p < 0.05$). The mean of two replicates is reported for resident chironomids (no SD calculated). Selenium standards included in the fit are selenite (SeO_3^{2-}), elemental Se (Se^0), selenomethionine (R-Se-R), and selenocystine (R-Se-Se-R).

C. dilutus and resident chironomids. Organic selenides, modeled as selenomethionine, accounted for 61% of the total selenium present in resident chironomids at UL1 and 66% for *C. dilutus* larvae exposed to biofilm from this site. Organic diselenides, modeled as selenocystine, was the predominant form of selenium in larvae that were fed reference detritus (low selenium concentration) and the whole-sediment treatment at 72% and 73%, respectively (Figure 3.7). Small amounts of selenite were also present in most larval samples, but the relative abundance ranged from only 4 to 6% of the total selenium content. The selenium speciation data for *C. dilutus* larvae from the detritus treatment were not available due to low sample mass leading to poor signal to noise during XAS analysis.

3.4 Discussion

Treated effluent released from the Key Lake uranium milling operation has contributed to elevated concentrations of selenium in the surface water and sediments in several downstream lakes compared to background levels (Robertson and Liber, 2007; Muscatello et al., 2008; Wiramanaden et al., 2010a). Based on past monitoring data from this location, three potential exposure routes were identified as likely contributing to elevated selenium concentrations in benthic invertebrates downstream from the Key Lake mill: uptake of selenium directly from surface water near the sediment-water interface, uptake of selenium from sediment pore water, and dietary accumulation from ingestion of sediment detritus/biofilm.

Results from 2008 caging study showed that *C. dilutus* larvae accumulate substantially less selenium when the source of exposure is limited to selenium present in the surface water. A difference of approximately 8 µg Se/L in the surface water between exposure lakes produced no apparent difference in whole-body selenium content. Furthermore, compared to selenium in the test larvae from the SW exposure at DL1, selenium in surface water in the exposure lakes

contributed only marginally (~ 2 µg/g) to the total whole-body selenium content in caged larvae, likely as a result of algal colonization inside the SW exposure chambers in Fox Lake and Unknown Lake. The low bioavailability of dissolved selenium in the SW treatments in the exposure lakes was observed despite 40 to 100-fold greater surface water selenium concentrations in Unknown Lake and Fox Lake compared to David Lake, respectively. A more in-depth investigation into selenium bioaccumulation in 2009 confirmed that surface water is not a significant exposure pathway for chironomid larvae. An average of 3.26 µg Se/g was accumulated by caged *C. dilutus* following 10 d of aqueous exposure at UL1, which compared well with whole-body selenium in *C. dilutus* from the same site in 2008 (2.03 µg Se/g).

Dissolved selenium in surface water is generally considered insignificant as a direct source of exposure to aquatic organisms (Luoma and Presser, 2009). However, aqueous uptake can contribute to selenium accumulation by invertebrates (Stewart et al., 2010), particularly where biogeochemical processes enhance the partitioning of more reduced forms of selenium from the sediments to the overlying water. At Key Lake, selenium predominantly enters the system in the form of selenate in the treated effluent, which is comparatively less bioavailable to many aquatic invertebrate species (Besser et al., 1993; Maier and Knight, 1993; Rosetta and Knight, 1995; Franz et al., 2011). Farther downstream, however, the selenium speciation in surface water is less certain. Analysis of total selenium in surface water and water collected just above the sediment surface in 2008 suggests that selenium may be partitioning from the surficial sediments/pore water back into the surface water, thereby slightly increasing the dissolved selenium concentration near the sediment water interface. More bioavailable forms of selenium (selenite and organic selenium) can diffuse into the surface water as a result of microbial degradation of sediment detritus (Martin et al., 2011). Additionally, bioturbation of sediments

by benthic organisms can form a locally oxidative environment, resulting in the release of more reduced selenium species from the sediment (Simmons and Wallschläger, 2005).

Sediments are the dominant sink for selenium in aquatic environments, particularly in lentic ecosystems characterized by low flow rates and high levels of primary productivity. Selenium accumulates in sediments largely via the uptake of dissolved selenium from the water column by primary producers, followed by sedimentation and decay of these organisms and directly by microorganisms inhabiting surface sediments (Fan et al., 2002; Orr et al., 2006). Consequently, as much as 90% of selenium can be found in the surficial sediment/detrital layer of aquatic systems where it may be available for incorporation into the benthic-detrital food web (Lemly, 2002). The importance of the sediment exposure pathway was evident by the noticeable increase in whole-body selenium in larvae from the sediment exposures in 2008. *Chironomus dilutus* larvae from the combined sediment and surface water exposures in Fox Lake accumulated in excess of 60 µg Se/g in 10 d, with the concentration in the test organisms at FL1 and FL2 above 80 µg Se/g dry wt. On average this was a 50-fold increase in whole-body selenium compared to *C. dilutus* larvae exposed primarily to selenium in the surface water. Farther downstream at Unknown Lake, the magnitude of the difference in whole-body selenium between the sediment and surface water exposure treatments decreased to approximately 6.5-fold.

Selenium partitioning to sediments is highly site-specific, resulting in substantial small-scale spatial variations in sediment selenium content among study sites. Within Fox Lake, sediment selenium was nearly five-fold greater at the inflow study location at FL1 compared to FL2 and FL3. An important factor influencing the accumulation of selenium in sediments is organic carbon content. High rates of primary productivity and longer hydraulic retention times

facilitate the accumulation of organic carbon in the sediments of lentic systems (Van Derveer and Canton, 1997). Previous research in this lake system found selenium concentrations in sediment to be positively correlated with the amount of organic carbon in the sediment (Wiramanaden et al., 2010a). The strongest correlation was observed when the TOC content was greater than 10% in the sediment, although this relationship was still apparent at sites with less TOC. In the present study, sediments from the in situ exposure site locations in 2008 all had less than 10% TOC, yet a strong site-specific correlation was apparent in each lake. Sediment from the study locations in Fox Lake had the strongest relationship between whole-sediment selenium and TOC (r^2 = 0.95), while the relationship at Unknown Lake was slightly weaker (r^2 = 0.72). This positive relationship between organic carbon and selenium was also observed for biofilm, detritus, and whole-sediment in the 2009 study, which suggests that TOC is the most important, practical determinant of selenium content in sediments regardless of the sediment composition or specific sediment fraction chosen for analysis.

Partitioning of selenium among surface water, sediments, and pore water is highly dependent on selenium speciation, which is influenced by the redox environment in the sediment. More reduced species of selenium have been shown to accumulate in the pore water and sediments under reducing conditions (Belzile et al., 2000). Red elemental Se, presumably synthesized by sediment microbes, was the most abundant form of selenium in the sediments collected from the in situ exposure sites in 2008, and the fraction of elemental selenium in Fox Lake remained relatively constant at 64 to 70%, despite differences in the whole-sediment selenium concentration among the sites. The high percentage of elemental selenium suggests that reducing conditions near the sediment-water interface are facilitating the reduction of inorganic selenium in surface water into more reduced forms of selenium, likely via microbial

transformation. In a recent study investigating sediment selenium speciation in the same lake system, elemental selenium was present at approximately 50% abundance in each lake, with no apparent correlation with total selenium in the sediment (Wiramanaden et al., 2010b). Elemental selenium has also been identified as a significant fraction of selenium in lake sediments in other studies (Belzile et al., 2000; Van Derveer and Canton, 1997; Martin et al., 2011), and its low solubility and mobility has historically led to the assumption of comparative unavailability to aquatic organisms (Andrahennadi et al., 2007). However, recent research by Gallego-Gallegos et al. (in review) has shown that elemental selenium is a potential source of bioavailable selenium to *C. dilutus*. Despite this observation, and in terms of identifying exposure pathways, whole-sediment selenium concentrations may overestimate the risk of bioavailable selenium exposure to aquatic invertebrates given the high percentage of elemental selenium that can occur in lake sediments.

Organic forms of selenium were the next most prevalent selenium species identified in the sediment at the various exposure sites in 2008. Primary producers (e.g., algae, aquatic macrophytes, periphyton) and bacteria efficiently biotransform inorganic selenium from the water column into organic selenium, and their eventual death and decay forms much of the surficial detrital layer in lentic systems. Increased TOC in the sediment at FL1 resulted in a higher concentration of organic selenium compared to sites FL2 and FL3, although the relative percent contribution (24 to 28%) was similar at each site. The similarity in percent organic selenium in the sediments, despite differences in TOC content, may be partly related to increased rates of biotransformation by sediment microorganisms. Higher TOC in the sediments generally increases microbial activity, which could enhance the synthesis of elemental selenium as microbes colonize sediment detritus (Siddique et al., 2006). Ultimately, the fraction of organic

selenium, either in the biofilm or detritus, represents a significant exposure pathway for benthic grazers and detritivores.

Based on selenium concentration data from 2008, both pore water selenium and sediment selenium normalized to the percent TOC were good predictors of selenium accumulation in test larvae. A previous study at Key Lake also identified pore water as being more predictive of selenium content in resident chironomids compared to sediment and surface water (Wiramanaden et al., 2010a). Despite the strong correlation, the 2008 experiment could not fully differentiate between dietary and aqueous pore water exposure as the likely pathway for selenium accumulation from sediments. Aqueous selenium speciation profiles at the sediment-water interface are unknown at this site, which makes it difficult to exclude aqueous uptake of selenium from pore water as a potential exposure pathway. A recent study investigating the mechanisms of selenium partitioning between surface water and sediment showed that reduced selenium species (selenite and organic selenium) can be released into the pore water of surficial sediment (Martin et al., 2011). Specifically, the presence of sub-oxic conditions near the sediment-water interface were found to greatly influence the formation and persistence of more reduced forms of selenium in the pore water. It was suggested in another study that the presence of organo-selenium in the pore water of surficial sediments could be a contributing factor to increased tissue selenium in benthic invertebrates (Orr et al., 2006). Selenite and organic forms of selenium are known to be more bioavailable to aquatic invertebrates (Rosetta et al., 1995; Besser et al., 1993; Maier et al., 1993), and in a recent study exposure to less than 2 µg/L dissolved organo-selenium (as selenomethionine) resulted in a total selenium body burden in *C. dilutus* larvae in excess of 30 µg/g dry weight after 10 d of exposure (Franz et al., 2011). By comparison, the study by Martin et al. (2011) measured approximately 3 µg Se/L as organic

selenium in the pore waters near the sediment-water interface, which would likely contribute substantially to the total accumulation by primary consumers inhabiting those sediments.

Further investigation of selenium bioaccumulation by chironomids from the sediment-detritus pathway was conducted in 2009 to address gaps in our understanding of the dominant pathways of selenium accumulation at this trophic level. Analysis of different fractions of the sediment revealed that the top 0.5 cm of the sediment was nearly 10 mg/kg higher in selenium than the homogenized 2.5 cm whole-sediment sample. Many benthic invertebrate species inhabit and feed within the top few millimeters of sediment, so analyzing the detritus separately may provide a more accurate assessment of selenium exposure. Sediment detritus is comprised of inorganic particles and organic matter in various states of decay, and algae, fungi, and bacteria colonize the detrital layer as part of the nutrient cycling process in aquatic systems (Orr et al., 2006). The biofilm had less total selenium than the surficial detritus, but organic selenium, modeled as selenomethionine, comprised more than 80% of the total selenium concentration in the biofilm. This finding supports the notion that accumulation and subsequent biotransformation of inorganic selenium to organic forms by primary producers and microorganisms represents the most important step in the trophic transfer of selenium through the aquatic food web (Luoma and Presser, 2009).

The whole-sediment treatment was the least manipulated of the treatments used in the 2009 in situ study, allowing larvae to accumulate selenium from dietary as well as pore water exposure pathways. The sediment detritus treatment was designed to be primarily a dietary exposure by isolating the top 0.5 cm of sediment for use as the substrate in the in situ cages. While some interstitial water may have been present in the 0.5 cm layer of detritus, the potential contribution of pore water as an exposure pathway for the test larvae was likely negligible. The

biofilm treatment was also designed primarily as a dietary exposure using the living microorganism component of the sediment as the substrate. Results from the 2009 study indicated that biofilm and detritus are the primary components of the sediment whereby *C. dilutus* accumulated selenium. Furthermore, *C. dilutus* larvae appear to have preferentially fed upon biofilm, as larvae from this treatment had significantly greater dry weights following 10 d of in situ exposure compared to both the whole-sediment and detritus treatments. *Chironomus* spp. are commonly associated with decomposing organic matter (detritus) and several species are known to preferentially feed on the biofilm component of sediment detritus (Pinder, 1986; Hax and Golladay, 1993; Alaimo et al., 1994). In the present study, whole-body selenium was elevated when the source of selenium exposure was primarily via biofilm, although larvae from the detritus and whole-sediment treatments had greater whole-body selenium concentrations after 10 d. The difference in larval selenium concentrations among treatments may be partly explained by growth dilution, as larval dry weights were significantly greater in the biofilm treatment relative to the detritus and whole-sediment treatments. Variability in larval dry weights among the various treatments likely corresponds to differences in the food quality and quantity among treatments; however, standardizing the food ration to ensure adequate nutrition for the test organisms would have been difficult given how little is known about the preferential feeding habits for most freshwater invertebrate species (Stewart et al., 2010).

Selenium speciation analysis of the caged midge larvae from the 2009 in situ experiment showed that the predominant selenium species were organic selenides (R-Se-R; modeled as selenomethionine) and organic diselenides (R-Se-Se-R; modeled as selenocystine). Selenium biotransformation mechanisms are largely unknown for many aquatic organisms; however, the presence of primarily R-Se-R and R-Se-Se-R in caged *C. dilutus* during this study is consistent

with other studies that have measured selenium speciation in aquatic invertebrates (Andrahennadi et al., 2007; Wiramanaden et al., 2010b). Resident chironomids collected from UL1 during the 2009 study showed R-Se-R, similar in structure to selenomethionine, were the dominant selenium species, with the remaining fraction comprised mainly of an organic diselenide compound similar to selenocystine. Larvae from the biofilm exposure treatment showed similar proportions of selenomethionine, selenocystine, and inorganic selenite compared to resident chironomids from this site, contributing further evidence that the biofilm component of the surficial sediment is a significant dietary selenium uptake pathway for benthic detritivores. As with other animals, aquatic invertebrates have no known mechanism for the synthesis of selenomethionine (Schrauzer, 2000); instead, they accumulate selenomethionine from dietary sources, such as bacteria, algae, and organic detritus (Alaimo et al., 1994; Fan et al., 2002; Orr et al., 2006). Animals are able to use selenomethionine from their diet for the de novo synthesis of the 21st amino acid selenocysteine, which is an essential component for proper functioning of several selenoproteins. Excess selenomethionine not used in the synthesis of selenocysteine is efficiently incorporated into proteins by non-specific substitution of methionine during protein synthesis (Schrauzer, 2000).

Caged *C. dilutus* from the biofilm and whole-sediment treatments showed significant quantities of organic selenides modeled as selenomethionine, suggesting that dietary selenium concentrations exceeded what are nutritionally required for *C. dilutus*. A similar observation was also made in a caging study on two small-bodied fish species (spottail shiner (*Notropis hudsonius*); lake chub (*Couesius plumbeus*)) conducted concurrently with the caged *C. dilutus* experiments in 2008 and 2009. In the caged small-bodied fish study, the authors noted that the fraction of selenomethionine was positively correlated with increasing whole-body selenium

concentrations (Phibbs et al., 2011a). Furthermore, there was an apparent physiological maximum of approximately 60 to 80% for the metabolism and storage of selenomethionine-like species by the fish (Phibbs et al., 2011a). The findings of these two studies, when integrated, strongly suggest that the concentration of organic selenides synthesized by primary producers and microorganisms at the base of the benthic-detrital food web exceed what is physiologically required by both invertebrates and small-bodied fish in successive trophic levels. The fraction of organic selenides transferred to higher trophic levels is critically important in predicting the risk to sensitive oviparous species because the maternal transfer of selenomethionine to developing eggs has been implicated as the causative agent in embryo mortality and teratogenicity in fish (Palace et al., 2004; Muscatello et al., 2006) and aquatic birds (Spallholz and Hoffman, 2002).

In contrast to larvae from the biofilm and whole-sediment treatments, no selenomethionine was detected in larvae from the surface water exposure in UL. This was likely the result of *C. dilutus* metabolizing trace levels of selenomethionine found in the reference detritus for normal physiological functions, resulting in very little excess selenium available for incorporation into body proteins. Larvae from this treatment accumulated selenium primarily as species resembling selenocystine (the dimer of two selenocysteine residues). Selenocysteine is a highly reactive molecule, which may explain why only selenocystine was found in test organisms from the surface water, biofilm, and whole-sediment treatments. In terms of biological relevance, all physiological functions of selenium were thought to be mediated through selenoproteins (e.g., glutathione peroxidases and thioredoxin reductases) containing a single selenocysteine residue at the active site; however, another family of selenoproteins (designated SeIL) was recently discovered, that contain a natural diselenide bond between two selenocysteine residues (Shchedrina et al., 2007). Interestingly, the selenoproteins with a

diselenide bond have so far only been identified in aquatic organisms, such as fish, invertebrates, and marine bacteria (Lobanov et al., 2009). The function of these proteins is currently unknown, but structural comparisons of SeIL to thioredoxin selenoproteins suggest they may function in redox regulation (Shchedrina et al., 2007). Ultimately, the presence of diselenides in *C. dilutus* suggests that the test organisms were efficiently metabolizing selenium from their diet for use in essential physiological functions.

The X-ray absorption spectroscopy data generated in this study highlight how selenium speciation in benthic detritivores is dependent on the source of exposure (sediment vs. surface water) and the chemical forms of selenium to which the organisms are exposed. The implications of the findings from the present study relate primarily to the trophic transfer of selenium to fish and aquatic birds via uptake and assimilation of organic selenium by *C. dilutus* from the benthic-detrital food web. In the present study, the concentrations of selenium (primarily selenomethionine) in both resident chironomids from Unknown Lake and caged *C. dilutus* from the in situ studies exceeded the threshold for potential adverse effects in fish (3 to 11 µg/g dry wt) and birds (5 µg/g dry wt) in near-field locations downstream from the Key Lake operation (Janz et al., 2010). In a recent multi-year research program investigating tree swallow (*Tachycineta bicolor*) reproduction in this lake system, selenium concentrations in benthic and aquatic invertebrates exceeded the dietary thresholds commonly used for reproductive effects (Weech et al., 2011). The authors also determined the selenium concentrations in the eggs were sufficiently high to suggest the potential for adverse effects on the developing embryo. However, despite these findings, no selenium-related deformities were noted in any of aquatic bird embryos during the three year study period, and overall tree swallow reproduction was similar between reference and exposure areas (Weech et al., 2011).

A similar study investigating the trophic transfer of selenium from invertebrates to caged small-bodied fish within this lake system determine whole-body fish selenium concentrations were below the proposed tissue based guideline of 7.91 μg Se/g dry wt (USEPA, 2004); although the authors reported that the duration of exposure was insufficient for the selenium concentration to reach steady-state (Phibbs et al., 2011a). The authors also observed that increased organic selenides in fish caged in the exposure areas compared to the reference area was a marker of elevated selenium exposure. Despite the apparent lack of effects in small-bodied fish and birds in the study area, the potential for adverse effects of elevated selenium on fish populations in this system were demonstrated for northern pike, as elevated egg selenium concentrations corresponded with in an increase frequency of deformities in pike fry (Muscatello et al., 2006). In this context, seemingly innocuous increases of organic selenium at the base of aquatic food webs may have the potential to significantly increase the risk of exposure to predatory large-bodied fish via the trophic transfer and biomagnification of dietary organic selenides.

3.5 Conclusions

In situ caging of *C. dilutus* larvae showed that aqueous uptake of selenium from surface water is not a significant exposure pathway for a benthic detritivore. Sediments represent the dominant sink for selenium in this system, and the partitioning of selenium into the sediment is related to the organic carbon content of the sediment. Once selenium partitions to the sediments, biogeochemical processes may enhance the formation of microbially synthesized elemental selenium, which is less directly bioavailable to chironomids than organic selenium. The dominant source of selenium exposure to benthic consumers was the organic fraction of selenium present in the surficial sediment layer. Combining whole-body selenium concentration data with XAS analysis of caged midge larvae provided strong evidence that the biofilm

component of the sediment detritus was the primary source of dietary selenium to *C. dilutus*. Furthermore, this study suggested that primary producers and microorganisms residing in the surficial sediments are responsible for introducing organic selenium (as selenomethionine) into the aquatic food web in the downstream environment of the Key Lake operation. Midge larvae are key prey items for a number of fish and bird species in this lake system, and defining the pathways of selenium accumulation at this trophic level will enable a more complete understanding of the risk selenium poses to higher trophic levels. More broadly, this study demonstrated the utility of in situ caging techniques for assessing contaminant exposure pathways in benthic species under site-specific conditions.

CHAPTER 4

GENERAL DISCUSSION

4.1 Project rationale and research objectives

Industrial mining operations are well-known for their potential to cause adverse effects on aquatic ecosystems (Lemly, 2004), and in northern Saskatchewan, decades of uranium mining have resulted in elevated selenium concentrations in nearby aquatic environments (Muscatello et al., 2008; Muscatello and Janz, 2009). Compared to research on the effects of selenium in warm-water environments, few large-scale selenium-related studies have been in conducted in cold freshwater ecosystems. Furthermore, the development of environmental guidelines has utilized data primarily from warm-water species and, as such, may not be representative and/or protective of northern Canadian aquatic ecosystems (USEPA, 2004). In light of the difficulty in predicting the environmental risks associated with selenium exposure based on a single generic guideline, site-specific assessments have been proposed as an effective way to accurately determine the bioaccumulation and potential ecological risks of selenium on a case-by-case basis (McDonald and Chapman, 2007; Ohlendorf et al., 2007).

This research project was undertaken as part of a collaborative multi-year study in the aquatic environment around the Key Lake uranium operation in northern Saskatchewan, with the overall objective of investigating the distribution, speciation, and potential effects of selenium. Previous work conducted in this lake system suggested that the sediment-detrital pathway plays an important role in the trophic transfer of selenium to top level predators (Muscatello et al., 2008). The importance of the dietary exposure pathway is well established for selenium

accumulation in fish and birds (Hamilton et al., 2004), but knowledge of selenium accumulation at the base of the aquatic food web is, in many respects, poorly characterized (Stewart et al., 2010). Research initiatives undertaken as a part of this thesis were designed to address specific knowledge gaps related to the bioaccumulation of selenium at the base of aquatic food webs. Using laboratory and field-based experimental approaches with the benthic invertebrate *Chironomus dilutus,* along with XAS analytical techniques, separate lines of evidence were generated to help identify the dominant exposure pathways and selenium species involved in the accumulation of selenium at this trophic level.

4.2 Summary and integration of project results

4.2.1 Laboratory bioaccumulation testing

The laboratory experiments presented in Chapter 2 were designed to calculate the uptake and elimination kinetics of selenium administered in the forms of selenate, selenite, and selenomethionine (seleno-DL-methionine, Se-met), in different life stages of the midge *C. dilutus*, and to determine the relationship between selenium bioavailability and selenium speciation using XAS. The comparative bioavailability of dissolved inorganic and organic selenium species is among the most well defined area of selenium ecotoxicology for freshwater invertebrates (Ingersoll et al., 1990; Rosetta and Knight, 1995); however, relatively little was known regarding how the speciation of selenium influences its bioavailability and subsequent biotransformation in aquatic invertebrates.

Analysis of whole-body selenium concentrations in test organisms following 10 d of exposure to different forms of selenium produced results that were consistent with other selenium bioavailability studies involving invertebrates; namely, that selenomethionine is more bioavailable to freshwater invertebrates than selenate and selenite (Ingersoll et al., 1990; Maier

and Knight, 1993). Selenate was the least bioavailable form of selenium, as midge larvae exposed to 4.3 µg/L as dissolved selenate showed negligible accumulation of selenium (indistinguishable from control organisms) during the 10 d exposure period. In contrast to the selenate treatment, larvae accumulated significantly more selenium when exposed to selenite 3.8 (µg/L) and Se-met (1.8 µg/L) (Figure 2.1). Whole-body selenium concentrations in *C. dilutus* from both of these treatments were measured in excess of the 3 to 11 µg/g dietary threshold for fish, which implies that low concentrations of selenite and organic selenium in surface water and/or pore water have the potential to increase the risk of dietary selenium exposure to fish via invertebrate prey items. Furthermore, most of the selenium accumulated by midge larvae exposed to selenite or Se-met was retained by the larvae after 10 d of elimination in clean water. A slight reduction in growth was observed for the test organisms from the Se-met treatment during the 10 d uptake period, but following 10 d of elimination in clean water, no apparent adverse effects on larval growth were observed.

Analysis of whole-body selenium concentrations in adult emerged insects following larval exposure to 4 µg Se/L as selenate, selenite, or selenomethionine showed that adult *C. dilutus* retain the majority of the selenium that is accumulated during their larval stage of development (Figure 2.2). A strong positive correlation was also found between the adult whole-body selenium concentration and the selenium concentration in the exuvia after emergence (Figure 2.3); however, the total selenium content in the exuvia was small compared with larvae and adult whole-body concentrations. The incorporation of selenium into exuvia does not appear to be a mechanism of detoxification or excretion of excess selenium; as opposed to non-essential metals (i.e., uranium) that have been shown to accumulate in the exuvia during molting as a mechanism of detoxification (Muscatello and Liber, 2009). The observation that selenium is

efficiently transferred from the larval stage to the adult stage suggests that selenium may be passed onto the next generation of *C. dilutus* larvae in a way analogous way to the maternal transfer of selenium in fish.

Speciation analysis via XAS provided important insights into the metabolism of selenium by *C. dilutus* larvae and adult insects following exposure to different forms of selenium. The current understanding of selenium metabolism and biotransformation for aquatic animals can be broadly separated into two physiological processes. First, trace amounts of selenium are required for normal physiological functions. These functions are mediated solely by the 21st amino acid selenocysteine for insertion into amino acids during the synthesis of selenoproteins. Secondly, excess selenium (primarily as selenomethionine) that is not metabolized for the *de novo* synthesis of selenocysteine is non-specifically incorporated into body proteins by the substitution of selenomethionine for methionine. Under conditions of moderate to low exposure to selenium, animals are capable of metabolizing the majority of the selenium they accumulate solely for use in normal biological processes, leaving only limited amounts of selenomethionine available for non-specific incorporation into proteins.

X-ray absorption spectra of *C. dilutus* larvae and adult insects from the selenate treatment, where there was very little bioaccumulation of selenium, showed that the dominant selenium species was selenocystine, the dimer of two selenocysteine residues linked by a diselenide bond. Selenocysteine is a highly reactive molecule, so the fact that it was not detected in any of the larvae, adults, or exuvia was not surprising. Initially, it was hypothesized that selenocystine serves as a proxy for estimating the abundance of selenocysteine in the sample. There is now compelling evidence that a selenoprotein family containing two selenocysteine residues linked by a diselenide bond may have physiological significance particularly in aquatic

organisms such as fish and invertebrates (Shchedrina et al., 2007). Speciation results presented in Chapter 2 substantiate the hypothesis that selenoproteins with a diselenide bond may have physiological significance. Comparing speciation spectra from each of the treatments for both the larval and adult insect samples showed that the fraction of organic diselenides (modeled as selenocystine) varied among treatments, but the actual concentration covered a relatively narrow range (0.35 – 1.54 μg/g dry wt). The observation that the concentration of organic diselenides remained fairly conserved among the different *C. dilutus* samples implies some physiological function, either as a biomarker for single selenocysteine molecules in the organisms or by the incorporation of two selenocysteine residues linked by a diselenides bond into selenoproteins.

Organic selenides, modeled as R-Se-R (i.e., selenomethionine), were the dominant forms of selenium in both the larval and adult insect stages for the selenite and Se-met treatments. The proportion and concentration of organic selenides (selenomethionine) increased in larvae and adults exposed to Se-met and selenite compared with larvae exposed to selenate (Figure 2.5). At the end of the 10 d uptake phase, selenomethionine-like compounds accounted for 76% and 79 % of the selenium accumulated by larvae from the selenite and Se-met treatments, respectively (Table 2.2). Furthermore, the fractions of R-Se-R compounds in the adult insects from the selenite and Se-met treatments were similar at 82% and 84%, respectively. These results point to an efficient transfer of organic selenides from the larval stage to the adult stage during metamorphosis. A companion study has reported an apparent maximum of between 60% and 80% of the selenium as selenomethionine for small-bodied fish during a 21 d caging study at Key Lake (Phibbs et al., 2011a). While the fraction of selenomethionine for selenite and Se-met exposed larvae and adults are similar to this range, it remains unclear whether there is a biological maximum fraction for selenomethionine in *C. dilutus*. Nonetheless, the observed

increase in the relative abundance and absolute concentration of selenomethionine for *C. dilutus* larvae and adult insects does substantiate the claim that increased fractions of selenomethionine are a biomarker of elevated selenium exposure for aquatic animals (Phibbs et al., 2011a).

4.2.2 In situ caging experiments at Key Lake

The in situ experiment conducted in 2008 was designed primarily to evaluate the importance of current effluent release (surface water) vs. historical contamination (sediment) on selenium bioaccumulation in caged *C. dilutus* larvae. Secondary objectives included assessing the spatial variability in selenium bioaccumulation within each exposure lake (i.e., Fox and Unknown Lakes) and investigating possible relationships between selenium in the abiotic environment and bioaccumulation in the caged test organisms. In situ caging was conducted using a method described previously by Robertson and Liber (2007) with modifications to allow for the concurrent comparison of surface water vs. sediment exposure pathways at each study location. Results from the 10 d in situ experiment conclusively demonstrated that sediment-associated selenium is the dominant pathway of accumulation by benthic detritivores (Figure 3.5). In comparison, surface water was found to contribute approximately 2 µg/g dry wt to the total selenium concentration in test organisms from Fox Lake (60 – 80 µg/g dry wt) lake and Unknown Lake (~ 14 µg/g dry wt) despite surface water selenium concentrations that were three times higher in Fox Lake (12 µg/L) compared to Unknown Lake (4 µg/L). At the study locations in Fox Lake, *C. dilutus* larvae accumulated between 20 and 90 times more selenium from exposure to sediment compared to larvae exposed only to selenium in the surface water. The magnitude of bioaccumulation was less in Unknown Lake, but the test organisms still accumulated approximately 7 times more selenium from sediment compared to surface water at both of the study site locations.

Sediment selenium concentrations were variable among the study site locations in the exposure lakes (Table 3.4). The concentration of selenium in sediment was highly correlated to the organic carbon content in the sediment (Figure 3.3a), consistent with results that were previously reported in this lake system (Wiramanaden et al., 2010a). Furthermore, when the sediment selenium concentration was normalized to organic carbon content at each site, a significant positive correlation was observed between the whole-body selenium concentrations in the test organisms and selenium in the sediment. A strong positive correlation was also observed between selenium in the caged organisms and pore water selenium (Figure 3.6a). Given that the partitioning of selenium between dissolved and solid phases in the sediment is a dynamic process, it was not surprising that both pore water and sediment selenium were predictive of whole-body selenium concentrations in midge larvae. Chironomids are thought to accumulate selenium primarily from their diet (Alaimo et al., 1994), but pore water may contribute as an exposure pathway depending on the form of selenium the organisms are exposed to (Fan et al., 2002; Orr et al., 2006). Ultimately, the results from the 2008 in situ experiment were unable to discriminate between pore water and particulate/dietary selenium as pathways of selenium bioaccumulation.

In light of the questions raised in the 2008 in situ bioaccumulation study, a follow-up experiment was conducted in 2009 at a single location in Unknown Lake to investigate the importance of dietary selenium (biofilm or detritus) vs. whole-sediment (including pore water) as an exposure pathway. In addition, XAS analysis of test organisms and sediment substrates from each treatment was conducted to help identify the selenium species involved in the trophic transfer of selenium at the base of the food web. It was found that larvae exposed to sediment detritus (top 2 – 3 mm of sediment) from the exposure site had the highest selenium

concentrations after 10 d of exposure (15.6 ± 1.9 µg/g) compared to larvae exposed to whole-sediment (12.9 ± 1.7 µg/g) or biofilm (9.9 ± 1.6 µg/g) (Figure 3.7). The observed difference in dietary selenium accumulation was largely attributed to growth dilution as test organisms from the biofilm treatment were significantly larger than larvae from the whole-sediment and detritus treatments. In addition, selenium speciation analysis revealed the presence of organic selenides (modeled as selenomethionine) in all of the sediment substrates. Biofilm had lower total selenium concentrations than the detritus and whole-sediment fractions, but 78% of the selenium was detected as organic selenides compared to only 35% in whole-sediment and 24% in detritus. Based on a comparison of selenium speciation profiles of the test organisms with resident chironomids collected from the same exposure site, organic selenides modeled as selenomethionine were shown to be the primary selenium species involved in the trophic transfer of sediment-associated selenium to *C. dilutus* larvae.

4.2.3 Integration of laboratory and field-based results

Research objectives outlined in Chapters 2 and 3 of this thesis generated multiple lines of evidence that were integrated to assess the bioaccumulation of selenium in benthic aquatic food webs. Integration of the project results led to two areas of consensus related to selenium in the aquatic environment at Key Lake: 1) surface water is not a direct source of bioavailable selenium to benthic invertebrates, and 2) dietary uptake of organic selenium from the sediment-detrital pathway is a significant process leading to the trophic transfer of bioavailable selenium to higher trophic level organisms. As a direct exposure pathway, surface water exposure (as selenate) was not a pathway leading to the accumulation of elevated selenium for *C. dilutus*. Exposure to selenate for 10 d under controlled laboratory conditions resulted in approximately 1 µg/g selenium in the test organisms, likely from the dietary accumulation of low-levels of selenium

from their food. The same duration of in situ exposure at Unknown Lake resulted in a similarly low uptake of selenium from the surface water by the caged test organisms (< 2 µg/g). Furthermore, there was no apparent difference in larval uptake of selenium from surface water exposure between the high and medium selenium exposure lakes (< 3 µg/g dry wt at all study locations), despite a three-fold difference in mean surface water concentrations between the exposure lakes (4 vs 12 µg/L). Selenium speciation data were not collected for the surface water at Key Lake due to analytical limitations; however, the similarity in *C. dilutus* whole-body selenium concentrations among the laboratory and in situ experiments strongly suggests, as other researchers have reported, that the dominant form of selenium in the exposure lakes is selenate (Phibbs, 2011).

Selenium concentration and speciation data from the in situ and laboratory bioaccumulation experiments provided convincing evidence that detritus/biofilm was responsible for increased uptake of selenium by chironomid larvae. Whole-body selenium concentrations were consistently higher when larvae were exposed in situ to site-specific sediment/detritus compared to larvae exposed only to surface water (Figures 3.5 and 3.7). In the 2008 study, midge larvae accumulated between 60 and 80 µg Se/g dry wt after only 10 d of exposure to sediment and surface water at the three study locations in Fox Lake. By comparison, chironomid larvae collected from several locations in Fox Lake in 2006 accumulated between 52 and 88 µg Se/g dry wt (Wiramanaden et al., 2010a). Furthermore, whole-body selenium concentrations observed for test organisms exposed in situ to sediment, detritus, or biofilm at Unknown Lake were similar to the concentrations previously reported in resident chironomids and other benthic detritivores in this lake (Muscatello et al., 2008; Wiramanaden et al., 2010a). While there may be species-specific differences in accumulation rates between resident and laboratory-reared *C.*

dilutus larvae, it appears as though steady-state may be achieved quite rapidly for midge larvae exposed to contaminated sediments.

Speciation data from the laboratory study and the 2009 in situ experiment provided additional lines of evidence that dietary uptake of organic selenium is the dominant exposure pathway for chironomid larvae. X-ray absorption spectra revealed similar proportions of organic selenides modeled as selenomethionine for test organisms from the biofilm treatment (66%) and resident chironomids (61%) from the in situ caging study in Unknown Lake (Figure 3.7). Organic selenides were the largest fraction of the total selenium in the biofilm sample collected from Unknown Lake (78%), and it seems likely that *C. dilutus* accumulated selenium primarily by grazing on the microorganisms that colonize the surficial sediment (Alaimo et al., 1994). Speciation results from the laboratory bioaccumulation experiments with selenate, selenite, and selenomethionine (Se-met) also demonstrated the importance of the dietary exposure pathway for *C. dilutus*, despite the use of a water-borne rather than dietary exposure method. Water-borne selenomethionine exposure has previously been shown to mimic dietary organo-selenium uptake in a freshwater bivalve (Adam-Guillermin et al., 2009), and XAS analysis of larval and adult *C. dilutus* provide additional evidence that this approach has merit. Similar fractions of organic selenomethionine-like compounds have been reported for invertebrates in other selenium-impacted watersheds (Andrahennadi et al., 2007), suggesting there may be a physiological similarity among different invertebrate species regarding the uptake and metabolism of elevated concentrations of organic selenium in the diet.

4.3 Recommendations

During the course of this research project, several areas of potential improvement were identified with respect to in situ bioaccumulation testing and abiotic sample collection and

processing. Specific recommendations and considerations to enhance site-specific ecological risk assessments for selenium in aquatic ecosystems are addressed in the following sections.

4.3.1 In situ caging methods using benthic invertebrates

A growing body of research has demonstrated that in situ testing can generate environmentally realistic response data; however, there are specific aspects of in situ testing that present challenges to designing and implementing them successfully. A recent Pellston Workshop held in Portland, Oregon, was organized to evaluate the use of field-based biological effects and exposure techniques, with the goal of improving their accuracy and relevance in ecological hazard and risk assessment (Baird et al., 2007). Liber et al. (2007) and Crane et al. (2007) conducted thorough reviews of current in situ methods and highlighted key areas where methods and quality control can be improved. Several specific considerations and recommendations are addressed here that apply directly to in situ caging methods used in this program.

Choosing suitable study locations that are amenable to caging methods is one of the main prerequisites when designing an in situ experiment. Sites that may be of interest from a toxicological perspective are often logistically difficult to investigate using caged invertebrates. Depositional areas in deep lakes are rarely investigated because of the difficulty in deploying and retrieving the cages, and for this reason, the in situ study locations in 2008 and 2009 were confined to locations in Fox, Unknown, and David Lakes that were less than 2 m in depth. Selecting appropriate reference sites is another important consideration when designing caging studies, as it is in any environmental investigation. Including reference locations in the study design is necessary to help identify possible effects due to the caging method that may otherwise be linked to the environment under investigation. While chambers are designed to reduce stress

on the test organisms while providing the most environmentally realistic exposures, physical confinement of the test organisms can contribute to stress and potentially confound the results of the study unless an appropriate reference location is included in the study design.

Several factors relating to how in situ tests are executed can affect the outcome of caging studies. In particular, careful handling of test organisms during transportation is essential to minimize the likelihood of mortality due to physical stress. For the 2008 in situ study, test organism survival was highly variable and poor at each of the exposure sites. The increased mortality was attributed to the young age of organisms at the start of the test, as well as handling stress caused by moving the test larvae from the aquaria to a sample bottle (10 ml) for transport to the study site locations. Chappie and Burton (1997) observed a similar effect of stress during transport causing increased mortality for chironomids. The authors suspected that when larvae were transported in the cages, they became trapped at the surface of the water, which contributed to stress on the organisms during transport. For the 2009 experiment, larvae were transported to Unknown Lake in their culture aquaria instead of in a separate bottle. In addition, the organisms were added to each in situ chamber while in their cases, affording them additional protection during deployment. These two modifications to the test method dramatically improved the success of the caging study, as survival rates were greater than 80% in all of the treatments.

Other factors that may affect the outcome of in situ testing are more difficult to control, such as predation by indigenous macroinvertebrates, food availability, and damage of the chambers by resident wildlife. Sediments used for in situ testing can be sieved to remove predator invertebrates, but this process destroys the sediment profile resulting in a less realistic exposure scenario. Maintaining the integrity of the collected sediments is of primary concern for in situ bioaccumulation experiments, since disrupting the structure of the sediment may change

the physicochemical and biological characteristics, which in turn could influence the partitioning, complexation, speciation and bioavailability of toxicants. Previous in situ work at this site involved collecting sediment with a hand-held corer and extruding the sediment core into the in situ chamber, which has the potential to disrupt the natural sediment horizons. An alternate way of collecting sediment was developed for the 2008 and 2009 studies where the in situ chamber was directly attached to the hand-held corer, thereby eliminating the step in which sediment was extruded from the core tube into the in situ chamber. Each core was visually inspected to confirm that the sediment profile remained intact during collection. This method proved successful for use at shallow sites (< 2 m deep), and future in situ studies could build upon this collection method for application at deeper locations.

Caging methods have the potential to alter the availability of food by confining the test organisms to a limited area, which can affect normal growth and survival (Crane et al., 2007). While supplemental feeding of the test organisms can enhance growth and survival, artificially adding food can influence the outcome of the tests by altering the natural foraging behavior of the organisms. During the 2009 experiment, variable growth was observed among the surface water, whole-sediment, detritus, and biofilm treatments. This difference was attributed to the quantity and quality of food naturally present in the sediment, detritus, and biofilm substrates, but standardizing the amount of biofilm, detritus, and whole-sediment to ensure equal nutrition would have proved challenging. The use of substrates for culturing biofilm described in Chapter 3 showed initial promise as a way of obtaining an environmentally relevant and site-specific source of food for benthic detritivores, and future research could be directed toward optimizing and refining this method of collecting sediment biofilm.

A great deal of information on the potential risk of contaminant exposure to benthic invertebrates can be generated using in situ test methods. The challenges inherent to conducting invertebrate caging studies highlight the need for a thorough assessment of the feasibility and merit of in situ testing prior to designing a study. Nonetheless, this research demonstrated that combining the results from well-designed in situ experiments with results from more conventional assessment methods such as laboratory toxicity tests, field surveys, and physicochemical characterization, can add to a weight-of-evidence approach for improved certainty during ecological risk and hazard assessments.

4.3.2 Environmental sampling methods

Environmental sampling for the purpose of exposure characterization is a critical component of any risk assessment. Several effective methods are available for the collection of surface water, pore water, and sediment in order to identify whether there is a risk of contaminant exposure to benthic invertebrates. For each environmental compartment, choosing an appropriate sampling method will depend on the specific objectives of the project. With respect to sediment sampling, the most recently introduced contaminants of concern are generally found in the upper 2 cm, which corresponds to the sediment horizon where most infaunal organisms dwell and epifaunal organisms feed (Environment Canada, 2011). Environment Canada (2011) recommends analyzing sediment for metals and radionuclides within the top 0 – 2 cm of sediment for monitoring programs specific to metal mining operations. Collecting sediment over a 2 cm interval ensures an adequate amount of sample for the analysis of various physical and chemical parameters at the expense of spatial resolution for detecting small vertical changes in sediment contaminant concentrations. This is particularly relevant for selenium, where mobilization/demobilization processes at the sediment-water interface can affect

the concentration and speciation of selenium over small vertical gradients (Martin et al., 2011). Results from the 2009 study at Key Lake verified that there is considerable variability in the concentration of sediment-associated selenium within the top 2.5 cm sediment horizon. Selenium concentrations in the top 0 – 2.5 cm of sediment from Unknown Lake site 1 ranged from 6.55 to 21.41 µg/g dry wt (mean = 13.89 µg/g), while the selenium concentration in the top 2 – 3 cm of sediment detritus had between 19.19 and 26.76 µg Se/g dry wt (mean = 23.71 µg/g). No statistical difference was observed between these two treatments (p = 0.11) due to the high degree of spatial variability among the three sediment samples. Nonetheless, these results provide evidence that the top few millimeters of sediment may be an especially enriched source of selenium in this lake system. Given that only one site was chosen for the 2009 in situ bioaccumulation study, it is recommended that further investigation into the small-scale spatial resolution of sediment selenium concentrations be conducted at other locations in the effluent-exposed lakes. This will help to determine if sediment sampling methods should be adapted to collect a narrower sediment horizon for selenium concentration and speciation analyses during site-specific selenium assessments.

Previous surveys in this lake system observed that the surface water selenium concentrations are homogenous within each lake (Wiramanaden et al., 2010a); however, this conclusion was based on surface water selenium concentrations collected from the same depth at various locations within the study lakes that were sampled. In order to assess whether selenium is homogenously distributed over a vertical gradient in the water column, water samples were collected at two depths at each study location in 2008 and 2009: 1) 30 cm above the sediment using a Van Dorn sampler, and 2) at the sediment-water interface (overlying water) using an in situ dialysis sampler. For the 2008 in situ bioaccumulation study, significantly higher selenium

concentrations were observed in overlying water compared to samples collected 30 cm above the sediment at four out of the five exposure locations in Fox Lake and Unknown Lake. At one location in Fox Lake, the average selenium concentration was 5 µg/L higher in the overlying water than in the water column sample. These results seem to indicate that selenium is partitioning from the sediment and/or pore water to the overlying water, possibly as the result of microbially-mediated biotransformation processes. As source of exposure to benthic invertebrates, this observed elevation in selenium concentration is potentially significant because the concentration of selenite and organic selenium has been shown to increase near the sediment-water interface in selenium-impacted lentic systems (Martin et al., 2011). Selenium speciation analysis was not conducted on water samples from the 2008 and 2009 studies; however, based on findings from Martin et al. (2011), there is cause to consider that trace amounts of dissolved bioavailable selenium may be contributing to the elevated concentrations of selenium observed in benthic invertebrates in the near-field exposure lakes. Provided that the logistics and design of the sampling program permit their use, it is recommended that dialysis samplers be used for collecting overlying water in order to more accurately assess the potential risk of metals exposure to benthic invertebrates.

4.4 Future research opportunities

Considerable progress has been made in the last decade toward understanding and predicting the risks associated with elevated selenium in aquatic ecosystems; however, there are still knowledge gaps that require further investigation. Two areas were highlighted during the course of this project: 1) full life-cycle assessments to help determine whether some benthic invertebrates are sensitive to the chronic effects of environmentally relevant concentrations of selenium, and 2) a better understanding of selenium accumulation in particulate material

(microorganisms and detritus) at the base of the food web. They are discussed in the following sections.

4.4.1 Life cycle assessments of selenium exposure to benthic invertebrates

Results from the laboratory experiments presented in Chapter 2 demonstrated that *C. dilutus* larvae rapidly accumulate selenium following exposure to selenite and selenomethionine, and that the majority of the selenium accumulated during larval development is retained by the adult insects following metamorphosis (Franz et al., 2011). Furthermore, XAS identified that organic selenides, similar to selenomethionine, were the dominant selenium species in larvae and adult insects, suggesting that organic selenium is efficiently transferred between life stages during *C. dilutus* development. The maternal transfer of selenomethionine from adult fish and birds to their eggs is well known for its potential to cause effects to the developing embryo (Palace et al., 2004), and recently two experiments with the mayfly *Centroptilum triangulifer* have provided the first evidence of the maternal transfer of selenium for benthic invertebrates (Conley et al., 2009; Conley et al., 2011). The authors observed that adult mayflies transferred an average of 46% of their selenium content to their eggs, with a reduction in fecundity (number of eggs) of 44% corresponding to a dietary selenium concentration of only 4 µg/g dry wt (Conley et al., 2011). Implicit within these findings is that reduced reproductive output can have significant effects on the abundance of some benthic invertebrate species. Furthermore, selenium accumulated by larvae via maternal transfer may have the potential to cause effects on growth and development during the larval stage, in a way that is analogous to the effects that have been observed for oviparous vertebrate species (Janz et al., 2010). As yet, no studies have investigated whether the maternal transfer of selenium by benthic invertebrates to their eggs results in adverse effects to successive generations. This area of research merits further

investigation, and results published in Chapter 2 suggest *C. dilutus* is an ideal test organism for exploring the potential adverse effects of selenium in full life-cycle assessments covering two or more generations.

4.4.2 Accumulation of selenium at the base of aquatic food webs

Accumulation of selenium by primary producers and microorganisms is the largest step in the bioconcentration and bioaccumulation of selenium in aquatic ecosystems. Particulate matter (abiotic and biotic) is the point of entry for selenium in aquatic food webs, and the concentration of selenium in this particulate matter determines the degree of bioaccumulation at the primary consumer trophic level (i.e., benthic detritivores). However, there are several knowledge gaps that need to be addressed regarding the accumulation of selenium within the benthic-detrital food web. First, there are virtually no data available on the uptake of selenium by sediment bacteria and the trophic transfer of selenium from bacteria to primary consumers. Second, only rough estimates of the selenium content of invertebrate diets are typically made due to the challenge of separating the biotic and abiotic fractions of the sediment. Third, the dietary preferences of many invertebrates are still poorly understood (Stewart et al., 2010).

Based on the results from the in situ bioaccumulation study presented in Chapter 3, there is merit to further investigating the uptake of selenium into biofilms, as well as their subsequent consumption by primary consumer invertebrates. Biofilms are a complex mixture of microorganisms (bacteria, algae, fungi, protozoa, and micrometazoa), exoenzymes, and detritus particles enmeshed in a gelatinous polysaccharide matrix, and their composition may differ substantially depending on whether the substrate is organic or inorganic (Hax and Golladay, 1993). Results presented in Chapter 3 showed that sediment biofilms collected from Unknown Lake were a highly enriched source of organic selenium available for dietary accumulation by

benthic detritivores. To our knowledge, this is the first study that has attempted to separate organic biofilms from other fractions of the surficial sediment for analysis of total selenium concentration and speciation, and overall the method proved successful. Nonetheless, this method was only utilized at one location, and future research is needed to optimize the biofilm collection method for identifying the assemblage of organisms within the biofilm. Given the importance of the lower trophic levels in determining the degree to which selenium accumulates in aquatic systems, continued research on selenium enrichment by microorganisms at the base of the food web would help accurately characterize the potential for selenium bioaccumulation. In addition, trophic transfer factors are also needed for different taxonomic groups within the broad category of "invertebrates" to accurately predict the risk of selenium exposure to higher trophic levels.

4.5 Summary

The use of laboratory and field-based caging studies generated independent lines of evidence that, when integrated, helped refine our understanding of selenium accumulation and uptake in benthic organisms. Controlled laboratory bioaccumulation experiments were primarily useful for assessing the link between selenium bioavailability, bioaccumulation, and biotransformation in *C. dilutus*, while the in situ bioaccumulation tests provided valuable insight into the site-specific bioaccumulation pathways that are involved in the accumulation and trophic transfer of selenium through the benthic-detrital food web. Overall, this research program demonstrated how the use of multiple experimental methods, with different levels of experimental control, can be successfully integrated in order to address knowledge gaps in the risk assessment of contaminant bioavailability and bioaccumulation for benthic invertebrates.

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APPENDIX

SUPPLEMENTARY DATA FROM CHAPTER 2

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Table A.1: Water quality parameters measured in new and old water during the selenium (Se) uptake and elimination experiments with *Chironomus dilutus*. Data are presented as the mean ± 1 SD.

Treatment	Water	Temperature (°C)	DO[a] (mg/L)	pH (units)	Conductivity (µS/cm)	Hardness (mg/L)	Alkalinity (mg/L)	Ammonia[b] (NH_3, mg/L)	Se[c] (µg/L)
Selenate and selenite uptake/elimination experiment									
Control	New	24.6 ± 0.2	7.2 ± 0.5	8.1 ± 0.1	573 ± 42	148 ± 2	92 ± 1	0.006 ± 0.007	0.50 ± 0.02
	Old	24.8 ± 0.1	6.3 ± 1.1	8.0 ± 0.2	652 ± 24	144 ± 6	92 ± 2	0.031 ± 0.030	0.49 ± 0.04
Selenate	New	24.9 ± 0.3	7.2 ± 0.3	8.1 ± 0.1	580 ± 44	159 ± 22	88 ± 4	0.003 ± 0.005	4.39 ± 0.41
	Old	24.9 ± 0.2	6.2 ± 1.4	8.0 ± 0.2	631 ± 23	140 ± 2	92 ± 4	0.021 ± 0.026	3.44 ± 1.62
Selenite	New	24.9 ± 0.2	7.2 ± 0.3	8.1 ± 0.1	574 ± 51	147 ± 4	90 ± 2	0.002 ± 0.003	4.55 ± 0.21
	Old	24.9 ± 0.2	6.3 ± 1.1	8.0 ± 0.2	618 ± 14	141 ± 6	90 ± 1	0.020 ± 0.025	2.55 ± 1.10
Seleno-DL-methionine uptake/elimination experiment									
Control	New	23.7 ± 0.1	7.6 ± 0.1	8.2 ± 0.0	394 ± 1	131 ± 1	85 ± 4	0.000 ± 0.000	-[d]
	Old	24.8 ± 0.1	7.3 ± 0.2	8.3 ± 0.1	410 ± 4	128 ± 10	78 ± 1	0.017 ± 0.010	-[d]
Se-met	New	23.8 ± 0.1	7.5 ± 0.1	8.3 ± 0.0	394 ± 1	134 ± 3	81 ± 2	0.000 ± 0.000	3.30 ± 0.85
	Old	24.7 ± 0.1	7.2 ± 0.2	8.3 ± 0.0	402 ± 3	131 ± 8	84 ± 5	0.023 ± 0.022	0.91 ± 0.25

[a] Dissolved oxygen.
[b] Calculated as unionized ammonia (NH_3) based on the method outlined by Environment Canada (2010).
[c] Dissolved Se.
[d] Not measured.

Table A.2: Water quality parameters measured in new and old water during the selenium (Se) retention and adult *Chironomus dilutus* emergence experiment. Data are presented as the mean ± 1 SD.

Treatment	Water	Temperature (ºC)	DO[a] (mg/L)	pH (units)	Conductivity (µS/cm)	Hardness (mg/L)	Alkalinity (mg/L)	Ammonia[b] (NH3; mg/L)
Control	New	23.7 ± 0.1	7.6 ± 0.1	8.2 ± 0.0	394 ± 1	131 ± 1	85 ± 4	0.000 ± 0.000
	Old	24.7 ± 0.2	7.1 ± 0.4	8.3 ± 0.1	406 ± 4	133 ± 6	85 ± 3	0.013 ± 0.016
Selenate	New	23.6 ± 0.0	7.7 ± 0.1	8.3 ± 0.0	393 ± 2	133 ± 1	82 ± 3	0.000 ± 0.000
	Old	24.6 ± 0.2	7.1 ± 0.3	8.3 ± 0.1	406 ± 3	133 ± 2	86 ± 2	0.014 ± 0.016
Selenite	New	23.6 ± 0.3	7.7 ± 0.0	8.3 ± 0.0	395 ± 1	134 ± 3	81 ± 2	0.000 ± 0.000
	Old	24.7 ± 0.2	7.2 ± 0.3	8.3 ± 0.1	407 ± 4	134 ± 3	85 ± 2	0.014 ± 0.017
Se-met	New	23.7 ± 0.1	7.5 ± 0.1	8.3 ± 0.0	394 ± 1	134 ± 3	81 ± 2	0.000 ± 0.000
	Old	24.7 ± 0.2	6.9 ± 0.6	8.2 ± 0.1	404 ± 4	132 ± 2	84 ± 3	0.014 ± 0.016

[a] Dissolved oxygen
[b] Calculated as unionized ammonia (NH_3) based on the method outlined by Environment Canada (2010).
Note: Dissolved Se concentrations were measured in the uptake and depuration experiment that was ran concurrently to this experiment.

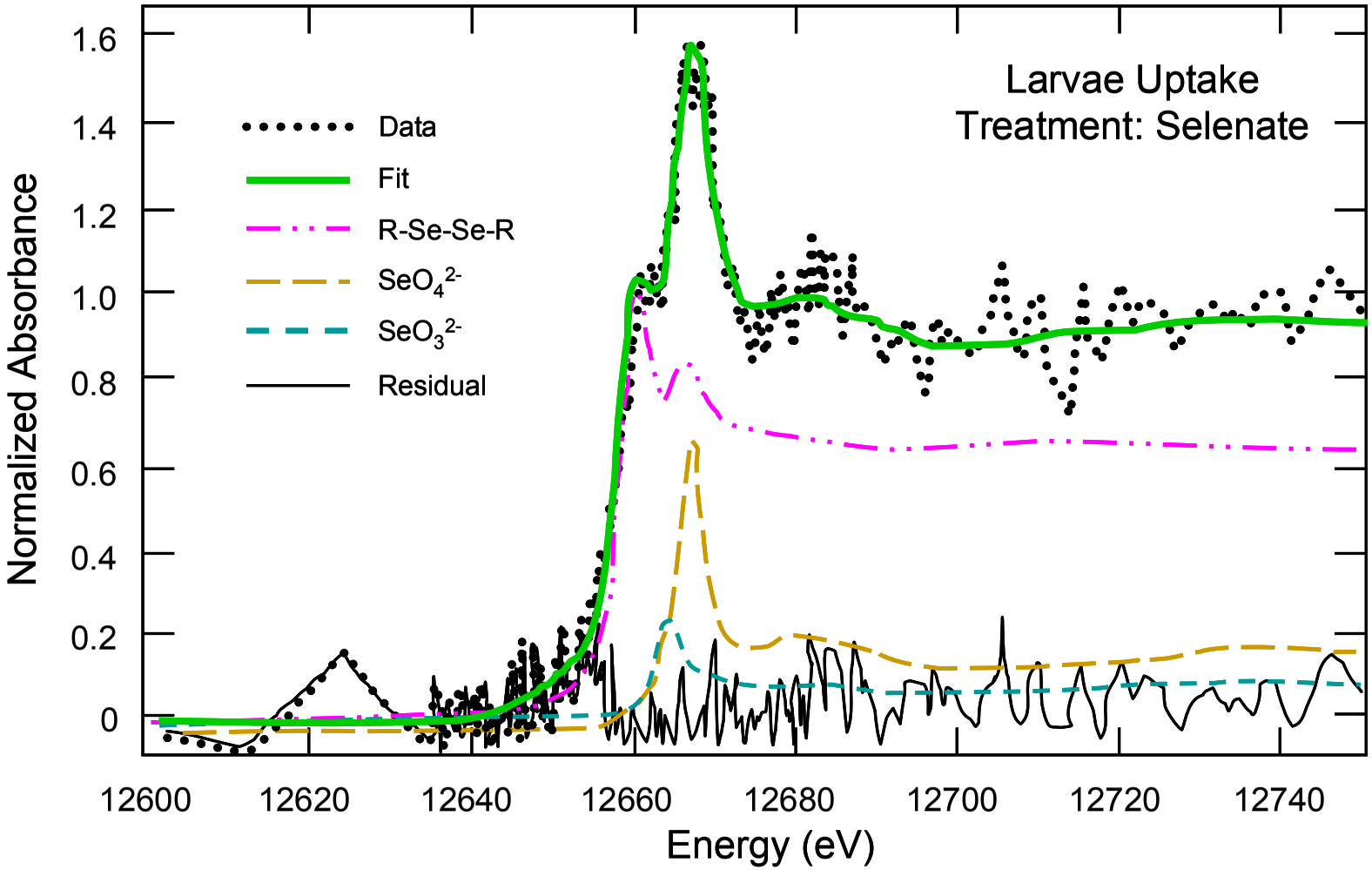

Figure A.1: X-ray absorption near-edge spectra of *Chironomus dilutus* larvae exposed to dissolved selenate for 10 days. Standards included in the fit are organic diselenides (R-Se-Se-R; i.e., selenocystine), selenate (SeO_4^{2-}), and selenite (SeO_3^{2-}).

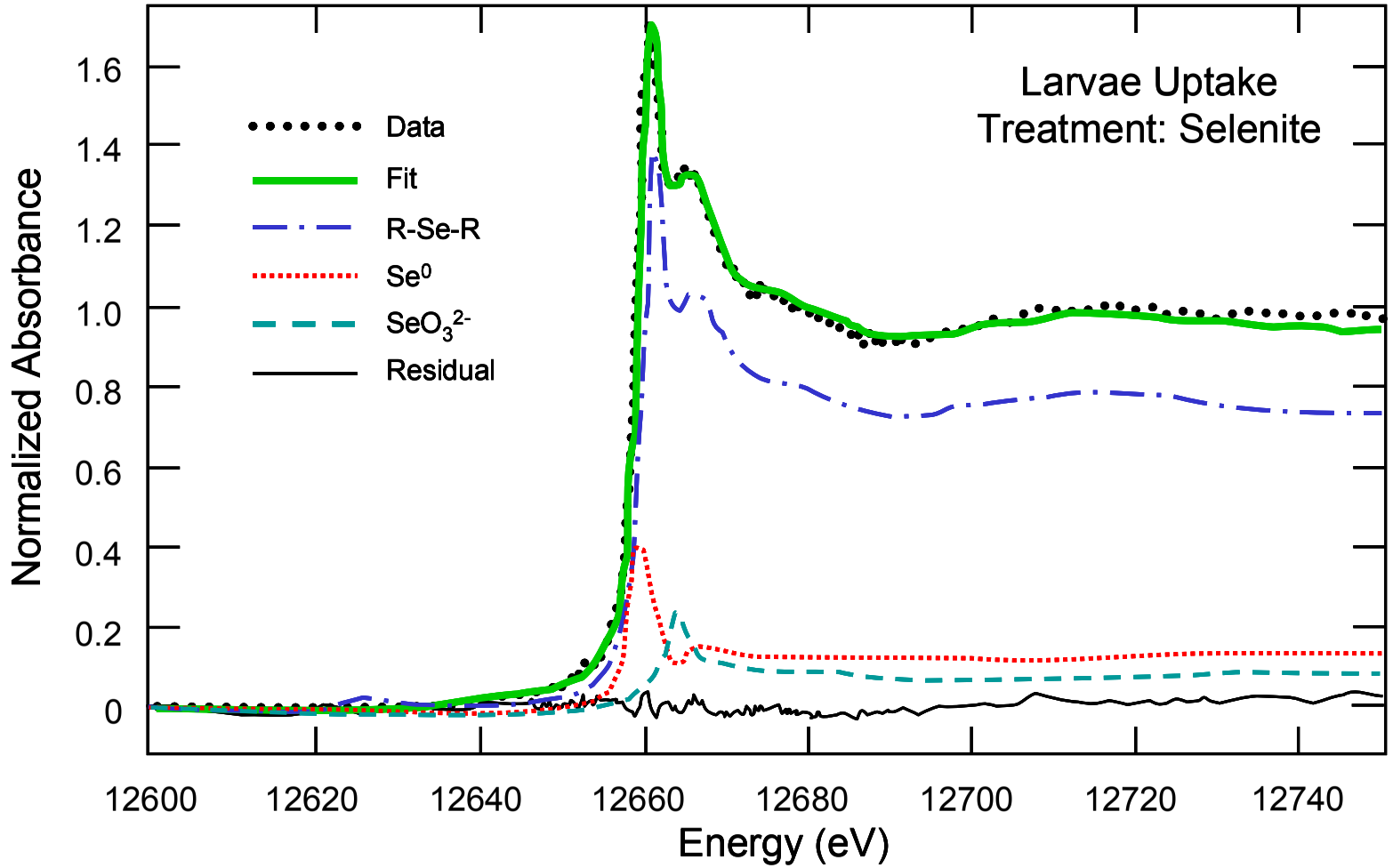

Figure A.2: X-ray absorption near-edge spectra of *Chironomus dilutus* larvae exposed to dissolved selenite for 10 days. Standards included in the fit are organic selenides (R-Se-R; selenomethionine), elemental selenium (Se^0), and selenite (SeO_3^{2-}).

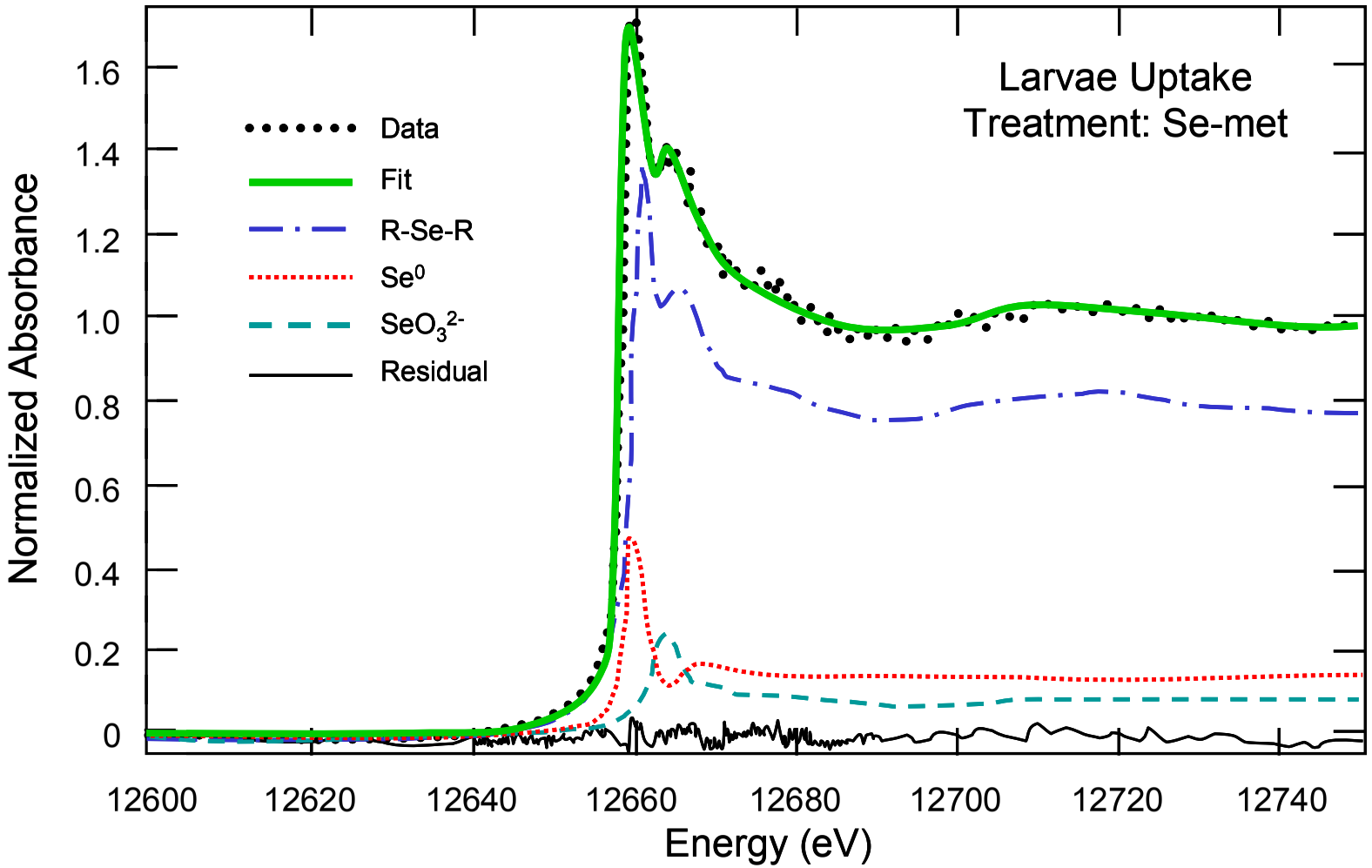

Figure A.3: X-ray absorption near-edge spectra of *Chironomus dilutus* larvae exposed to dissolved selenite for 10 days. Standards included in the fit are organic selenides (R-Se-R; selenomethionine), elemental selenium (Se^0), and selenite (SeO_3^{2-}).

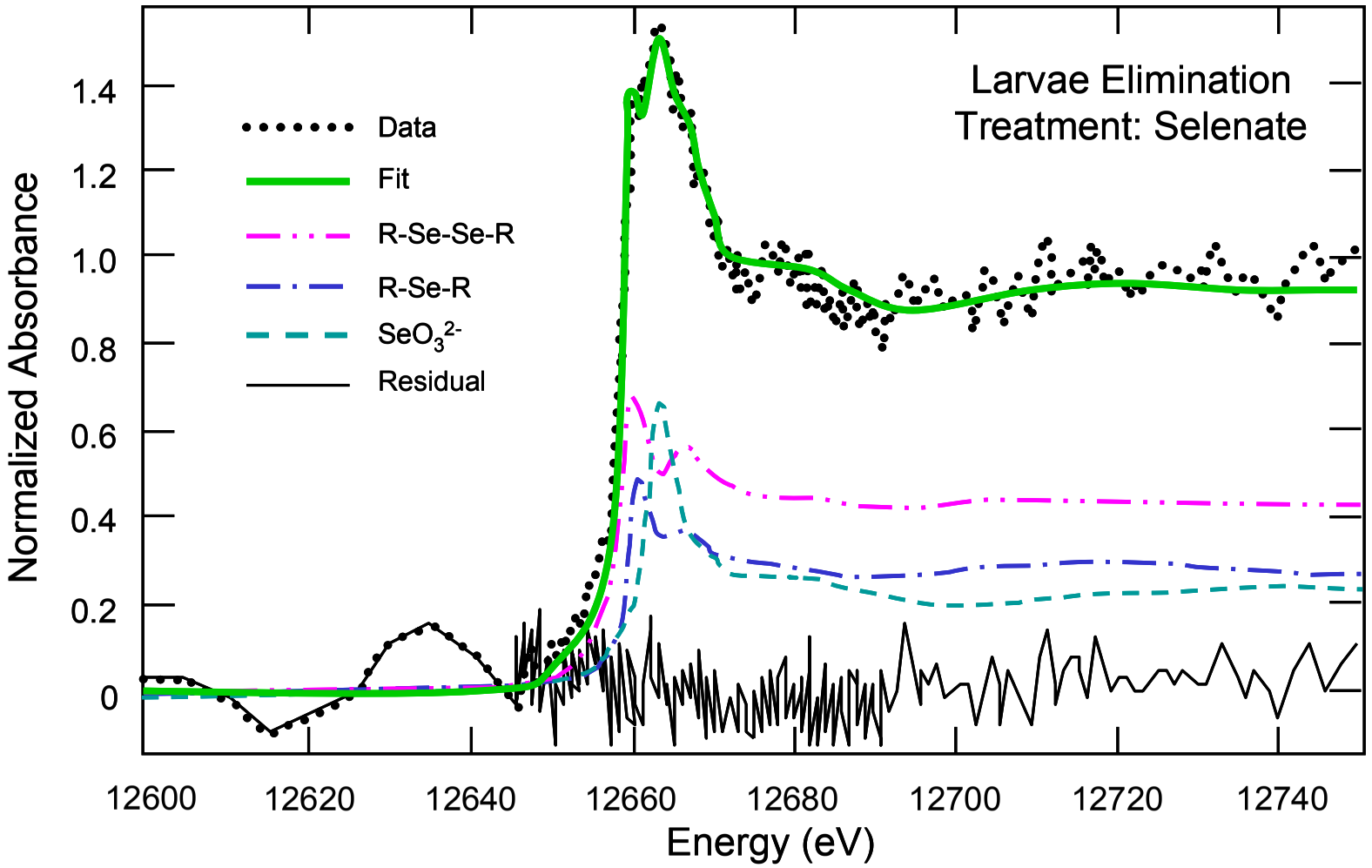

Figure A.4: X-ray absorption near-edge spectra of *Chironomus dilutus* larvae from the selenate treatment after 10 days of elimination in clean water. Standards included in the fit are organic diselenides (R-Se-Se-R; selenocystine), organic selenides (R-Se-R; selenomethionine), and inorganic selenite (SeO_3^{2-}).

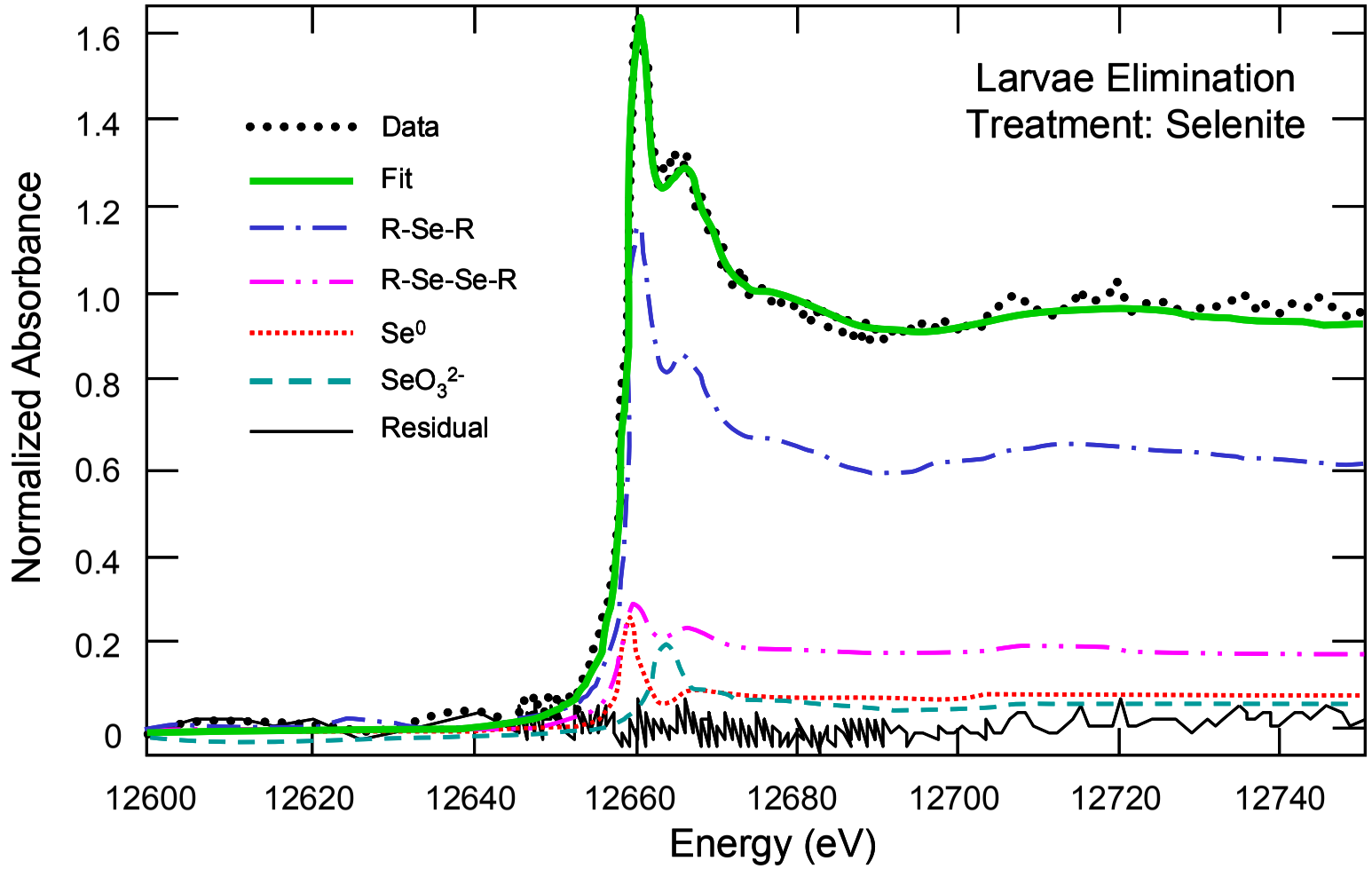

Figure A.5: X-ray absorption near-edge spectra of *Chironomus dilutus* larvae from the selenite treatment after 10 days of elimination in clean water. Standards included in the fit are organic selenides (R-Se-R; selenomethionine), organic diselenides (R-Se-Se-R; selenocystine), elemental selenium (Se^0), and selenite (SeO_3^{2-}).

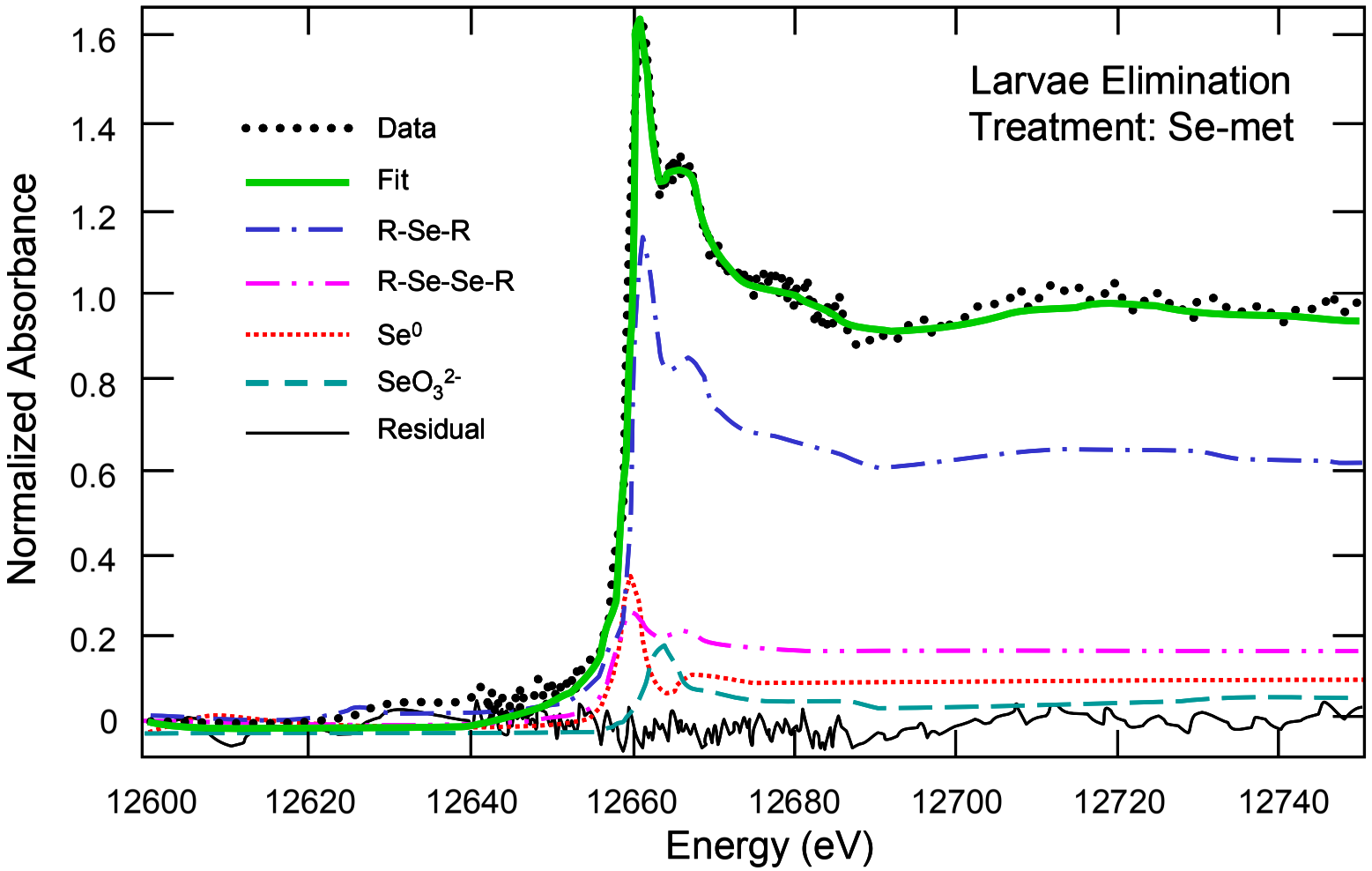

Figure A.6: X-ray absorption near-edge spectra of *Chironomus dilutus* larvae from the -DL-methionine (Se-met) treatment after 10 days of elimination in clean water. Standards included in the fit are organic selenides (R-Se-R; selenomethionine), organic diselenides (R-Se-Se-R; selenocystine), elemental selenium (Se^0), and selenite (SeO_3^{2-}).

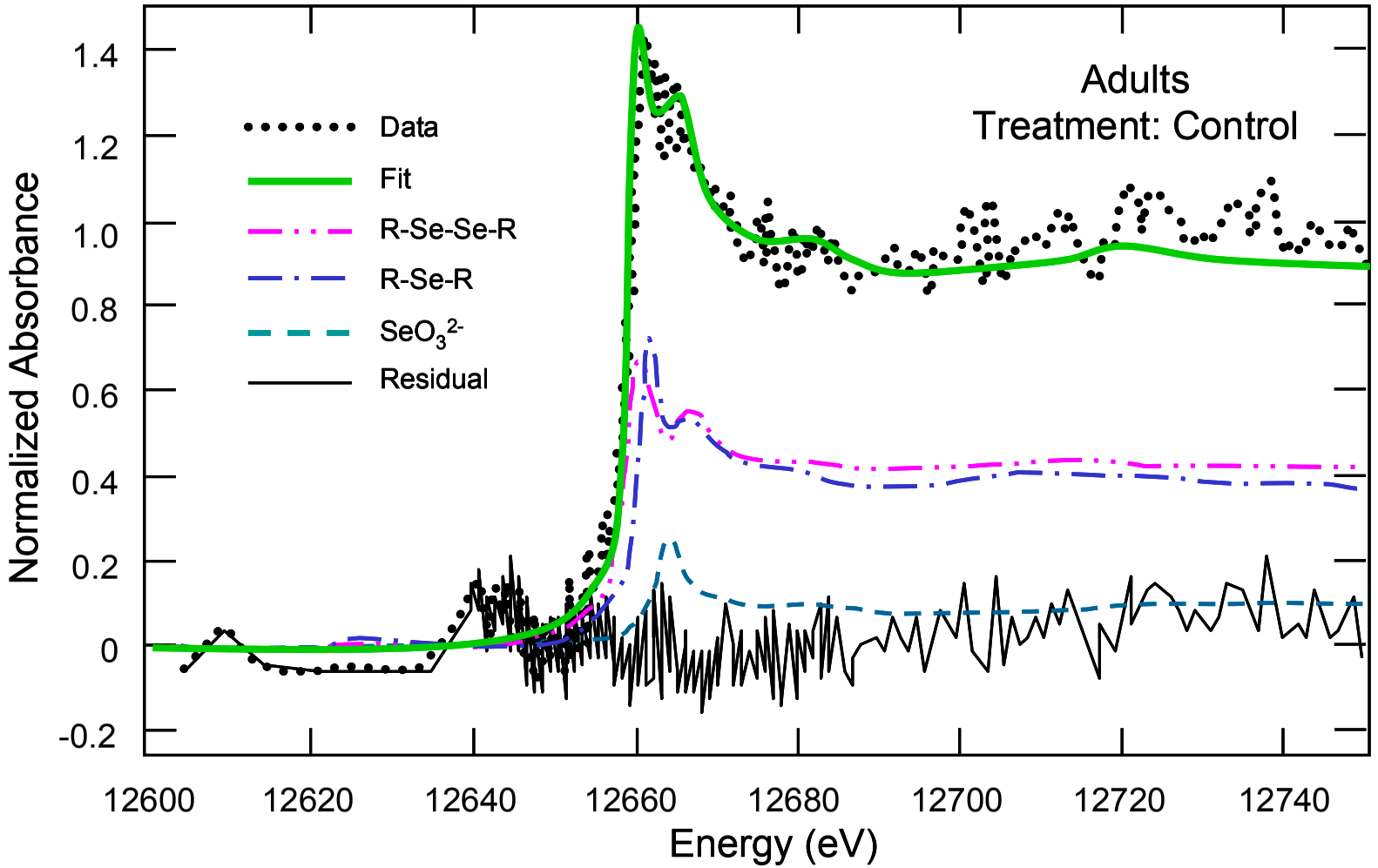

Figure A.7: X-ray absorption near-edge spectra of *Chironomus dilutus* larvae from the control treatment after continuous larval exposure until emergence. Standards included in the fit are organic diselenides (R-Se-Se-R; selenocystine), organic selenides (R-Se-R; selenomethionine), and inorganic selenite (SeO_3^{2-}).

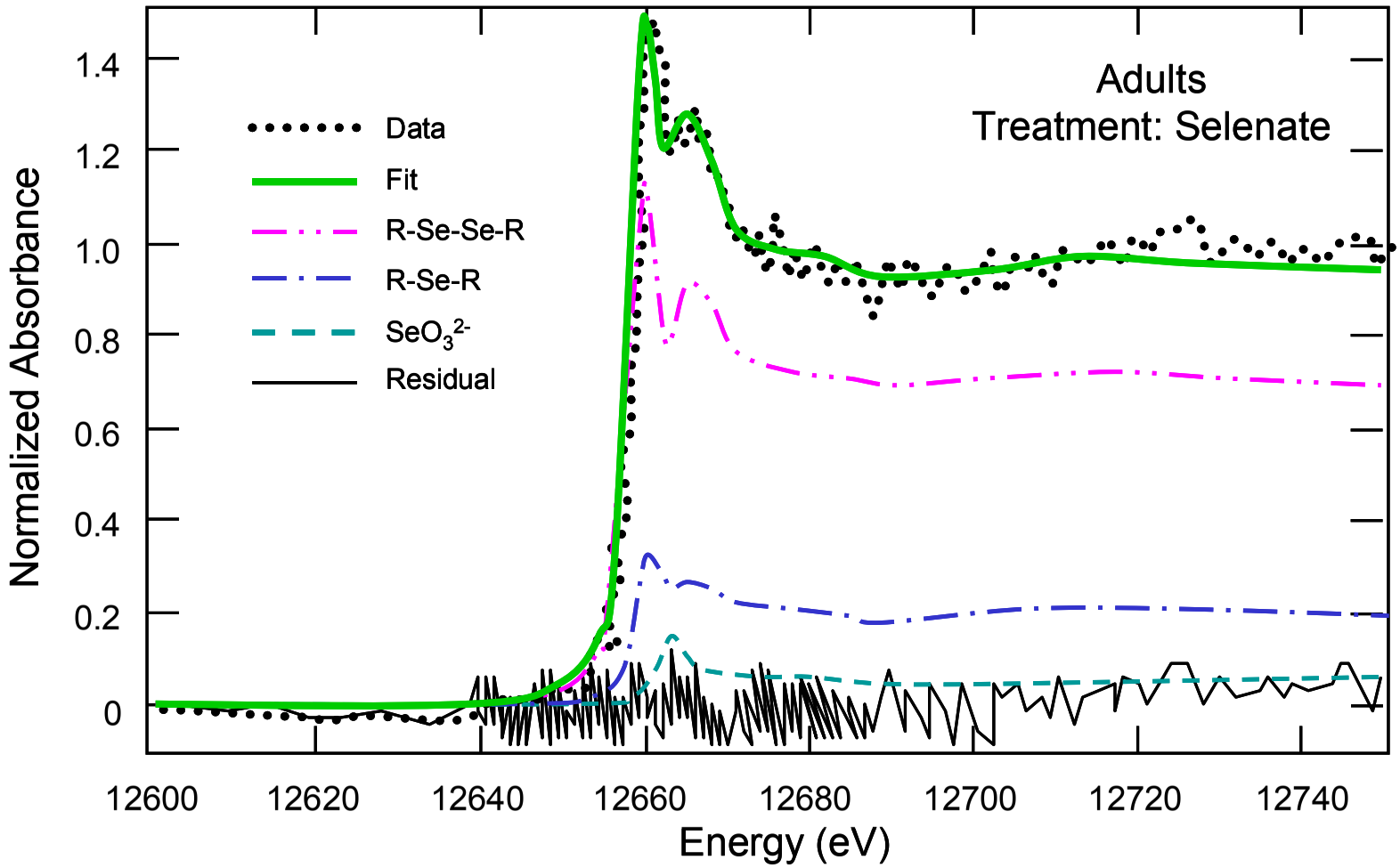

Figure A.8: X-ray absorption near-edge spectra of *Chironomus dilutus* adult insects from the selenate treatment after continuous larval exposure until emergence. Standards included in the fit are organic diselenides (R-Se-Se-R; selenocystine), organic selenides (R-Se-R; selenomethionine), and inorganic selenite (SeO_3^{2-}).

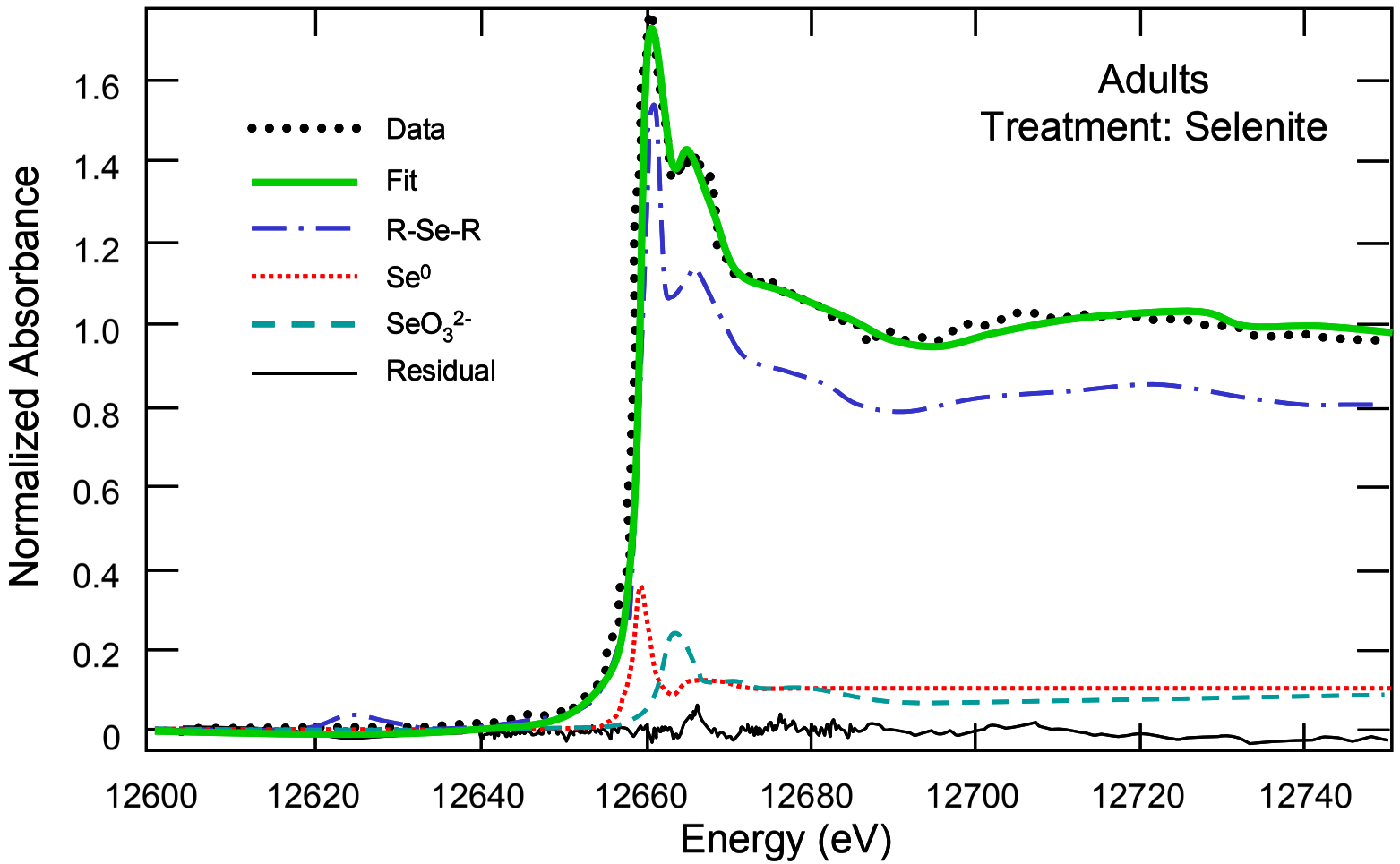

Figure A.9: X-ray absorption near-edge spectra of *Chironomus dilutus* adult insects from the selenite treatment after continuous larval exposure until emergence. Standards included in the fit are organic selenides (R-Se-R; selenomethionine), elemental selenium (Se^0), and selenite (SeO_3^{2-}).

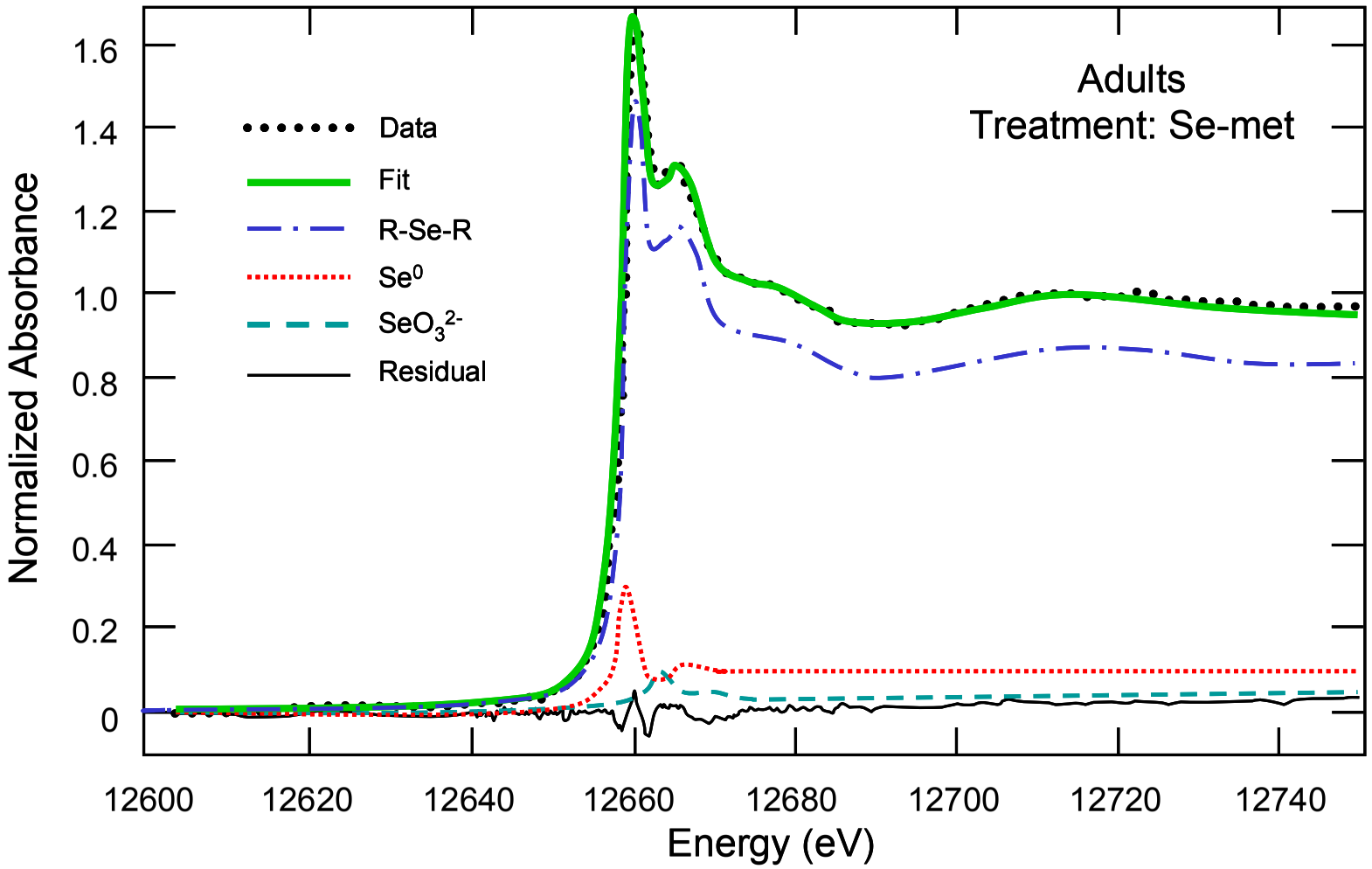

Figure A.10: X-ray absorption near-edge spectra of *Chironomus dilutus* adult insects from the seleno-DL-methionine treatment after continuous larval exposure until emergence. Standards included in the fit are organic selenides (R-Se-R; selenomethionine), elemental selenium (Se^0), and selenite (SeO_3^{2-}).

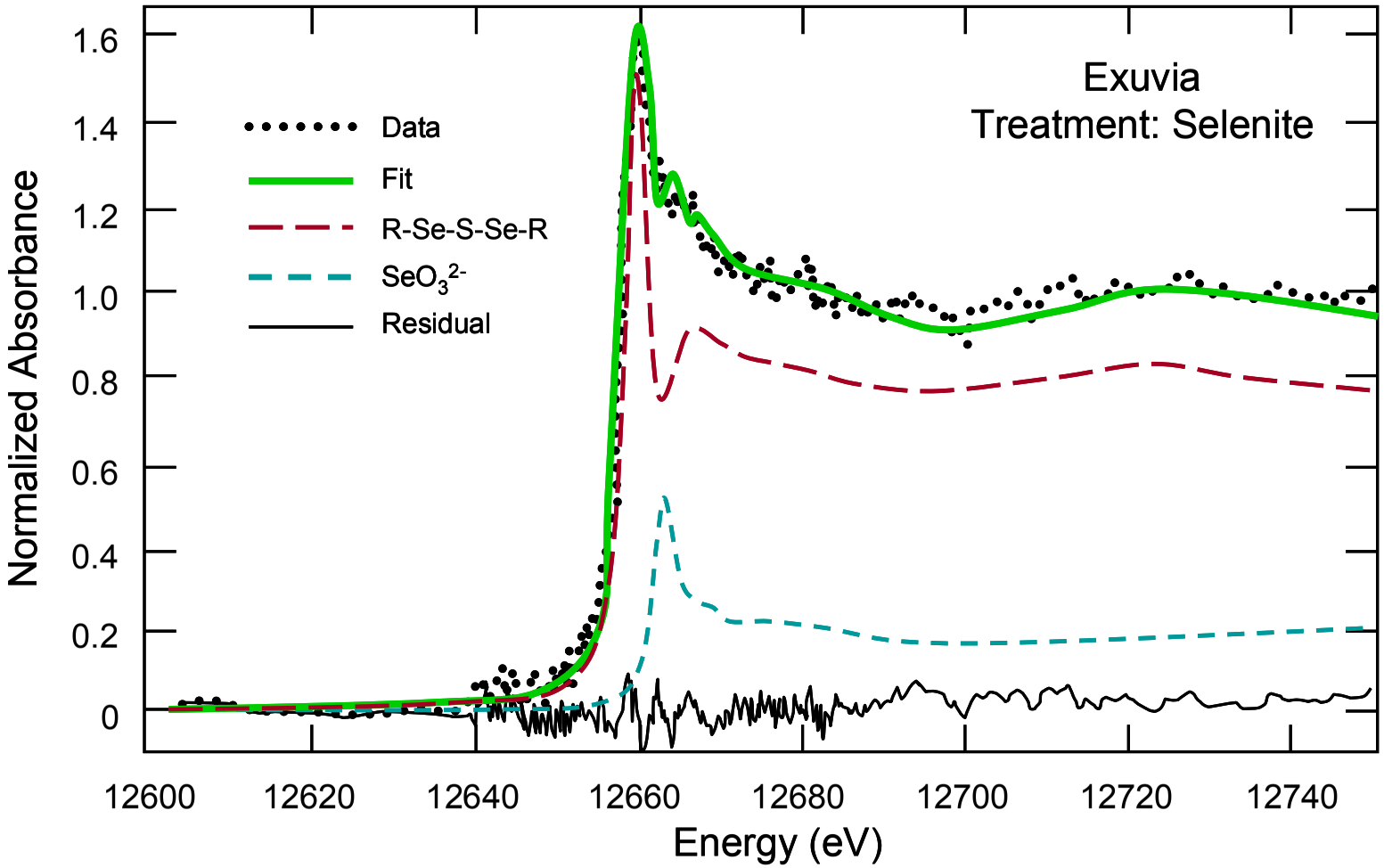

Figure A.11: X-ray absorption near-edge spectra of *Chironomus dilutus* exuvia from the selenite treatment. Standards included in the fit are seleno-*bis*-glutathione (R-S-Se-S-R), and selenite (SeO_3^{2-}).

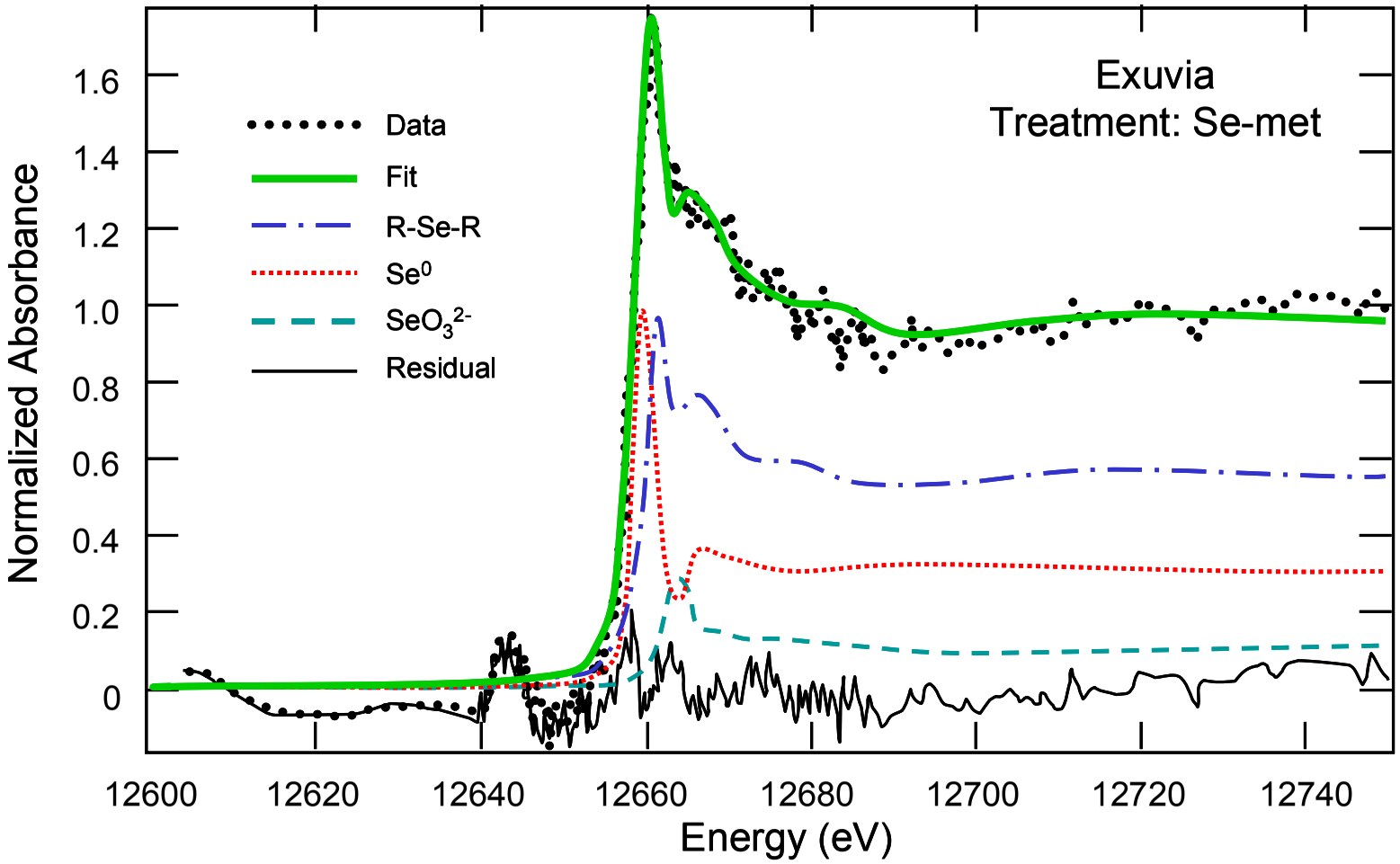

Figure A.12: X-ray absorption near-edge spectra of *Chironomus dilutus* exuvia from the seleno-DL-methionine (Se-met) treatment. Standards included in the fit are organic selenides (R-Se-R; selenomethionine), elemental selenium (Se^0), and selenite (SeO_3^{2-}).