ASSESSING THE EFFECT OF SELENIUM ON THE LIFE-CYCLE OF TWO AQUATIC INVERTEBRATES: *CERIODAPHNIA DUBIA* AND *CHIRONOMUS DILUTUS*

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RÉSUMÉ

Le ruissellement de surface ainsi que les effluents miniers ont mené à des concentrations élevées de sélénium dans les écosystèmes aquatiques. Il est connu que la biodisponibilité dépend de la spéciation chimique. Même si les espèces inorganiques dissoutes du Se ne sont pas toxiques, la prise en charge par les producteurs primaires et la transformation subséquente du Se en espèces organiques augmentent le risque et le potentiel de bioaccumulation, ce qui en bout de ligne peut affecter la reproduction des organismes plus haut dans la chaine trophique. Les effets de l'exposition au Se via la consommation de nourriture contenant du Se, a été déterminée pour 2 invertébrés aquatiques: *Ceriodaphnia dubia* et *Chironomus dilutus*. Deux générations de ces organismes ont été exposées à des algues ayant poussé dans un milieu contenant 0-40 µg L^{-1} de sélénate. Le sélénate dissout a été facilement absorbé par les algues et concentré par *Pseudokirchneriella subcapitata et Chlorella kesslerii*. Le Se n'a toutefois pas eu d'effet sur la survie des algues. De plus, l'exposition à la nourriture contenant du Se a eu peu d'effets sur le système de reproduction des invertébrés étudiés, ce qui suggère que ces deux invertébrés sont capables de réguler les effets chroniques provenant du Se dans leur alimentation. Ces résultats fournissent de l'information importante sur le rôle de la nourriture contenant du Se pour les invertébrés aquatiques.

ABSTRACT

Runoff and effluent discharge from mining activities has resulted in elevated concentrations of selenium in aquatic ecosystems. Bioavailability is dependent on chemical speciation. Although dissolved inorganic Se species are not directly toxic to organisms, uptake by primary producers and subsequent biotransformation to organo-selenium species substantially increase risk and bioaccumulation potential, potentially impairing reproduction in high-order organisms. The effects of dietary selenium exposure were assessed in two aquatic invertebrates: *Ceriodaphnia dubia* and *Chironomus dilutus*. Two generations of these organisms were exposed to seleniferous algae grown in 0-40 µg L^{-1} selenate. Dissolved selenate was readily absorbed and concentrated by algal species *Pseudokirchneriella subcapitata and Chlorella kesslerii*. Se had no effect on survival at treated concentrations. Dietary selenium exposures had little effect on reproductive endpoints in either invertebrate species, suggesting that invertebrates have the ability to regulate chronic Se exposures from dietary sources. These results provide valuable information concerning the effects of dietary selenium in aquatic invertebrates.

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PREFACE

This thesis is organized as manuscripts for publication. As such, the chapters may contain some repetition of the Introduction of this thesis.

CHAPTER 1

INTRODUCTION

Selenium is a naturally occurring trace element present in the earth's crust, soil and minerals. The metalloid has an atomic number of 34 and an atomic mass of 78.96. Selenium shares chemical properties with other members of the chalcogen group, including oxygen and sulphur; similarities to sulphur account for the many analogous chemical and biological interactions between the two elements (Unrine *et al.* 2007, Moroder 2005). Se occurs in the natural environment in four oxidation states as elemental selenium (Se^0), oxidized selenate (SeO_4^{-2}) and selenite (SeO_3^{-2}), and reduced selenides (Se^{-2}). Reduced forms exist as volatile methylated selenides, seleno-amino acids (selenomethionine and selenocysteine) and their associated proteins, and inorganic selenides (Martens and Saurez 1998). Site-specific biotransformation pathways contribute to the complex speciation and biogeochemical cycling patterns observed in aquatic environments (Chapman *et al.* 2010). Selenium is also an important elemental component of silver, copper, nickel and uranium ore deposits (CCME 2007).

1.1 Selenium in the aquatic environment

1.1.1 Sources of Se contamination

Selenium mobilization and distribution occur through natural processes as well as a result of anthropogenic activities (Presser *et al.* 1990). Se concentrations in aquatic environments are variable and dependent on biotic and abiotic factors of the region (Lemly 2002). Natural weathering of rock and soil contribute to elevated Se loads, though human activities are primarily responsible for Se mobilization to aquatic environments (Lemly 2004). Selenium is present in surface waters at background concentrations below 1 $\mu g\ L^{-1}$ (CCME 2009), but has been found at elevated concentrations at many locations in the vicinity of mining operations, petroleum refineries, coal-fired power plants and agricultural irrigation (Presser *et al.* 2004). These anthropogenic disturbances contribute to elevated selenium levels in aquatic ecosystems receiving effluent/runoff.

Selenium became a contaminant of potential concern due to two well-publicized cases of adverse effects on fish and wildlife populations (Hamilton 2004). In Belews Lake, North Carolina, the disappearance of the majority of fish species was linked to the discharge of selenium-rich effluent from a nearby coal-fired power plant (Lemly 1985). Se levels in surface water ranged between 5 and 10 μg L^{-1}, resulting in the bioaccumulation of organo-Se species in the food-web (Lemly 1985). The processing and combustion of coal is the primary anthropogenic activity responsible for the mobilization of selenium in the environment. As such, the increased reliance of the global population on coal is an environmental safety concern (Lemly 2004).

The most documented case of selenium contamination occurred in the San Joaquin Valley, California, which has undergone extensive agricultural development since the 1950s (Chapman *et al.* 2010). As part of a reclamation project, the Kesterson National Wildlife Refuge built a subsurface drainage system and reservoir to collect agricultural drainage. Concentrations of selenium in irrigation drainage entering the reservoir were not initially detected (Presser and Ohlendorf 1987), which allowed it to be discharged into surrounding ecosystems. Se was later identified in the water-column at concentrations up to 1400 μg L^{-1}, predominantly as selenate species (Presser and Ohlendorf 1987). Mass mortality in fish and birds was caused by the bioaccumulation of Se in the food-web (Ohlendorf *et al.* 1988). Congenital deformities and reproductive impairment in fish and aquatic bird species contributed to population decline and included missing or abnormal eyes, wings, limbs and beaks in addition to malformed major organs such as heart, liver and brain (Ohlendorf *et al.* 1986, Presser 1994). The series of events which occurred at the Kesterson Reservoir furthered the understanding of ecological risks associated with selenium-rich soils. The culmination of geological, biogeochemical and anthropogenic factors leading to biological effects was termed the "Kesterson Effect" (Presser 1994). In the 1980s, the Kesterson Reservoir was filled and capped to limit Se leaching from contaminated sediments (Chapman *et al.* 2010).

1.1.2 Se partitioning

The majority of aquatic selenium is located in surface waters and sediments (Andrahennadi 2007). As a result, aquatic organisms may be exposed to bioavailable

selenium via multiple pathways. Predicting exposure risk and toxicity to aquatic organisms requires site-specific knowledge of biogeochemical processes (Lemly 2002). Selenium can exist in four oxidation states (selenate, selenite, elemental selenium, and selenide), in addition to different chemical forms (inorganic and organic). Inorganic forms of selenium are taken up by microbes, algae, and plants and converted to organic forms (Freeman *et al.* 2006; Sors *et al.* 2005; Terry *et al.* 2000; White *et al.* 2007). This conversion occurs through the substitution for sulfur in amino acids methionine and cysteine (Moroder 2005). Selenium species can be grouped into four major categories: (1) inorganic Se, (2) volatile and methylated Se, (3) protein and amino acid Se, and (4) non-protein amino acids and biochemical intermediates. Bioavailability and toxicity of Se-species is dependent on its chemical properties, while site-specific biogeochemical factors dictate partitioning and transfer of Se species between biotic and abiotic environmental compartments (Lemly 2002).

1.1.2.1 Se in surface waters

Dissolved or particulate adsorbed selenium species in discharge streams are usually present as selenate and selenite (Chapman *et al.* 2010). The abundance of each oxyanion depends on the industrial process and treatment prior to the effluent discharge. Discharge from agricultural irrigation results primarily in selenate (Gao *et al.* 2007), while coal fly ash leeching results in selenite being released (Wang *et al.* 2007). Some industrial processes produce other Se species, which are treated prior to discharge, resulting in the formation of selenate and selenite (Sayre 1980). Municipal wastewater contains dissolved and suspended particulate Se. The majority of these species are removed by biological and water treatment processes (Knotek-Smith *et al.* 2006, Zhang and Frankenberger 2006, Lenz *et al.* 2008). Effluent discharge into the environment is regulated to preserve ecological integrity.

Selenate and selenite are the most common forms of inorganic dissolved Se (Besser *et al.* 1993). Selenate is the dominant species in well-aerated waters due to kinetic stability and slow reduction to selenite (USEPA 2004). As such, selenate has been characterized as the most mobile Se species in aquatic environments. In moderate oxidizing conditions, selenium occurs as selenite. Both species are stable in natural

waters (Kumar and Riyazuddin 2011, Torres *et al.* 2011), but are effectively removed from the water column by biotic factors. Both inorganic Se species have been shown to be absorbed by phytoplankton present in the water column (Riedel *et al.* 1996). Selenite uptake by some algal species has been characterized as non-biological adsorption to the cell surface (Riedel *et al.* 1991). This is explained by the polar nature of selenite, which adsorbs preferentially to suspended particulate matter within the water column. Selenate species are rapidly removed from the water column by microbial reduction to insoluble elemental Se (Se^0) and subsequent binding to organic matter at the sediment level (Zhang and Moore 1997). Organo-Se species are less abundant in surface waters than inorganic species (Orr *et al.* 2006). These compounds are poorly characterized, though they are most likely the result of microbial decomposition of biological material. Organic Se species are likely present as seleno-amino acids incorporated into proteins and peptides, and not as free amino acids in water (Fan *et al.* 2002).

1.1.2.2 Se in sediments

Once in sediments, Se is cycled between particulate and dissolved phases. Partitioning between aqueous and solid phases greatly influences the distribution of selenium in aquatic ecosystems. Sediments are a complex phase, where selenium speciation is dependent on chemical and physical properties of the sediments and biotic components (Chapman *et al.* 2010). Sediments are the dominant sink for selenium in aquatic environments, and partitioning occurs through adsorption and precipitation mechanisms (Myneni *et al.* 1997, Duc *et al.* 2003).

Partitioning to sediments occurs primarily as a result of death and decomposition of Se-enriched organisms. In contaminated aquatic ecosystems, up to 90% of selenium can be found in the upper few centimetres of the sediments and overlying detritus (Simmons and Wallschlager 2005). As a result, Se can be found in sediments long after input to the system has ceased (Swift 2002). In a simulated experiment, an artificial stream receiving 10 μg selenite L^{-1} contained 5-10 μg g^{-1} two years after dosing ceased (Swift 2002). Once Se has been introduced, contamination persists. Lentic environments are more conducive to the accumulation of selenium in sediments than lotic systems due

mainly to longer water retention and larger deposits of organic material in the sediments (Canton and Van Derveer 1997).

Redox potential, influenced by microbial activity, has a significant influence on partitioning of selenium to sediments (Schink 1997). Low redox conditions favor Se species with low solubility such as iron selenide and elemental selenium (Tokunaga *et al.* 1997). Insoluble Se compounds are stable, facilitating their precipitation and accumulation in sediments. However, Gallego-Gallegos *et al.* (2013) claim that elemental Se nanoparticles are an available source of Se to benthic invertebrates. Exposure of *C. dilutus* benthic invertebrates to Se nanoparticles resulted in high Se bioaccumulation and larval growth inhibition (Gallego-Gallegos *et al.* 2013). Elemental Se nanoparticles may no longer be considered environmentally inert, and should be further evaluated as a potentially bioavailable Se species in aquatic ecosystems.

There are a variety of organisms, microorganisms, and aquatic plants which influence the cycling of inorganic and organic selenium species. They play an important part of the biogeochemical cycle in aquatic ecosystems (Siddique *et al.* 2006). Organic species accumulate in sediments via decomposition and decay of primary producers that have absorbed and transformed inorganic Se to organic forms (Orr *et al.* 2006). Accumulation of bioavailable organic selenides in sediments accounts for a significant portion of total selenium in a contaminated system (Cutter *et al.* 1990).

1.2 Bioaccumulation and trophic transfer

The propensity for Se to bioaccumulate in the food web increases risk to aquatic organisms. Selenium uptake at the base of the food web by primary producers and microorganisms is the largest bioconcentration step (Luoma and Presser 2009). Dietary exposure constitutes the primary pathway of Se bioaccumulation, toxicity and trophic transfer (Muscatello and Janz 2009). Accumulation can occur through water-borne pathways, but compared to dietary exposure levels, aquatic exposure does not pose a significant risk to most species (Janz 2011). Relying solely on monitoring of dissolved Se concentrations is not an effective predictor of exposure levels. Despite data indicating that dietary exposure pathways are more toxic to aquatic organisms, ecotoxicological regulations regarding permissible Se exposure levels are based on water-borne Se

concentrations (1 μg L^{-1}, Canadian Water Quality Guidelines for the Protection of Aquatic Life 1987). In British Columbia, the Ministry of Environment has released a draft guideline for selenium which includes water and tissue levels (British Columbia Ministry of Environment 2012). The document outlines specific regulations for water and dietary Se exposures, as well as tissue concentrations in fish (egg/ovary, whole-body, muscle) and birds (egg). In the United States, the USEPA has recognized that basing regulations solely on dissolved Se exposure concentrations is insufficient and is considering a national fish-tissue criterion for managing Se contamination (USEPA 2004). These criterions are still under revision, which indicates the difficulty associated with the protection of aquatic organisms at Se contaminated sites.

1.2.1 Se uptake by primary producers

Selenium is an essential micronutrient for algal growth (Hoffman 2002). It is generally agreed that sulfur and selenium follow the same metabolic pathway in plant species (Freeman *et al.* 2006; Sors *et al.* 2005; Terry *et al.* 2000; White *et al.* 2007). Selenate is actively absorbed from water by a sulfate transport protein, metabolized by sulfur pathways and incorporated into sulfur-containing biomolecules. The conversion of selenate to organic Se compounds in plants proceeds through reduction to selenite, selenide (-II), selenocysteine, and finally selenomethionine. Following their synthesis, selenomethionine and selenocysteine are incorporated into proteins (White *et al.* 2007). While selenate is actively transported into the cell by membrane proteins, selenite is passively absorbed (Riedel 1991). Concentration by primary producers from water is the most important step of bioaccumulation in the aquatic food web (Luoma and Presser 2009). Selenium concentrations in algae are typically 100-500 times higher than dissolved selenate concentrations (Luoma 2009). Primary producers are the only organisms that can synthesize selenomethionine (Spallholz and Hoffman 2002). Selenium accumulation in primary producers has the potential to become toxic by concentrating Se species in a biologically available form that can accumulate through the food chain (Muscatello and Janz 2009).

1.2.2 Se accumulation in invertebrates

As the majority of Se in contaminated sediments is found in the upper few centimetres of sediments and detritus (Simmons and Wallschlager 2005), benthic invertebrates inhabiting this layer of sediments are exposed to high dietary Se concentrations. Although the food chain is the most important route of exposure for upper trophic level organisms (Muscatello and Janz 2009), studies assessing the accumulation and toxicity of selenium in lower order organisms are limited. This information is important, as primary producers are the link between dissolved inorganic Se and organo-Se consumers (Spallholz and Hoffman 2002). Dissolved inorganic selenium species are taken up by algae, which is then ingested by filter-feeders, leading to bioaccumulation of Se and transfer to higher trophic levels. It is important to consider low trophic-level sublethal effects in order to identify and manage potential environmental health impacts from Se exposure. Se uptake by invertebrates is dependent on the concentration and bioavailability in the sediments and surface waters (Orr *et al.* 2006). Habitat preferences, feeding patterns and kinetics account for differences between invertebrate species (Presser and Luoma 2010). Therefore, knowledge of site-specific food-web interactions is critical to assess risk to aquatic organisms at all levels. Limited understanding of Se bioaccumulation in freshwater invertebrates is due to poor characterization of Se concentration and species in sediment detritus ingested by benthic invertebrates. Dietary preferences of many invertebrate species are not well established (Chapman *et al.* 2010).

1.3 Physiological role of selenium

Selenium is an essential micronutrient required for growth and development in virtually all species of bacteria, plants and animals (Hoffman 2002). The metabolism of selenium is highly specific and involves several inorganic and organic Se-compounds and intermediate metabolites in the formation of selenium-containing proteins (Daniels 1996). There are three types of selenium-containing proteins: (1) proteins containing seleno-cysteine, (2) proteins containing non-specifically incorporated selenium, and (3) proteins that bind selenium (Janz 2011).

1.3.1 Selenoproteins

Physiological functions of Se are mediated through selenoproteins that specifically incorporate selenocysteine at the active site during proteins synthesis (Lobanov 2007). Selenocysteine is only incorporated when it is required for protein function, specifically as a key functional group in enzymes involved in antioxidant and other functions (Gladyshev 2001). These enzymes are fully ionized as physiological pH, making them efficient catalysts (Reilly 2007). Compared to proteins with cysteine at the active site, selenocysteine is 1000 times more effective as a redox catalyst (Lobanov 2009).

1.3.2 Metabolism

The primary pathways involved in Se metabolism produce seleno-amino acids selenocysteine and selenomethionine (Daniels 1996). These amino acids are homologous to cysteine and methionine, with Se substituting for sulphur during amino acid synthesis (Moroder 2005). Selenocysteine synthesis is tightly regulated for each selenoprotein and governed by a UGA codon on mRNA that is specific to selenocysteinyl-tRNA (Daniels 1996). Selenocysteine metabolism is unique compared to other amino acids; free selenocysteine is not found within the cell because its highly reactive properties would result in cellular damage (Daniels 1996).

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). Thus, in aquatic ecosystems primary producers are responsible for the introduction of selenomethionine into the food web. Unlike selenocysteine, selenomethionine is not required for any specific physiological function and can be non-specifically incorporated into proteins (Schrauzer 2000). The enzyme that charges methionyl-tRNA does not discriminate between methionine and selenomethionine (Moroder 2005, Schrauzer 2000), and synthesis is dependent on the relative abundance of Se versus S within the cell (Schrauzer 2000). Although animals are incapable of synthesizing selenomethionine, they are capable of metabolizing the species to selenite, which can be used in the synthesis of selenocysteine (Schrauzer 2000). Selenomethionine has gained attention in the risk assessment of

contaminated systems due to its bioavailability and toxic effects in egg-laying vertebrates (Hamilton 2004).

1.3.3 Se toxicity

There is a narrow margin between essential versus toxic Se concentrations (Hoffman 2002). Predators readily accumulate selenium from Se-contaminated food (Bertram and Brooks 1986, Hamilton *et al*. 1990, Misra *et al.* 2012). Nutritional Se requirements are species specific. For fish, dietary Se exposures below 3 $\mu g\ g^{-1}$ dry wt. is optimal, while slight increases (7-30 times) significantly elevate the risk of embryonic toxicity (Janz 2011). Optimal dietary intake for birds should not exceed 5 $\mu g\ g^{-1}$ dry wt. (Ohlendorf *et al.* 2011). Toxicity is dependent on both concentration and speciation of Se. Regardless of the Se species introduced into an aquatic system, organic forms of Se (predominantly selenomethionine) are the primary compounds found in whole-body tissues (Gallego-Gallegos *et al.* 2013). Egg laying vertebrates exposed to a chronic selenium-enriched diet have demonstrated reproductive failure and teratogenesis, resulting in substantial population impacts (Ohlendorf *et al.* 1988, Janz 2011). Effects occur due to embryonic exposure to Se as a result of maternal transfer to offspring (Simmons and Wallschlager 2005). Selenomethionine is the cause of embryonic mortality and teratogenic effects in egg-laying vertebrates, as it is the primary form of Se deposited in eggs during reproduction (Janz 2011).

The proposed mechanism of Se toxicity is thought to be related to altered enzyme function due to the substitution of selenomethionine for methionine during embryonic development (Lemly 1997). Although, more recently, protein folding has been shown to be largely unaffected by the substitution (Schrauzer 2000). Altered enzyme activity has been observed in *E. coli* when selenomethionine replaces methionine near the active site of some enzymes (Schrauzer 2000). Alternatively, oxidative stress caused by the metabolism of selenomethionine may be responsible for toxic response in fish and birds (Spallholz and Hoffman 2002, Palace 2004). Although there is no direct evidence that the metabolism of selenomethionine generates reactive oxygen species, the metabolism of selenomethionine to reactive Se metabolites has been proposed to generate free radicals causing DNA damage and altered protein function (Lenz and Lens 2009). In addition,

dietary exposure to selenium has been linked to an increased stress response (Wiseman 2011), and altered movements in fish (Thomas and Janz 2011). These responses to Se have the potential to cause significant population-level effects in aquatic ecosystems.

Studies have focused primarily on fish and birds, while invertebrates have been relegated to a source of dietary Se to higher trophic levels. According to deBruyn and Chapman (2007), invertebrate species may be more sensitive to the toxic effects of Se. The risk of reproductive sublethal effects on aquatic invertebrates is of concern. Additional research is required to assess whether invertebrates are protected by current water quality guidelines.

1.4 Algal species: *Pseudokirchneriella subcapitata and Chlorella kesslerii*

Pseudokirchneriella subcapitata and *Chlorella kesslerii* are non-motile, unicellular green algae (8-14 µm and 2-10 µm, respectively) (Environment Canada 2007a). These species are easily cultured in the laboratory as they reach exponential growth 3-4 days after inoculation, and their morphology makes them ideal for enumeration with an electronic particle counter. *P. subcapitata* and *C. kesslerii* are particularly suited to assess the potential toxicity of selenium concentration from water, as algal species are a major component of food chains due to their ubiquity in aquatic ecosystems (Environment Canada 2007a). As primary producers, they are capable of producing selenomethionine via concentration and transformation of inorganic Se species from water (Simmons and Wallschlager 2011). These algal species were specifically chosen as they have been successfully cultured and implemented in the diet of the invertebrates to be used for testing: *Ceriodaphnia dubia* and *Chironomus dilutus*.

1.5 *Ceriodaphnia dubia*

Ceriodaphnia dubia is a small (< 1mm), planktonic crustacean (Environment Canada 2007b). A healthy culture consists of solely female organisms as males are indicators of environmental stressors such as low temperature, drought conditions, or environmental contaminants. Daphnia reproduce parthenogenetically (Environment Canada 2007b); fully mature females are capable of producing several broods over a lifetime. Newly hatched daphnia molt their exoskeleton several times during growth into

adulthood. Daphnids inhabit various aquatic environments such as freshwater lakes, ponds and marshes and feed on suspended particulate matter and detritus at the sediment surface. This invertebrate is often used in laboratory testing of effluent from municipal and industrial sources. They are particularly useful as laboratory testing organisms due to their small size, short lifespan, reproductive capabilities, and sensitivity to aquatic contaminants.

Most standardised ecotoxicological tests evaluating aquatic exposures to invertebrates have been performed with crustaceans. Planktonic crustaceans, such as daphnids, are an important food and energy link between primary producers (algae) and secondary consumers (fish). It is important to consider low trophic-level sublethal effects in order to identify and manage potential environmental health impacts from Se exposure. Daphnia species are the most commonly used invertebrate species in regulatory chemical testing and several guidelines exist for both acute and chronic tests (Environment Canada 2007b, USEPA 2002, CSIRO 1997). No standardized tests exist to address the effects of dietary exposures, but as current tests incorporate algal feeding regimens, the use of Se-enriched algae is an appropriate modification to explore this exposure pathway.

1.6 *Chironomus dilutus*

Chironomus dilutus (formerly *Chironomus tentans*) is a non-biting midge often used in both field and laboratory tests. The benthic invertebrate progresses through three aquatic life stages: egg, larvae and pupae, followed by a terrestrial adult stage. Eggs are laid in gelatinous egg masses and typically hatch within 2 days of oviposition (Benoit *et al.* 1997). Larvae develop through four instars, each lasting between four and seven days (Environment Canada 1997). Pupation occurs at the end of the fourth instar. The pupal stage typically lasts no more than 24 hours, and is followed by emergence of the adult insect. Emergence follows a bimodal distribution with peak male emergence occurring prior to female emergence, with the complete cycle taking approximately 23-30 days at 23°C under laboratory conditions (Benoit *et al.* 1997). Female midges generally produce a single egg mass within 24 hours of mating.

The midge *C. dilutus* is a major component of aquatic food chains due to its ubiquity in aquatic ecosystems (Environment Canada 1997). A short life-cycle, ease of

use, tolerance to a wide-range of physical and chemical conditions in both sediments and water and large number of developmental and reproductive endpoints make it a preferred choice for chronic sediment toxicity testing. The benthic macroinvertebrate *C. dilutus* is particularly suited to assess the potential toxicity of contaminated sediments as it burrows in the upper layer of sediment and feeds on detritus at the sediment surface during larval development. Ingestion of organic material is expected to contribute to the bioaccumulation and toxicity of Se for larvae.

1.7 Research goals and objectives

The goal of this research was to assess the multi-generational effects of dietary selenium on the life-cycle of aquatic invertebrates. To address this goal, the research was divided into three objectives:

1. Assess selenium uptake and speciation in algal species: *Pseudokirchneriella subcapitata* and *Chlorella kesslerii* based on exposure to dissolved selenate under laboratory conditions.
2. Determine the multi-generational effects of selenium exposure in *Ceriodaphnia dubia* based on exposure via two key pathways: aquatic (dissolved selenate) and dietary (selenium-enriched algae).
3. Establish an environmentally relevant method for life-cycle testing to determine the multi-generational effects of dietary selenium exposure in *Chironomus dilutus* based on exposures to selenium-enriched algae.

CHAPTER 2

ASSESSMENT OF SELENIUM UPTAKE IN ALGAL SPECIES: *PSEUDOKIRCHNERIELLA SUBCAPITATA* AND *CHLORELLA KESSLERII*

2.1 Introduction

Selenium is an essential micronutrient with a narrow margin between nutritionally optimal and toxic dietary exposures (Hoffman 2002). Selenium is present in surface waters at background concentrations below 1 μg L^{-1} (CCME 2009), but has been found at elevated concentrations at many locations in the vicinity of mining operations, petroleum refineries, coal-fired power plants, and agricultural irrigation (Presser *et al*. 2004). These anthropogenic disturbances contribute to elevated selenium levels in aquatic ecosystems receiving effluent/runoff. In Southeast British Columbia, open-pit coal mining has led to effluent concentrations exceeding 300 μg Se L^{-1} (Martin *et al.* 2008). Se concentrations in Elk River were 9.6 μg L^{-1} in 2008, and continued to rise, resulting in reproductive failure and teratogenesis in egg laying vertebrates (Elk Valley Se Task Force 2008).

Selenium can exist in four oxidation states (selenate, selenite, elemental selenium, and selenide), in addition to different chemical forms (inorganic and organic). Dissolved or particulate adsorbed selenium species in discharge streams are usually present as selenate and selenite (Chapman *et al.* 2010). The abundance of each oxyanion depends on the industrial process and treatment prior to effluent discharge. Discharge from agricultural irrigation results primarily in selenate (Gao *et al.* 2007), while coal fly ash leeching results in selenite being released (Wang *et al.* 2007). Some industrial processes produce other Se species, which are treated prior to discharge, resulting in the formation of selenate and selenite (Sayre 1980). Municipal wastewater contains dissolved and suspended particulate Se. The majority of these species are removed by biological and water treatment processes (Knotek-Smith *et al.* 2006, Zhang and Frankenberger 2006, Lenz *et al.* 2008). Effluent discharge into the environment is regulated to preserve ecological integrity.

Selenate is the dominant species in well-aerated waters due to kinetic stability and slow reduction to selenite (USEPA 2004). As such, selenate has been characterized as the most mobile Se species in aquatic environments. In moderate oxidizing conditions,

selenium occurs as selenite. Both species are stable in natural waters (Kumar and Riyazuddin 2011, Torres *et al.* 2011), but are effectively removed from the water column by biotic factors. Both inorganic Se species are taken up by microbes, algae and plants and converted to organic forms (Freeman *et al.* 2006; Sors *et al.* 2005; Terry *et al.* 2000; White *et al.* 2007). This conversion occurs through the substitution for sulfur in amino acids methionine and cysteine (Moroder 2005). Bioavailability and toxicity of Se-species is dependent on its chemical properties, while site-specific biogeochemical factors dictate partitioning and transfer of Se species between biotic and abiotic environmental compartments (Lemly 2002). Lentic environments are more conducive to the accumulation of selenium than lotic systems due mainly to longer water retention and larger deposits of organic material in the sediments (Canton and Van Derveer 1997).

It is generally agreed that sulfur and selenium follow the same metabolic pathway in plant species (Freeman *et al.* 2006; Sors *et al.* 2005; Terry *et al.* 2000; White *et al.* 2007). Selenate is actively absorbed from water by a sulfate transport protein, metabolized by sulfur pathways and incorporated into sulfur-containing biomolecules. The conversion of selenate to organic Se compounds in plants proceeds through reduction to selenite, selenide (-II), selenocysteine, and finally selenomethionine. Following their synthesis, selenomethionine and selenocysteine are incorporated into proteins (White *et al.* 2007). Se uptake in *Chlorella kesslerii* (formerly *Chlorella vulgaris*) has been investigated by Simmons and Wallschlager (2011). They found that significant changes in Se species occurred with the onset of exponential growth; selenate species were gradually reduced to selenite. While there is some understanding of the process for biological transformation within algae it has been suggested that different algal species have varying uptake capacities for selenium (Chapman *et al*. 2010). While selenate is actively transported into the cell by membrane proteins, selenite is passively absorbed (Riedel 1991). Selenium concentrations in algae are typically 100-500 times higher than dissolved selenate concentrations (Luoma and Presser 2009). Primary producers are the only organisms that can synthesize selenomethionine (Spallholz and Hoffman 2002). Environmental concentrations of dissolved selenite and selenate are not directly toxic to aquatic organisms (Lemly 1985, Luoma 1992), while organic selenides pose a substantial risk to aquatic organisms at low exposure concentrations (Ingersoll 1990).

The propensity for Se to bioaccumulate in the food web increases risk to aquatic organisms. Selenium uptake at the base of the food web by primary producers and microorganisms is the largest bioconcentration step (Luoma and Presser 2009). Dietary exposure constitutes the primary pathway of Se bioaccumulation, toxicity and trophic transfer (Muscatello and Janz 2009). Accumulation can occur through water-borne pathways, but compared to dietary exposure levels, aquatic exposure does not pose a significant risk to most species (Janz 2011). As selenium accumulation in algae from water has the potential to bioconcentrate Se species in a highly bioavailable form it is important that the link between dissolved inorganic Se and organo-se consumers be investigated further.

Two algal species were used to investigate bioconcentration of selenium in this study: *Pseudokirchneriella subcapitata* and *Chlorella kesslerii*. Both species are nonmotile, unicellular green algae (8-14 µm and 2-10 µm, respectively) (Environment Canada 2007a). *P. subcapitata* and *C. kesslerii* are particularly suited to assess the potential toxicity of selenium concentration from water, as algal species are a major component of food chains due to their ubiquity in aquatic ecosystems. Algae are a food source for several aquatic organisms including aquatic invertebrates such as daphnia. These species are easily cultured in the laboratory and their morphology makes them ideal for enumeration with an electronic particle counter. A well-established method exists to assess direct toxicity of aquatic contaminants to *P. subcapitata* (Environment Canada 2007a). Unfortunately, no standard method currently exists for the determination of Se toxicity in *C. kesslerii*.

The objectives of this study were (1) to determine selenium species transformation and influence on uptake in two algal species, (2) to determine Se bioconcentration in algae at various concentrations of exposure, and (3) to develop a selenium-enriched algal food source for use in invertebrate toxicity tests.

2.2 Materials and methods

2.2.1 Culturing

Algal species *Pseudokirchneriella subcapitata* and *Chlorella kesslerii* were obtained from the Canadian Phycological Culture Centre at the University of Waterloo

(Waterloo, ON) and cultured separately in modified Bristol's Medium (Bold 1949) under constant aeration for 7-days in an incubator chamber at 24±1°C under continuous "cool white" fluorescent light with an intensity of 4000 lux. Algal species *P. subcapitata* and *C. kesslerii* were cultured under aseptic conditions in Erlenmeyer flasks at final volumes of 3100 and 1600 mL, respectively. Differences between culturing volumes was based on cell density requirements for each species in the preparation of an algal food source (3.5 million cells mL^{-1}, 3:1 ratio *P. subcapitata*:*C. kesslerii*).

2.2.2 Media preparation

Selenium stock solutions were prepared by adding analytical reagent grade $NaSeO_4$ (99.8+%, Alfa Aesar) to ultrapure water (Milli-Q, Millipore Corp.). Nominal test concentrations (0-327.68 mg L^{-1}, 0-100 µg Se L^{-1}) were prepared by adding appropriate aliquot volumes to modified Bristol's Medium. Due to space limitations, algal exposures to aquatic Se could not be replicated; sampling consisted of three pseudo-replicates per flask at each time point. Seleniferous media samples (10 mL) were collected to determine total Se exposure concentrations and speciation at inoculation (t=0), during and following growth (t=1, 3, 7 days). All media samples were frozen.

2.2.3 Selenium toxicity testing

Selenium toxicity in *P. subcapitata* was assessed according to the standard Environment Canada method (2007a). Algal cells were exposed to selenate at 0.0025, 0.005, 0.01, 0.02, 0.04, 0.08, 0.16, 0.32, 0.64 and 1.28 mg L^{-1} in triplicate (N=3). The control consisted of no exposure to selenate (0 mg L^{-1}, N=8). Algal cell concentrations were determined using a Coulter Counter (Beckman Coulter Inc.) prior to, and following the 3-day exposure period.

2.2.4 Algal Se exposures

Se-enriched algae was produced from the addition of selenate in the growth medium at concentrations of 0, 0.625, 1.25, 2.5, 5, 10, 20, 40, or 100 µg L^{-1} prior to inoculation. Following growth, algal cells were pelleted by centrifugation at 2000 rpm (Sorvall RC-5B, Dupont Instruments) for 15 minutes. Algal cells were re-suspended in

ultrapure water and centrifuged to ensure removal of seleniferous growth media. One algal cell sample (20 mL) from each exposure was collected to determine total Se uptake; sampling could not be replicated, as algal cells were required for the preparation of a seleniferous algal feedout. Algal cell concentration was determined using a Coulter Counter (Beckman Coulter Inc.) and an algal feedout was prepared with a concentration of 3.5 million cells mL^{-1} and a 3:1 ratio of *P. subcapitata:C. kesslerii*.

2.2.5 Se uptake

To evaluate Se uptake in algal cells, samples (20 mL) were dried and incubated at room temperature with 4 mL of concentrated trace-metal grade HNO_3 (Fisher Scientific) for seven days, followed by the addition of 3 mL of 30% H_2O_2 (Fisher Scientific) for 24 hrs. Digests were diluted to 10 mL with ultrapure water and filtered (0.45 µm) prior to analysis. Tissue digestion was verified using apple leaves standard reference material (National Institute of Standards and Technology). Digested standard reference material, method blanks and algae samples were analyzed using liquid chromatography mass spectrometry (LC-MS). Standard reference material samples yielded >100 % recovery of the 0.050±0.009 µg Se g^{-1} certified value. Total selenium concentration in modified Bristol's Medium was measured (ICP-MS). Selenium species in growth medium were determined by ion chromatography inductively coupled plasma mass spectrometry (IC-ICP-MS).

2.2.6 BCF calculation

Bioconcentration factors (BCF) were calculated using the ratio of algal concentration (µg Se g^{-1} wet wt) and aquatic exposure concentration (µg L^{-1}) (McGeer *et al.* 2003). Dry weight data was converted to wet weight with a dry-to-wet conversion ratio of 0.1 for alga (McGeer *et al.* 2003). The relationship between aquatic selenium exposure and bioconcentration in algal species was assessed using a regression analysis with curve fit estimation (SPSS, IBM Inc.). The mean of all data values were considered to determine the BCF for *P. subcapitata* and *C. kesslerii*.

2.2.7 Data analysis

Selenium toxicity data for *P. subcapitata* was analyzed using CETIS (Tidepool Scientific LLC.). A growth endpoint (IC_{25}, indicating a 25% decrease in growth) and 95% confidence intervals were calculated using a non-linear regression. Selenium uptake in algal species was analyzed by linear regression, using Excel (Microsoft Inc.); Se bioconcentration in algal species was analyzed by SPSS (IBM Inc.). Selenium speciation was analyzed using SPSS (IBM Inc.). Levine's test was conducted on speciation results to determine homogeneity of variance of data sets. If not significant ($p>0.05$) data were analysed using a one-way Analysis of Variance. If significant ($p<0.05$) data were analysed using a non-parametric Kruskal-wallis test on the concentration of each species to assess differences between sampling dates. Dunnett's T3 post-hoc test was conducted to identify the days at which concentrations differed significantly from inoculation (day 0).

2.3 Results

2.3.1 Toxicity test

Selenium toxicity in *P. subcapitata* was assessed by exposing algal cells to selenate at 0-0.64 mg L^{-1}. Growth was measured by using a Coulter Counter to determine algal cell concentrations prior to, and following the 3-day exposure period. A significant non-linear regression was observed ($p=0.0031$) with a coefficient of regression (R^2) of 0.7919. Growth endpoints were measured; a 25% growth inhibition (IC_{25}) corresponds to a 116 µg L^{-1} exposure (Figure 1). Measured concentrations of selenium in algal exposures were consistent with nominal values.

2.3.2 Selenium uptake and speciation

2.3.2.1 *P. subcapitata*

Selenium speciation and uptake into algae was assessed by exposing cells to selenate at 0-40 µg L^{-1}. The addition of selenium as selenate to algal growth media prior to inoculation was confirmed (Figure 2). At exposures of 2.5, 5, 10, 20 and 40 µg L^{-1} (Figure 2D-H) Se concentrations and species remained stable through day 3 (KWALLIS, $p>0.05$); a significant decrease in total Se occurred between days 3 and 7 (KWALLIS, $p=0.020$, $p<0.001$, $p=0.004$, $p=0.003$, $p<0.001$ respectively). At day 7, selenate species

were completely depleted, while the presence of selenite was detected in algal growth media**.** The loss of selenium from solution was accompanied by concentration of Se in algae by the end of the growth exposure (Figure 3).

Algal growth media spiked with lower concentrations of selenate did not exhibit the typical speciation curve. The control displayed an increase in selenate in the algal growth media at day 3 (Figure 2A). This trend was not observed in any other exposure concentration. No significant decrease in total Se was observed in algal growth media spiked with 0.46 µg L^{-1} (KWALLIS, $p>0.05$, Figure 2B). Selenate decreased over the 7-day incubation, but selenite was not detected. Similarly, at the 1.27 µg L^{-1} exposure, total selenium levels remained constant, while selenate decreased (Figure 2C). In this case, selenite was detected on day 7. Regardless of the speciation profile, Se uptake into algal cells was positively correlated to exposure concentration ($R^2=0.985$, Figure 4).

2.3.2.2 *C. kesslerii*

Se speciation profiles for *C. kesslerii* growth media varied, depending on exposure concentration (Figure 5). Selenium species were not detected (<0.1 µg L^{-1}) in the control (Figure 5A). Media Se concentrations remained stable through day 3 (KWALLIS, $p>0.05$). At an exposure of 10.12 µg L^{-1} a significant decrease in total Se occurred by day 7 ($p<0.001$, Figure 5F). Selenium species in the algal growth media remained as selenate through day 7. Selenite was not detected at any exposure concentration during the 7-day exposure period. At exposures of 0.53, 1.12, 5.65, 19.45 and 39.20 µg L^{-1}, a significant increase in total Se occurred by day 7 ($p=0.029$, $p=0.001$, $p=0.020$, $p<0.001$, and $p=0.047$ respectively, Figures 5B, C, E, G, H)). High variability in total Se and selenate concentrations at day 7 does not permit proper analysis of media containing 2.77 µg L^{-1} (Figure 5D). Se concentrations for this exposure do not appear to have changed following inoculation with algal cells.

A strong positive correlation was observed between Se exposure levels and uptake in algal cells ($R^2=0.931$, Figure 6). As a result of an increase in total selenium concentrations for several treatments over the 7-day algal growth period, it is difficult to assess the relationship between changes in Se levels in media and uptake by *C. kesslerii*

(Figure 7). Media treated with 10.12 µg L^{-1} displayed a 9.38±0.60 µg loss over the 7-day exposure, coupled with a 2.3 µg uptake into algal cells.

2.3.3 Se bioconcentration

Bioconcentration factors were calculated following a 7-day exposure to dissolved selenate. Bioconcentration in *P. subcapitata* showed some variability at lower exposure concentrations, averaging 148±60 (Figure 8). A significant inverse regression was observed (p=0.045) with a coefficient of regression (R^2) of 0.585. *C. kesslerii* showed variability, particularly at low exposure concentrations below 3 µg L^{-1}, where bioconcentration averaged 248±37. At exposure concentrations above 6.5 µg L^{-1}, bioconcentration was steady at 70±4. A significant logarithmic relationship (p=0.016) was observed between bioconcentration and Se exposure concentrations (R^2=0.721, Figure 9).

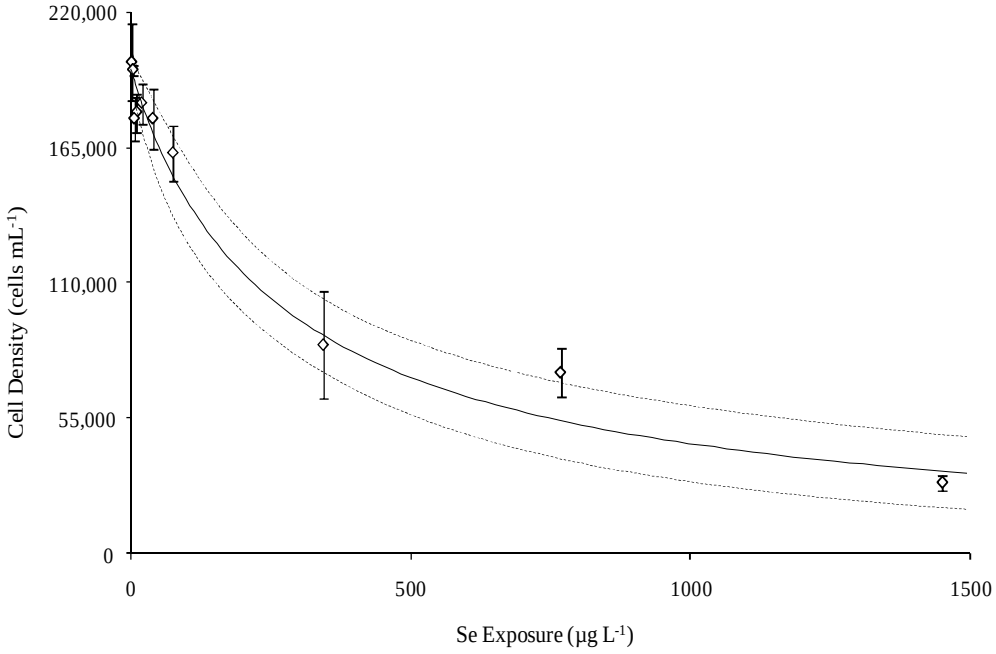

Figure 1 Se growth inhibition in *P. subcapitata*. Algal cell concentrations following a 3-day exposure to selenate (N=3). Non-linear regression (—) with 95 % confidence intervals (---). The coefficient of regression (R^2) for Se is 0.7919. Error bars represent standard deviation from the mean.

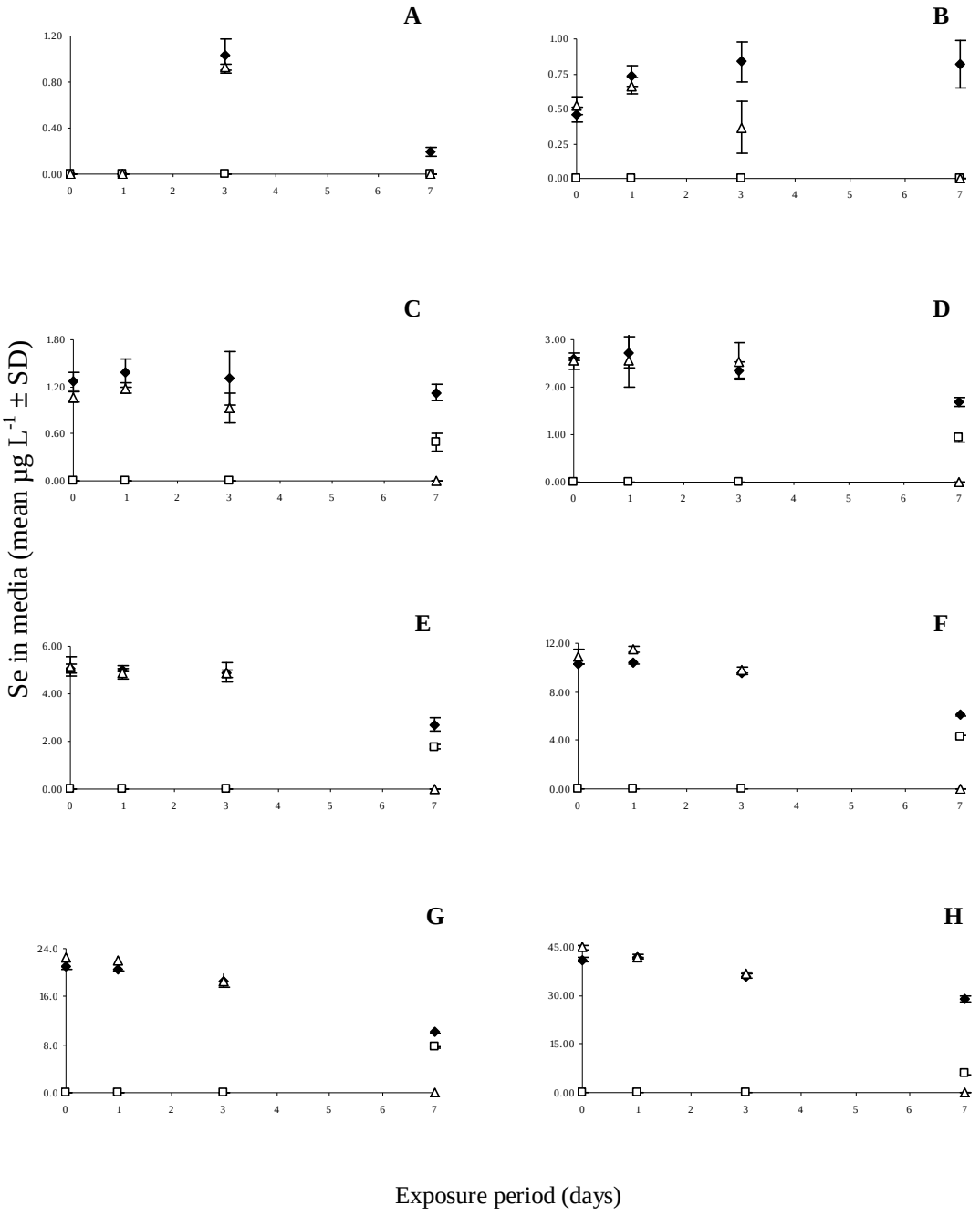

Figure 2 Biotransformation of selenium species during 7-day growth of *P. subcapitata*. Total selenium (♦), selenate (△) and selenite (□) species in algal growth media during selenate exposure: (A) 0 µg L^{-1}, (B) 0.46 µg L^{-1}, (C) 1.27 µg L^{-1}, (D) 2.59 µg L^{-1}, (E) 5.04 µg L^{-1}, (F) 10.33 µg L^{-1}, (G) 20.99 µg L^{-1}, and (H) 41.09 µg L^{-1}. Error bars represent standard deviation from the mean (N=3).

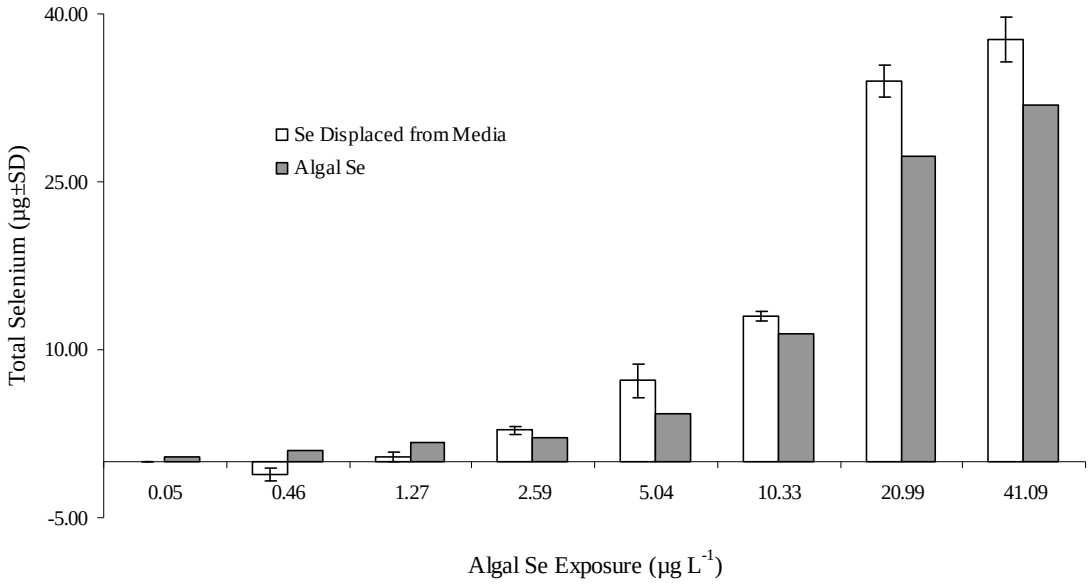

Figure 3 Selenium absorption by *P. subcapitata* during 7-day growth exposure. Comparison of total Se loss from growth media (N=3) and total Se uptake into algal cells (N=1). Error bars represent standard deviation from the mean. For Se concentration below the detection limit (control, <0.1 µg L^{-1}), half the value was used.

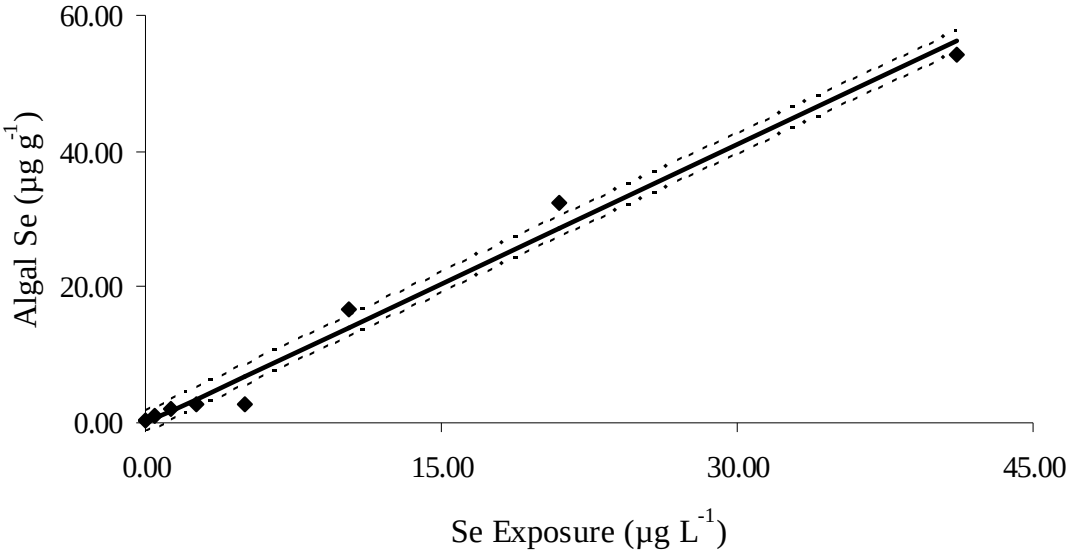

Figure 4 Selenium uptake by *P. subcapitata*. Algal Se concentration following 7-day exposure to selenate in growth media. Linear regression (—) with 95 % confidence intervals (---): y = 1.37x. The coefficient of regression (R^2) is 0.985.

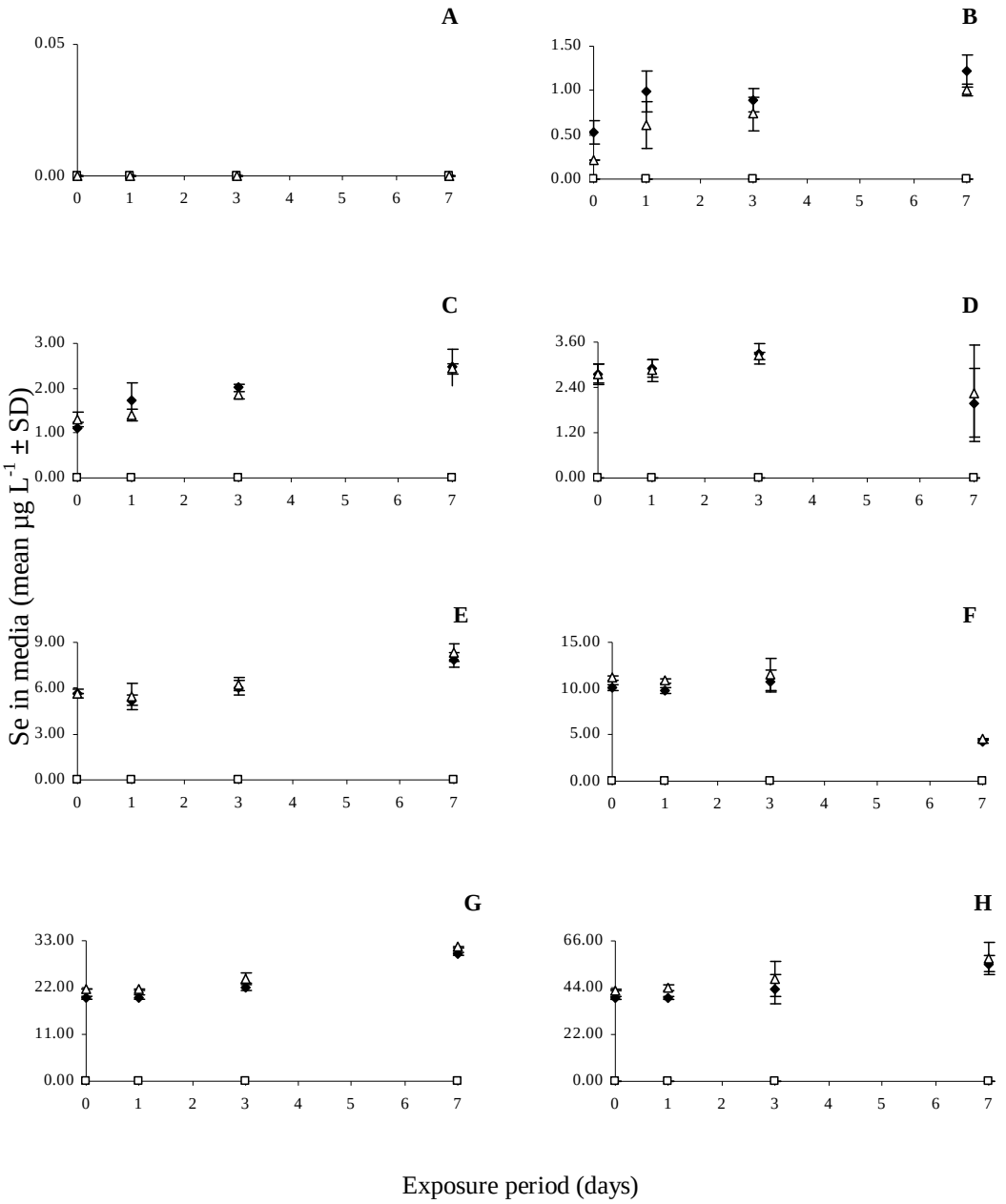

Figure 5 Biotransformation of selenium species during 7-day growth of *C. kesslerii*. Total selenium (♦), selenate (△) and selenite (□) species in algal growth media during selenate exposure: (A) 0 µg L^{-1}, (B) 0.53 µg L^{-1}, (C) 1.12 µg L^{-1}, (D) 2.77 µg L^{-1}, (E) 5.65 µg L^{-1}, (F) 10.12 µg L^{-1}, (G) 19.45 µg L^{-1}, and (H) 39.20 µg L^{-1}. Error bars represent standard deviation from the mean (N=3).

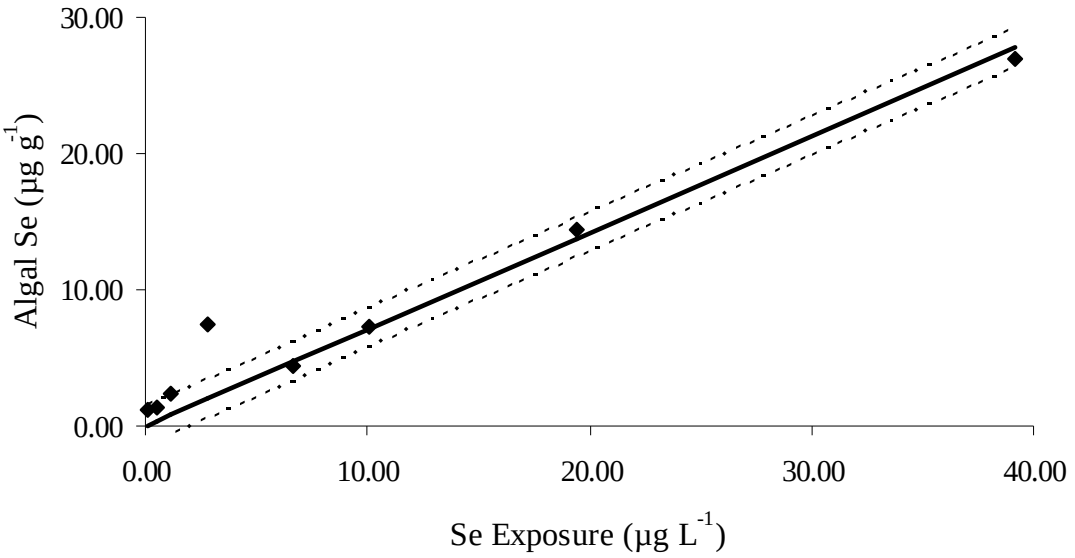

Figure 6 Selenium uptake by *C. kesslerii*. Algal Se concentration following a 7-day exposure to selenate in the growth media. Linear regression (—) with 95 % confidence intervals (---): y = 0.71x. The coefficient of regression (R^2) for Se is 0.931.

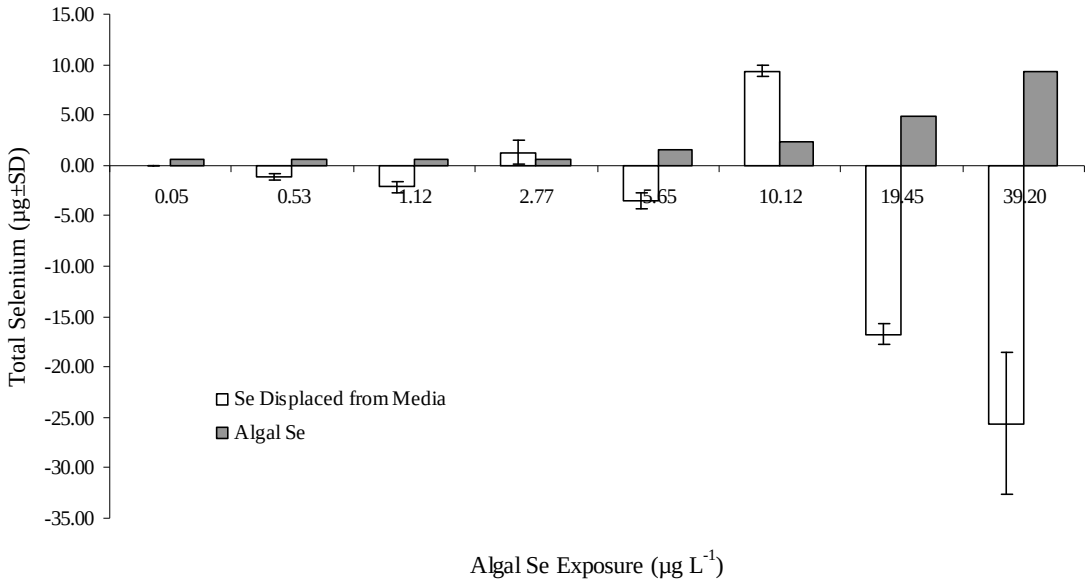

Figure 7 Selenium absorption by *C. kesslerii* during 7-day growth exposure. Comparison of total Se loss from growth media (N=3) and total Se uptake into algal cells (N=1). Error bars represent standard deviation from the mean. For Se concentration below the detection limit (control, <0.1 µg L^{-1}), half the value was used (0.05 µg L^{-1}).

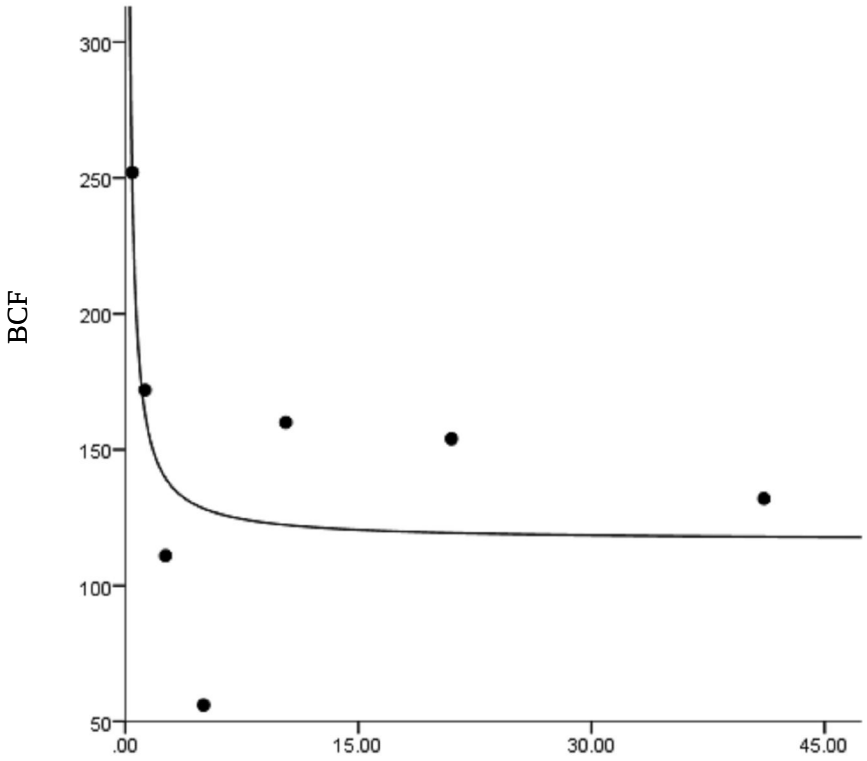

Figure 8 Bioconcentration (BCF) of selenium in *P. subcapitata*. Algal concentration following a 7-day exposure to selenate in the growth media followed an inverse regression (—) (R^2=0.045).

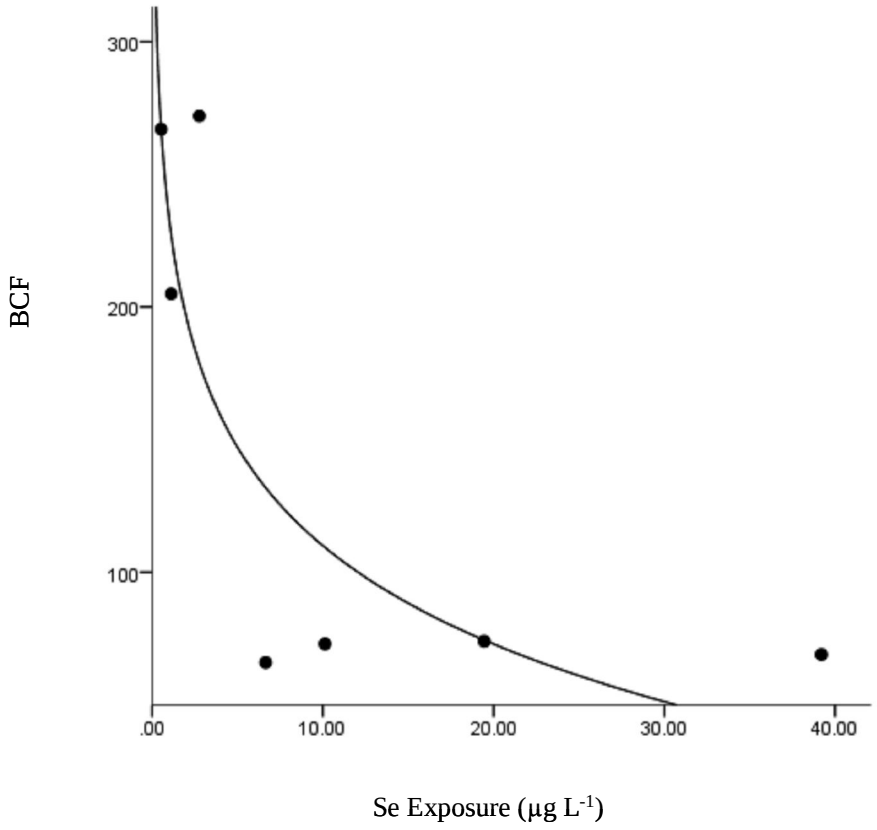

Figure 9 Bioconcentration (BCF) of selenium in *C. kesslerii*. Algal concentration following a 7-day exposure to selenate in the growth media displayed a logarithmic relationship (—) (R^2 = 0.721).

2.4 Discussion

Selenium is an essential micronutrient for algal cells, with a narrow margin between nutritionally optimal and toxic concentrations (Hoffman 2002). Growth inhibition (25%) in *P. subcapitata* occurs at aquatic selenate concentrations of 104 μg L^{-1}. In Southeast British Columbia, open-pit coal mining has caused Se concentrations in Elk River to reach 9.6 μg L^{-1} in 2008, and continues to rise (Elk Valley Se Task Force 2008). Similarly, in North Carolina selenium-rich effluent was discharged into Belews Lake from a nearby coal-fired power plant; Se levels in surface water ranged between 5 and 10 μg L^{-1} (Lemly 1985). While some sites demonstrate high levels of Se in receiving waters, they do not reach a concentration that could detrimentally affect these algal species. The predominant risk exists with the uptake of dissolved inorganic species and biotransformation to bioavailable organic species.

Selenium speciation in growth media was measured during algal exposure. Se exposures in *P. subcapitata* produced typical speciation profiles. A decrease in selenate species was coupled with an increase in selenite. The detection of selenite in the growth media reflects the metabolism of Se by plants (Terry *et al*. 2000). Plants reduce selenate after uptake, first to reduced inorganic species and then to reduced organic species. Reduced species are released into surrounding growth media as cells end their natural growth cycle and rupture. This is supported by Simmons and Wallschlager (2011) who observed significant changes in Se species occurring with the onset of exponential growth. Selenate species were gradually replaced by selenite in the growth media. The discrepancy between total selenium concentrations and selenite levels indicate the presence of other Se species. As the predominant organic selenide, seleno-methionine incorporated into proteins or peptides is most likely released (Fan *et al.* 2002). Additional speciation work is necessary to confirm its presence in media.

C. kesslerii treatment with 10 μg L^{-1} produced a speciation profile similar to *P. subcapitata*, while exposure to 2.5 μg L^{-1} caused no significant changes in speciation over the 7-day growth period. Several Se treatments of *C. kesslerii* produced unexpected speciation profiles where increases in total selenium were observed in some exposure concentrations. These significant increases over the exposure period cannot be explained. An influx of Se into the media could only come from the algal cells. According to the

control, each untreated culture contains an average of 0.54 µg selenium. Massive cell death and release of cellular contents into the surrounding media could account for an increase in aquatic selenium. This hypothesis is simply not supported by the algal cell densities measured following the exposure period; treated cultures were healthy.

The decrease in total selenium levels in media over the exposure period indicates Se uptake by algal cells. As observed with *P. subcapitata*, algal tissue concentrations were comparable to Se displaced from the media. Selenium loss between days 4 and 7 corresponds to the exponential growth phase of algal species (Simmons and Wallschlager 2011). An increase in algal cell density would cause a corresponding increase in Se absorption from the surrounding media. There is a marked difference in uptake capacities between algal species. *P. subcapitata* absorbs selenium at almost twice the rate of *C. kesslerii*. It has been suggested that algal species have varying uptake capacities for selenium (Chapman *et al*. 2010). However, the biological processes which regulate Se uptake and transformation within algal cells are not fully understood.

The strong relationship between exposure and uptake in algal species indicates the possibility of risk to consumers. Selenium uptake by primary producers, such as algae, is the largest bioconcentration step (Luoma and Presser 2009) and has been observed both in the laboratory and field assessments. For example, in Elk River, British Columbia, exposure to 9.6 µg L^{-1} in lentic receiving waters resulted in Se concentration in periphyton to 5 mg kg^{-1} dw (Orr *et al.* 2006). In Belews Lake, Se concentrations in phytoplankton ranged from 41-97 mg kg^{-1} dw following exposure to Se at 10 µg L^{-1} (Lemly 1985). Interestingly, although both populations were exposed to similar aquatic Se concentrations, lotic site exposures resulted in substantially higher uptake. Comparatively, laboratory assessments to determine bioconcentration factors in *P. subcapitata* and *C. kesslerii* resulted in BCFs of 148±60 and 70±4 respectively. Bioaccumulation factors in these tests more closely resemble those observed in lotic environments. It is important that site-specific biotic and abiotic factors be taken into account when not only designing laboratory tests but also analysing data. Methods which more closely mirror environmental conditions are necessary to accurately assess the risk associated with Se contamination. Selenium bioconcentration in *P. subcapitata* was independent of exposure concentration, while bioconcentration in *C. kesslerii* was

slightly inverse at low exposure concentrations. This inverse relationship occurs because Se is as essential micronutrient. At lower exposure concentrations, Se uptake is active, while uptake regulation occurs at higher exposure concentrations.

Unfortunately, due to the digestion method, it was not possible to determine Se speciation in tissue samples. Speciation could be preserved with the use of an enzymatic digestion method (Polatajko *et al.* 2006), or detection by X-ray absorption spectroscopy using a Synchrotron beam line (Gallego-Gallegos *et al.* 2013). In addition, it is recommended that speciation, uptake and bioconcentration studies be completed with algal exposure to selenite in the growth media. It may also be possible to assess the fate of selenium using alternative techniques, such as radioisotopes (^{75}Se), which have been applied in various studies using other algal species (Besser *et al.* 1993, Bertram and Brooks 1986).

CHAPTER 3

ASSESSING THE EFFECT OF SELENIUM OF THE LIFE-CYCLE OF THE AQUATIC INVERTEBRATE *CERIODAPHNIA DUBIA*

3.1 Introduction

Selenium is an essential micronutrient with a narrow margin between nutritionally optimal and toxic dietary exposures. Selenium is present in surface waters at background concentrations below 1 µg L^{-1} (CCME 2009), but has been found at elevated concentrations at many locations in the vicinity of mining operations, petroleum refineries, coal-fired power plants and agricultural irrigation (Presser *et al*. 2004). These anthropogenic disturbances contribute to elevated selenium levels in aquatic ecosystems receiving effluent/runoff. In Southeast British Columbia, open-pit coal mining has led to effluent concentrations exceeding 300 µg Se L^{-1} (Martin *et al.* 2008). Se concentrations in Elk River were 9.6 µg L^{-1} in 2008, and continued to rise, resulting in reproductive failure and teratogenesis in egg laying vertebrates (Elk Valley Se Task Force 2008).

Selenium occurs in the natural environment in several oxidation states. Selenium typically enters surface waters as selenite (SeO_3^{2-}) and selenate (SeO_4^{2-}). Organic selenides are produced by biological reduction of these inorganic forms (Wrench *et al* 1978). Environmental concentrations of dissolved selenite and selenate are not directly toxic to aquatic organisms (Lemly 1985, Luoma 1992), while organic selenides present in the food-web pose a substantial risk to aquatic organisms at low exposure concentrations (Ingersoll 1990). Although low levels of selenium can be taken up directly from water, Se accumulation and toxicity are the result of dietary intake. Predators readily accumulate selenium from Se-contaminated food. Hamilton *et al.* (1990) examined the accumulation of dietary selenium in Chinook salmon. Reduced growth and survival, and increased whole-body Se were correlated to increased dietary exposure concentrations. Dietary exposures to selenomethionine in rainbow trout resulted in the accumulation of selenomethionine, selenocysteine and selenocystine species in all tissues (Misra *et al.* 2012). High levels of selenocystine were detected in kidney and liver tissues. Selenium can accumulate and be biologically magnified to toxic levels even though water-borne concentrations are below regulatory requirements (Muscatello and Janz 2009). Relying

solely on monitoring of water concentrations is not an effective predictor of exposure levels. Despite data indicating that dietary exposure to organo-selenium compounds is more toxic to aquatic organisms than dissolved inorganic species, ecotoxicological regulations regarding permissible Se exposure levels are based on water-borne Se concentrations (1 μg L^{-1}, Canadian Water Quality Guidelines for the Protection of Aquatic Life 1987). Recommendations for future tissue-based guidelines are underway in Canada and the United States. The USEPA has recognized that basing regulations solely on dissolved Se exposure concentrations is insufficient and is considering a national fish-tissue criterion for managing Se contamination (USEPA 2004). In Canada, a review is in progress which aims to translate fish tissue-Se concentrations to water-Se concentrations (Deforest *et al.* 2012, Deforest 2012). In British Columbia, the Ministry of Environment has released a draft guideline for selenium which includes water and tissue levels (British Columbia Ministry of Environment 2012). The document outlines specific regulations for water and dietary Se exposures, as well as tissue concentrations in fish (egg/ovary, whole-body, muscle) and birds (egg).

Current field and laboratory studies indicate that selenium toxicity is manifested primarily as sublethal effects. Egg laying vertebrates exposed to a chronic selenium-enriched diet have demonstrated reproductive failure and teratogenesis, resulting in substantial population impacts (Elk Valley Se Task Force 2008). Studies have focused primarily on fish and birds, while invertebrates have been relegated to a source of dietary Se to higher trophic levels. According to deBruyn and Chapman (2007), invertebrate species may be more sensitive to the toxic effects of Se. Toxic dietary concentrations vary 100-fold between taxa, while sublethal effects occur at 10-fold lower concentrations than lethality (deBruyn and Chapman 2007). The risk of reproductive sublethal effects on aquatic invertebrates is of concern. Assessing whether invertebrates are protected by current water quality guidelines should be a priority.

Most standardised ecotoxicological tests evaluating aquatic exposures to invertebrates have been performed with crustaceans. Planktonic crustaceans, such as daphnids, are an important food and energy link between primary producers (algae) and secondary consumers (fish). Dissolved inorganic selenium species are taken up by algae, which are then ingested by filter-feeders, leading to bioaccumulation of Se and transfer to

higher trophic levels (Environment Canada 2007b). It is important to consider low trophic-level sublethal effects in order to identify and manage potential environmental health impacts from Se exposure. Daphnia species are the most commonly used invertebrate species in regulatory chemical testing and several guidelines exist for both acute and chronic tests (Environment Canada 2007b, USEPA 2002, CSIRO 1997). No standardized tests exist to address the effects of dietary exposures, but as current tests incorporate algal feeding regimens, the use of Se-enriched algae is an appropriate modification to explore this exposure pathway. In addition, facilities which release effluent into aquatic environments must conduct regulatory toxicity testing with sample wastewater on living aquatic organisms, such as *C. dubia*, as part of federal monitoring of environmental effects. Besser *et al.* conducted similar bioaccumulation experiments with *D. magna* using algae dosed with Se species (1993). Following dietary exposure, selenium concentrations in daphnids were equivalent to algal Se concentrations (CF=1).

An important area of research involves examining the multi-generational effects of dietary selenium exposure to aquatic organisms. Initiating tests with neonates from unexposed parental organisms ignores the potential sublethal effects of exposure during oogenesis and embryognesis, as well as Se transfer from parent to offspring (Van Leeuwen *et al* 1985). In addition, this practice excludes the examination of second-generation viability in terms of survival and reproductive capacity. By using a modified *C. dubia* 7-day life-cycle test, it was possible to: (1) compare food- and water-borne exposures to selenium and (2) determine the effects of dietary Se exposures on second-generation organisms.

3.2 Materials and Methods

3.2.1 Test Organism

Ceriodaphnia dubia were obtained from Aquatic Research Organisms Inc. (Hampton, NH) and cultured individually in borosilicate glass test tubes containing reconstituted soft water (EPA 2002), YCT[1] and algae feedout[2] according to Environment Canada guidelines (2007b). Methods for testing followed Environment Canada

[1] YCT = Yeast + Cerophyll™ + trout chow (Aquatic Organisms Inc.)
[2] Algae feedout = 3.5 million cells mL^{-1} as 3:1 ratio of *Pseudokirchneriella subcapitata:Chlorella kesslerii*

Biological Test Methods (2007b), except for changes in test exposure methods as described in the text. Survival and reproductive output were recorded daily for the duration of the 7-day tests. Tests consisted of water or food-based exposures to selenium run in parallel (Figure 10). On the final day of the test, neonates from food-exposed organisms (F_0) were used to initiate two second generation (F_1) tests. Offspring from each exposure concentration underwent continued dietary exposure or no exposure to Se. In water-based exposures, dissolved selenate (SeO_4^{2-}) was present in reconstituted soft water at concentrations of 0, 0.625, 1.25, 2.5, 5, 10, 20, and 40 µg L^{-1}. For dietary exposures, Se was present as seleniferous algae feedout and was prepared as described below. Each source of Se exposure consisted of 10 replicates per treatment.

3.2.2 Algal Food Source

Algal species *Pseudokirchneriella subcapitata* and *Chlorella kesslerii* were cultured separately in modified Bristol's Medium (Bold 1949) under constant aeration for 7-days in an incubator chamber at 24±1°C under continuous "cool white" fluorescent light with an intensity of 4000 lux. Seleniferous algae were produced from the addition of selenate in the growth medium at concentrations of 0, 0.625, 1.25, 2.5, 5, 10, 20, and 40 µg L^{-1} prior to inoculation. Following growth, algal cells were pelleted by centrifugation at 2000 rpm (Sorvall RC-5B, Dupont Instruments) for 15 minutes. Algal cells were re-suspended in ultrapure water (Milli-Q, Millipore Corp.) and centrifuged to ensure removal of seleniferous growth media. Algal cell concentration was determined using a Coulter Counter (Beckman Coulter Inc.) and an algal feedout was prepared with a concentration of 3.5 million cells mL^{-1} and a 3:1 ratio of *P. subcapitata:C. kesslerii*. To evaluate Se uptake in algal cells, samples (20 mL) were dried and incubated at room temperature with 4 mL of concentrated trace-metal grade HNO_3 (Fisher Scientific) for seven days, followed by the addition of 3 mL of 30% H_2O_2 (Fisher Scientific) for 24 hrs. Digests were diluted to 10 mL with ultrapure water and filtered (0.45 µm) prior to analysis. Tissue digestion was verified using apple leaves standard reference material (National Institute of Standards and Technology). Digested standard reference material, method blanks and algae samples were analyzed using liquid chromatography mass

spectrometry (LC-MS). Standard reference material samples yielded 100.0±0.0 % recovery of the 0.050±0.009 µg Se g^{-1} certified value.

Selenium stock solutions were prepared by adding analytical reagent grade $NaSeO_4$ (99.8+%, Alfa Aesar) to ultrapure water. Nominal test concentrations (0-40 µg Se L^{-1}) were prepared by adding appropriate aliquot volumes to dilution media (reconstituted soft water). Seleniferous media samples (10 mL) were collected to determine total Se exposure concentrations. Total selenium concentration in reconstituted soft water was measured by inductively coupled plasma mass spectrometry (ICP-MS).

3.2.3 Data Analysis

Selenium exposure data were analyzed using CETIS (Tidepool Scientific LLC.) and SPSS (IBM Inc.). Survival endpoints (EC_{50}) could not be calculated due to a lack of mortality at tested concentrations. Levine's test was conducted on neonate production results to determine homogeneity of variance in the data sets. If not significant ($p>0.05$), data were analyzed using a two-way Analysis of Variance (ANOVA) for reproduction to assess treatment, source and generational effects. If Levine's test was significant ($p<0.05$), non-parametric two-way ANOVAs were conducted using the Scheirer-Ray-Hare extension of the kruskal-wallis test (Sokal and Rohlf 2003). If a significant interaction was observed, data sets were investigated independently by one-way analysis of variance, where no significant variance was observed or non-parametric Kruskal-wallis tests, where significant variance was observed. Post-hoc Tukey and Dunnett's T3 tests were conducted to identify the treatments which differed significantly from the control.

3.3 Results

3.3.1 Water quality

Measured water-quality parameters for all tests are summarized in Table 1. Test pH, temperature, and dissolved oxygen were within the accepted range as defined by Environment Canada (2007b). Parameters were measured before (t=0) and after (t=24) daily transfers to ensure consistent water quality; values did not differ significantly

(p>0.05). Measured concentrations of Se in reconstituted soft water (RSW) were consistent with nominal values (Table 2). Selenium concentration in the control was below the detection limit (<0.5 ppb).

3.3.2 Algal and dietary Se

Final dietary exposure concentrations are listed in Table 3. Se in modified Bristol's Medium and corresponding algae represents initial algal exposure concentrations and final algal uptake following the 7-day exposure period. Se in algae feedout represents *C. dubia* dietary exposure concentrations. Selenium concentration in the control was below the detection limit (<0.5 ppb). Selenium concentration in YCT was measured at 1.30±0.003 µg Se g^{-1}.

3.3.3 *C. dubia* life-cycle test

3.3.3.1 First generation Se exposures

Water and food-borne exposures were conducted to compare Se source and treatment effects. EC_{50} values could not be determined for *C. dubia*, as no significant levels of mortality occurred at tested concentrations. It was not possible to determine whether method of exposure (i.e. dietary versus aquatic) had an effect on either survival or reproduction due to a significant interaction within the test (p=0.025). However, when analysed independently, water-borne exposures to selenate (0-40 µg L^{-1}) had no impact on reproductive output (KWALLIS, p>0.05), while first generation (F_0) dietary exposures to algae grown in 20, 40 µg Se L^{-1} caused a significant increase in neonate production (24.7% and 18.7%, respectively) compared to the control (KWALLIS, p=0.037, p=0.007) (Figure 11).

3.3.3.2 Second generation Se exposures

Similarly to first generation tests, survival was not affected by Se exposure levels and an EC_{50} could not be established. Using neonates produced from *C. dubia* exposed to several dietary concentrations of Se allowed us to determine the effect of food-borne Se on the viability and reproductive capacity of the second generation (F_1): without any further exposure, or with continued dietary exposure to the same levels as F_0. Non-

exposed F_1 organisms showed no significant changes in neonate production, regardless of F_0 exposure levels (KWALLIS, $p>0.05$). Similarly, second generation (F_1) organisms exposed to dietary Se showed no significant reproductive differences (KWALLIS, $p>0.05$). Although the second generation had a higher reproductive output than F_0 (2-way ANOVA, $p<0.001$, Figures 12, 13), this trend included the control organisms and was not caused by dietary Se exposures. Unlike the first generation (F_0), which saw a significant increase in offspring production at higher Se exposure concentrations, second generation (F_1) organisms showed no reproductive differences between treatment concentrations.

Table 1 Summary of water quality parameters (mean±SD) for Se exposure tests. Measurements of fresh renewal media and media following a 24 hr exposure were recorded daily.

Test	pH	Temperature (°C)	Dissolved Oxygen (%)	Conductivity (µS/cm)
F_0: Seleniferous RSW	6.91±0.13	22.7±0.8	90.3±7.6	129±1
F_0: Seleniferous algae	6.84±0.17	22.8±0.6	87.6±6.1	128±1
F_1: No Se	6.88±0.17	22.8±0.4	85.9±6.7	131±2
F_1: Seleniferous algae	6.94±0.11	22.8±0.4	87.6±4.3	131±2

Table 2 Nominal and measured concentrations (µg L^{-1}) of total Se in reconstituted soft water test media.

Nominal Se	Measured Se
0	<0.05
0.625	0.7
1.25	1.23
2.5	3.2
5	4.8
10	10.1
20	21.6
40	40.6

Table 3 Nominal and measured concentrations (µg L^{-1}) of total Se in modified Bristol's Medium, Se uptake into algae (µg g^{-1}) and total dietary exposure (µg g^{-1}).

Nominal	*P. subcapitata*		*C. kesslerii*		Algal	Total
Se	Media Se	Algal Se	Media Se	Algal Se	feedout Se	exposure[a]
0	0.5	<0.01	<4.55	<0.01	<0.01	1.305[b]
0.625	1.1	0.73	<4.55	0.10	<0.01	1.305[b]
1.25	1.9	1.22	1.6	0.19	<0.01	1.305[b]
2.5	2.7	2.14	4.0	0.49	0.85	2.150
5	6.5	4.01	7.2	1.47	1.93	3.230
10	15.8	7.76	12.1	2.61	5.08	6.380
20	27.2	16.22	24.5	4.98	10.99	12.29
40	52.1	46.59	46.9	10.62	22.42	23.72

[a] – All dietary exposures taken into account (algal and Tetramin).
[b] – For algal feedout concentrations below the detection limit (0.01 ppb) half the value was used (0.005 ppb).

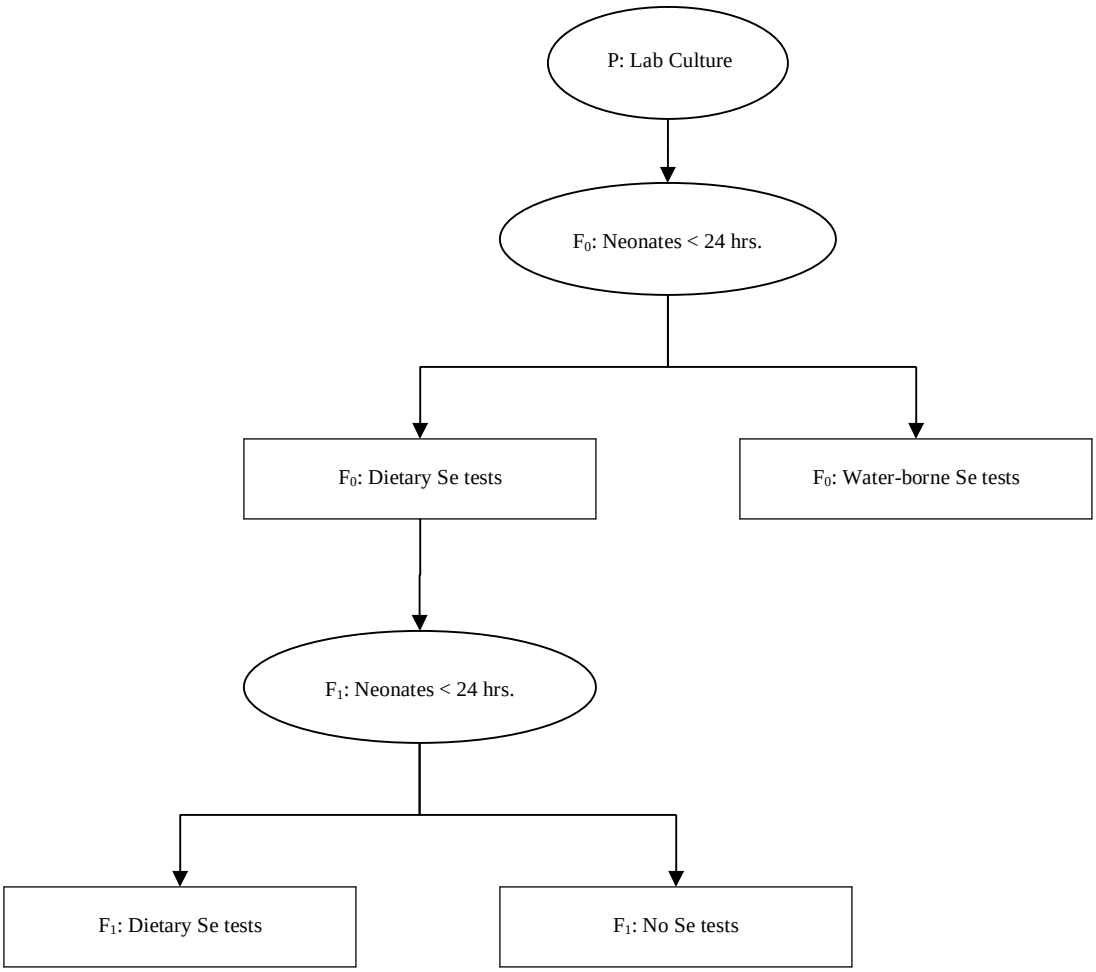

Figure 10 *Ceriodaphnia dubia* testing schedule for first (F_0) and second generation (F_1) Se exposure. Second generation dietary tests were started with neonates produced by F_0 exposed to same Se-enriched diet.

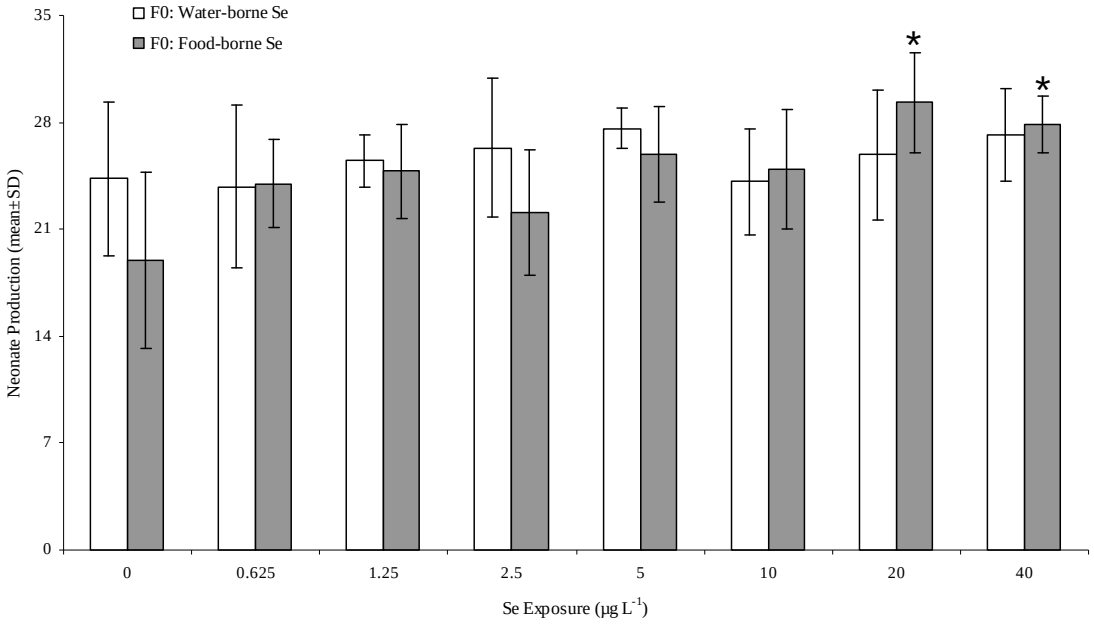

Figure 11 Reproduction in first generation (F_0) *C. dubia* exposed to water or dietary selenium. Se exposure corresponds to dissolved concentrations for water-borne exposures, and algal growth exposures for dietary exposure. Asterisk denotes significant difference from control (0 µg/g), where * = $p<0.05$.

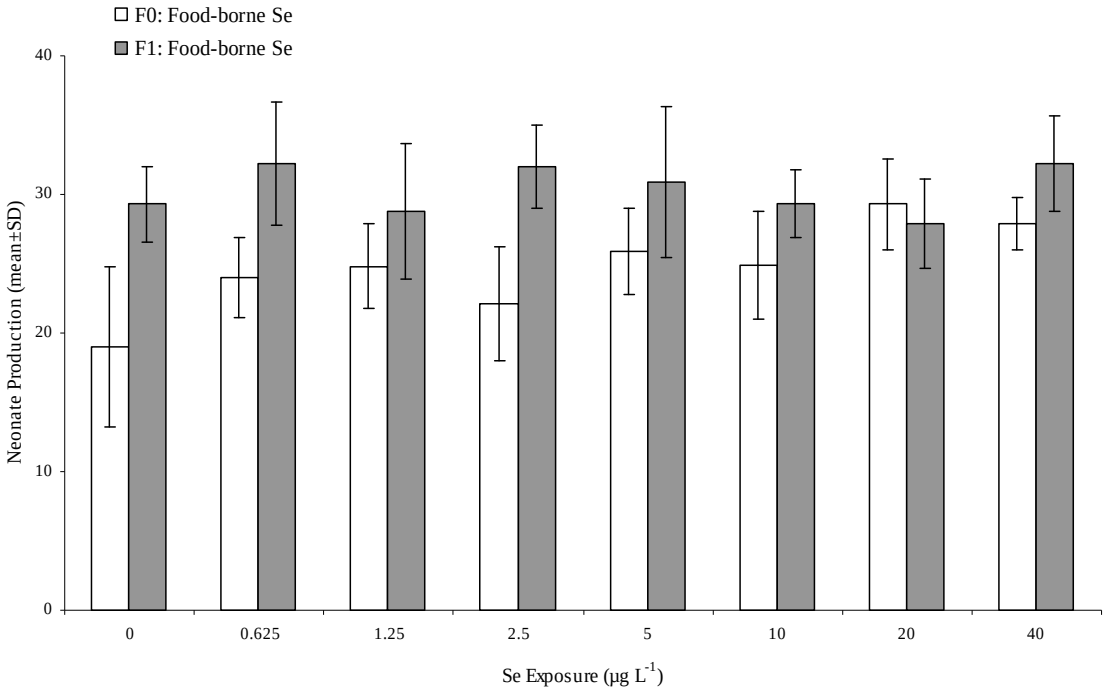

Figure 12 Reproduction in first (F_0) and second (F_1) generation *C. dubia* exposed to dietary selenium. Se exposure corresponds to algal growth exposures.

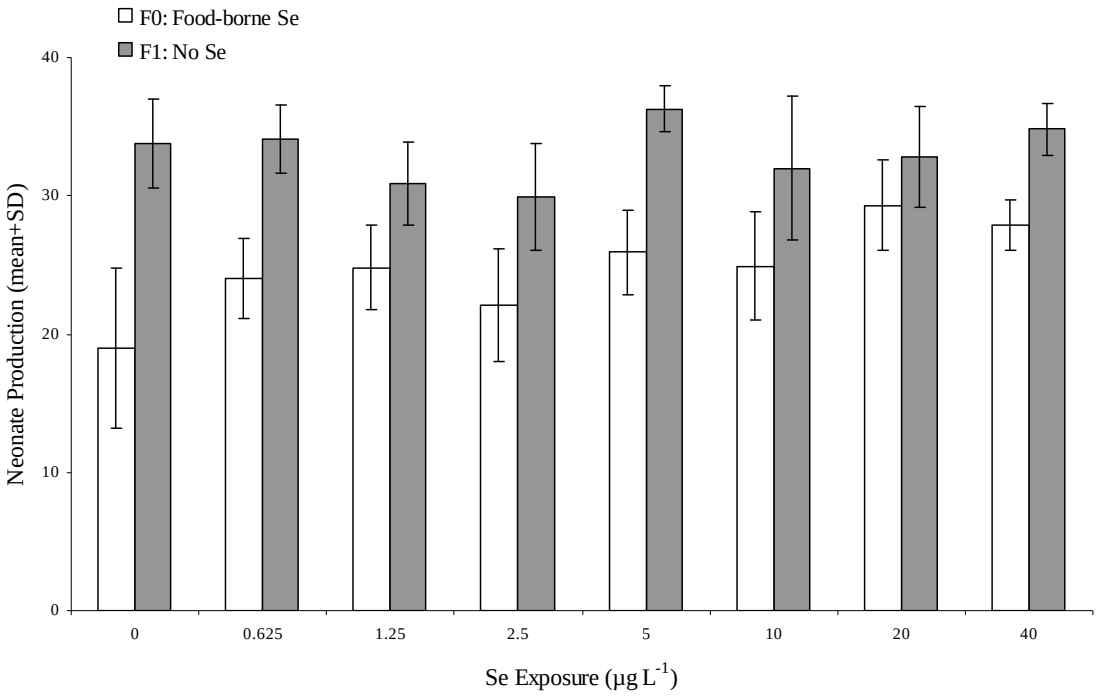

Figure 13 Reproduction in first generation (F_0) *C. dubia* exposed to dietary selenium and second generation (F_1) not exposed to Se. Se exposure corresponds to algal growth exposures for dietary exposure of F_0.

3.4 Discussion

Although selenium enters aquatic environments primarily as highly soluble selenate and selenite species, organic selenides are produced by biological reduction of inorganic species. In this case, seleno-amino acids and selenoproteins are being incorporated into algal cells grown in a seleniferous environment. Despite low dissolved concentrations in water, invertebrates such as *C. dubia* may be exposed to dietary concentrations of organo-Se. Relying on water-based measurements of Se may not reflect true organismal exposure levels or toxic risk potential.

Survival of *C. dubia* exposed to aquatic selenium up to 40 µg L^{-1} and dietary selenium up to 22.42 µg g^{-1} is consistent with the belief that environmental concentrations of Se are not responsible for acute toxicity to exposed organisms. Brix *et al.* measured acute toxicity in *C. dubia* at a much higher aquatic exposure of 1.92 mg L^{-1} (2001). It is difficult to compare dietary exposure results due to a lack of research conducted in this area. To our knowledge, these tests are one of the first to investigate dietary exposures to selenium in *Ceriodaphnia dubia*.

Results indicate that this invertebrate species is not sensitive to water or dietary selenium up to 40 µg L^{-1} and 22.42 µg g^{-1}, respectively. An increase in neonate production in F_0 suggests a potential effect at 10.99 and 22.24 µg g^{-1} dietary concentrations, but this trend was not observed in the second generation. These generational differences in reproductive responses to Se-enriched algae may indicate adaptation. Under chronic exposure scenarios, cladocerans have been shown to develop tolerance (Leblanc *et al.* 1982) either through physiological acclimation or genetically based mechanisms (Klerks and Weis 1987). Exposure of successive generations of daphnids to copper resulted in the development of significant resistance as a result of adaptation (Leblanc *et al.* 1982).

Unfortunately, due to lack of tissue, it was not possible to measure the accumulation of selenium in *C. dilutus* and therefore we could not confirm that uptake occurred. In order to determine if bioconcentration or bioaccumulation is occurring, more tissue is required. *Daphnia magna*, a larger cladoceran, is a viable alternative for determining uptake and calculating a trophic transfer factor (TFF). It is recommended

that multi-generational dietary exposure tests employing *D. magna* be conducted to determine the fate of selenium. It may also be possible to assess the fate of selenium with *C. dubia* using alternative techniques, such as radioisotopes (^{75}Se), which have been applied in various studies using other invertebrate species (Besser *et al.* 1993, Conley *et al.* 2009).

CHAPTER 4

ASSESSING THE EFFECT OF SELENIUM OF THE LIFE-CYCLE OF THE AQUATIC INVERTEBRATE *CHIRONOMUS DILUTUS*

4.1 Introduction

Selenium is an essential micronutrient with a narrow margin between nutritionally optimal and toxic dietary exposures. Selenium is present in surface waters at background concentrations of 1 μg L^{-1} (CCME 2009), but has been found at elevated concentrations at many locations in the vicinity of mining operations, petroleum refineries, coal-fired power plants, and agricultural irrigation (Presser *et al*. 2004). These anthropogenic disturbances contribute to elevated selenium levels in receiving environments. In Southeastern British Columbia, open-pit coal mining has led to effluent concentrations exceeding 300 μg Se L^{-1} (Martin *et al.* 2008). Se concentrations in Elk River were 9.6 μg L^{-1} in 2008, and continued to rise, resulting in reproductive failure and teratogenesis in egg laying vertebrates (Elk Valley Se Task Force 2008).

Selenium bioavailability is dependent on the biogeochemical transformation of Se species. Selenium typically exists in aquatic environments as dissolved inorganic selenate (SeO_4^{2-}) and, to a lesser extent, selenite (SeO_3^{2-}) (Martin *et al.* 2008). Organic selenides are produced by biological reduction of these inorganic forms (Wrench *et al* 1978). Environmental concentrations of dissolved selenite and selenate are not directly toxic to aquatic organisms (Lemly 1985, Luoma *et al.* 1992), while organic selenides present in the food-web pose a substantial risk to aquatic organisms at low exposure concentrations (Ingersoll *et al.* 1990). Selenium concentrations in algae are typically 100-500 times higher than dissolved selenate concentrations (Luoma and Presser 2009).

Sediments are the dominant sink for selenium in aquatic environments, and partitioning occurs primarily as a result of death and decomposition of Se-enriched organisms. In contaminated aquatic ecosystems, up to 90% of selenium can be found in the upper few centimetres of sediment and overlying detritus (Simmons and Wallschlager 2005). As a result, Se can be found in sediments long after input to the system has ceased (Swift 2002). In a simulated experiment, an artificial stream receiving 10 μg selenite L^{-1} contained 5-10 μg g^{-1} two years after dosing ceased (Swift 2002). Once Se has been introduced, contamination persists. Lentic environments are more conductive to the

accumulation of selenium in sediments than lotic systems due mainly to longer water retention and larger deposits of organic materials in the sediments (Canton and Van Derveer 1997).

The benthic macroinvertebrate *Chironomus dilutus* is particularly suited to assess the potential toxicity of contaminated sediments as it burrows in the upper layer of sediments and feeds on detritus at the sediment surface during larval development. The midge *C. dilutus* is a major component of food chains due to its ubiquity in aquatic ecosystems. A short life-cycle (28 days) and large number of developmental and reproductive endpoints make it a preferred choice for chronic sediment toxicity testing.

Several guidelines exist for the assessment of contaminated sediments with *Chironomus dilutus.* These include relatively brief 10-day exposures that encompass only a portion of the life cycle and focus on survival and growth of the organism (Environment Canada 1997) to life-cycle methods, which assess potential chronic toxicity and various sublethal endpoints, including reproduction, and better represent environmental conditions (OECD 2010, USEPA 1994). To understand chronic effects of selenium, the developing organism must be considered. Efforts must be made to understand exposure during embryological development through adulthood. Exposed parental organisms have the potential to pass on seleno-proteins to offspring during embryogensis, which may result in deformities and reproductive failure (Van Leeuwen *et al.* 1985). Multi-generational testing allows sublethal effects to be properly examined in contaminated ecosystems.

Currently employed life-cycle toxicity tests are conducted in beakers with automated or manual water renewal (Benoit *et al.* 1997*)*. Water renewal has the potential to flush toxic chemicals out of the test chamber and does not allow the establishment of equilibrium between water and sediments but is necessary to maintain water quality (Ankley *et al*. 1993). Borgmann and Norwood have discussed alternative testing under static conditions using Imhoff cones, in which a large volume of water overlies a smaller sediment volume (1999). Large water to sediment ratio ensures that acceptable water quality (pH, ammonia) is maintained for the duration of the toxicity test. Unfortunately this method has only been evaluated for use in 10-day sediment toxicity testing using *C.*

dilutus. As sublethal effects are manifested as a result of chronic exposure to a toxicant, life-cycle exposure scenarios are necessary for proper risk assessment.

The objectives of this study were (1) to develop a life-cycle test with *C. dilutus* using Imhoff cones, (2) to modify the feeding regime to include an algal food source to allow for assessment of dietary exposures and (3) to determine the sublethal effects of dietary selenium on two generations of *C. dilutus* using the methods developed. The proposed test design seeks to harmonize the current life-cycle beaker test with the simplicity of the Imhoff cone test for future use in life-cycle sediment toxicity testing. Various sublethal endpoints are investigated, including teratogenic development and reduced egg hatchability, in accordance with observed effects in fish and birds (Chapman *et al.* 2010).

4.1.1 Materials and Methods

4.1.2 Culturing *Chironomus dilutus*

Chironomus dilutus were obtained from Aquatic Research Organisms Inc. (Hampton, NH) and cultured in 55 L tubs (61x40.6x22.2 cm, Rubbermaid) containing 3 cm of flint silica sand and 35 L of dechlorinated City of Ottawa water (Soft-Minder Twin, Culligan International Co.) under constant aeration (Figure 14). Dechlorinated water was on a flow-through system with a renewal rate of 17.3 L per day using a peristaltic pump (PumpPro, Watson Marlow). Cultures were fed 75 mL of algae feedout, and 45 mL Tetramin (Tetra Werke) slurry (100 g L^{-1}) per week. Egg cases produced as a result of adult mating were removed to prevent overpopulation. The *C. dilutus* culturing environment was kept at 26±2 °C under a 16 hr. light: 8 hr. dark photoperiod.

4.1.3 Food preparation

Tetramin slurry was prepared by grinding Tetramin flakes (Tetra Werke) in a coffee grinder until homogeneous and mixed in ultrapure water (Milli-Q, Millipore). In order to test the effect of dietary selenium on the life-cycle of *C. dilutus* a seleniferous food source was prepared. Selenium is readily absorbed by algae from its environment and serves as an ideal dietary source. A typical Tetramin slurry diet is easily supplemented by

addition of algae to the chamber. Algal cells dispensed near the sediments are available for consumption by *C. dilutus* feeding at the sediment surface.

Algal species *Pseudokirchneriella subcapitata* and *Chlorella kesslerii* were cultured separately in modified Bristol's Medium (Bold 1949) under constant aeration for 7-days in an incubator chamber at 24±1°C under continuous "cool white" fluorescent light with an intensity of 4000 lux. Seleniferous algae was produced from the addition of selenate in the growth medium at concentrations of 0, 0.625, 1.25, 2.5, 5, 10, 20, and 40 µg L^{-1} prior to inoculation. Following growth, algal cells were pelleted by centrifugation at 2000 rpm (Sorvall RC-5B, Dupont Instruments) for 15 minutes. Algal cells were resuspended in ultrapure water (Milli-Q, Millipore) and centrifuged to ensure removal of seleniferous growth media. Algal cell concentration was determined using a Coulter Counter (Beckman Coulter Inc.) and algae feedout was prepared with a concentration of 3.5 million cells mL^{-1} and a 3:1 ratio of *P. subcapitata:C. kesslerii*. Algae feedout and Tetramin slurry were stored at 4°C.

4.1.4 Testing apparatus

The use of various apparatus was required for successful life-cycle testing. Several vessels are required to house and transfer *C. dilutus* during hatching, larval growth, reproduction and oviposition. Equipment was chosen based on ease of use and construction. The details and figures required for assembling all vessels are discussed below.

4.1.4.1 Testing Chamber

The 1 L Imhoff cone (Nalgene, Wheaton) was chosen as a suitable chamber for larval development due to its ease of use. Traditional life-cycle tests employ beakers, which necessitate manual or automated renewal of overlying water in order to maintain water quality. The Imhoff cone's unique shape allows testing under static conditions due to a large volume of water overlying a smaller sediment volume (Figure 15). A large water to sediment ratio results in greater dilution of waste products, and thus higher water quality during testing. This method better reflects environmental conditions, as it retains

potential toxic chemicals released from the sediments and reaches equilibrium conditions within the testing chamber.

Graduated Imhoff cones were fitted with silicone stoppers to minimize sediment volumes required; 80 mL sediment was added and dechlorinated water was filled to the 1000 mL mark, providing ample airspace between the 1000 mL water mark and the top edge of the cone for emerging adults. In previous tests plastic cups fitted with mesh were used, however, this proved unnecessary and created an escape route for emerged adults once airline tubing was added. An improved method was adopted in later experiments whereby cones were covered directly with mesh secured by bungee cord in order to trap emerged adults. As adults were collected on a daily basis, crowding was not a concern.

4.1.4.2 Insect aspirator and oviposit chambers

The collection and transfer of emerged organisms from Imhoff cones to reproduction chambers was conducted using an insect aspirator (Figure 16). The aspirator unit was constructed from a size 9 stopper, a short glass tube, two lengths of PVC tubing (3/16" ID, Nalgene), a 1000 µL pipette tip and fine mesh. Two holes were bored through the rubber stopper to fit the short glass tube and the length of tubing. A piece of fine mesh was affixed to the bottom of the glass tube to prevent organisms from being sucked into the inhalant tubing. The second length of tubing, with a trimmed 1000 µL pipette tip as a mouthpiece, was attached to the top of the glass tube. This setup ensured gentle aspiration of emerged organisms by mouth. The stopper was used to seal a 1000 mL Erlenmeyer flask containing a fine mesh strip and approximately 50 mL of dechlorinated water (Figure 17). These vessels served as reproduction and oviposit chambers.

4.1.4.3 Hatching vessel

Initiation of the second generation life-cycle tests necessitated individual hatching of egg masses by Se exposure treatment. One vessel per treatment served to hatch and rear *C. dilutus*. Hatching vessels were designed as disposable rearing vessels constructed from relatively inexpensive and readily available materials (Figure 18). 4 L Cubitainers (I-CHEM) were modified to resemble culturing chambers by removing one side with scissors. The cap was replaced by fine mesh affixed with a Quick Serve Tap

(Waddington & Duval Ltd.), which, when in the open position, acted as filter and overflow drain, respectively. The Cubitainers were set up similarly to the culture growth tanks, with dechlorinated water renewal on a flow-through system and constant aeration through a 200 µL pipette tip.

4.1.5 Method development

An environmentally relevant method for the life-cycle assessment of *C. dilutus* was developed. Initial life-cycle tests were designed in accordance with Borgmann and Norwood (1999) and Benoit *et al.* (1997), while subsequent tests were modified in order to accommodate deficits in survival and emergence (Table 4). The life-cycle method was developed by modifying the following parameters: (1) feeding regime, (2) population density, (3) temperature, and (4) sediment volumes.

All method tests were conducted in 1 L Imhoff cones under a 16 hr. light: 8 hr. dark photoperiod. Laboratory lighting over the exposure system consisted of cool-white fluorescent lights with an intensity of 500 to 1000 lux at the water surface. All testing apparatuses were kept within the exposure system to ensure emergence occurred within an acceptable timeframe. Water quality (pH, temperature, dissolved oxygen content, conductivity and ammonia) was monitored once per week and maintained within Environment Canada guidelines (1997). The method was evaluated by assessing (1) survival, (2) time to emergence, (3) adult health and reproductive capacity, and (4) production of viable egg masses. Several tests were conducted in order to obtain a viable method (Table 5).

4.1.5.1 Method development stage 1 – Feeding regimes

The initial life-cycle tests conducted in Imhoff cones was designed in accordance with Borgmann and Norwood (1999) and Benoit *et al.* (1997). Surface water temperature was maintained at 23ºC in order to complete a life-cycle in 28 days (Benoit *et al.* 1997). The test was initiated with 15 larvae per cone, as Borgmann and Norwood (1999) concluded that a population density (5-15 larvae per cone) had no effect on growth or survival. First instar larvae were used according to Benoit *et al.* (1997). A diet consisting of Tetramin slurry (9 or 12 mg wk^{-1}) was supplemented with algal feedout (0, 2.5, or 5

mL wk^{-1}) in order to introduce a source of dietary Se during testing. Different amounts of Tetramin slurry and algal feedout were tested in order to determine optimal feeding concentrations. A 15 mL sediment volume was used according to the Borgmann and Norwood (1999) method.

4.1.5.2 Method development stage 2 – Population density

Testing parameters were modified in order to address deficits in growth and survival. Temperature was maintained at 23ºC, while overcrowding was rectified by decreasing population density to 5 larvae per cone. In addition, survival was improved by initiating life-cycle tests with older organisms (15-day) within burrowing cases. Use of older larvae facilitated handling and reduced the risk of death due to unrelated reasons, as observed with first instar larvae. Low and high algal (7 and 35 mL wk^{-1}) and Tetramin (15 and 30 mg wk^{-1}) feeding regimens were tested to improve larval growth. A 15 mL sediment volume was maintained.

4.1.5.3 Method development stage 3 - Temperature

In order to reduce temperature fluctuations during the testing period, cones were placed in a heated water bath. Surface water temperature was increased to 25ºC in order to decrease time to emergence. A population density of 5 larvae per cone was maintained. The life-cycle test was initiated with slightly younger organisms (13-day), as they were large enough to handle. Low and high algal (7 and 35 mL wk^{-1}) and Tetramin (15 and 30 mg wk^{-1}) feeding regimens were continued from the previous test. Sediment volume was increased slightly to 20 mL.

4.1.5.4 Method development stage 4 – Sediment volume

In order to optimize testing parameters, temperature and population density were maintained at 25ºC and 5 larvae per cone (12-day old), respectively. The high (35 mL wk^{-1}) algal treatment was applied to all four cones. Low and high Tetramin (11.67 and 28 mg wk^{-1}) feeding regimens were modified in order to correspond with those from Borgmann and Norwood (1999) and Benoit *et al.* (1997) (10 and 28 mg wk^{-1}, respectively). Sediment volumes were increased (80 and 130 mL) in order to address unpredictable

emergence and aggressive behaviour between organisms. Increasing sediment volumes in Imhoff cones resulted in an increase in sediment surface area and the space allotment per organism. Final testing parameters chosen for dietary exposures were (1) surface water temperature of 25ºC, (2) test initiation with five 10-day old larvae, (3) feeding 35 mL algal feedout and 28 mg Tetramin per week and (4) an 80 mL sediment volume.

4.1.6 Dietary Se exposure

4.1.6.1 Testing parameters

Evaluation of the 28-day life-cycle test was conducted at 25±1 °C under a 16 hr. light: 8 hr. dark photoperiod. Laboratory lighting over the exposure system consisted of cool-white fluorescent lights with an intensity of 500 to 1000 lux at the water surface. All testing apparatuses were kept within the exposure system to ensure emergence and reproduction occurred within an acceptable timeframe. Water quality (pH, temperature, dissolved oxygen content, conductivity and ammonia) was monitored once per week and maintained within Environment Canada guidelines (1997). The effects of dietary selenium exposure were evaluated by (1) survival, (2) emergence, (3) time to initial emergence, (4) sex ratio, (5) adult lifespan, (6) number of egg masses, (7) number of eggs per mass, and (8) hatching success.

4.1.6.2 Test setup

Twelve days prior to initiating the life-cycle test, 10 female and 3 male *C. dilutus* adults were isolated in a reproduction chamber and covered with a black plastic bag. The following day, three egg cases with the healthiest appearance were transferred to a rearing vessel. Date of embryo hatching was recorded and served as day 1 in the timing of the life-cycle test. Larvae were fed 1-2 mL of Tetramin slurry (100 g L^{-1}), as required. Larval growth was continued until *C. dilutus* survival and size were adequate for test initiation (8-12 days post-hatch).

The newly developed method was used to determine the effect of dietary selenium on two generations of *C. dilutus*. Life-cycle tests consisted of 28-day dietary selenium exposures run in triplicate. One day prior to test initiation, the cones were fitted with size 4 silicone stopper and filled with 80 mL flint silica sand and dechlorinated water to the

1000 mL mark. Cones were covered with mesh secured by bungee cord. 1 mL glass pipettes fixed with airline tubing were secured to each cone by puncturing mesh and submerging to the 200 mL mark. Cones were aerated for 24 hours prior to test initiation and for the duration of the life-cycle test to ensure dissolved oxygen levels were adequate. Cones were placed in a heated water bath to prevent temperature flux.

4.1.6.3 Test initiation

Prior to test initiation, Tetramin slurry and algae feedout were added to each cone using Fast-Release Pipette Pump II (Bel-Art Scienceware) and glass pipettes. Larvae were fed 1 mL Tetramin slurry (4 g L^{-1}) and 5 mL seleniferous algae feedout (grown in 0-40 ppb SeVI, described below) daily. Food was dispensed close to the sediments without disturbing the surface. The same daily feeding regimen was followed for the course of the test.

The life-cycle test was initiated by the addition of 5 larvae within burrowing cases to each cone using a disposable plastic transfer pipette with a trimmed tip. Larvae were chosen based on size (largest) and perceived health (color and movement). Use of 10-day old larvae facilitated handling and reduced the risk of death due to unrelated reasons, as observed with first instar larvae.

4.1.6.4 Emergence & collection of adults

Emerged adults were collected daily and isolated by treatment in reproduction flasks using the insect aspirator. The collection tubing is slipped under the mesh screen and positioned at the base of the emerged adult. Enough suction is applied through the mouthpiece to gently lift the organism into the reproduction chamber. Due to the small number of larvae, emerged adults are pooled by treatment into reproduction chambers.

Records indicating emergent adult sex and lifespan were kept for each individual. Pupal exuviae were removed from cones following transfer of emerged adults. Unsuccessful emergence, where the organism was unable to completely shed the exuvia, was observed and recorded. Immobile pupae were observed at the water surface and, more frequently, at the sediment surface. Mortality was confirmed following a 24 hour

waiting period. In some cases, pupae at the sediment surface were eaten by larvae. It is unknown whether the death was a result of the cannibalism. All occurrences were noted.

4.1.6.5 Reproduction

Reproduction chambers were checked daily for adult mortality and egg cases. Dead adults were removed with the use of tweezers. Using a trimmed transfer pipette, egg cases were transferred individually to Petri dishes containing dechlorinated water. Over 90% of females will oviposit within 24 hours of fertilization (Benoit *et al.* 1997). In some cases, oviposition occurred in the absence of adult males. These unfertilized eggs were not included in reproductive estimates.

4.1.6.6 Egg counts & hatch determination

Egg cases were transferred to individual Petri dishes containing dechlorinated water. The number of eggs per case was determined using a ring count method or by direct counting under a dissecting microscope. Typical C-shaped egg masses were estimated using the mean number of eggs in four rings selected at approximately equal distances along its length and multiplying by the total number of rings. The number of eggs in unusually shaped or uncoiled egg masses was determined by counting directly.

Egg cases were incubated in their respective Petri dishes with overlying water until hatched. Hatching success was determined by subtracting the number of unhatched eggs remaining at 3 days following oviposition. As dietary selenium exposure tests required egg masses to initiate the second generation, hatching success was not available for every egg case.

4.1.6.7 Second generation initiation

Egg cases from food-exposed *C. dilutus* (F_0) were used to generate the second generation (F_1) tests. One egg case from each exposure concentration was transferred to hatching chambers (Figure 18). Larval growth parameters (lighting, temperature, feeding) and second generation (F_1) test initiation were identical to those used for the first generation (F_0). Offspring from each exposure concentration underwent continued

exposure to dietary selenium for the duration of the 28-day life-cycle. Larvae survival, time to emergence, sex, oviposition and death were monitored throughout the test.

Due to the small number of organisms employed, not all treatment concentrations yielded enough egg masses in order to initial second generation testing in addition to determining hatching success. The single egg mass produced from treatments 2.5 and 5 was used to determine hatching success, while egg masses from treatment 20 had poor hatching success or was unfertilized. Second generation testing was not initiated at these treatment concentrations.

4.1.6.8 Terminating life-cycle test

The length of the life-cycle is dependent on treatment components. Under control conditions (clean sediments, food and water), completion of a life-cycle requires approximately 28 days in addition to set-up time. In the presence of environmental stressors, reduced larval growth and delayed emergence result in a longer life-cycle. Depending on emergence patterns, treatments may need to be terminated separately. Red *C. dilutus* larvae are easily located in cones. Following mortality, larvae became colorless and indistinguishable from sediment. Each cone was terminated when all organisms had either emerged or died, or when no live larvae or pupae were visible within the cone. In the event that larvae remained, but were unable to undergo pupation due to reduced growth, the test was terminated when no further emergence was observed over a period of seven days (Benoit *et al.* 1997). At termination, contents of the cones were sieved through a 250 µm mesh screen to recover remaining larvae and pupae. Survival and mortality of larvae were noted.

4.1.6.9 Selenium preparation and analysis

Selenium stock solutions were prepared by adding analytical reagent grade $NaSeO_4$ (99.8+%, Alfa Aesar) to ultrapure water. Nominal test concentrations (0-40 µg Se L^{-1}) were prepared by adding appropriate aliquot volumes to modified Bristol's Medium. Seleniferous media samples (10 mL) were collected to determine total Se exposure concentrations. To evaluate Se uptake in algal cells, samples (20 mL) were dried and incubated at room temperature with 4 mL of concentrated trace-metal grade HNO_3

(Fisher Scientific) for seven days, followed by the addition of 3 mL of 30% H_2O_2 (Fisher Scientific) for 24 hrs. Digests were diluted to 10 mL with ultrapure water and filtered (0.45 µm) prior to analysis. Tissue digestion was verified using apple leaves standard reference material (National Institute of Standards and Technology). Digested standard reference material, method blanks and algae samples were analyzed using liquid chromatography mass spectrometry (LC-MS). Standard reference material samples yielded 127±31% of the 0.050±0.009 µg Se g^{-1} certified value. Total selenium concentration in modified Bristol's Medium was measured by inductively coupled plasma mass spectrometry (ICP-MS).

4.1.7 Data analysis

C. dilutus life-cycle tests resulted in a large number of developmental and reproductive endpoints: emergent sex ratio, time to emergence (male and female), adult midge lifespan (male and female), egg production and hatching success. Selenium exposure data were analyzed using SPSS (IBM Inc.). Levine's test was conducted on growth and reproduction results to determine homogeneity of variance in the data sets. If not significant ($p>0.05$) data were analysed using a one-way Analysis of Variance (ANOVA) for growth and reproduction to assess treatment effects. If Levine's test was significant ($p<0.05$) a Kruskal-wallis test was conducted. In addition, a two-way ANOVA was conducted to assess generational effects on emergence and hatching success. If Levine's test was significant ($p<0.05$), non-parametric two-way ANOVA was conducted using the Scheirer-Ray-Hare extension of the Kruskal-wallis test (Sokal and Rohlf 2003). If a significant interaction was observed, data sets were investigated independently by either one-way ANOVA, where Levine's test was non significant ($p>0.05$), or non-parametric Kruskal-wallis tests, where Levine's test was significant ($p<0.05$). Post-hoc Tukey and Dunnett's T3 tests were conducted with all analysis to identify the treatments which differed significantly from the control.

Table 4 Testing parameters for life-cycle (Benoit *et al.* 1997) and 10-day (Borgmann and Norwood 1999) sediment toxicity tests.

Method	Testing vessel	(1) Feeding Regimes		(2) Population Density	(3) Temperature	(4) Sediment Volume
		Algal (mL/wk)	Tetramin (mg/wk)	(larvae/cone)	(°C)	(mL)
Benoit *et al.* (1997)	Beaker	---[a]	28	12	23	100
Borgmann and Norwood (1999)	Imhoff cone	---[a]	10	5-15	23-25	15
Developed method	Imhoff cone	35	28	5	25	80

[a]- Method did not include algal feedout in diet.

Table 5 Life-cycle method development tests. Highlighted row indicates parameters chosen for life-cycle testing.

Experiment	Temp.	Population	Algae	Tetramin Slurry	Sediment	Survival	Emergence	First Emergence
	(°C)	(larvae/cone)	(mL/wk)	(mg/wk)	(mL/cone)	(%±SD)	(%±SD)	(day±SD)
(1) Feeding Regimes	23	15	2.5	9	15	50±5	0±0	---
	23	15	5	12	15	20±28	3±5	29±0
	23	15	0	12	15	50±33	10±14	34±0
(2) Population Density	23	5	7	15	15	73±12	27±12	33±5
	23	5	35	15	15	87±12	20±0	38±2
	23	5	7	30	15	93±12	60±0	36±3
	23	5	35	30	15	60±53	33±42	37±9
(3) Temperature	25	5	7	15	20	53±10	0±0	---
	25	5	35	15	20	80±0	13±12	35±3
	25	5	7	30	20	53±23	40±20	30±1
	25	5	35	30	20	80±35	47±31	31±6
(4) Sediment Volume	25	5	35	11.67	80	87±12	20±20	25±0
	25	5	35	28	80	87±23	87±23	24±2
	25	5	35	11.67	130	67±42	20±0	26±3
	25	5	35	28	130	87±23	87±23	22±3

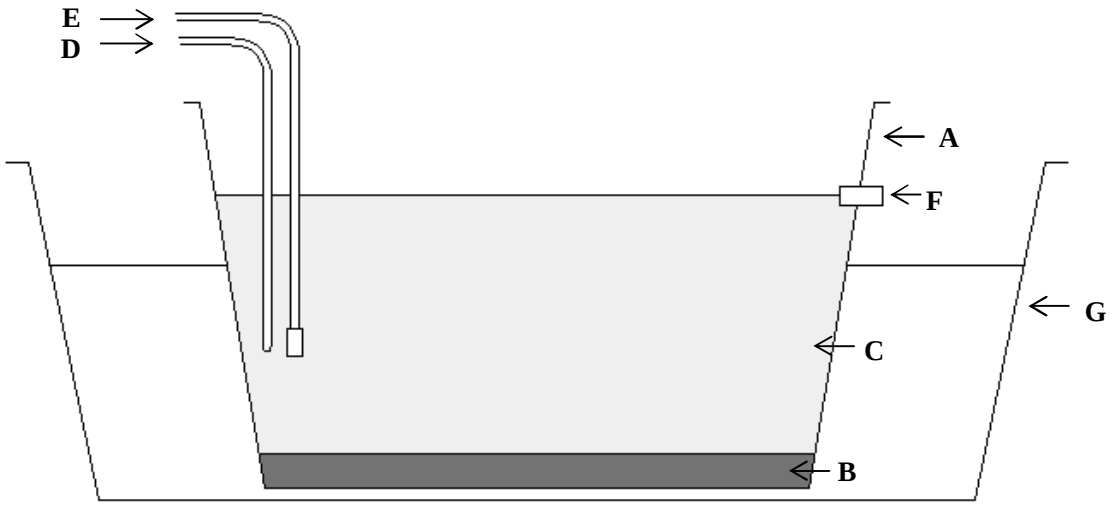

Figure 14 Culturing vessel for larvae prior to test initiation. A, plastic tub; B, 3 cm flint silica sand; C, dechlorinated water; D, water inlet from pump through plastic tubing; E, airline tubing with aerating stone; F, overflow drain; G, heated water bath.

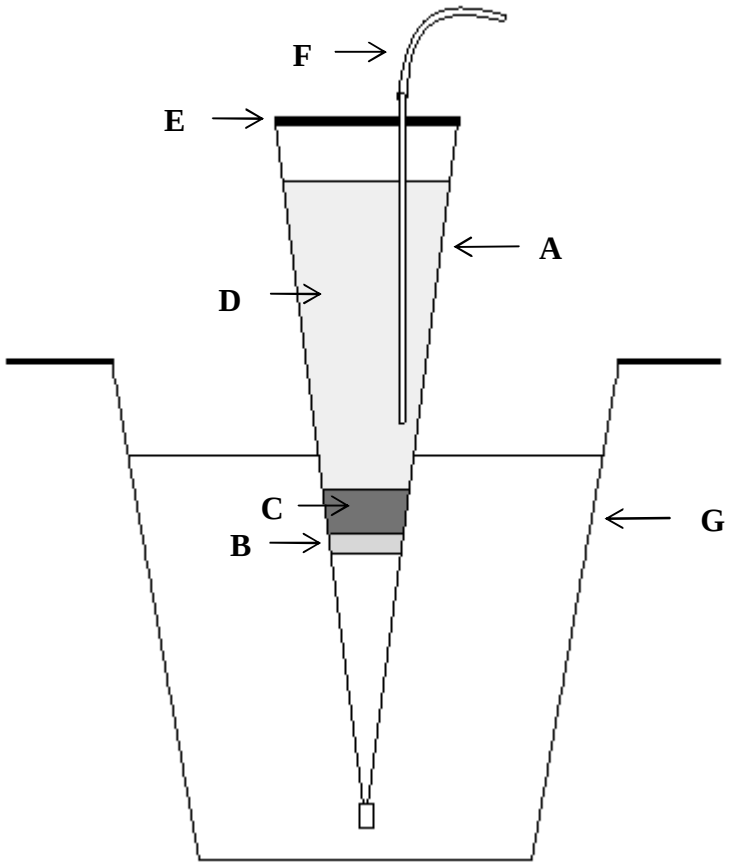

Figure 15 Imhoff cone for *C. dilutus* life-cycle test. A, 1000 mL Imhoff cone; B, silicone stopper; C, 3 cm sediment; D, dechlorinated water to 1000 mL mark; E, mesh screen fixed with bungee cord; F, 1 mL glass pipette with airline tubing; G, heated water bath.

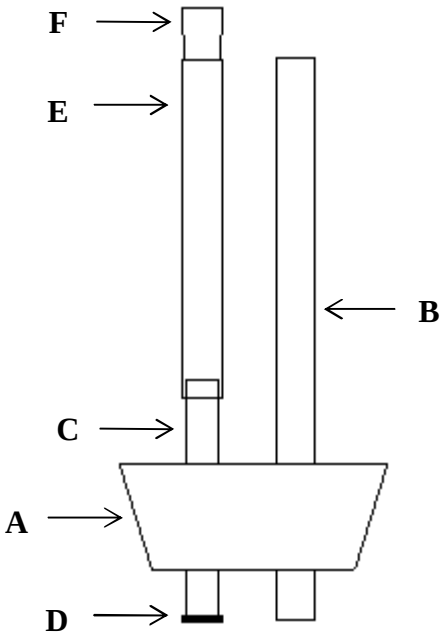

Figure 16 Insect aspirator for collection of emerged adults. A, stopper with two holes; B, plastic collection tubing; C, short glass tube with; D, safety mesh attached to lower end; E, plastic inhaler tubing with; F, plastic mouthpiece.

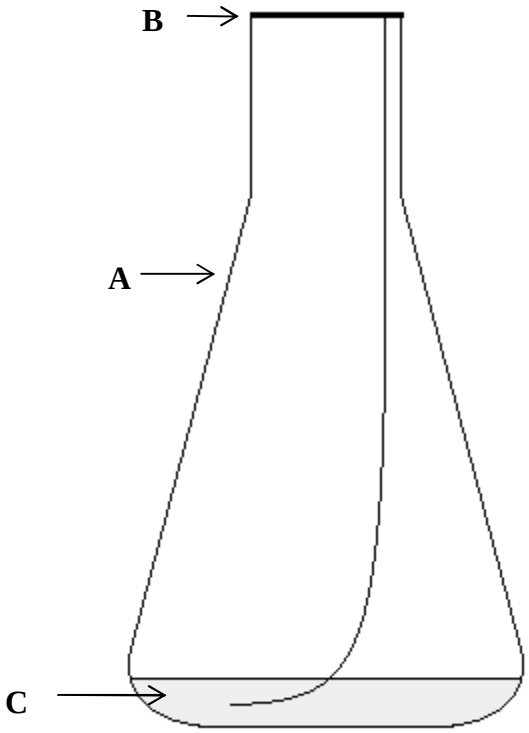

Figure 17 Reproduction and oviposit chamber. A, 1000 mL Erlenmeyer flask wit wide-mouth opening; B, strip of fine mesh affixed with tape to neck of flask; C. 50 mL dechlorinated water.

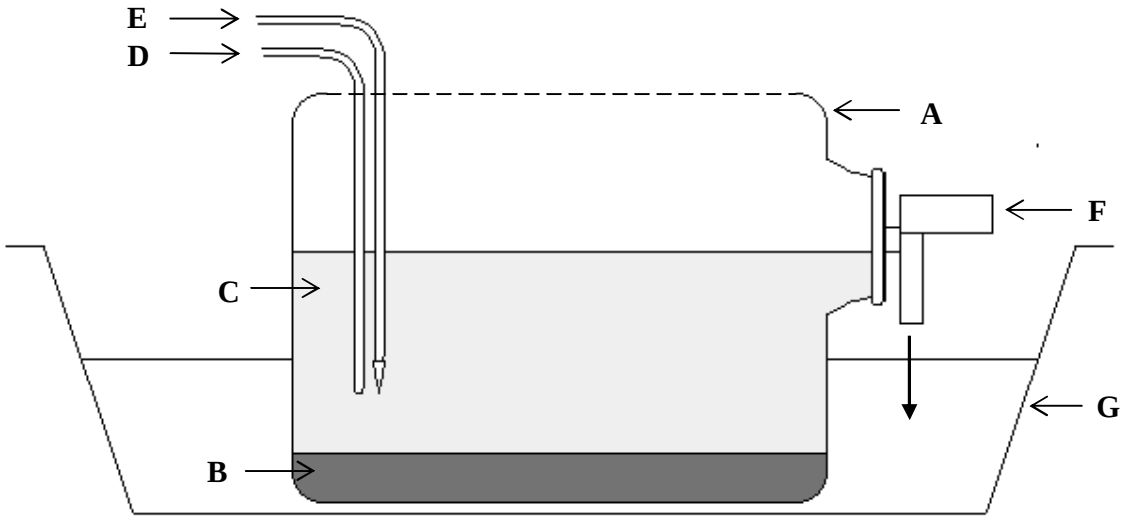

Figure 18 Disposable hatching vessel for rearing of second generation larvae. A, 4L Cubitainer with trimmed side (---); B, sediment; C, dechlorinated water; D, water inlet from pump; E, airline tubing with 200 µL pipette tip; F, fine mesh affixed with Quick Serve Tap; G, heated water bath.

4.2 Results

4.2.1 Life-cycle method development

4.2.1.1 Temperature

A series of experiments were conducted to evaluate the use of Imhoff cones in life-cycle testing (Table 5). Several testing parameters were modified in order to improve *C. dilutus* survival and emergence. Temperature fluctuations within cones was identified as a possible cause for delayed emergence. All subsequent tests were conducted in heated water baths to ensure temperature was maintained at 25±2°C. While heated water baths eliminated temperature fluctuation within cones, delayed emergence remained a problem.

4.2.1.2 Population density

Unpredictable emergence was improved by increasing sediment surface area. The Organization for Economic Co-operation and Development (OECD, 2010) states that 2-3 cm^2 are required per larvae, for proper growth and development. In an effort to harmonize a life-cycle beaker method (Benoit *et al.* 1997) and short-term cone method (Borgmann and Norwood 1999), preliminary tests were initiated with 15 larvae. This corresponds to 0.64 cm^2 per organism, well below the OECD requirement. Following 50 day growth in Imhoff cones, survival was recorded at 40±25% (Figure 19). The majority of larvae recovered suffered from reduced growth, and less than 5% of organisms emerged into adulthood. In addition, larvae were observed attempting to climb the sides of the vessel, which is often a sign of overcrowding.

Testing parameters were modified in order to improve survival and emergence. Decreasing population density to 5 organisms per cone eliminated overcrowding behaviour. A 15 mL sediment volume designates 1.93 cm^2 per organism, just under the minimum requirement. The population density caused a marked improvement in survival (60±53%) and emergence (33±42%), although variability between replicates was high. Time to emergence exceeded 35 days (Figure 20). A subsequent sediment volume increase to 20 mL met the OECD requirement (2.29 cm^2 per organism) but did not produce adequate emergence. Smaller sediment surface areas promote aggressive behaviour between organisms. Surface area increases to 3.35 cm^2 and 4.87 cm^2 per organism (80 mL and 130 mL sediment, respectively) caused a significant increase in *C.*

dilutus survival. As no significant differences in emergence patterns were observed between 80 mL and 130 mL sediment volumes (2-way ANOVA, $p>0.05$), the smaller volume was a practical choice.

4.2.1.3 Feeding regimens

Dietary tests with both (80 mL and 130 mL) sediment volumes were conducted under varying feeding regimens to determine an adequate feeding ratio between Tetramin and algae for the dietary exposure tests. Various volumes of algae and Tetramin were tested to try to establish optimal survival and emergence. A low (11.67 mg) or high (28 mg) Tetramin diet was supplemented with 35 mL algal feedout per week. When larvae were fed 35 mL of algae feedout and 28 mg of Tetramin per week survival and emergence was 87±21% (Figure 21), with emergence beginning at 24±2 days post hatch (Figure 22). All females from the chosen test successfully mated and produced viable egg cases. Final testing parameters included (1) initiation with five larvae, (2) an 80 mL sediment volume, (3) 35 mL algae feedout per week, and (4) 28 mg Tetramin per week.

4.2.1.4 Water Quality

Sediment volume adjustments made in order to increase sediment surface area reduced potential water to sediment ratios. An increase in sediment surface area was necessary to ensure proper growth and development in *C. dilutus*. As such, a large water to sediment ratio (67:1, Borgmann and Norwood 1999) could not be conserved. Regardless of control sediment volume, water quality was within acceptable range (Table 6, Environment Canada 1997). No ammonia was detected for the duration of the tests.

4.2.2 Dietary Se exposure

Dietary exposure tests were conducted in Imhoff cones fitted with size 4 silicone stoppers and 80 mL of sediment. No ammonia was detected for the duration of the tests. Measured water-quality parameters for Se exposure tests are summarized in Table 4. Test pH, temperature, and dissolved oxygen were within the accepted range as defined by Environment Canada (1997).

The effects of dietary Se exposure on the life-cycle of *C. dilutus* were assessed using the newly developed cone method. Final dietary exposure concentrations for first (F_0) and second (F_1) generation *Chironomus dilutus* are listed in Table 5. Selenium in modified Bristol's Medium and corresponding algae represents initial algal exposure concentrations. Selenium in algal feedout represents *C. dilutus* dietary exposure concentrations in first (F_0) and second (F_1) generations. Selenium concentration in the control feedout was below the detection limit (<0.24 ppb). Due to the variability associated with selenium uptake in algal cells, dietary exposure concentrations differed slightly between generations. Second generation *C. dilutus* larvae from treatment 0.625 were exposed to dietary Se concentrations of 9.9 μg Se g^{-1} dw. This differs significantly from the first generation exposure to Se levels below the detection limit. Although both algal species were exposed to similar concentrations of dissolved selenate, cellular uptake by one or both species increased, resulting in elevated tissue concentrations. Selenium concentration in Tetramin was measured at 2.38±0.21 μg Se g^{-1}

4.2.2.1 First generation

Larval survival, time to pupation and emergence and sex ratios of first generation emerged adults were not affected by dietary exposures to Se (ANOVA, $p>0.05$) (Figure 23, 24). Lifespan of adult male midges was not affected (ANOVA, $p>0.05$), while female midge lifespan was significantly decreased by exposure to algal feedout grown at 40 μg Se L^{-1} compared to the control (ANOVA, $p=0.030$) (Figure 25). There appears to be an increase in egg case production at algal exposures of 0.625 and 10 μg Se L^{-1} (Figure 26). This does not appear to be related to selenium exposure.

The average number of eggs per mass did not differ significantly between treatments (ANOVA, $p>0.05$) (Figure 27). A decrease in hatching success was observed with exposure to algal feedout grown in 20 μg Se L^{-1} in comparison to 0.625 μg L^{-1} (ANOVA, $p=0.000$) and 1.25 μg L^{-1} (ANOVA, $p=0.015$) (Figure 28). No significance was observed in relation to the control due to variability between samples (N=2). Deformities were observed in female midges and identified as misshapen abdomens (Figure 29, 30). Affected organisms were unable to reproduce and died within 24 hrs of emergence. The high variability observed in the highest dietary exposure (40μg L^{-1}) and

the lack of deformities in the 20 μg L^{-1} treatment make it difficult to assess whether these occurrences were due to selenium exposure.

4.2.2.2 Second generation

Dietary exposure to selenium had no significant effect on second generation larval survival or emergence of organisms (ANOVA, $p>0.05$) (Figure 23, 31). There was a lack of adult female emergence in the control treatments resulting in an abnormal sex ratio for this treatment; this endpoint is therefore difficult to assess statistically. Misshapen abdomens were also observed in second generation female midges in the 0.625 and 1.5 μg L^{-1} treatments (Figure 32). Affected organisms were unable to reproduce and died within 24 hrs of emergence. The high variability of female emergence at all exposure concentrations and the lack of female organisms in the control make it difficult to assess whether these occurrences were due to selenium exposure.

Premature emergence was observed in second generation males exposed to algae grown in 40 μg Se L^{-1} (ANOVA, $p=0.024$). No significant difference in male lifespan was observed (ANOVA, $p>0.05$). Reproduction occurred with organisms exposed to 1.25 and 10 μg L^{-1} treatments (Figure 33). Emergence and reproductive patterns in female organisms could not be determined due to a lack of females in the control. Comparison of the first and second generation produced no differences in survival and emergence (2-way ANOVA, $p>0.05$).

Table 6 Summary of water quality parameters (mean±SD) for final method test. Measurements were recorded weekly.

Experiment	Ammonia (ppm)	pH	Temperature (°C)	Dissolved Oxygen (%)	Conductivity (µS/cm)
4	0±0	7.3±0.5	23.6±0.8	90.8±1.6	185±32

Table 7 Summary of water quality parameters (mean±SD) for Se exposure tests. Measurements were recorded weekly.

Test	pH	Temperature (°C)	Dissolved Oxygen (%)	Conductivity (µS/cm)
F_0: Seleniferous algae	7.63±0.14	25.0±1.0	92.6±2.0	156±17
F_1: Seleniferous algae	7.44±0.18	25.9±0.7	89.5±12.8	144±14

Table 8 Nominal and measured concentrations (µg L^{-1}) of total Se in modified Bristol's Medium, Se uptake into algae (µg g^{-1}) and total dietary Se exposure (µg g^{-1}). F_0 corresponds to first generation dietary Se exposure; F_1 corresponds to second generation exposure.

Nominal Se	*P. subcapitata*	*C. kesslerii*	Algae feedout Se	Total exposure[a]
	Media Se	Media Se		
F_0: 0	< 0.76	< 0.76	< 0.24	2.50[b]
0.625	< 0.76	< 0.76	< 0.24	2.50[b]
1.25	1.40	2.00	< 0.24	2.50[b]
2.5	3.50	3.68	0.74	3.12
5	7.20	4.50	3.30	5.68
10	11.40	10.00	9.30	11.68
20	24.20	23.00	18.90	21.28
40	44.90	44.40	24.60	26.98
F_1: 0	< 0.24	0.15	< 0.24	2.50[b]
0.625	0.62	0.68	9.9	12.28
1.25	1.38	1.64	1.04	3.42
2.5	2.63	3.04	2.60	4.98
5	5.50	5.84	3.40	5.78
10	10.20	11.60	6.70	9.08
20	22.40	22.70	15.30	17.68
40	41.30	48.30	33.80	36.18

[a] – All dietary exposures taken into account (algal and Tetramin)

[b] – For algal feedout concentrations below the detection limit (0.24 ppb), half the value was used (0.12 ppb)

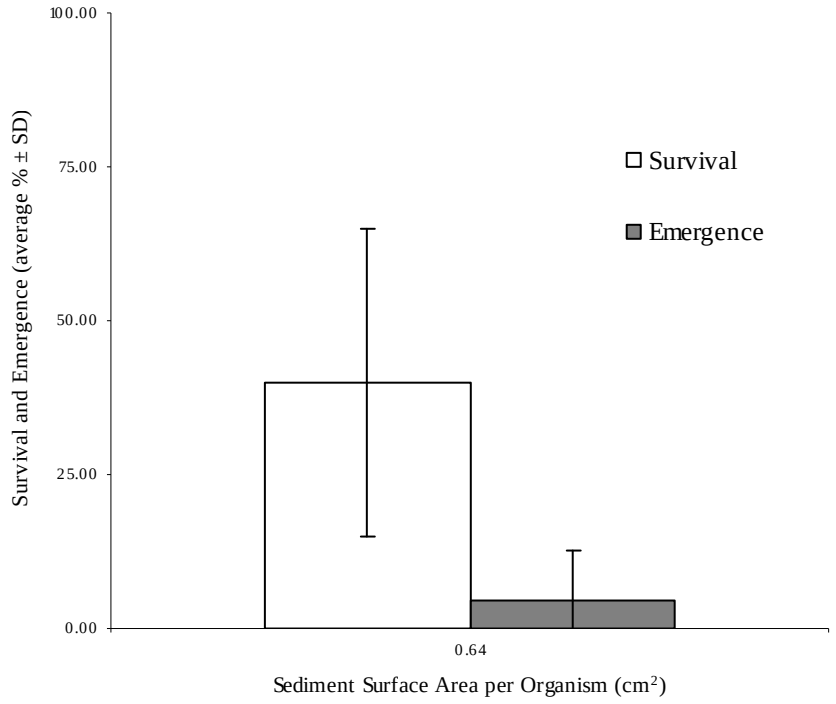

Figure 19 Survival and emergence of *C. dilutus* when grown at high population density. Error bars represent standard deviation from the mean (n=3).

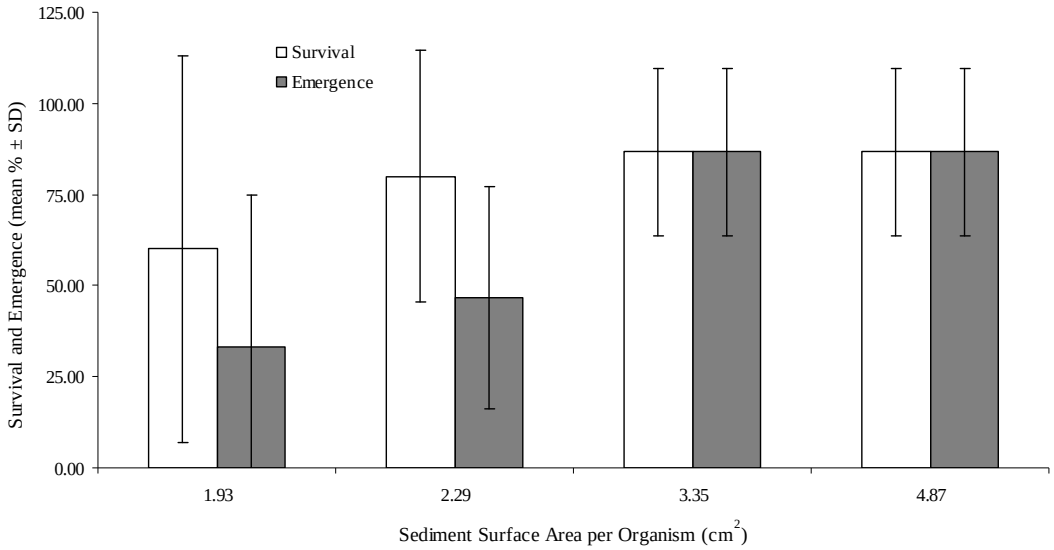

Figure 20 Survival and emergence of *C. dilutus* when grown in varying sediment surface areas. Error bars represent standard deviation from the mean (n=3).

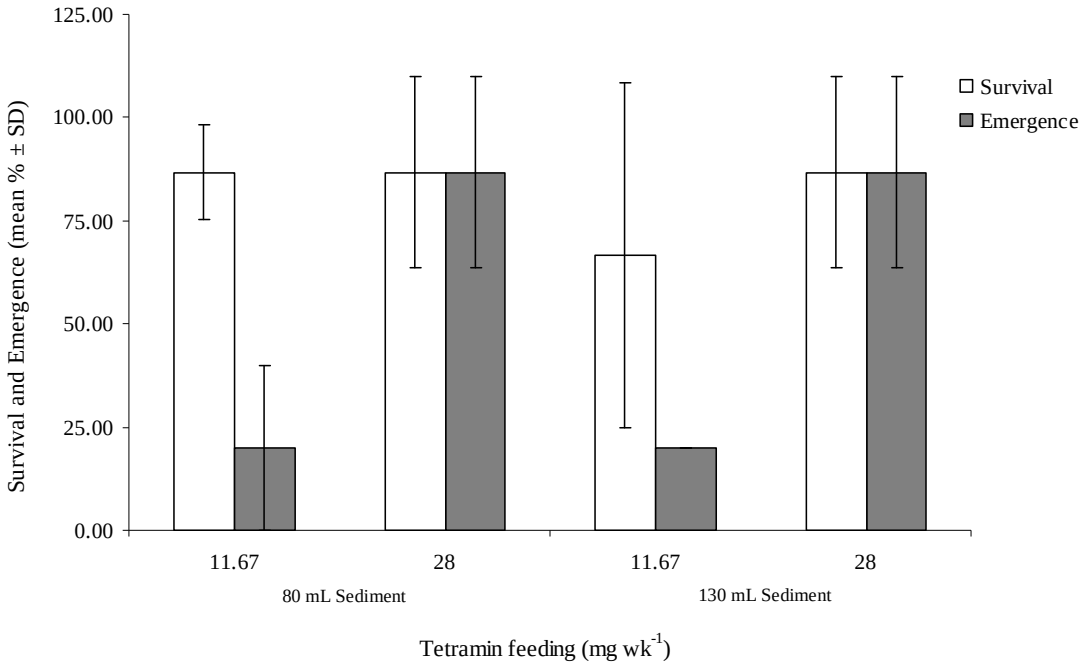

Figure 21 Survival and emergence of *C. dilutus* fed varying feeding regimens (algal, Tetramin) in two sediment volumes. High and low algal concentration were supplemented with 35 mL algal feedout in each sediment volume. Error bars represent standard deviation from the mean.

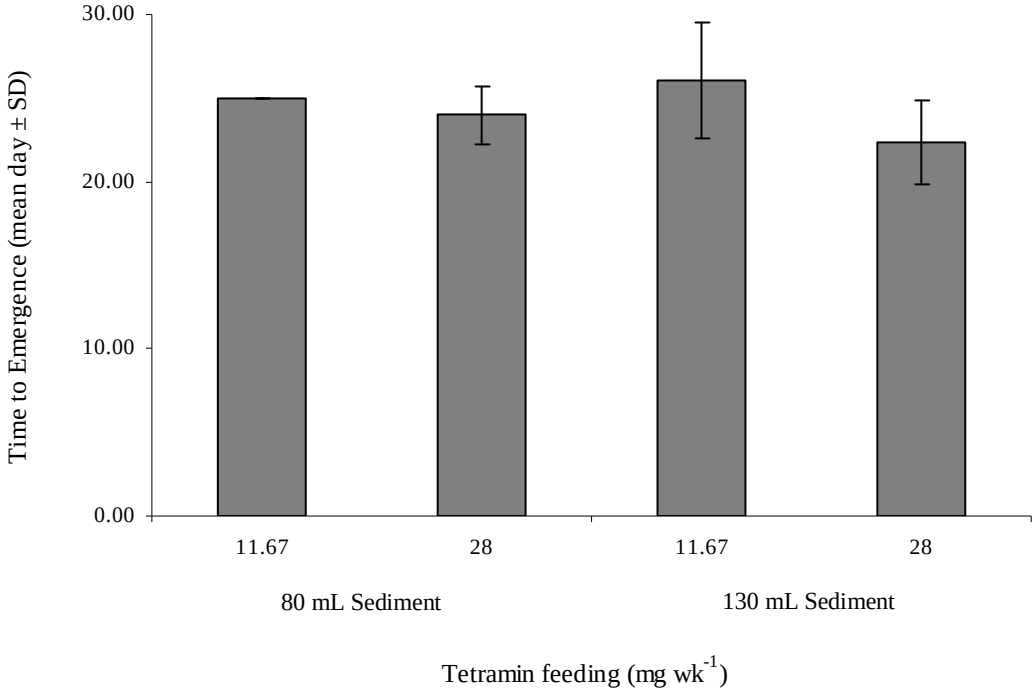

Figure 22 Time to emergence in *C. dilutus* when fed varying amounts of Tetramin in two sediment volumes. Error bars represent standard deviation from the mean.

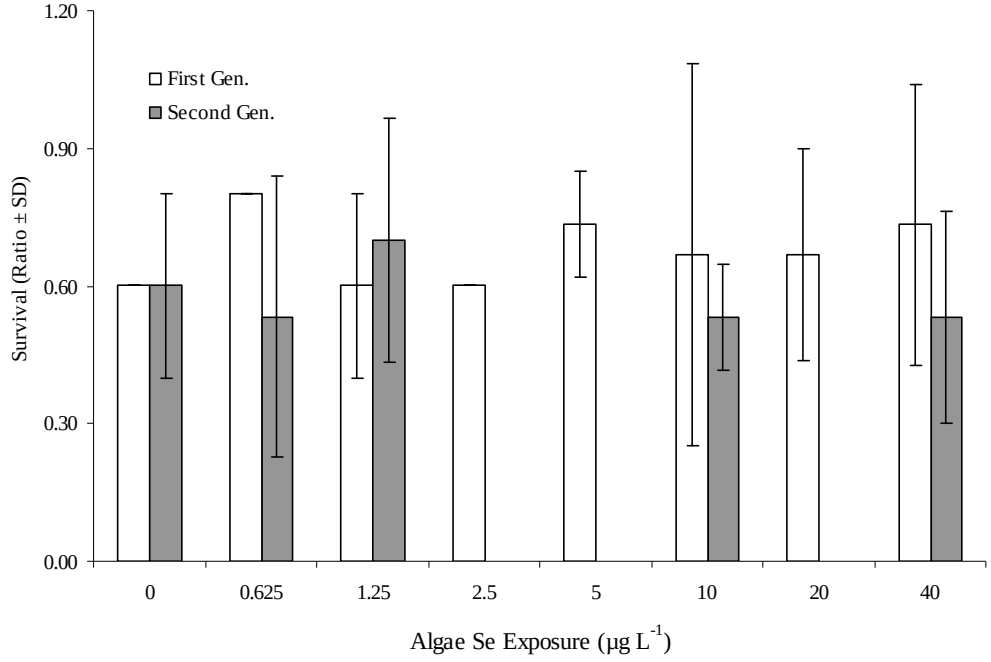

Figure 23 Survival (ratio±SD) of first and second generation *C. dilutus* exposed to seleniferous algae (grown in 0-40 µg Se L^{-1}) during larval development. Error bars represent standard deviation from the mean.

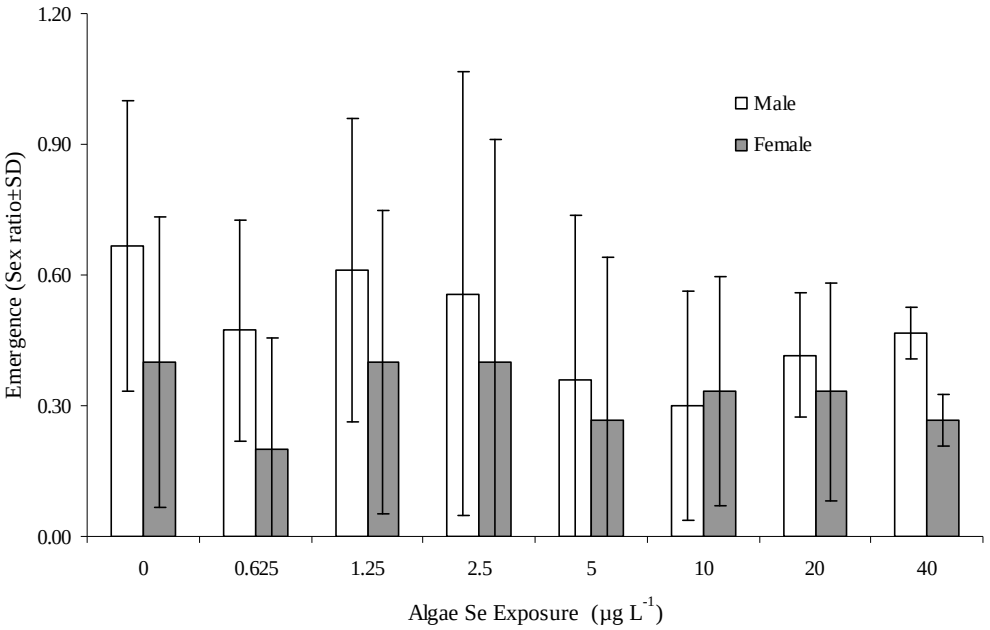

Figure 24 Emergent sex-ratio for first generation organisms exposed to seleniferous algae (grown in 0-40 µg Se L^{-1}) during larval development. Error bars represent standard deviation from the mean.

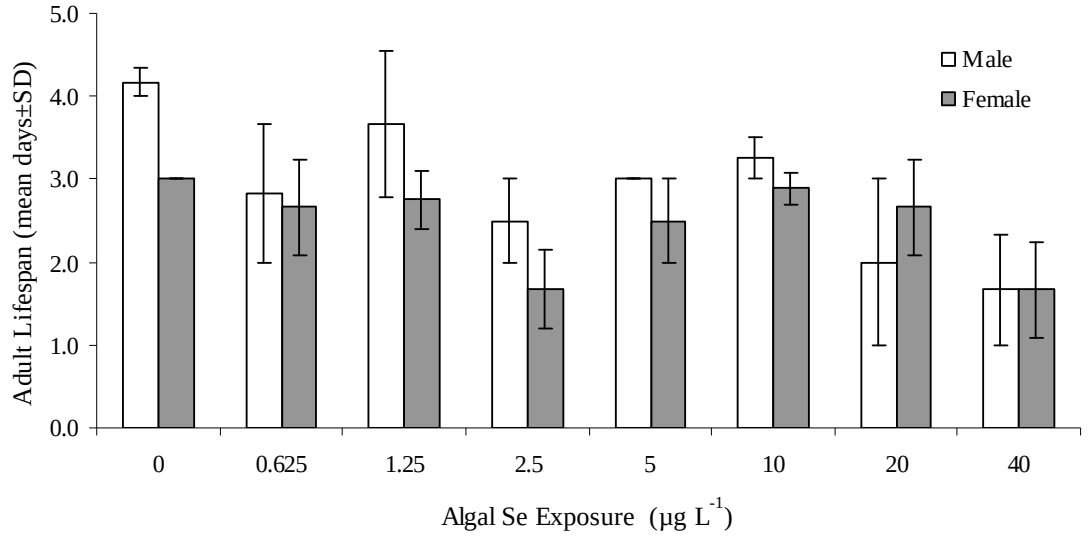

Figure 25 Lifespan (mean days+SE) of first generation *C. dilutus* adult organisms exposed to seleniferous algae (grown in 0-40 µg Se L^{-1}) during larval development. Error bars represent standard deviation from the mean.

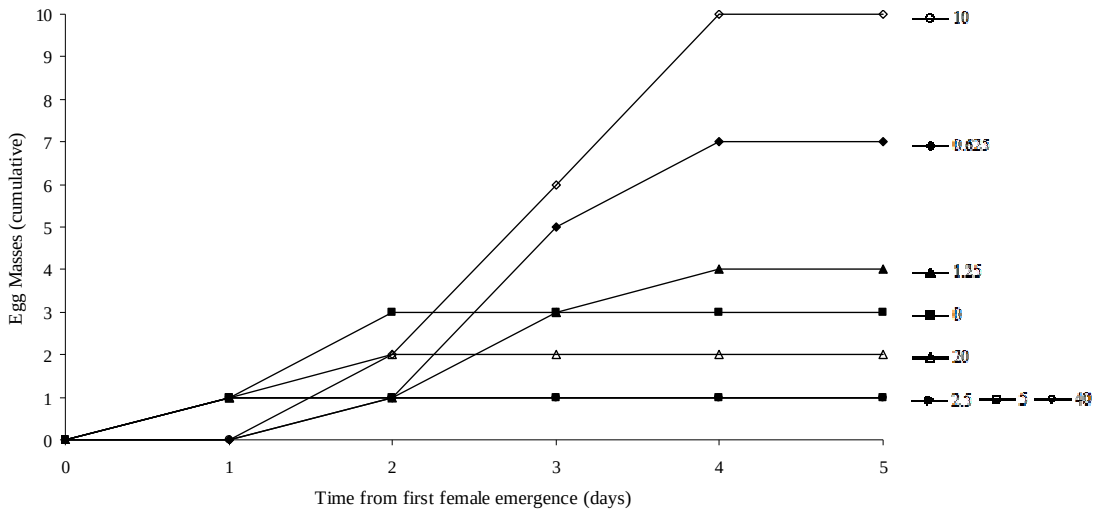

Figure 26 Cumulative egg masses by first generation *C. dilutus* exposed to seleniferous algae (grown in 0-40 µg Se L^{-1}) during larval development. Day 0 corresponds to first female emergence in treatment.

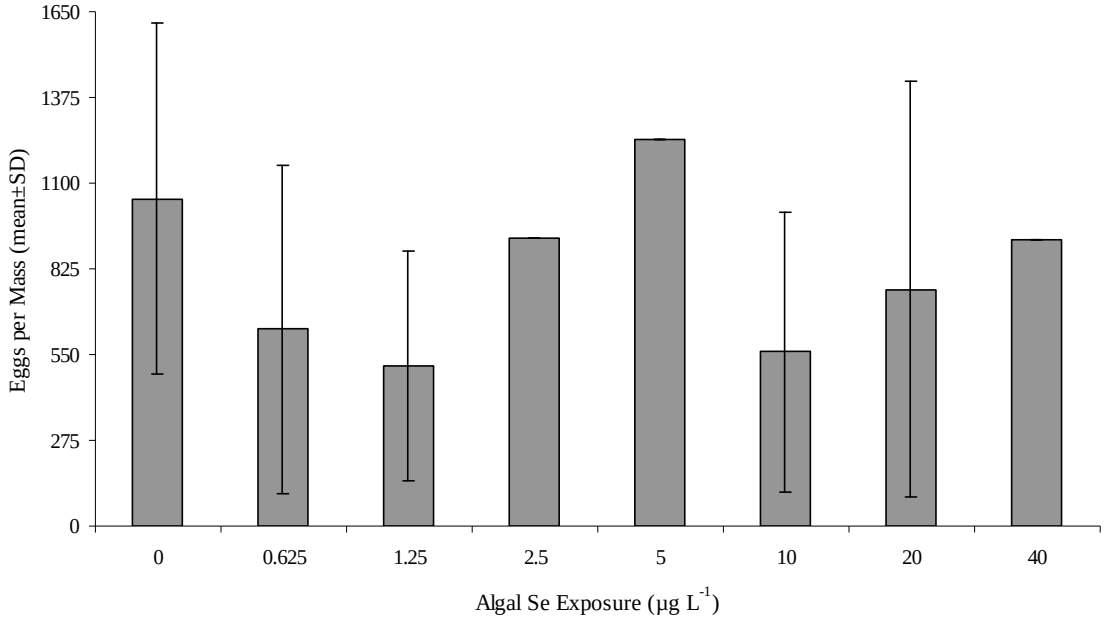

Figure 27 Eggs per mass (mean %+SD) produced by first generation *C. dilutus* exposed to seleniferous algae (grown in 0-40 µg Se L^{-1}) during larval development. Error bars represent standard deviation from the mean.

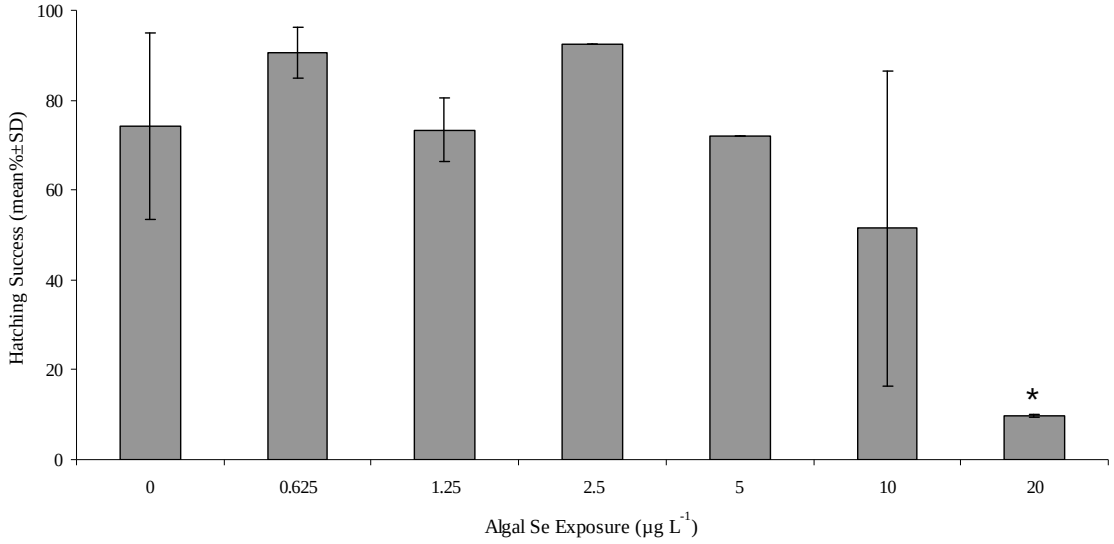

Figure 28 Hatching success (mean %+SD) in egg cases produced by first generation *C. dilutus* exposed to seleniferous algae (grown in 0-40 µg Se L^{-1}) during larval development. Significance at 20 µg L^{-1} when compared to treatments 0.625 and 1.25 µg L^{-1} denoted by asterisk (*) where *= $p<0.05$. Error bars represent standard deviation from the mean.

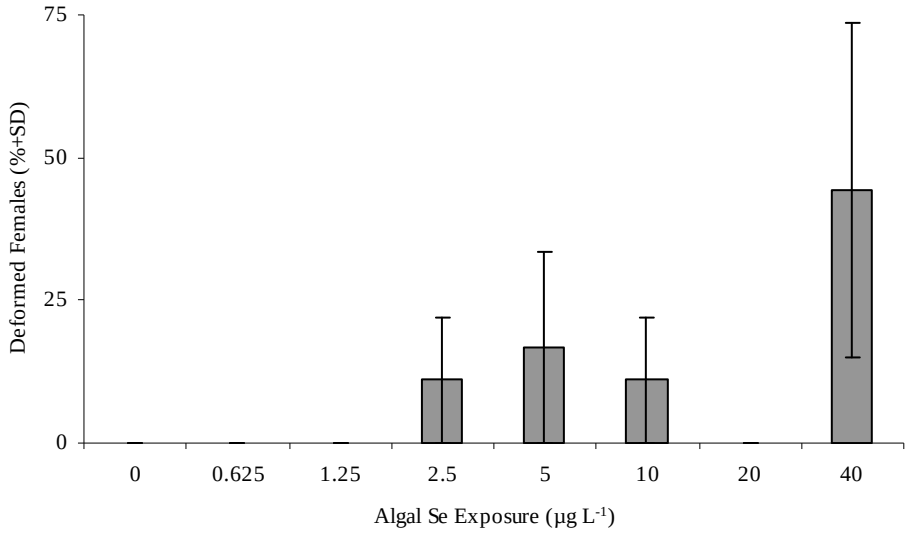

Figure 29 Deformities in first generation *C. dilutus* females (mean%+SD) exposed to algal feedout grown in 0-40 µg Se L^{-1} during larval development. Deformed females presented as a percentage of total emerged females in treatment. Error bars represent standard deviation from the mean.

Figure 30 Misshapen abdomen in first generation *C. dilutus* female exposed to a Se-enriched diet (2.5 µg Se L^{-1} algal exposure) during larval development.

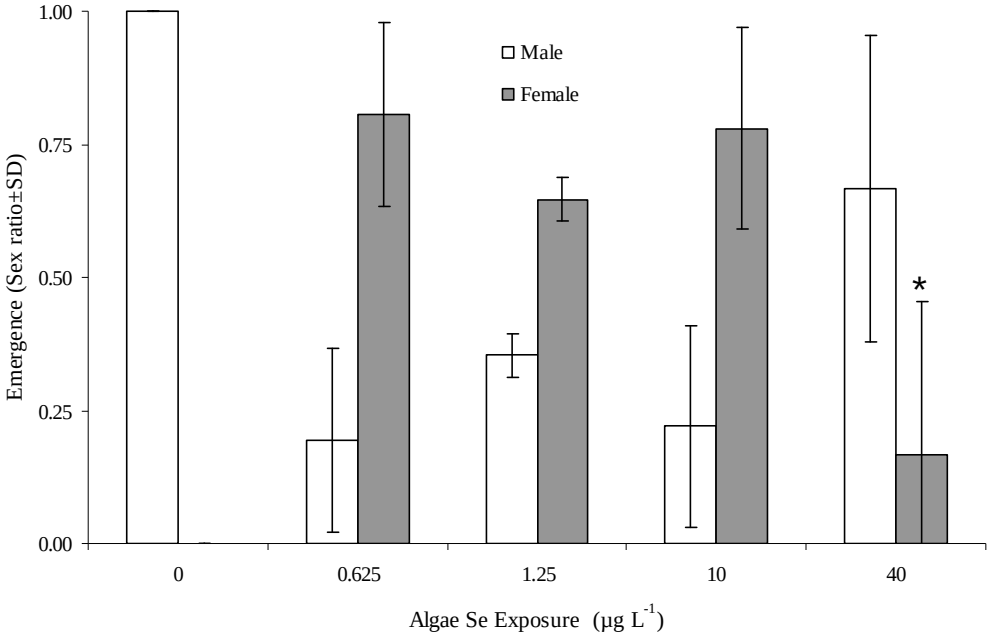

Figure 31 Emergent sex-ratio for second generation organisms exposed to seleniferous algae (grown in 0-40 µg Se L^{-1}) during larval development. Asterisk (*) denotes significant difference from treatment with 0.625 µg Se L^{-1}, where * = $p<0.05$. Error bars represent standard deviation from the mean (n=3).

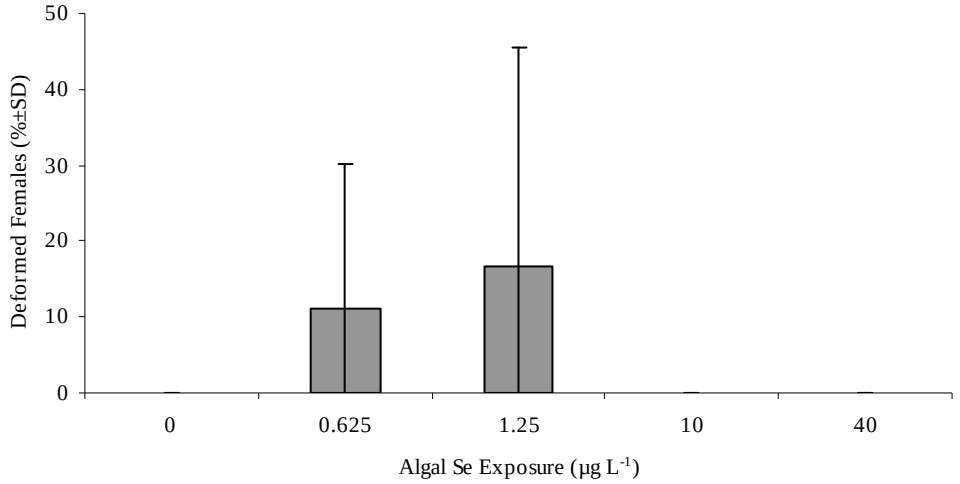

Figure 32 Deformities in second generation *C. dilutus* females (mean%+SD) exposed to algal feedout grown in 0-40 µg Se L^{-1} during larval development. Deformed females presented as a percentage of total emerged females in treatment. Error bars represent standard deviation from the mean.

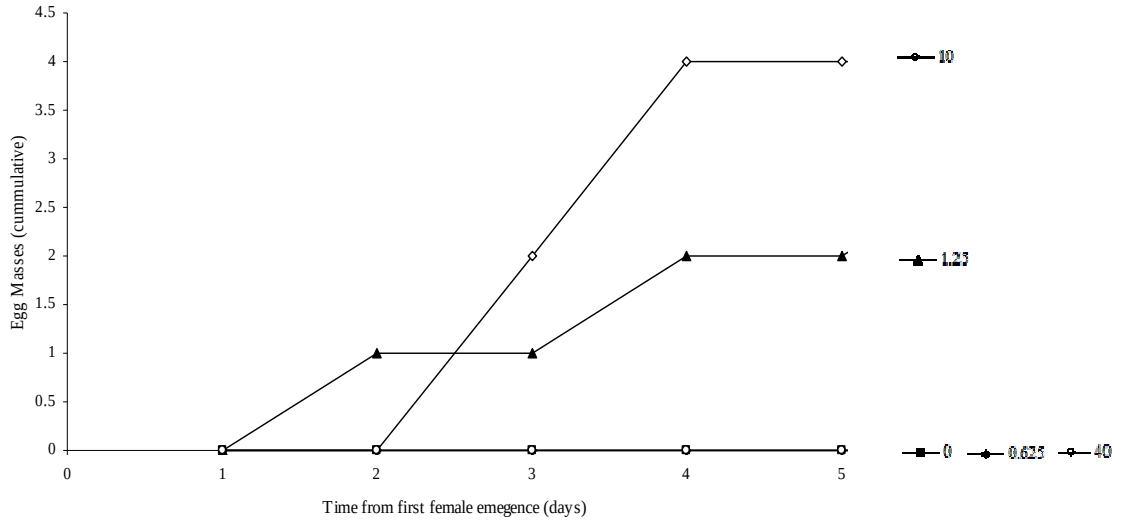

Figure 33 Cumulative egg masses by second generation *C. dilutus* exposed to seleniferous algae (grown in 0-40 µg Se L^{-1}) during larval development. Day 0 corresponds to first female emergence in treatment.

4.3 Discussion

4.3.1 Life-cycle method development

The development of a life-cycle method for *C. dilutus* was dependent on several parameters: (1) temperature, (2) sediment surface area and (3) feeding regimen. Several feeding regimens were tested in order to optimize growth and emergence. Algal feedout was introduced to the diet to facilitate dietary Se exposures. A diet of algal feedout was supplemented with ground Tetramin, as *C. dilutus* could not survive on algae alone.

Population density and sediment volume were dependent on one another. Larvae require at least 2 cm^2 per organism for proper growth and development (OECD 2010). Although Borgmann and Norwood (1999) were able to complete a 10-day sediment test using 15 mL sediment (1.93 cm^2 per organism), this was not feasible for the duration of a life-cycle test. By decreasing the number of organisms used to initiate a test, and increasing the sediment volume (and thus the sediment surface area), survival and emergence increased to sustainable levels. It should be noted that sediment surface area, not sediment volume, is the important requirement. Sediment volume is only important insofar that larvae have enough depth available for burrowing. A 2.5 cm sediment depth is adequate for *C. dilutus* growth and survival. By using a larger silicone stopper, sediment depth is decreased while maintaining surface area requirements. Using a larger stopper decreases the amount of sediment used, and increases the water to sediment ratio. It is recommended that the a size 7 stopper be used as this would decrease the sediment volume to 45 mL and nearly double the water:sediment ratio as recommended by Borgmann and Norwood (1999).

The Borgmann and Norwood (1999) method employed a large water to sediment ratio (67:1) in order to eliminate the need for water renewal. The need to increase the sediment surface area was a major concern, as a decrease in the water-sediment ratio can lead to the deterioration of overlying water quality. Water quality did not become an issue during method development tests, and remained within the acceptable range, as defined by Environment Canada (1997). Static conditions allow equilibrium to be established between water and sediment phases, more accurately reflecting environmental conditions. In addition, this method retains dissolved and particulate toxicants within the

test chamber. This is particularly important for dietary exposures, as algal cells have the tendency to travel between the sediment surface and the water column and would otherwise be lost as a result of water renewal.

Further tests should be performed to determine the validity of the cone method for use with field sediment samples. Completion of a comparative study with the beaker method (Benoit *et al.* 1997) would further support the use of the newly developed cone method in life-cycle assays.

4.3.2 Dietary Se exposure

The newly developed method was used to assess the effects of dietary selenium exposures in *C. dilutus*. A diet supplemented with Se-enriched algal feedout had little effect on survival or reproductive endpoints. Unfortunately, due to a lack of tissue, it was not possible to measure Se accumulation in *C. dilutus*, and therefore we could not confirm that uptake occurred. *C. dilutus* larvae were observed feeding on detritus at the sediment surface, though specific dietary preferences are not well established (Chapman *et al.* 2010). Algae are only one component of sediment detritus, and may have been selectively avoided by the organism. In a similar study, Se-enriched *P. subcapitata* (formerly *Selenastrum capricornutum*) was fed to *Chironomus decorus* (Malchow *et al.* 1995). Short-term (96 hr) exposures resulted in Se bioaccumulation from dietary exposure pathways and significantly reduced larval growth; the trophic transfer factor (TTF) for this food-chain was 1 (Malchow *et al.* 1995). A more recent investigation into trophic transfer factors detected very limited Se uptake in *C. dilutus* larvae, adults and exuvia when exposed to Se-enriched algae (Rickwood and Jatar 2012). This study measured a TTF of <0.35 in the lowest exposure concentrations and demonstrated an inverse relationship with dietary exposure. This indicates that *C. dilutus* were either selectively feeding or were able to regulate Se body burden. This study and the limited trophic-transfer of Se, validates the lack of survival and reproductive effects observed for the multi-generational life-cycle study. There is evidence to suggest *C. dilutus* is capable of developing resistance to environmental contaminants. Wentsel *et al.* (1978) demonstrated that larvae developed a degree of resistance to heavy metal contaminated sediments present in their habitat. Little effect was also observed by *C. dubia* fed Se-

enriched algal feedout (see previous chapter), indicating a similar regulatory response from two invertebrate species with different feeding strategies.

The occurrence of deformities in adult *C. dilutus* midges is an interesting result. Some occurrences of deformities are expected within a population. It is necessary to establish a baseline, as it is presently unclear whether the observed results are due to selenium exposure. Based on TTF values (Rickwood and Jatar 2012), it is unlikely that tissue concentrations were high enough to cause the observed physical malformations. The incidence of abdomen deformities should remain an established endpoint and be further investigated as it could be a related effect. Larger scale testing is required in order to increase the number of organisms and improve replication.

In order to determine if bioconcentration or bioaccumulation is occurring, more tissue is required. Larger scale testing should be conducted in order to generate enough tissue for determination of Se uptake in larvae and adult organisms. An increase in number of organisms would also resolve issues with lack of females emerged. An assessment of Se-enriched sediments and their effect on the life-cycle of *C. dilutus* should be undertaken to compare and contrast exposure mediums. Further work should be undertaken to determine the route of exposure for benthic organisms. It may also be possible to assess the fate of selenium using alternative techniques, such as radioisotopes (^{75}Se), which have been applied in various studies using other invertebrate species (Besser *et al.* 1993, Conley *et al.* 2009).

SUMMARY

The present study was completed in order to assess the multi-generational effects of dietary selenium exposure in two aquatic invertebrates: *Ceriodaphnia dubia* and *Chironomus dilutus*. A Se-enriched algal food source was successfully prepared; dissolved selenate was readily absorbed and concentrated by algal species *Pseudokirchneriella subcapitata and Chlorella kesslerii*. The strong relationship between exposure and uptake in algal species indicates the possibility of risk to consumers. There was evidence of Se metabolism in algal cells, indicating biotransformation to organo-Se species. The predominant risk exists with the uptake of dissolved inorganic species and biotransformation to bioavailable organic species.

No standardized tests exist to address the effects of dietary exposures in *C. dubia*, but as the standard Environment Canada test incorporates algal feeding regimens, the use of Se-enriched algae was an appropriate modification to explore this exposure pathway. Dietary Se exposures were examined over two generations to determine the effects on second-generation survival and reproductive capacity.

A life-cycle method for *C. dilutus* was successfully adapted from Borgmann and Norwood (1999). Imhoff cones eliminated the need for water renewal by employing a large water to sediment ratio. Static conditions allowed equilibrium to be established between water and sediment phases, more accurately reflecting environmental conditions. In addition, this method retained dissolved and particulate toxicants within the test chamber, which would otherwise have been lost as a result of water renewal. Growth and survival was dependent on three key factors: (1) temperature, (2) feeding and (3) population density. Larvae required adequate space in order to complete their life-cycle. This method which more closely mirrors lentic environmental conditions was necessary to accurately assess the risk associated with Se contamination.

Dietary selenium exposures had little effect on survival or reproductive endpoints in either invertebrate species, suggesting that invertebrates have the ability to regulate chronic Se exposures from dietary sources. These results are validated by separate studies which measured limited trophic transfer of Se from dietary sources. This study provides valuable information concerning the effects of dietary selenium in aquatic invertebrates.

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