CHRONIC TOXICITY AND ACCUMULATION OF URANIUM IN THE AQUATIC INVERTEBRATE *CHIRONOMUS TENTANS*

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By

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ABSTRACT

Northern Saskatchewan is home to several active uranium (U) mines. Discharges of U to the aquatic environment during mining operations may produce measurable increases in U levels that could pose a potential hazard for aquatic organisms. Aqueous U toxicity has been investigated in a number of aquatic invertebrates, but limited data are available on the effects of U exposure on benthic macroinvertebrates. The objective of this study was to evaluate the toxicity and accumulation of U over the life cycle of the aquatic invertebrate, *Chironomus tentans*, and to determine the rates of uptake and depuration for U in *C. tentans* larvae.

Chronic U toxicity was evaluated by exposing the animals to three different U concentrations (40, 200, and 1000 μg/L) and an untreated control. Dry weight and U tissue concentration were measured at all life stages and for the exuvia remaining after adult emergence. In addition, some *C. tentans* adults collected from the treatments were mated with un-exposed adults from an in-house colony to evaluate hatching and mating success. After 10 d of U exposure, *C. tentans* larvae showed a significant decrease in growth (as dry weight) at mean U concentrations ≥ 157 μg/L. The no observable effect concentration and lowest observable effect concentration for growth in U exposed *C. tentans* larvae were 39 and 157 μg/L, respectively. Ten-day larval growth retardations correlated strongly with emergence reductions (r^2 = 0.88) and also led to significant delays in time to adult emergence. Reductions of approximately 30 to 40 % in larval growth corresponded to decreases of 40 to 60 % in adult

emergence. This strongly indicates that reductions in *C. tentans* emergence can be predicted by 10-d growth data. Furthermore, the effects of U on larval growth were observed not only in the directly exposed F_0 larvae, but also in the unexposed F_1 generation larvae. Therefore, U exposure during the paternal generation can significantly influence the growth and reproductive potential of the next generation. Uranium that accumulated during *C. tentans* immature stages was partially excreted (approximately 50%) during metamorphosis to the adult stage. The process of U elimination can be explained both by physiological changes taking place during metamorphosis, as well as by the shedding of the exoskeleton when molting from the pupal to the adult stage. However, the elimination of U was not complete and, as a result, transfer of U to the adult midges was observed.

In order to investigate U uptake and depuration, 10-d old *C. tentans* larvae were exposed to 300 μg U/L. After 9 d, larvae were transferred to clean water to calculate the U depuration rate. Animal samples were collected every 3 d to evaluate U tissue concentration and dry weight. Steady state conditions during U exposure were approached in 9-11 d. However, accumulated U was rapidly depurated (within 3 d) when larvae were transferred to U free water. The calculated uptake (K_u) and depuration rate constants (K_d) were 20.3 and 0.36, respectively. A separate experiment that measured U uptake in dead and live larvae revealed that accumulation of U in *C. tentans* larvae could be explained by a passive mechanisms of uptake coupled with an active mechanisms of U depuration.

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DEDICATION

To my family. Without their everlasting love and constant support of every "loca idea" I have, I would not be able to keep going.

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α = alpha

> = greater than

< = less than

$\geq$ = greater than or equal to

$\leq$ = less than or equal to

μg/g = micrograms per gram

μg/L = micrograms per litre

μg/ml = microgram per milliliter

μm = micrometer

ANOVA = analysis of variance

ASTDR = Agency of Toxic Substances and Disease Registry

BAF = bioaccumulation factor

BCF = bioconcentration factor

C = concentration of uranium in the animal

$^{\circ}$C = centigrade

CANDU = Canadian Deuterium Uranium

Css = mean concentration of uranium at steady state concentration

Cw = concentration of uranium in the water

d = day

DL = analytical detection limit

DO = dissolved oxygen

EC_{50} = median effect concentration

EDTA = ethylenediamine tetraacetic acid

F_0 = parental generation

F_1 = first filial generation

h = hour

ICP-MS = Inductive-Coupled Plasma Mass Spectometry

K_d = constant of depuration

K_u = constant of uptake

L = liter

LC_{20} = lethal dose, 20% kill

LC_{50} = median lethal concentration

ln = natural logarithm

LOEC = Lowest Observable Effect Concentration

log = base 10 logarithm

M = molar

mg = milligram

mg/kg = milligrams per kilogram

mg/kg dw = milligrams per kilogram dry weight

mg/L = milligrams per litre

min = minute

ml = milliliter

MS-222 = 3-aminobenzoic acid ethyl ester

n = number of samples

N = normal

NOEC = No Observable Effect Concentration

OECD = Organization for Economic Co-operation and Development

SD = Standard Deviation

t = time

$t_{½}$ = half time

t_d = depuration sampling time

t_u = uptake sampling time

U = uranium

$UO_2(NO_3)_2 \cdot 6H_2O$ = uranium (VI) dinitrate oxide hexahydrate

USEPA = United States Environmental Protection Agency

Chapter 1. Introduction

1.1. The uranium mining industry

1.1.1. Past and present uranium mining operations

Uranium (U) exploration, mining and milling have taken place over extensive areas of the subarctic and arctic Canadian Precambrian Shield, particularly in northern Saskatchewan (Melville, 1995). Mining of uranium in Saskatchewan began in the Beaverlodge district on the north shore of Lake Athabasca in 1962. The larger mines (Eldorado, Gunnar and Lorado) had mills to process their ore and to custom mill ore for the smaller producers (Swanson, 1997). By 1964, falling U prices and depletion of existing ore bodies left Eldorado as the sole producer in the Beaverlodge area where production continued until June 1982.

Exploration for U continued in Saskatchewan in the intervening years, primarily in the Athabasca sand region, leading to the development of open-pit mining at Rabbit Lake in the Wollaston area in 1975 and at Cluff Lake in 1979. Other major ore bodies have since been discovered at Midwest Lake, Collins Bay, McClean Lake and Keefe Lake in the Wollaston area, and at Maurice Bay on Lake Athabasca (Salaff, 1983; Swanson, 1997).

Today, Canada is in the midst of a transition from the second-generation U mines, started in 1975, to new third-generation mines. All of these mines

are located in northern Saskatchewan (World Nuclear Association, 2003; Cameco, 2001). The major U mining company, Cameco Corporation, owns the Key Lake and Rabbit Lake mines. Key Lake has been mined out, so all production comes from ore stockpiles. Rabbit Lake has been on standby since March 2001. The other major U mining company is Cogema Resources Inc. It's 20-year old Cluff Lake mine is also producing from stockpiles only, and is pending decommissioning (World Nuclear Association, 2003; Cameco, 2001).

In addition to the above, there are two new U mines: McClean Lake (owned by Cogema Resources Inc.), which started production in July 1999, and McArthur River (owned by Cameco Corp.), which opened at the end of 1999. There are also three new U projects in early stages of development: Cigar Lake, Midwest Joint Venture, and Dawn Lake (World Nuclear Association, 2003; Cameco, 2001). Finally, there are five decommissioned or abandoned mines in the region (Environment Canada and Health Canada, 2001).

1.1.2. Uranium mining, milling and refining

Canada is home to some of the richest U deposits in the world (Pyle *et al*, 2002). In 2001, Canada produced 14,762 tonnes of U, which represents one third of the world's U output. The production of U by 2005 is expected to double from 2001 levels as the new high-grade mines increase Canada's production (World Nuclear Association, 2003; Cameco, 2001).

The U-containing ores are usually mined by conventional extraction methods, such as open-pit mining, *in situ* leaching and/or underground mining (ASTDR, 1997; OECD Nuclear Energy Agency, 1999). The mined U ore is crushed and leached in a U mill. Uranium mills extract triuranium octoxide (U_3O_8) from crushed, ground ores with acid (H_2SO_4) and O_2 leaching processes. After the leaching process, U is separated from the waste solids by washing and chemical treatment. The resultant solution containing U goes through a solvent extraction process (NH_3) where the U is purified, concentrated and precipitated as yellowcake (Environment Canada and Health Canada, 2001; ASTDR, 1997). Yellowcake, which is the end product of the milling process, is then shipped from northern Saskatchewan to a U refinery located in Ontario. The residual tailings (crushed ore minus most of the U) are pumped as slurry to a liquid retention impoundment in a tailings management facility. In the tailings ponds, the solids settle out and the effluent is treated with $BaCl_2$ and lime to reduce radionuclide and metal levels in the water (Environment Canada and Health Canada, 2001; Swanson, 1997). The resultant effluent and slurry goes to the monitoring ponds and tailing dam respectively.

There are two major processing steps involved in U refinement: the purification of yellowcake to uranium trioxide (UO_3) and the conversion of UO_3 to uranium hexafluoride (UF_6). It is in the form of UF_6 that U is shipped to foreign markets, where U metal can be produced from uranium tetrafluoride (UF_4) drawn from the UF_6 circuit. In another processing stage,

uranium dioxide (UO_2), an intermediate product of the UO_3 circuit, is converted to reactor-grade UO_2 for use in Canadian Deuterium Uranium (CANDU) reactors (Environment Canada and Health Canada, 2001).

1.2. Chemical properties of uranium

Uranium is a member of the actinide series and is the heaviest natural-occurring element. It occurs naturally in the earth's crust at an average concentration of about 2 mg/kg (ASTDR, 1997). Uranium is able to adopt four valences resulting in the ions, U^{3+} (III), U^{4+} (IV), UO_2^{+} (V), and UO_2^{2+} (VI), the latter of which is also known as the uranyl ion (Ribera *et al*, 1996).

Uranium has 10 radioactive isotopes, but only three are naturally-occurring: ^{238}U, ^{235}U, and ^{234}U. The predominant isotope of U commonly found in nature is ^{238}U, which has a physical half-life of 4.5×10^9 years, giving it a low specific activity. For this reason, U is generally more chemotoxic than radiotoxic (Environment Canada and Health Canada, 2001; Ribera *et al*, 1996).

Tetravalent U is reasonably stable and forms hydroxides, hydrated fluorides and phosphates of low solubility. Hexavalent U is the most stable form and the most commonly occurring form of U under environmental conditions (ASTDR, 1997; Clark *et al*, 1995). The U (VI) uranyl form is potentially quite mobile in the environment (Chisholm-Brause *et al*, 2001).

However, transition from U (VI) to insoluble salts (U (IV)) may occur at low pH (~3) and/or in reductive environments (Shock *et al*, 1997; ASTDR, 1997).

Since U^{3+} and its complexes are not stable in water, and since U (V) is rapidly oxidized to U (VI), trivalent (U^{3+}) and pentavalent (UO_2^+) U species are minor constituents of aqueous uranium solutions (Shock *et al*, 1997; Allard *et al*, 1984).

1.3. Uranium in the aquatic environment

1.3.1. Aqueous uranium chemistry and bioavailability

Aqueous actinide chemistry in environmental systems is complicated by a variety of phenomena, including the co-occurrence of dissolved species in multiple valence states. The chemical speciation of U, like other metals, may be influenced by water quality variables such as hardness, alkalinity, pH and natural organic matter (Environment Canada and Health Canada, 2001; Riethmuller *et al*, 2001; Hyne *et al*, 1992a). Hardness and alkalinity influence toxicity through different mechanisms. In the case of hardness, Ca^{2+} and/or Mg^{2+} competitively inhibit U uptake at biotic ligands thereby reducing its toxicity, whereas in the case of alkalinity, complexation of U with carbonate modifies metal speciation and reduces the concentration of the toxic/bioavailable metal species (Environment Canada and Health Canada, 2001; Riethmuller *et al*, 2001; Hyne *et al*, 1992a).

The chemistry of U in aqueous systems is dominated by the difference in behaviour of the predominant U (VI) and U (IV) ions. The tetravalent form

generally has low solubility, whereas the hexavalent form is relatively soluble as the uranyl ion and its complexes, such as hydroxides and carbonates, which are dominant actinide complex-forming agents. Thus, transport of U is greatly enhanced if it is present in solution as the uranyl ion and associated complexes (Burns and Finch, 1999).

The transport and dispersion of U in ponds, lakes and rivers is also affected by adsorption and desorption of U on sediments. In anoxic environments, U (VI) will be reduced to U (IV) and deposited into the sediments, due to the insolubility of the resulting U (IV) salts (ASTDR, 1997). In most water-bodies, sediments will act as a sink for U so that the concentration in sediments and suspended solids will be several orders of magnitude higher than in the surrounding water (ASTDR, 1997; Environment Canada and Health Canada, 2001).

Uranium sorption to minerals and organic matter may precede reduction to U (IV). Dissolved organic matter, in the form of humic and fulvic acids, is known to form stable complexes with the uranyl ion in natural waters, which contributes to the migration of uranyl, as well as ameliorating its toxicity to aquatic organisms (Markich *et al*, 1996). In contrast, the decrease in sorption of U (VI) in aqueous solutions at pHs above 8 can be attributed to its strong affinity for carbonate ligands, which effectively compete with sorption to minerals and organic matter (Burns and Finch, 1999; Kim, 1986; Allard *et al*, 1984).

1.3.2. Uranium contamination of aquatic environments

Uranium may enter the environment via human activities, such as mining and milling, nuclear industries, medicine, waste management, and food industries, which use ionizing radiation sources (Labrot *et al*, 1999). However, the major environmental problems pertaining to U are related to U mines, and tailings and process effluents resulting from the milling of U ore. Effluents from U mines generally contain elevated levels of metals (e.g., As, Pb, Cd, Mo, Ni, U) and radionuclides (e.g., ^{226}Ra, ^{210}Pb, ^{228}Th, and ^{210}Po). Solid and liquid wastes have been directly discharged into lakes, streams and nearby water bodies (Goldstick, 1980). In addition, leaching from mill tailings into surface and groundwater may occur due to the breakdown of dams and liners, or flooding during the rainy season (Burns and Finch, 1999)

Prior to 1970, no treatment of liquid effluent from tailings took place. Accidental spills of tailings were frequent and were often left untouched when they occurred (Swanson, 1997). In the mid 1970's, regulations were put in place requiring proper containment of tailings and treatment of effluents (Swanson, 1997).

Each U mine or mine-mill complex in Saskatchewan is required to monitor the quality of downstream water as part of the license to operate. Consequently, an extensive body of physical-chemical monitoring data exists for several regions of the Canadian Precambrian Shield. These data indicate that substantial changes in water quality and invertebrate

communities have occurred, not only in tailings ponds, but also in a number of adjacent northern lakes (e.g., Island Lake, Rabbit Lake) (Melville, 1995). Furthermore, the higher U concentrations found in water and sediment in downstream areas indicate that these are important areas of potential impact of U released from mines and mills to the aquatic environment.

In northern Saskatchewan, the background median aqueous U concentration ranges from 0.05 to 0.35 μg/L and the background median sediment concentration ranges from 3.7 to 29.5 mg U/kg dry weight (dw) (Environment Canada and Health Canada, 2001). However, downstream of U mines and mills the U concentration in water could range from 100 to approximately 500 μg/L (Environment Canada and Health Canada, 2001; Hynes *et al*, 1987; Melville, 1995) and the U concentration in sediment is often more than 1000 mg/kg dw (Environment Canada and Health Canada, 2001; Hynes *et al*, 1987; Cooley and Klaverkamp, 2000).

Uranium toxicity has been shown to be strongly dependent on the distribution of uranyl species. The uranyl ion and the hydrated metal ion (UO_2OH^+) are the species most responsible for the toxic effects of U, while carbonate and sulphate complexes are not believed to contribute to toxicity (Environment Canada and Health Canada, 2001; Markich *et al*, 1996; Semaan *et al*, 2001).

As U has a high propensity for solubilisation and migration in natural waters, it poses a potential hazard to aquatic organisms (Markich *et al*, 1996). Moreover, since uranium is a non-essential metal for biological

processes, concerns for the protection of aquatic biota exposed to U contaminated waters must be considered (Semaan *et al*, 2001). The recommended interim threshold effect values for U in water and sediments are 218 µg/L and 94 mg/kg dw, respectively (Environment Canada and Health Canada, 2001). However, no formal water and sediment quality guidelines for U presently exist and consequently there is a priority need to develop sound guidelines for acceptable U levels in Canadian waters.

1.3.3. Uranium toxicity to aquatic biota

1.3.3.1. Pelagic organisms

The aquatic system is often the major system affected by the release of uranium from mines and mills (Ribera *et al*, 1996). Effluents from U mines generally contain elevated concentrations of various metals (e.g., cadmium, nickel, arsenic, copper, molybdenum), in addition to U, that could result in a potentially toxic concentration in the aquatic receiving environment (Liber and White Sobey, 2004; Environment Canada and Health Canada, 2001). However, U has been identified as the prime ecotoxic contaminant in some mine waste waters (Holdway, 1992; Semaan *et al*, 2001).

Aqueous uranium toxicity has been investigated in a number of aquatic invertebrates, including crustaceans, worms, hydra, insects and bivalves. In most cases, reported toxicity endpoints, such as the median effect concentration (EC_{50}) and no observed effect concentration (NOEC) / lowest observed effect concentration (LOEC), are in the range of 0.2 to 10 mg/L

(Liber and White Sobey, 2004). Acute and chronic U toxicity data for several fish and aquatic invertebrate species found in Canada are summarized in Tables 1.1 and 1.2, respectively.

Holdway (1992) reported that older and larger fish were less sensitive to U toxicity than younger smaller fish. In the same study, Holdway calculated a toxicity threshold for growth in larval tropical fish, *Morgurnda morgurnda* and *Melanotaenia splendida inornata,* of 0.20 mg/L in soft water. Bywater *et al* (1991) reported U median lethal concentration (LC_{50}) values ranging from 0.73 to 3.46 mg/L for six tropical fish and from 0.14 to 0.90 mg/L for four species of cladocerans in water low in hardness and alkalinity. In contrast, fish species tested in water at higher hardness and alkalinity had LC_{50} values several times higher. Tazwell and Henderson (1960) reported 96-h LC_{50} values for *Pimephales promelas* of 2.8 mg/L in water with a hardness of 20 mg/L and 135 mg/L in a hardness of 400 mg/L. Parkhurst *et al.* (1984) also reported 96-h LC_{50}s for brook trout (*Salvelinus fontinalis*) exposed to U of 5.5 and 23 mg/L at hardnesses of 35 and 208 mg/L, respectively.

Hyne *et al.* (1992a) investigated the effect of U on the growth of freshwater hydras (*Hydra viridissima* and *H. vulgaris*). Results from this study showed that U concentrations as low as 150 μg/L may have a direct inhibitory effect on population growth of *H. viridissima*. *H. vulgaris* showed

Table 1.1. Acute toxicity of uranium to fish species found in Canada.

Species	Test endpoint[a]	U Conc. (mg/L)	Hardness (mg $CaCO_3$/L)	Reference
Pimephales promelas	96-h LC_{50}	2.8	20	Tarzwell and Henderson (1960)
Pimephales promelas	96-h LC_{50}	135	400	Tarzwell and Henderson (1960)
Salvelinus fontinalis	96-h LC_{50}	5.5	35.1	Parkhurst *et al.* (1984)
Salvelinus fontinalis	96-h LC_{50}	23	208	Parkhurst *et al.* (1984)

[a] Median lethal concentration

Table 1.2. Acute and chronic toxicity of uranium to aquatic invertebrates found in Canada.

Species	Test endpoint	U Conc. (mg/L)	Hardness (mg $CaCO_3$/L)	Reference
Daphnia magna	5-d LOEC [a]	0.52	66 - 73	Poston *et al.* (1984)
Ceriodaphnia dubia	7-d NOEC [b] 7-d LOEC [a]	0.002-0.003 0.003-0.008	3-6 1-4	Picket *et al.* (1993)
Ceriodaphnia dubia	48-h LC_{50} [c]	0.43	27	Liber and George (2000)
Ceriodaphnia dubia	7-d NOEC [b] 7-d LOEC [a]	0.010 0.035	60	Liber and George (2000)
Ceriodaphnia dubia	7-d EC_{50} [d]	0.22	120	Liber and George (2000)
Hyallela azteca	96-h LC_{50} [c]	8.2	126	Liber and White Sobey (2004)
Chironomus tentans	96-h LC_{50} [c]	33.5	126	Liber and White Sobey (2004)
Tubifex tubifex	24-h EC_{50} [d] 48-h EC_{50} [d] 96-h EC_{50} [d]	8.6 7.9 2	245	Khangarot (1991)

[a] Lowest observable effect concentration
[b] No observable effect concentration
[c] Median lethal concentration
[d] Median effect concentration

growth reductions at U concentrations ranging from 400 μg/L to 550 μg/L. In this study, U toxicity to hydra was shown to be pH-dependent in the presence of carbonate.

Semaan *et al.* (2001) reported no differences in the sensitivity of three populations of the tropical cladoceran, *Moinodaphnia macleayi,* exposed to U. The 48-h EC_{50} (immobilization-lethality) for U ranged between 160 to 390 μg/L. Reproductive impairment was observed for all three populations following exposure to U concentrations of 20 to 49 μg/L in soft water (~ 2 mg/L). The LOEC for reproduction reported by Poston *et al.* (1984) for *Daphnia magna* exposed to U for 5 d was 520 μg/L at a water hardness higher than 60 mg/L. The apparent differences in sensitivity between *D. magna* and *M. macleayi* may be due to the difference in water hardness used in these studies and the genetic differences between the two species (Semaan *et al*, 2001).

Picket *et al.* (1993) derived a chronic value (geometric mean of the NOEC and LOEC) of 3 μg/L when testing the effects of U on reproduction of *Ceriodaphnia dubia* in soft water (~ 4 mg/L as $CaCO_3$). Liber and George (2000) also investigated the toxicity of U to *C. dubia* reporting a 48-h LC_{50} of 430 μg/L at a water hardness of 27 mg/L and a pH of 7.3. In a 7-day chronic toxicity test with a water hardness of 60 mg/L, the NOEC and LOEC values were 10 and 35 μg/L, respectively. Uranium was less toxic at a water hardness of 120 mg/L with an effect concentration (7-d EC_{50}) for reproduction of 218 μg/L.

1.3.3.2. Benthic invertebrates

In aquatic ecosystems, benthic invertebrates are likely among the organisms most highly exposed to U due to the propensity of sediments for accumulating and storing this metal. Sediment-associated contaminants may also have adverse effects on fish that feed on contaminated invertebrates (Environment Canada and Health Canada, 2001; Ribera *et al*, 1996). There are, however, limited data available on the effects of uranium on benthic macroinvertebrates, especially with respect to reproduction, which is one of the most relevant endpoints for ecological risk assessment.

Other work has shown that juvenile *Hyallela azteca* were much more sensitive to U than adults when exposed to U-spiked sediments. BEAK International Inc. (1998) exposed both juvenile and adult amphipods, reporting a 96-h LC_{20} of 15 mg U/kg dw sediment and a 96-h LC_{50} of 57 mg U/kg dw sediment, respectively.

Khangarot (1991) reported 24, 48, and 96-h EC_{50} values for the freshwater worm *Tubifex tubifex* exposed to U of 8.6, 7.9, and 2.1 mg/L, respectively. Liber and White Sobey (2004) calculated aqueous 96-h LC_{50} values for U of 8.2 and 33.5 mg/L for *H. azteca* and *Chironomus tentans*, respectively. In the same study, sediment U toxicity tests yielded LC_{50} values of 2,442 and 10,551 mg U/kg dw sediment for *H. azteca* and *C. tentans,* respectively. The mean inhibition concentration (IC_{50}) for growth was 1,918 and 2,695 mg U/kg dw sediment for *H. azteca* and *C. tentans*, respectively.

In another study, Peck *et al* (2002) reported 72-h LC_{50} values for the tropical chironomid, *Chironomus crassiforceps,* of 36 and 58 mg U/L at pH 6 and 4, respectively. The decrease in the toxicity response in this study may be explained by the increase in H^+ activity, which may compete for uptake with U at biological surfaces.

1.3.3.3. Uranium accumulation in aquatic organisms

Like many other metals, U can be taken up and accumulated to relatively high concentrations by various aquatic organisms, including invertebrates and fish. Waite *et al* (1988) found that the tissues of northern pike (*Exos lucius*) and lake whitefish (*Coregonus clupeaformis*) exposed to U mine effluents (0.5-200 μg U/L) generally contained higher metal concentrations (0.1-0.7 μg U/g) than fish from control areas. Nichols and Scholz (1989) evaluated U uptake in rainbow trout, *Oncorhynchus mykiss,* exposed to U mine effluents (mean U concentration 142-464 μg/L) and found the average fish U concentration ranged from 0.67 to 0.58 μg/g. In a different study, exposure of juvenile brook trout, *Salvelinus fontinalis,* to U concentrations ranging from 0.16 to 10 mg/L displayed mean bioconcentration factors (BCFs) ranging from 1.9 to 4.3 (Parkhrust *et al*, 1984). The BCF values found in this study were very small when compared to the BCFs of other metals in fish (e.g., arsenic, cadmium, lead).

More recently, Peterson *et al.* (2002) reported U concentrations in macroinvertebrates collected immediately downstream from a U mill-tailing

site located in Montezuma Creek, Utah, that were 10 times higher than concentrations in similar invertebrates from reference sites. Hyne *et al.* (1992b) noted that U accumulation might be seen as aggregated crystals in epidermal regions adjacent to nematocysts in *Hydra viridissima*. These regions were reported to be collagenous in nature, and like many other collagens, may have an affinity for U. Another study by Hyne *et al.* (1993) investigated U accumulation in the nematocysts of *H. viridissima* when exposed to U concentrations of 200 and 350 μg/L. The accumulation of U correlated with reduced population growth and feeding dysfunction in the freshwater hydra.

There is no evidence that U biomagnifies through the food web, probably because of its very low rate of uptake through the gut of most organisms (Environment Canada and Health Canada, 2001; Ribera *et al*, 1996). As a result, the concentration of U in upper trophic level organisms is often much lower than in bottom trophic level organisms (Labrot *et al*, 1999; Mahon, 1982). Since many lower trophic level organisms spend more time in contact with sediments they tend to be exposed to higher U concentrations than upper trophic level organisms (Environment Canada and Health Canada, 2001). Mahon (1982) reported high levels of U in plankton and benthos from a U polluted area in central British Columbia. However, the fish species *O. mykiss* and *Catastomus catastomus* sampled from this U impacted area, showed low concentrations of this metal in their tissues. In general, data showed a decrease of at least one order of

magnitude in U BCFs with increasing trophic level. Labrot *et al.* (1999) evaluated U accumulation and BCFs in the clam, *Corbicula fluminea,* and the fish, *Brachydanio rerio.* In this study, low BCFs (less than 1) were found for both species.

1.4. Life cycle toxicity assessment

The tests most commonly utilized to assess the potential toxicity of contaminants focus primarily on survival rather than sublethal endpoints such as reproduction and growth (Benoit *et al*, 1997; Liber *et al*, 1996; Rand and Petrocelli, 1985). However, population or community level impacts due to contaminants often result from chronic, sublethal effects rather than acute toxicity (Benoit *et al*, 1997).

Chronic toxicity tests are designed to measure the effects of toxicants to organisms over a significant portion of the organism's life cycle. They generally provide a more sensitive measure of chemical toxicity than acute tests, because concentrations that produce chronic effects (e.g., growth and reproductive effects) are usually lower than, or at least equal, to those that produce acute effects, such as mortality (Rand and Petrocelli, 1985; Giesy and Graney, 1989). A comparison of the chemical concentration that causes significant deleterious effects in a chronic study (e.g., the LOEC) with the chemical concentration that is expected to occur in the aquatic environment (e.g., expected environmental concentration) permits an evaluation of the potential hazard that the chemical poses to aquatic biota (Rand and

Petrocelli, 1985). Moreover, the reduced number of confounding variables found in laboratory toxicity tests produces less ambiguous results, leading to the demonstration of cause-effect relationships (Hare, 1992; Dickson, 1995).

One of the primary strengths of life cycle tests is that effects on survival, growth and various reproductive endpoints can be measured in a single assay (Benoit *et al*, 1997; Hoffman *et al*, 2003). Nevertheless, effects on survival are rarely the main objective of such a study (Hoffman *et al*, 2003; Rand and Petrocelli, 1985).

The evaluation of a variety of reproductive and developmental endpoints may be important for several reasons in terms of assessing toxicity. Firstly, reproductive output by itself is often very sensitive to contaminant stress. Secondly, examination of reproductive output may provide insights regarding effects observed on growth. Finally, impaired reproductive output can be directly related to changes at the population level (Benoit *et al*, 1997; Giesy and Graney, 1989, Pery *et al*, 2002).

The recurrent problem, however, is the predictive value of such laboratory tests for extrapolation to real world situations. There are uncertainties in extrapolating from laboratory toxicity tests to biological effects in natural environments. However, such potential sources of error are often taken into account by applying safety factors to the data (Crossland, 1992). Safety factors applied to chronic data are usually 10 to 100 times lower than those applied to acute data, since chronic studies always provide more extensive and reliable results (Crossland, 1992). From the results of

such tests it is possible to define potential effect concentrations in the environment with greater accuracy. Some examples of chronic aqueous U toxicity studies include tests with brook trout (*S. fontinalis*) (Parkhrust *et al*, 1984), rainbow trout (*O. mykiss*) (Poston, 1982; Nichols and Scholz, 1989), fathead minnow (*P. promelas*) (Pyle *et al*, 2002), daphnids (*D. magna, C. dubia, M. macleayi*) (Melville, 1992; Semaan *et al*, 2001; Poston *et al*, 1984; Liber and George, 2000), and freshwater hydra (*H. viridissima* and *vulgaris*) (Riethmuller *et al*, 2001; Hyne *et al* 1992a, 1992b).

Although the use of fish in full life cycle tests provides more accurate information on potential toxicant effects, the time and cost associated with such tests are much greater than with acute tests. Life cycle tests with invertebrates are also significantly longer than acute tests, however, the time and cost are much less than for fish life cycle test (Rand, 1995).

1.5. *Chironomus tentans* as a laboratory test organism

The benthic macroinvertebrate, *Chironomus tentans,* has often been used to assess the potential toxicity of contaminated water and sediment (Environment Canada, 1997). Several important factors have contributed to the widespread use of this midge as a test species: it is easy to culture in the laboratory, it normally completes its life cycle in a relative short period of time (Benoit *et al*, 1997; Armitage *et al,* 1995; U.S. EPA, 2000; Environment Canada, 1997), a variety of developmental and reproductive endpoints can be monitored (U.S. EPA, 2000; Benoit *et al*, 1997), it is widely distributed,

and chironomids, in general, are frequently the most abundant insects in freshwater systems (Armitage *et al*, 1995; Benoit *et al*, 1997; Environment Canada, 1997). Therefore, *C. tentans* is ecologically and environmentally relevant (Environment Canada, 1997; Craig *et al*, 1998).

Chironomids are also sensitive to a broad range of contaminants in water and sediments (Environment Canada, 1997; Liber *et al*, 1996; Sibley *et al*, 1997; Craig *et al*, 1998). In comparative chronic sediment and water-only tests using a number of species of freshwater invertebrates, the sensitivity of third or fourth instar chironomid larvae was ranked low. However, comparative chronic tests using first or second instar midge larvae have shown that early-instar chironomids are often more sensitive than daphnids and amphipods (Environment Canada, 1997; Cairns *et al*, 1984; Ingersoll *et al*, 1990; Taylor *et al*, 1991; Phipps *et al*, 1995).

It can also be important to use a test organism that eventually emerge from the aquatic environment, since the transport of contaminants out of the aquatic system may have harmful effects on terrestrial, insectivorous predators (Timmermans and Walker, 1989). *Chironomus tentans* is a holometabolous insect with two aquatic immature stages (larvae and pupae) and a short terrestrial adult stage. Therefore, transport of contaminants from the aquatic to the terrestrial environment may occur after emergence (Timmermans and Walker, 1989, Menzie, 1980).

1.6. Research goal and objectives

1.6.1. Goal

The goal of this work was to evaluate the toxicity and bioaccumulation of uranium over the life cycle of the aquatic invertebrate, *Chironomus tentans*. Since there are gaps in the assessment of risk for aquatic species, especially invertebrates, it is unclear what uranium concentrations in water and sediments can affect growth, reproduction and development in this representative macroinvertebrate.

1.6.2. Objectives

The specific objectives of this research were:

I. To evaluate U toxicity over the life cycle of the aquatic invertebrate *C. tentans* via aqueous exposure. This included assessment of:

   (i) uranium effects on *C. tentans* growth and reproduction;

   (ii) effects of parental U exposure on the F_1 generation larvae; and

   (iii) uranium accumulation in *C. tentans* tissues at all major life stages.

Null Hypothesis: Uranium does not affect growth and reproduction in *C. tentans.*

II. To determine differences in U accumulation in *C. tentans* larvae organs/tissues using x-ray microprobe analysis.

Null Hypothesis: There is no distinct pattern in uranium accumulation in *C. tentans* larvae.

III. To evaluate the rates of U uptake and depuration, and to determine the possible mechanism of U uptake, in *C. tentans* larvae.

Null Hypothesis: There is no U uptake in *C. tentans* larvae.

Chapter 2. Bioaccumulation and chronic toxicity of uranium at different life stages of the aquatic invertebrate *Chironomus tentans.*

2.1. Introduction

Uranium (U) exploration, mining and milling activities have been extensive in the subarctic Precambrian Shield area of northern Saskatchewan. Effluents from U mines generally contain elevated concentrations of metals (e.g., U, molybdenum, nickel, arsenic, and copper), which occasionally result in toxic concentrations in the aquatic receiving environment (Environment Canada and Health Canada, 2001; Liber and White Sobey, 2004). These activities can therefore cause substantial changes in both water quality and aquatic communities downstream of U mines (Melville, 1992).

The background median aqueous U concentration upstream of U mines in northern Saskatchewan, is in the range of 0.05 to 0.35 μg/L and the median sediment concentration is in the range of 3.7 to 29.5 mg/kg dry weight (dw) (Environment Canada and Health Canada, 2001). However, downstream of U mines the U concentration in surface water can range from 100 to approximately 500 μg/L (Environment Canada and Health Canada, 2001; Melville, 1995; Hynes *et al*, 1987) and the U concentration in sediment is often more than 1000 mg/kg dw (Environment Canada and Health Canada,

2001; Hynes *et al*, 1987; Cooley and Klaverkamp, 2000). The higher U concentrations found in water and sediment in downstream areas indicate that these are important areas of potential impact of U released from mines and mills to the aquatic environment.

Uranyl species of importance under environmental conditions include the dissolved uranyl ion (UO_2^{+2}), the free hydrated ion (UO_2OH^+), and carbonate complexes (e.g., UO_2CO_3) (Poston *et al*, 1984; Environment Canada and Health Canada, 2001). Uranium toxicity is, however, primarily governed by the activity of the uranyl ion and its hydrated complexes (Environment Canada and Health Canada, 2001). The complexation of the uranyl ion with carbonate ions in neutral to alkaline waters explains the high mobility of U in many freshwater systems. This propensity of U to solibilize and be transported poses a potential hazard to aquatic organisms exposed to effluents from U mines (Poston *et al*, 1984).

Short-term aqueous uranium toxicity has been investigated in a number of aquatic invertebrates, including crustaceans, worms, hydra, insects and bivalves. In most cases, reported toxicity endpoints, such as the median effects concentration (EC_{50}) and the no-observed-effect-concentration/lowest-observed-effect-concentration (NOEC/LOEC), are in the range of 0.2 to 10 mg/L (Liber and White Sobey, 2004). Benthic invertebrates are possibly among the most highly exposed organisms due to the capacity of sediments for accumulating U. However, limited data are available on the effects of uranium exposure on benthic macroinvertebrates, especially regarding reproduction,

which is considered one of the most relevant endpoints for ecological risk assessment.

The midge, *Chironomus tentans,* is a good candidate for assessing the toxicity of U to benthic macroinvertebrates, as well as for chronic toxicity testing because it normally completes its life cycle in a relatively short period of time, and a variety of developmental and reproductive endpoints can be easily monitored (Sibley *et al*, 1997). In addition, comparative chronic sediment and water-only tests using a number of species of freshwater invertebrates have shown that early-instar chironomids, are often more sensitive to contaminants than daphnids or amphipods (Environment Canada, 1997; Cairns *et al*, 1984; Ingersoll *et al*, 1990; Taylor *et al*, 1991; Phipps *et al*, 1995).

Since uranium is a non-essential metal for biological processes, concerns for the protection of aquatic biota exposed to U contaminated waters must be considered (Semaan *et al*, 2001). Moreover, there is a priority need to develop guidelines or objectives for acceptable U levels in Canadian waters for the protection of aquatic organisms. The primary objective of this study was to determine bioaccumulation and long-term effects of chronic U exposure at difference life stages of *C. tentans,* as well as the significance of gut clearance on U bioaccumulation. This information could be used to help develop a U water quality guideline or objective for the protection of aquatic life.

2.2. Materials and methods

2.2.1. Test organism

2.2.1.1. Biology of *Chironomus tentans*

Chironomus tentans lives in tubes on or in the substrate in depositional habitats, and has four distinct life stages: egg, larvae, pupae, and adult. The invertebrate completes metamorphosis in approximately 23-30 d at 23 °C under laboratory conditions. At this temperature, eggs begin to hatch within 2 d of oviposition and can require up to 6 d to complete the hatch (Benoit *et al*, 1997).

Hatched larvae remain with the egg case for approximately 24 h and appear to use the gelatinous component of the egg mass as an initial source of food. The larvae pass through four instars. Towards the end of the fourth instar, approximately 23 d after hatch, the larvae cease feeding and pupation commences (Benoit *et al*, 1997). The pupal stage generally lasts less than 1 d. Emergence usually exhibits a bimodal pattern with the peak of male emergence occurring before female emergence. Females will generally produce a single egg mass within 24 h of mating. Males and females die within 7 d of emergence (Benoit *et al*, 1997).

2.2.1.2. Culturing procedures

Chironomus tentans were obtained from a laboratory culture maintained at the Toxicology Centre, University of Saskatchewan, Saskatoon, SK. Organisms were cultured in a Coldmatic Model W.I.C. environmental chamber with a set photoperiod of 16:8 h light:dark and a temperature of 23 ± 1 °C. The culture

was maintained using performance-based techniques according to culturing protocols outlined by Environment Canada (1997). Organisms were fed three times per week with 10 ml of 6 mg/L Tetramin® fish food slurry.

The culture water was dechlorinated, carbon-filtered, Saskatoon municipal water. Prior to use, the water was aerated for a minimum of 24 h in a 50-L Nalgene® carboy. Water quality variables (mean ± SD) were as follows: temperature 22.6 ± 0.7 °C; dissolved oxygen (DO) 7.4 ± 0.5 mg/L; pH 8.2 ± 0.2; hardness 131.5 ± 9.9 mg/L as $CaCO_3$; and alkalinity 79.12 ± 14.58 mg/L as $CaCO_3$.

2.2.1.3. Acquisition of test organisms

To obtain *C. tentans* larvae for the life cycle and gut clearance tests, fully emerged adults were removed from the main colony by aspirating them into a 500-ml Erlenmeyer flask. After adults were collected, the flask was inverted over a glass jar containing a plastic platform partially submerged in culture water and plastic nets to provide mating surfaces (breeding chamber). Each morning, breeding chambers were inspected for new egg masses.

New egg masses were individually transferred to large surface area tanks containing aerated culture water and a thin layer of silica sand with a particle size of 250-425 μm. Feeding started 48 h after the egg masses were placed in the tanks. Animals were fed three times per week with 2.5 ml of Tetramin® fish food slurry with a concentration of 6 mg/L. After 8 to 10 d, second instar larvae,

were transferred to a glass pan containing culture water where larvae were gently teased out of their sand cases for use in the tests.

2.2.2. Experimental procedures

Tests were conducted in a controlled environment chamber set at 23 ± 1 °C with a photoperiod of 16:8 light:dark and an illumination intensity of 800 -1300 lux. Uranium stock solutions, as $UO_2(NO_3)_2 \cdot 6H_2O$ (Strem Chemicals Inc., MA, USA), with a concentration of 100 mg/L were prepared with deionized water (Milli-Q®). To generate the desired U tests concentrations, the stock solution was diluted using test water. The test water used was carbon-filtered Saskatoon municipal water, the same as that used for culturing the organisms.

The tests were static-renewal tests performed in 250-ml glass beakers. Each beaker contained test water and a thin layer of sand (particle size 250-425 μm) to allow case building. Gentle aeration was not initiated until 2 h after the larvae were placed in the test beakers to allow larvae time to settle into the sand. Test solutions were replaced every 2 d with new solutions to maintain adequate water quality and constant U concentrations. Before the water was changed, water quality variables such as pH, DO, alkalinity, hardness, ammonia, and temperature were measured in three replicate beakers per treatment. Water samples for measurement of U exposure during the life cycle test were taken from three beakers at each treatment every 4 d, prior to water renewal. Previous work in this laboratory has clearly shown that U concentration in test water does not change over a 2 to 3 d water renewal

period (Liber *et al*, 2004 unpublished data; Liber and George, 2000). Thus, U concentration in new water was only measured at the beginning of the study.

Test organisms were fed daily using 1 ml of a Tetramin® fish food slurry at 6 mg/L. Food was added after sampling of animals to ensure that organisms with relatively empty guts were sampled (Timmermans *et al*, 1992).

2.2.3. Life cycle test

To evaluate chronic U effects on *C. tentans,* animals were exposed to three different U concentrations (40, 200 and 1000 μg/L) and an untreated control. Each treatment had ten replicates with ten *C. tentans* larvae per replicate. Animals dying at the beginning of the experiment due to stress of handling were replaced after 4 h of being placed in the test beakers. A total of seven *C. tentans* larvae were replaced for this test.

After 10 d of exposure larval survival, growth (as dry weight), and U tissue concentration were evaluated in three randomly selected beakers from each treatment. The rest of the beakers (seven per treatment) continued with the U exposure. When animals reached the pupal stage they were collected from two randomly selected beakers from each treatment for determination of dry weight and U tissue concentrations.

On day 18, emergence traps, constructed using a transparent plastic sheet tube with a mesh-screen (140 μm diameter) covering one end, were placed with the screen facing up over the five remaining beakers in each treatment. The traps fitted tightly with the rim of the 250-ml beakers to prevent gaps and consequently the loss of adults. Daily records of adult emergence were

maintained and the exuvia remaining after emergence were collected for U measurement and dry weight determination. Two replicates from each treatment were used to evaluate adult (sex specific) U tissue concentrations and adult dry weight. As well, "mating success" of the *C. tentans* adults exposed to U during their immature stages was evaluated. Adults collected from the three remaining beakers per treatment (exposed females and males) were mated with unexposed adults from the main culture colony (unexposed males and females) in a breeding chamber using a 1:5 (Female:Male) sex ratio.

Breeding chambers were checked every day and new egg masses collected and transferred to plastic petri dishes to determine the number of eggs using the ring count method (Benoit *et al*, 1997). After counting the number of eggs, the egg masses were transferred individually to jars with clean dechlorinated water, aeration, and a layer of sand (250-425 μm) to evaluate hatching success. Three days later, the egg masses were removed from the jars to count the number of unhatched eggs and, subsequently, carefully returned to the jars using a plastic pipette. Hatching success of *C. tentans* larvae was evaluated by subtracting the number of unhatched eggs remaining in the gelatinous egg mass after the 3-d period, from the original estimated number of eggs (Benoit *et al*, 1997). After 10 d, larvae dry weight and U tissue concentration were evaluated.

Chironomus tentans larvae are know to settle down in the sediment shortly after leaving the egg mass. Therefore, to prevent disturbance and loses of early instar larvae, only half of the water in the jars was replaced with new culture

water every 2 d (aereated for a period of 12 h). The animals were fed daily after hatching with 1 ml of Tetramin® fish food slurry. The life cycle test was terminated when no further emergence was recorded over a period of 7 d.

2.2.4. Gut clearance test

During the gut clearance test, animals were exposed to two U concentrations (200 and 1000 μg/L) plus a control. Each treatment had six replicates with ten *C. tentans* larvae per replicate. After 10 d of exposure, larval survival, growth (as dry weight) and U tissue concentration were evaluated in three randomly selected beakers from each treatment. The *C. tentans* larvae in the remaining beakers were gently teased out of their sand tubes and transferred to beakers containing clean water (culture water) and a thin layer of sand (250-425 μm). Subsequently, the animals were fed 1 ml of Tetramin® fish food slurry to stimulate gut clearance. Four hours later, the water and sand were changed to prevent coprophagy. Five hours after that, larval dry weight and U tissue concentration were evaluated (Groenendijk *et al*, 1999; Hare *et al*, 1989).

2.2.5. Sampling procedures and processing

2.2.5.1. Test materials

All sampling materials were pre-cleaned before use. Eight-ml Nalgene® bottles used for animal and water samples for U analysis were rinsed with nitric acid (1N) and then thoroughly rinsed with deionized water (Milli-Q®). Plastic

vials used for water quality samples were washed with labtone soap for at least 12 h and then rinsed with deionized water (Milli-Q®).

2.2.5.2. Animal samples

To evaluate the dry weight of *C. tentans* larvae, pupae, and exuvia, samples were collected and dried for 24 h at 60 °C. Dry weight was measured to 0.01 mg using a Sartorious scale model BP211D (Sartoriuos North America Inc., Edgewood, NY, USA). Adults *C. tentans* were immobilized by exposing them to 4 °C for at least 30 min before drying.

2.2.5.3. Water samples

Water temperature and DO levels in test beakers were measured with an ORION® dissolved oxygen meter model 835 (ORION Research, Beverly, MA, USA); pH was measured with an ORION® PerpHect LogR meter model 370. Water samples for water quality measurements were removed from the test beakers with a sterilized 20-ml plastic syringe and placed in pre-cleaned plastic vials (~20 ml). Water hardness and alkalinity were evaluated with a Hach Digital Titrator model 16900 (Hach Company, Loveland, CO, USA). Ammonia was measured with an ORION® Aquafast II Ammonia Photometer.

2.2.5.4. Uranium samples

2.2.5.4.1. Water samples

Water samples for U analysis were removed from test beakers with a 20-ml plastic syringe and placed in pre-cleaned 8-ml Nalgene® bottles following filtration through a 0.45 μm Nalgene® filter. Samples were acidified with double distilled (ultra-pure) nitric acid (1N) to pH 2 and stored at 4 °C until they were analyzed. Elemental analysis was performed in the Department of Geological Sciences at the University of Saskatchewan using Inductively Coupled Plasma-Mass Spectrometry (ICP-MS) with a detection limit within 0.04 to 0.1 μg U/L, based on three determinations of blanks each time samples were measured.

2.2.5.4.2. Tissue samples

In order to evaluate U accumulation in *C. tentans*, metal accumulation was measured at all major life stages. Larvae, pupae and exuvia were washed with Milli-Q water for 10 min, then left in an EDTA (1M) solution for 15 min, and finally rinsed thoroughly with Milli-Q water to eliminate any U bound to the surface of the insect.

Chironomus tentans adults used the exuvia as a platform to emerge, but they were not in full contact with the U solution. Thus, adult samples were only rinsed with EDTA (1M) and then with Milli-Q water to eliminate any U that could have bound to their surface during emergence. Following dry weight evaluation, animal samples were transferred to pre-cleaned 8-ml Nalgene® bottles and stored at 4 °C until analyzed. Whole animal U concentrations were measured in

the Department of Geological Sciences at the University of Saskatchewan by ICP-MS following tissue digestion.

2.2.6. Statistical analysis

Statistical analyses were performed using the computer program SigmaStat®, version 2.03 (SPSS Inc., Chicago, IL, USA) with a 95% (α= 0.05) level of confidence. Significant differences among treatments in survival, dry weight and U tissue concentrations of larvae and exuvia were assessed using one-way ANOVA. Pupae dry weight and U tissue concentration failed the test and the transformations for homogeneity of variances so Kruskal-Wallis one-way ANOVAs on ranks were used to evaluate differences among treatments; multiple pairwise comparisons were evaluated by Dunn's test.

Comparison between adult males and females for variables such as dry weight, emergence, U tissue concentration, egg production and hatching success were determined using two-way ANOVA, with sex and U exposure concentration as the two factors. Significant differences in the time to emergence were evaluated using one-way repeated measures ANOVA. Dry weight and U tissue concentration of F_1 generation larvae were evaluated using a two-way ANOVA, with parental sex and parental U exposure concentration as the two factors. Differences in the U tissue concentration among *C. tentans* life stages were evaluated by one-way ANOVA. Adult and exuvia data failed tests for normality so data were transformed using the $\log_{(10)}$ transformation. Comparison between growth of F_0 and F_1 generation 10-d old larvae was

performed by using a parallelisms test (Statistical® software, version 4.2, StatSoft Inc., Tulsa, OK, USA). For all parametric ANOVA, individual comparison of means was conducted using Dunnett's test, except for U tissue concentration within *C. tentans* life stages were Tukey's Post Hoc test was used.

Larvae dry weight during the gut clearance test was evaluated using separate *t*-tests for each U exposure concentration. Uranium tissue concentration was evaluated using two-way ANOVA with gut (empty or non-empty) and U treatment as the two factors.

2.3. Results

2.3.1. Life cycle test

Water quality and U exposure concentrations data are presented in Table 2.1. There were only negligible differences among U treatments in water quality variables such as pH, hardness, alkalinity, temperature, dissolved oxygen, and ammonia throughout the test. Generally, measured U concentrations over the duration of the test were in reasonable agreement with nominal concentrations, although mean measured concentrations were generally slightly lower than nominal. A mean exposure value for U was also calculated separately for the first 10 d of the study to be used together with dry weight and U bioaccumulation measurements for larvae after 10 d of exposure. The small traces of U measured in control water were due to naturally occurring U in Saskatoon municipal water.

No U effects on survival (p = 0.23) were observed in *C. tentans* immature and adult stages throughout the experiment. Survival was greater than 80 % at all of the U concentrations tested.

2.3.1.1. Larvae

An increase in the U exposure concentration resulted in a significant reduction in the dry weight of larvae. Larval dry weight showed a significant decline at mean U concentrations ≥ 157 μg/L ($p < 0.05$), with larval growth retardations relative to the control of 30 to 40% (Figure 2.1). Furthermore, mean 10-d growth of *C. tentans* larvae displayed a strong inverse relationship with measured U concentration (r^2 = 0.96; Figure 2.1). It is recognized that this regression is a practical over-simplification of the true relationship where growth will level off below some critical lowest effect threshold and disappear above a different highest effect threshold.

An increase in U concentration in *C. tentans* larvae was observed at all U treatments, however, significant differences were found only at U exposure concentrations ≥ 157 μg/L ($p < 0.05$; Figure 2.2). Uranium concentrations in *C. tentans* increased with increasing level of U exposure and was best described by the equation Y = 0.06X + 0.39 (r^2 = 0.99). The calculated bioaccumulation factor (BAF) for U in the exposed larvae was 67 ± 20 (Table 2.2). The relationship between BAF and U water concentration expressed on a log-log scale showed a gentle linear decrease (r^2 = 0.79; Figure 2.3), with a slope of – 0.13 (p = 0.30).

Table 2.1. Measurements of water quality at each treatment of the life cycle test. Data represents mean values ± SD (n = 3).

Variable	Unit	DL[e]	Control	40 µg/L	200 µg/L	1000 µg/L
U (10 d)[a]	µg/L	0.04 - 0.1	0.29 ± 0.02	39 ± 8	157 ± 25	835 ± 38
U (entire test)[b]	µg/L	0.04 - 0.1	0.39 ± 0.15	31 ± 9	175 ± 25	856 ± 87
pH	pH	0.05	7.75 ± 0.17	7.80 ± 0.22	7.81 ± 0.25	7.83 ± 0.18
Total-Hardness	mg/L[c]	10	135 ± 5	136 ± 5	133 ± 5	132 ± 4
Alkalinity	mg/L[c]	10	66 ± 8	65 ± 6	66 ± 5	65 ± 5
DO[d]	mg/L	0.1	7.3 ± 0.3	7.1 ± 0.3	7.2 ± 0.3	7.3 ± 0.3
Temperature	°C	0.1	22.9 ± 0.4	23.1 ± 0.7	23.1 ± 0.7	23.1 ± 0.7
Ammonia	mg/L	0.005	0.73 ± 0.49	0.20 ± 0.11	0.10 ± 0.04	0.48 ± 0.43

[a] Mean U concentration calculated for the first 10 d of exposure.
[b] Mean U concentration measured over the entire test.
[c] mg/L as $CaCO_3$.
[d] Dissolved Oxygen.
[e] Analytical Detection Limit.

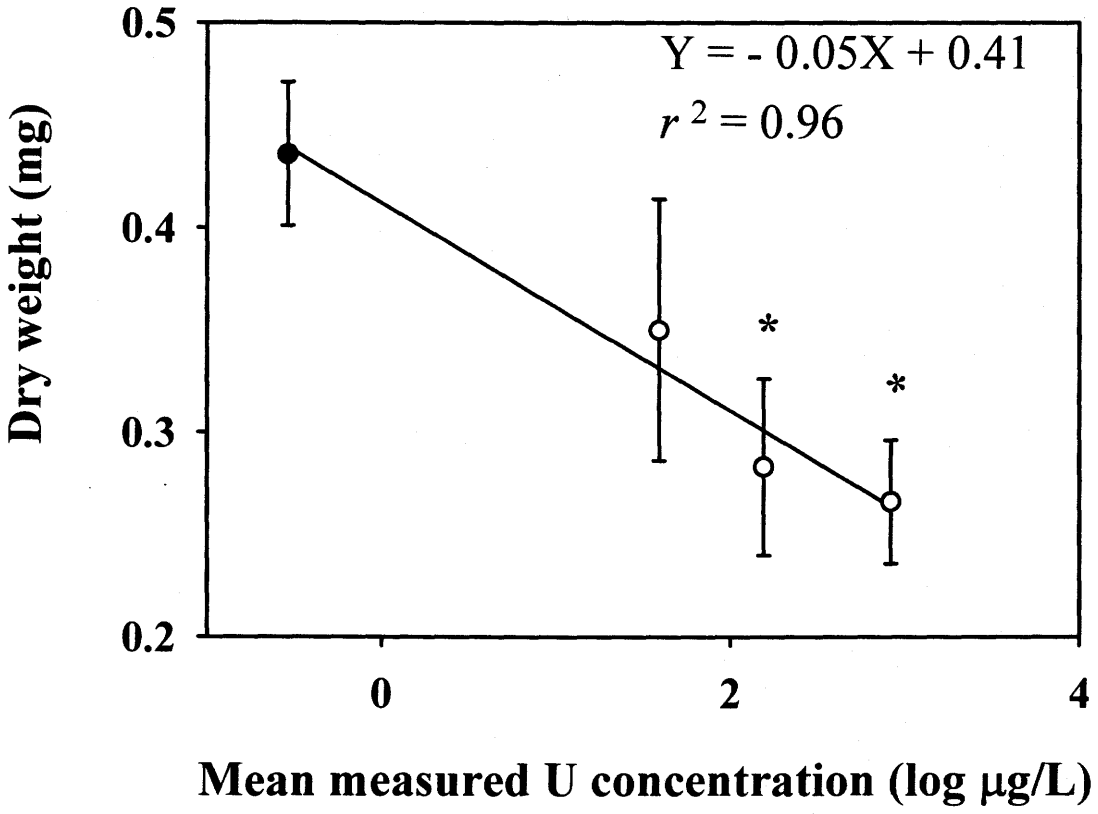

Figure 2.1. Relationship between measured uranium concentration and body weight (as dry weight) of 10-d old *Chironomus tentans* larvae. Each point represents the mean ± SD of three replicate samples. * Significantly different from control (solid symbol; $p < 0.05$).

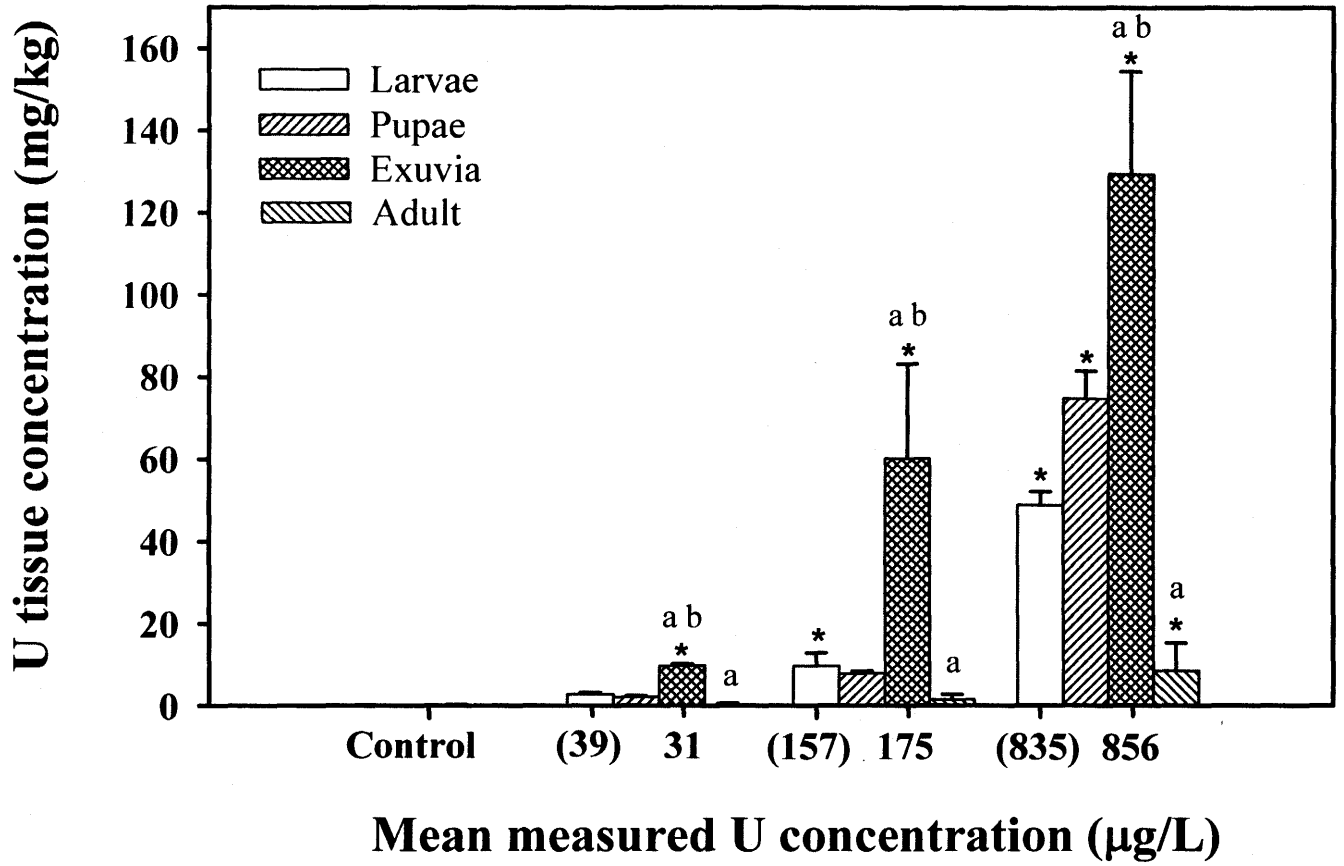

Figure 2.2. Uranium (U) tissue concentration (± SD) at different *Chironomus tentans* life stages. A mean exposure value was calculated separately for larvae during the first 10 d of the study (in parenthesis). * Significantly different from the comparable control life stage ($p < 0.05$).
a = Significantly different ($p < 0.05$) from larvae and pupae life stages within individual U treatments. b = Significantly different from the adult stage within individual U treatments.

Table 2.2. Bioaccumulation factor (BAF) for different *Chironomus tentans* life stages after continuous uranium (U) exposure. Values (mean ± SD) are calculated as the ratio between the mean U concentration in the animal's body and the mean measured U concentration in the exposure solution.

Life stage	BAF
Larvae	67 ± 20
Pupae	65 ± 21
Exuvia	264 ± 114
Adult	10 ± 3

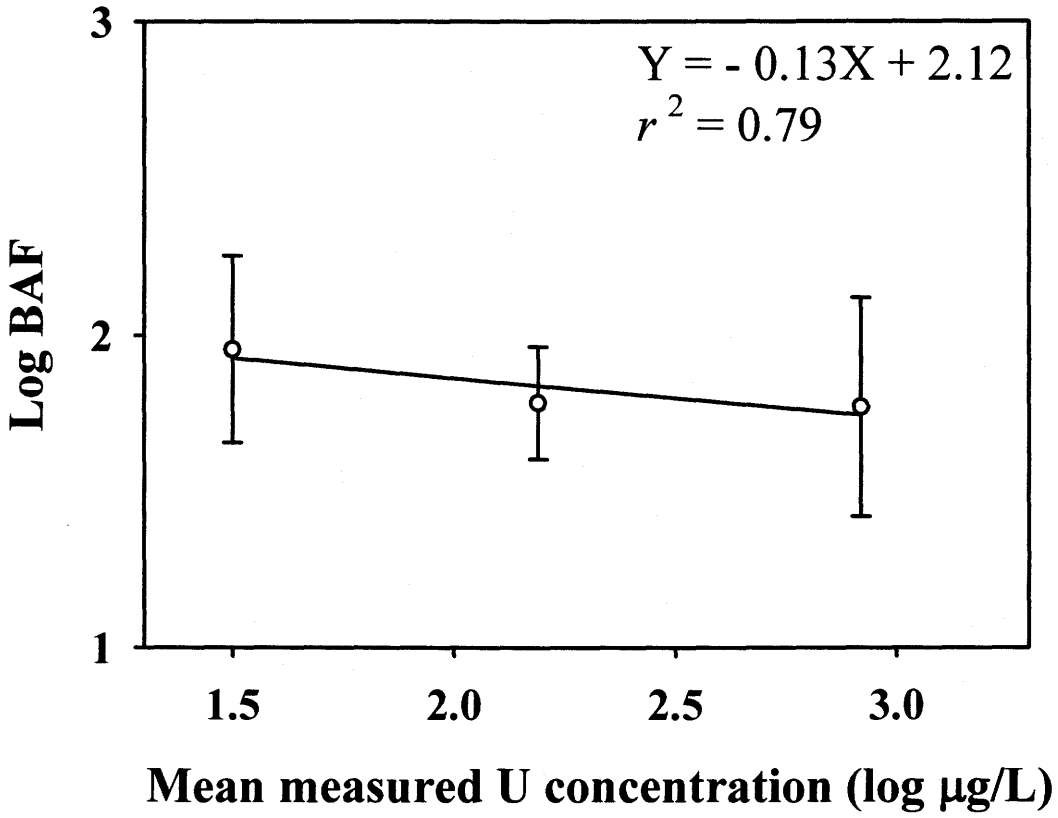

Figure 2.3. Uranium (U) bioaccumulation factors (BAF) for 10-d old *Chironomus tentans* larvae as a function of the U concentration in the water. Each point represents the mean ± SD of three replicate samples.

2.3.1.2. Pupae and exuvia

No effects were observed in pupae and exuvia dry weight at all U exposure concentrations (p = 0.21 and 0.20, respectively). However, pupae and exuvia dry weights appeared to decrease slightly with increasing U concentrations (Figure 2.4). Significant differences in the U tissue concentration of *C tentans* pupae were found only at the highest mean U exposure concentration, 856 μg/L ($p < 0.05$; Figure 2.2). The relationship between U tissue concentration and U exposure concentration in the pupae was described by the equation $Y = 0.09X - 2.44$ ($r^2 = 0.99$).

There was a steady increase in the amount of U in *C. tentans* exuvia with increasing U exposure, which resulted in significant differences from the control at all U exposure concentrations ($p < 0.05$; Figure 2.2). The relationship was best described by the equation $Y = 0.14X + 12.12$ ($r^2 = 0.89$). The BAF for the exuvia was four-fold higher than for pupae (Table 2.2).

2.3.1.3. Adults

2.3.1.3.1. Growth and emergence

No significant differences were found in either male or female dry weights among treatments (p = 0.18). However, the dry weight of adult females was significantly greater than that of males for all evaluated U concentrations ($p < 0.05$; Figure 2.5).

There was a strong relationship between the mean number of emerging adults and the level of U exposure. The number of successfully emerged adults

declined significantly with increasing level of U exposure ($p < 0.05$; Figure 2.6). It is recognized that this straight-linear relationship was chosen only for simplicity as an over-simplification of the respective quadratic relationship. The percent emergence reduction relative to control was 8%, 42%, and 58% for mean measured U concentrations of 31, 175, and 856 μg/L, respectively.

Time to emergence relative to the day of larval hatching was also significantly affected by U exposure ($p < 0.001$). The time to emergence increased with increasing level of U exposure, with control animals emerging approximately 10 d before (day 26) than all exposed animals (day 36; Figure 2.7).

The sex ratio of adult *C. tentans* was close to unity at most U exposure concentrations (Figure 2.8). Although a skewed sex ratio favoring males was observed in the 175 μg/L treatment group, there was not a trend towards a higher proportion of males with increasing U concentrations ($p = 0.065$).

Adult female *C. tentans* dry weight showed a decline (not statistically significant) of approximately 20 to 30% at U concentrations ≥ 175 μg/L; corresponding 10-d larval growth reductions at these U concentrations were 30 to 40% (Figure 2.9). Data also suggested that a decrease in larval growth, after 10 d of exposure, correspond to a decrease in adult emergence (Figure 2.10). The relationship between 10-d larval growth (as dry weight) and adult emergence success was best described by the equation $Y = 1.45X - 38.8$ ($r^2 = 0.88$).

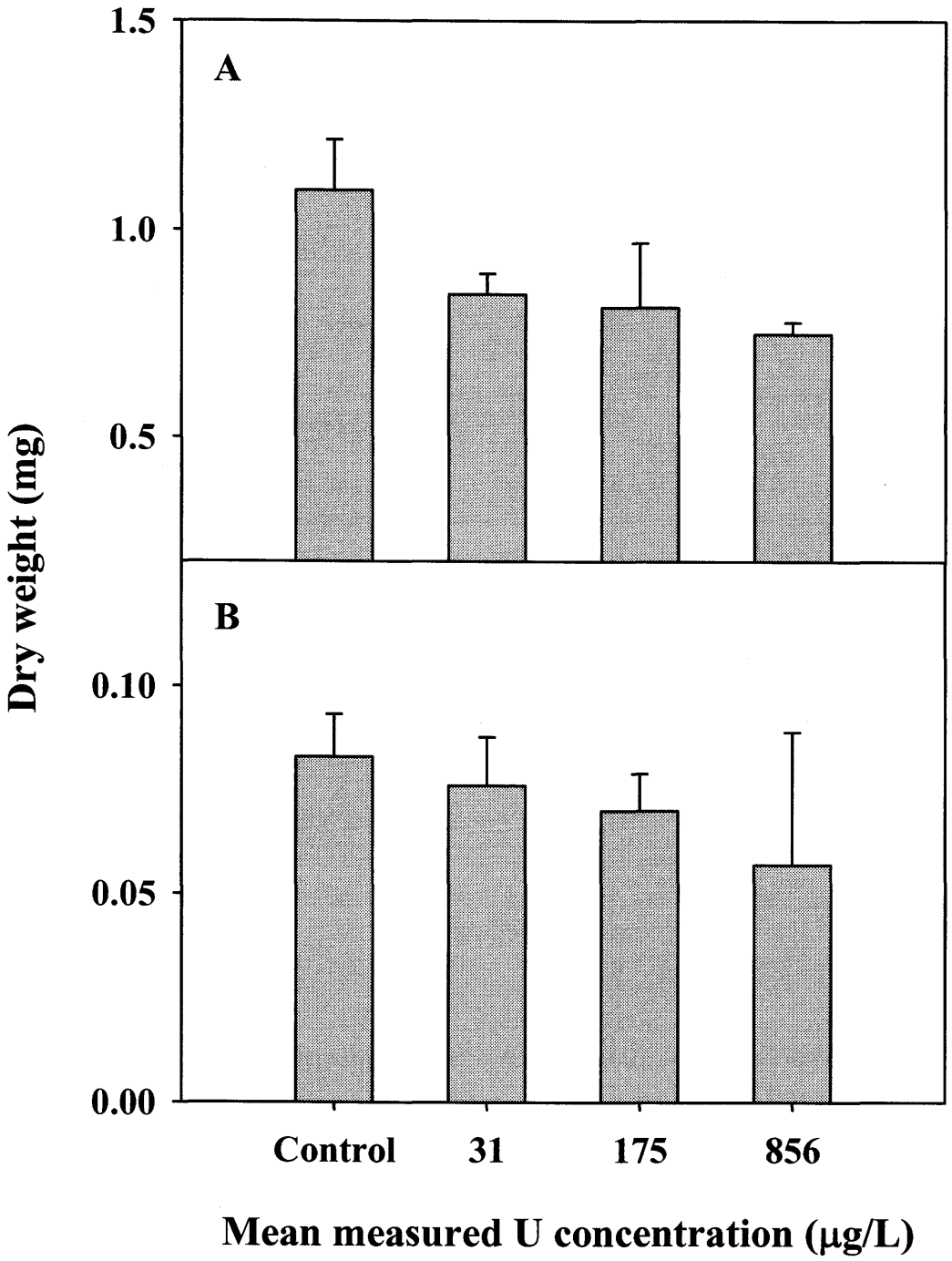

Figure 2.4. Dry weights of *Chironomus tentans* pupae (A) and exuvia (B). Data represent the mean ± SD of two and five replicates for pupae and exuvia, respectively.

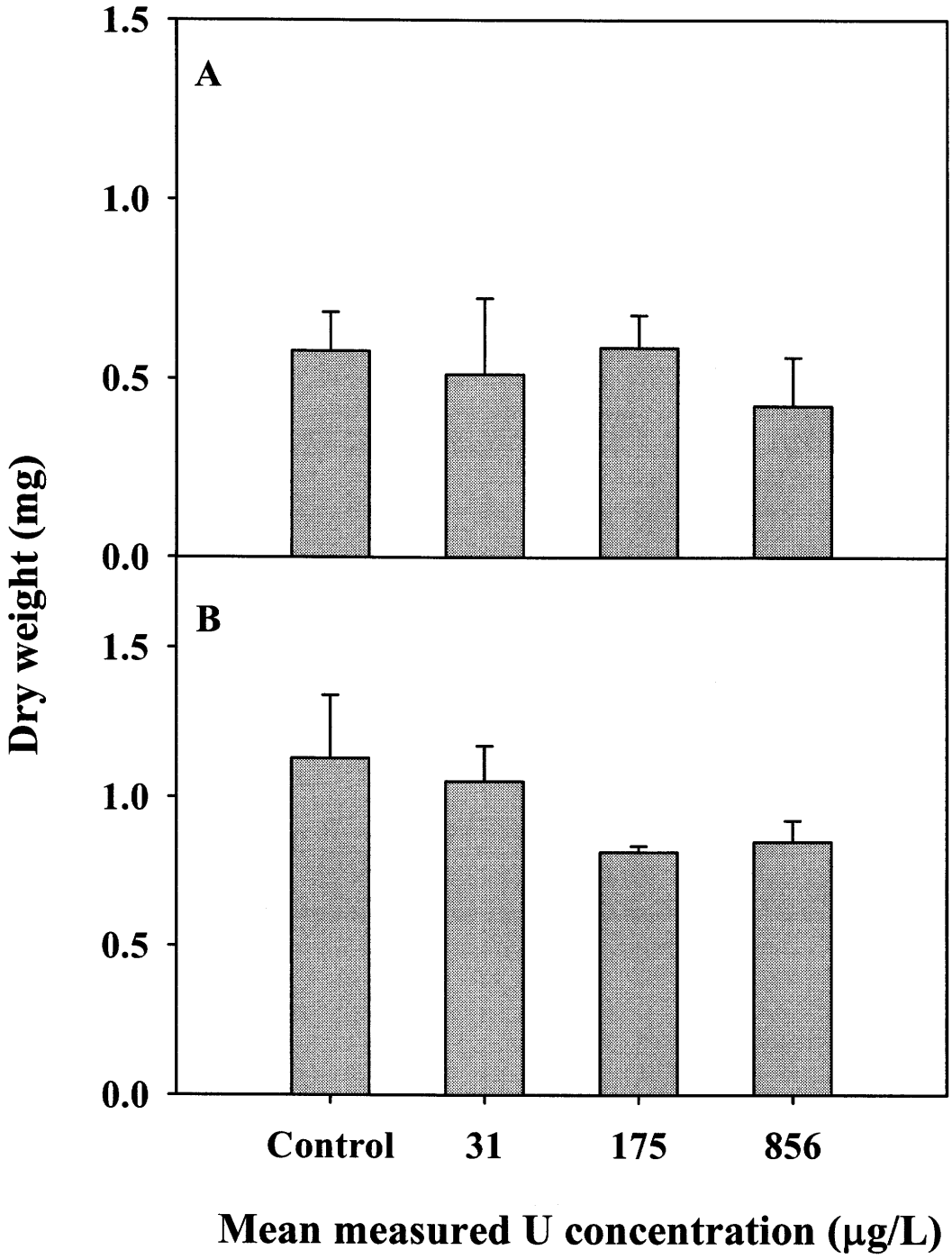

Figure 2.5. Dry weight of male (A) and female (B) adult *Chironomus tentans* emerging from different uranium treatments. Each bar is the mean (± SD) of two replicate samples.

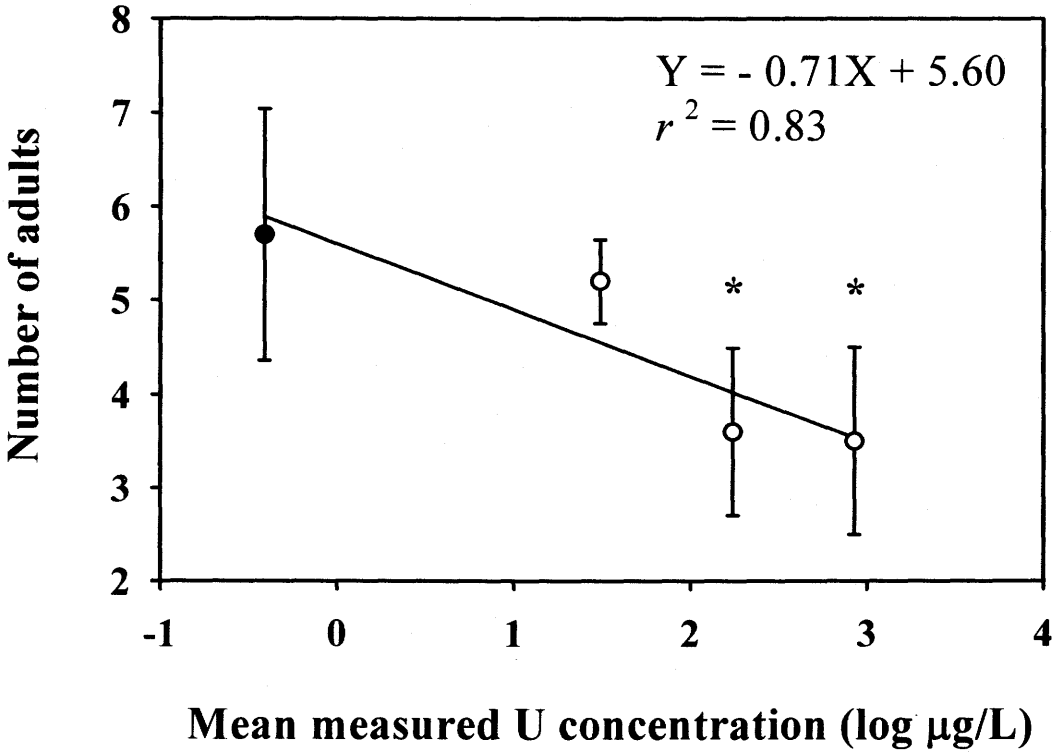

Figure 2.6. Number of *Chironomus tentans* adults that successfully emerged from each beaker of 10 larvae at the different uranium treatments. Each point represents the mean ± SD of five replicate samples per treatment.
* Significantly different from control (solid symbol; $p < 0.05$).

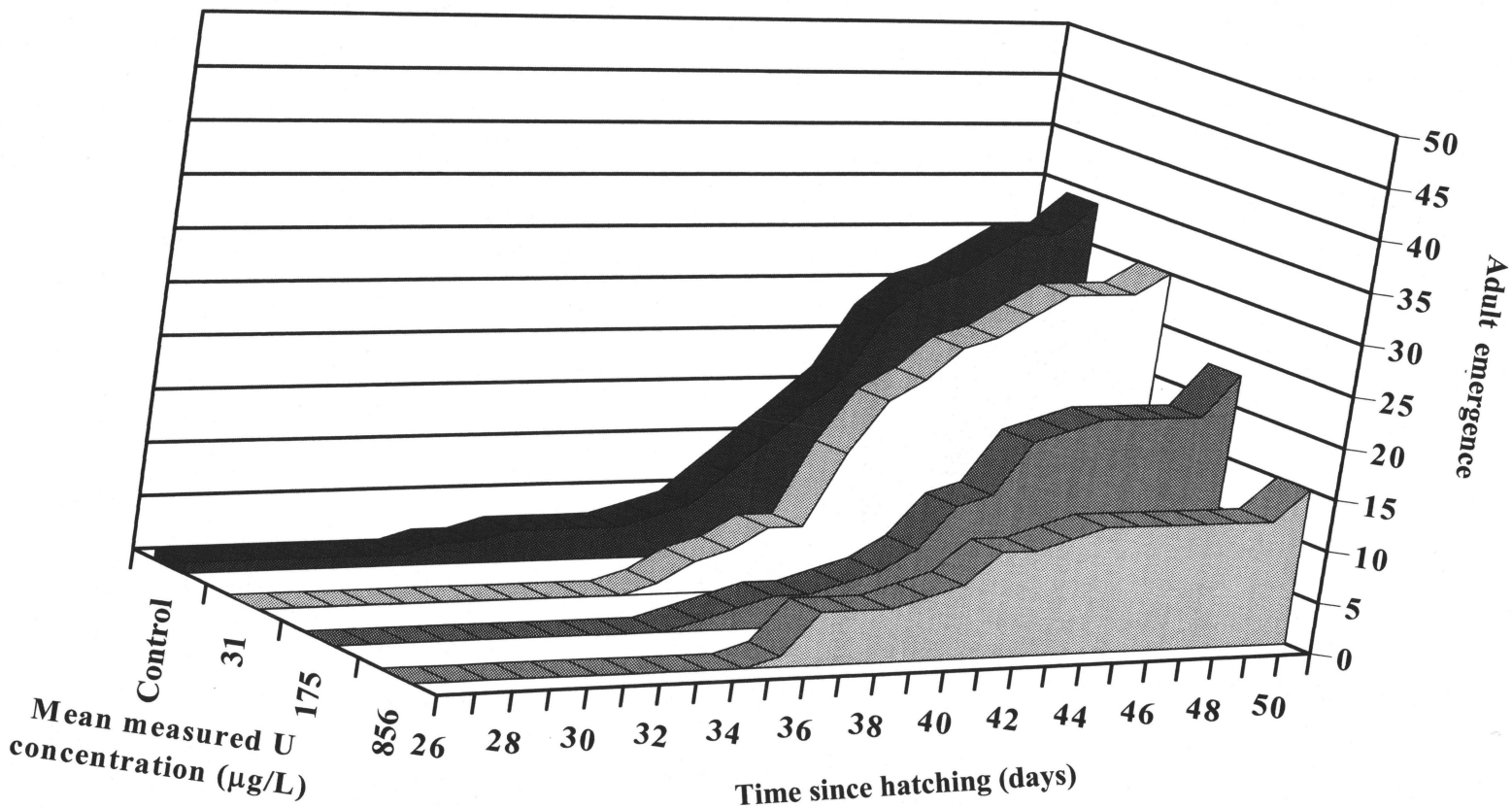

Figure 2.7. Cumulative successfully emerged adults and time to emergence measured relative to the day of hatching for 50 *Chironomus tentans* larvae at the evaluated uranium concentrations.

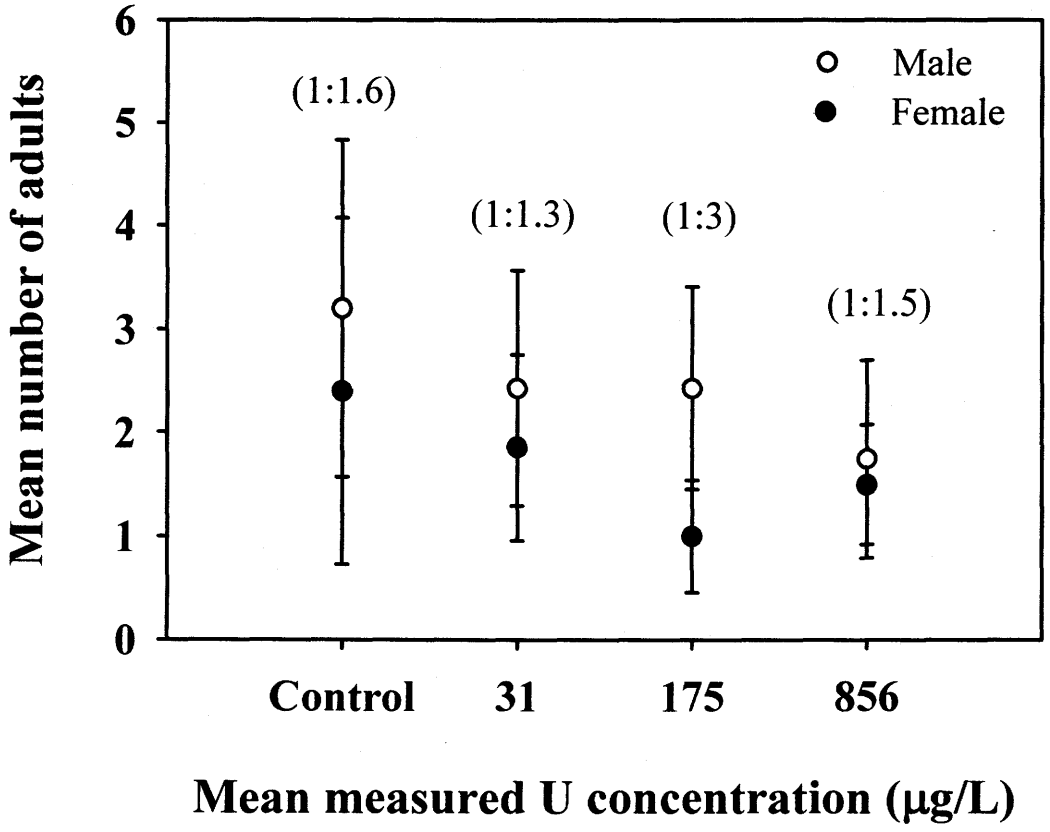

Figure 2.8. Number of female and male *Chironomus tentans* at evaluated uranium test concentrations. Points represent mean ± SD of five replicate samples. Female:Male ratios are shown in parenthesis.

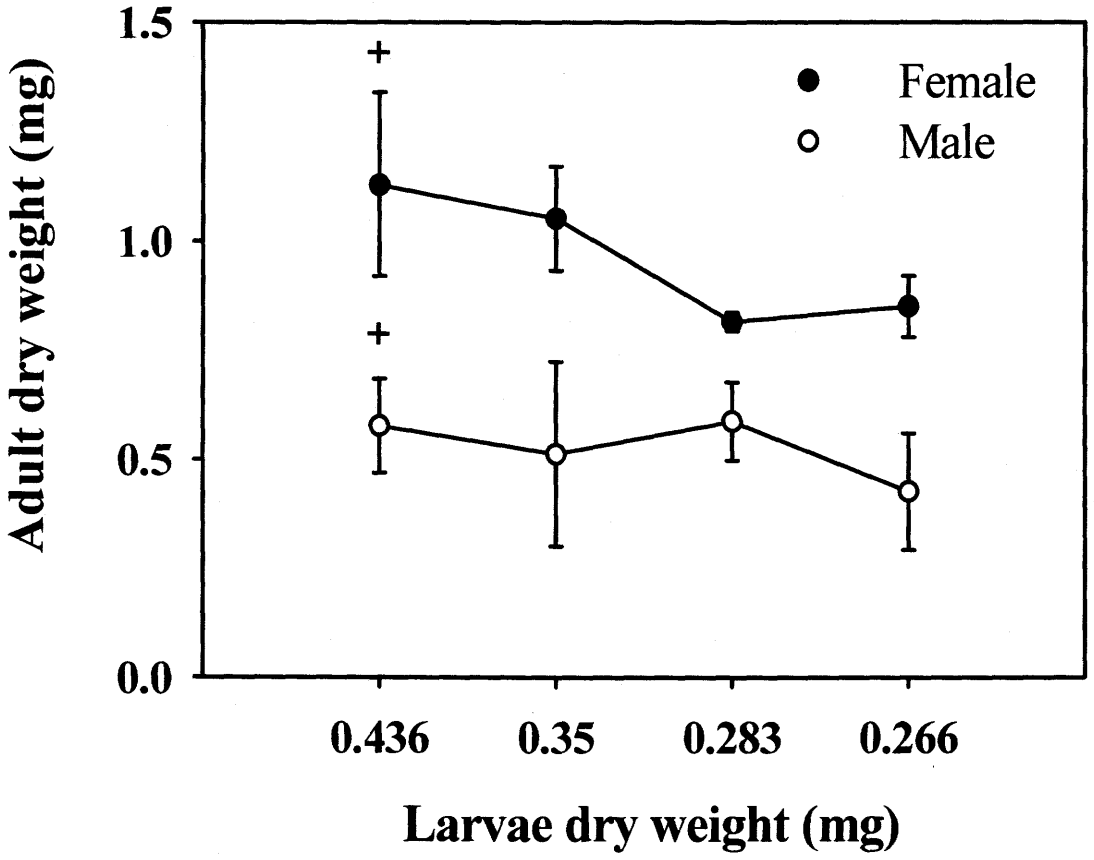

Figure 2.9. Relationship between *Chironomus tentans* adult dry weight and dry weight of larvae after 10 d of uranium exposure. Each point is the mean ± SD of five replicate samples. + Represents control treatment.

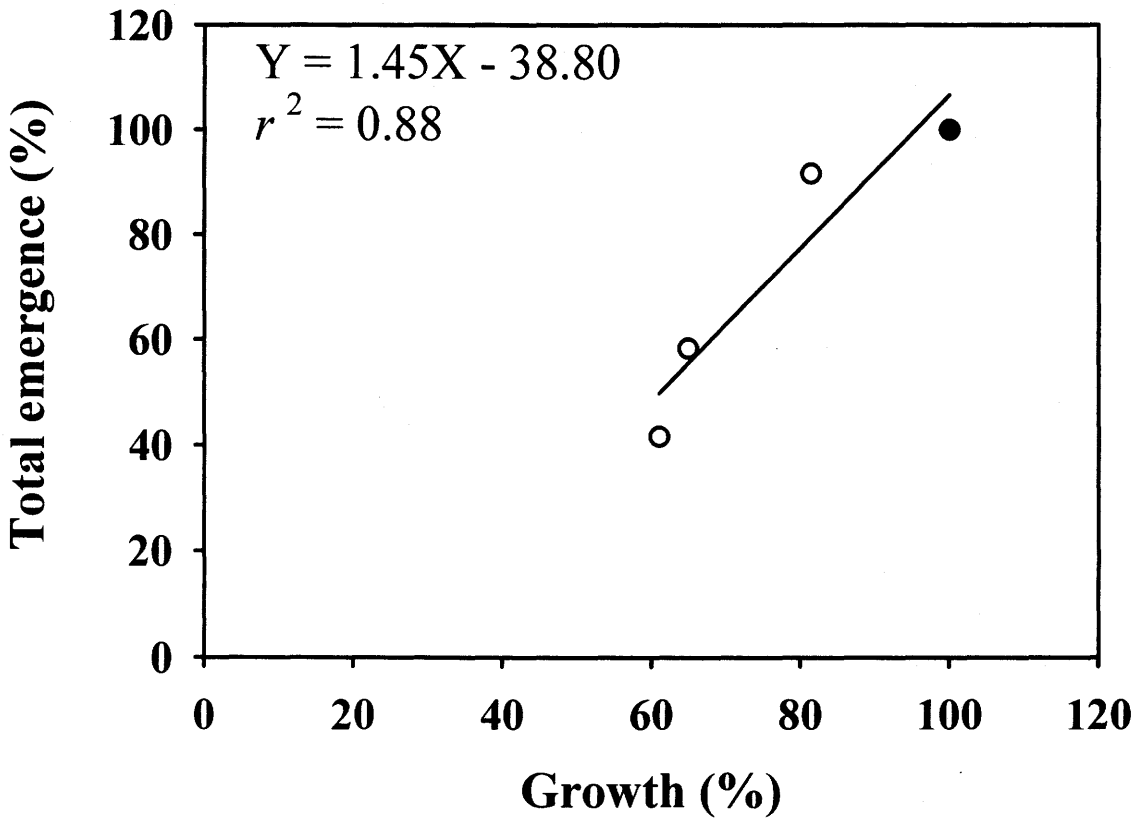

Figure 2.10. Relationship between mean growth (dry weight) of 10-d old *Chironomus tentans* larvae and adult emergence success. Both variables were measured relative to the control group (solid symbol).

2.3.1.3.2. Uranium tissue accumulation

Despite the significant increase in U accumulation in *C. tentans* larvae, much of the U that accumulated in larval tissues was eliminated during metamorphosis to the adult stage. Mass balance calculations estimated that losses of U via the exuvia was approximately 50 ± 27 % of the concentration in larvae. As a result, U accumulation in adult midges dropped to relatively low levels at all treatments (Figure 2.2). There was only a significant difference in U accumulation in *C. tentans* adults at a U exposure concentration of 856 µg/L ($p < 0.05$; Figure 2.2), but a general relationship between U accumulation and exposure could be expressed by the equation, $Y = 0.01X - 0.03$ ($r^2 = 0.99$). No sex related differences were found in U accumulation by adult midges ($p = 0.56$). Adult midges had a lower BAF than larvae, pupae and exuvia with a calculated mean value (± SD) of 10 (± 3) (Table 2.2).

2.3.1.3.3. Reproduction

2.3.1.3.3.1. Egg production and hatching success

There were no significant differences in the mean number of eggs and the number of hatched eggs per egg mass among treatments ($p = 0.66$ and 0.38, respectively; Figure 2.11). Similarly, no significant differences were found in the number of eggs per egg mass and hatched eggs coming from exposed females (mated with unexposed males) versus unexposed females (mated with exposed males) ($p = 0.78$ and 0.80, respectively). The percent hatchability was comparable in all treatments (74.1 ± 2.6 %).

2.3.1.3.3.2. Embryo viability and uranium tissue concentration

The F_1 generation larvae were used in a 10-d larval growth test following a similar experimental procedure to that used for the F_0 10-d larval growth test described earlier (section, 2.2.3), except that F_1 generation larvae were not exposed to U. Results from this test suggested than the initial exposure of F_0 larvae to U had effects on F_1 larval growth (Figure 2.12). Growth (as dry weight) of F_1 larvae originating from both exposed males or exposed females decreased with increasing F_0 U exposure concentration, showing a 10-d larvae growth pattern similar to that observed in F_0 larvae (p = 0.61 and 0.52, respectively). The mean dry weight relative to respective control of F_1 larvae coming from exposed females was approximately 12% lower than that for F_1 larvae originating from exposed males (p = 0.04). Moreover, there was a significant difference in the dry weight of F_1 generation larvae coming from females previously exposed to a mean U concentration of 856 μg/L, relative to the control ($p < 0.05$).

The concentration of U found in F_1 larvae was similar to control values at all evaluated treatment groups (mean ± SD = 0.038 ± 0.013 mg/kg). No significant differences were found among treatments (p = 0.56).

2.3.1.4. Uranium fate during the metamorphosis of *Chironomus tentans*

A steady increase in U accumulation occurred at all life stages of *C. tentans* with increasing level of U exposure (Figure 2.2). The U accumulation in larvae after 10 d did not differ from the U accumulation in pupae ($p > 0.05$). This may

indicate that larvae had reached steady-state conditions after approximately 10 d of exposure. Although, the exuvia showed the highest U concentrations, and adults *C. tentans* displayed significantly lower U tissue accumulation than 10-d old larvae ($p < 0.05$), the elimination of U as a result of molting was not complete. Consequently, transfer of U to the adult midges was observed.

2.3.2. Gut clearance test

Overlying water chemistry was acceptable throughout the test; mean water quality measurements are presented in Table 2.3. Survival of *Chironomus tentans* larvae was > 80% for all treatments and was not adversely affected during the experiment.

The gut clearance of midge larvae that had been exposed to U was measured after 10 d of exposure. Gut content represented 2 to 9% of the whole animal dry weight. However, no statistically significant differences in the dry weight of larvae before and after gut clearance were found at U exposure concentrations of 200 and 1000 μg/L ($p = 0.81$ and 0.33, respectively; Table 2.4). Uranium concentration in the whole insect body before gut clearance did not differ from the U tissue concentration after gut clearance at either treatment ($p = 0.95$; Figure 2.13). Therefore, any potential influence from gut content on interpretation of U in whole *C. tentans* would be negligible.

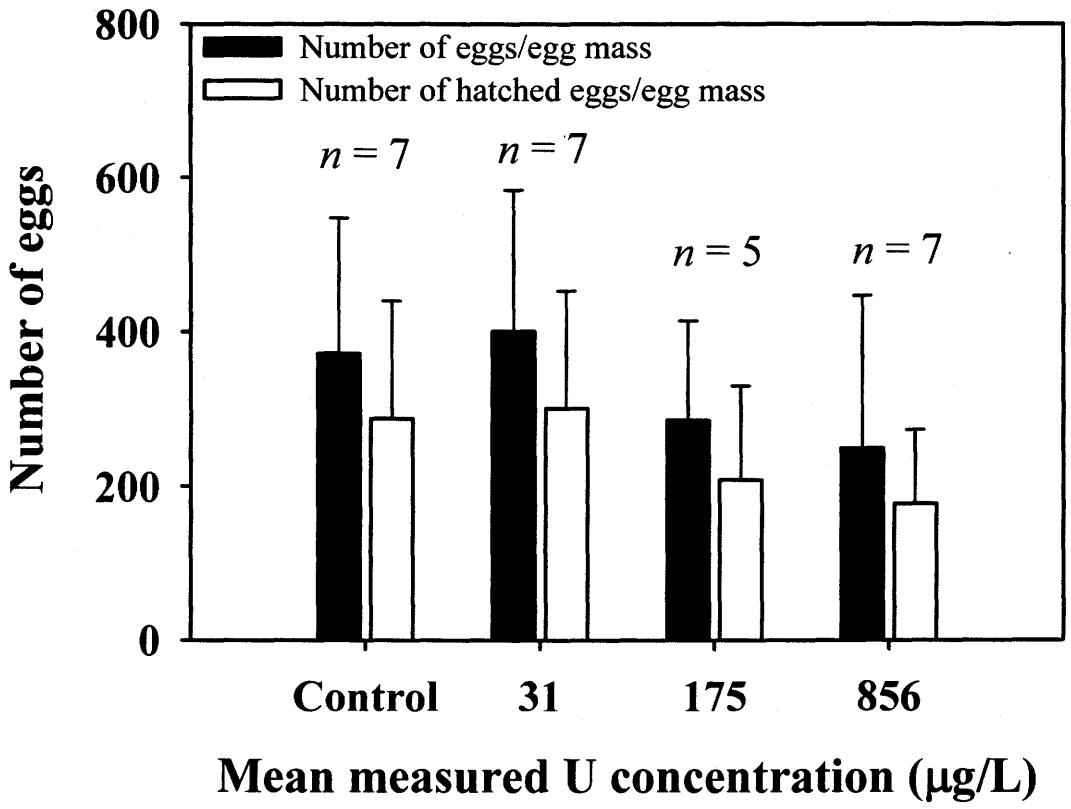

Figure 2.11. Number of eggs and hatched eggs produced by adult *Chironomus tentans* exposed to uranium during their immature stages. Values are the mean ± SD.

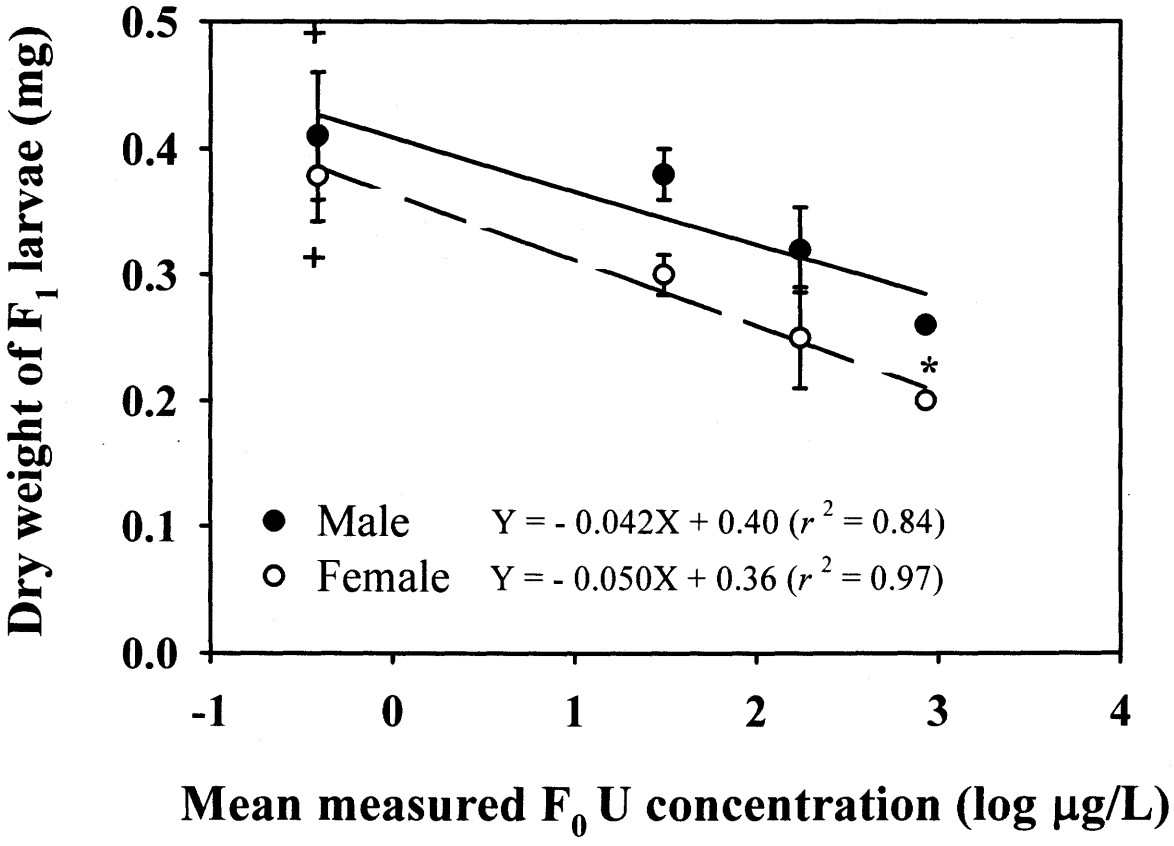

Figure 2.12. Relationship between uranium exposure concentration for F_0 generation animals and growth of F_1 generation *Chironomus tentans* larvae (mean ± SD). + Represents control group. * Significantly different from control (p < 0.05).

Table 2.3. Water quality measurements during the gut clearance test. Data represent the mean (± SD) of three replicate samples.

Variable	Unit	DL[c]	Mean (±SD)
pH	pH	0.05	8.34 ± 0.13
Total-Hardness	mg/L[a]	10	136 ± 8
Alkalinity	mg/L[a]	10	85 ± 4
DO[b]	mg/L	0.1	7.8 ± 0.3
Temperature	∘ C	0.1	22.2 ± 0.7
Ammonia	mg/L	0.005	0.40 ± 0.21

[a] mg/L as $CaCO_3$.
[b] Dissolved Oxygen
[c] Analytical Detection Limit.

Table 2.4. Dry weight of 10-d old *Chironomus tentans* larvae before and after gut clearance and corresponding *p*-value value. Data represent the mean ± SD of three replicate samples. Dry weight measurements after gut clearance are showed in parenthesis.

U concentration (µg/L)	Dry weight (mg)	*p*-value
200	0.79 ± 0.07 (0.76 ± 0.05)	0.81
1000	0.68 ± 0.02 (0.63 ± 0.12)	0.32

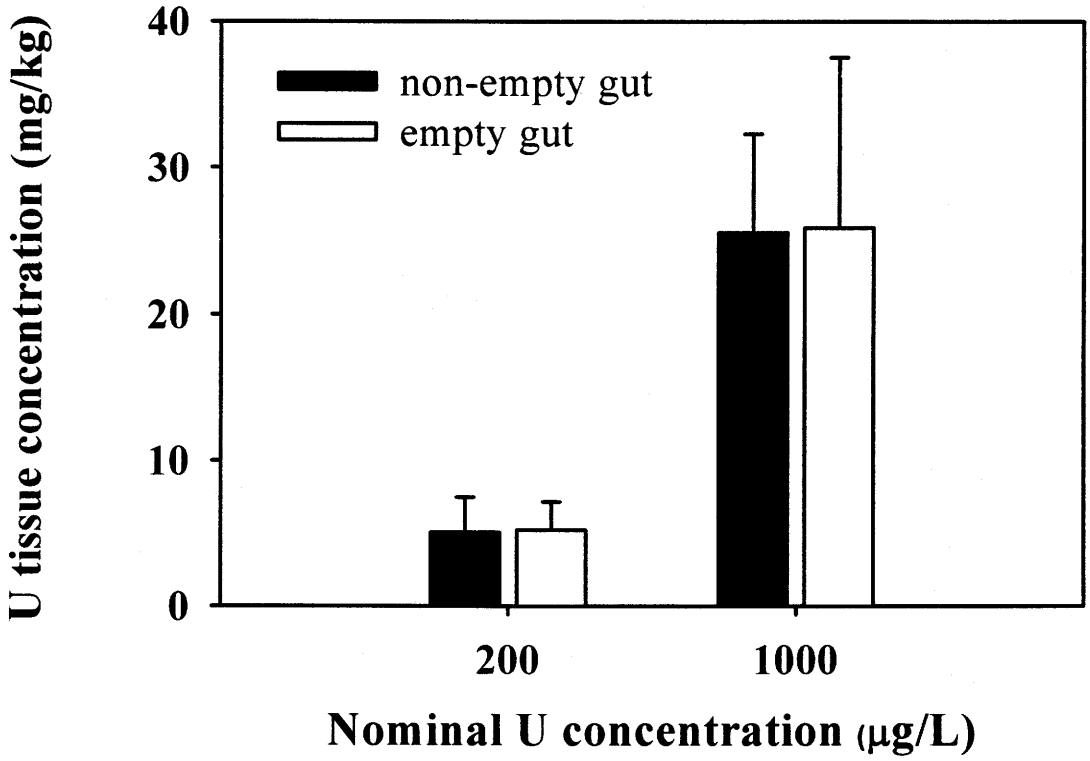

Figure 2.13. Comparison of the mean uranium (U) tissue concentration (± SD) in 10-d old *Chironomus tentans* larvae at the evaluated U exposure levels before and after gut clearance.

2.4. Discussion

2.4.1. Life cycle test

Although U is actively mined in Canada and is on the Canadian Priority Substances List, there has been a limited amount of information published on the acute and chronic toxicity of this metal to aquatic organisms. Therefore, it is unclear what U concentrations can affect growth, reproduction and development of most aquatic invertebrates.

Studies using the cladocerans, *Moinodaphnia macleayi*, reported NOEC and LOEC (reproduction) values for U in soft water (~ 2 mg/L $CaCO_3$) ranging from 8 to 31 μg/L and 20 to 49 μg/L, respectively (Semaan *et al*, 2001). Poston *et al.* (1984) reported a LOEC (reproduction) value of 520 μg/L for *Daphnia magna* exposed to U for 5 d at a water hardness of 60 mg/L. In a 7-d chronic test with a water hardness of 60 mg/L, the NOEC and LOEC (reproduction) values for *Ceriodaphnia dubia* were 10 and 35 μg/L, respectively (Liber and George, 2000). In the present study, the NOEC and LOEC (growth) values for *C. tentans* exposed to U were 39 μg/L and 157 μg/L, respectively, in good agreement with other published data for freshwater invertebrates.

Relationship between growth, emergence and reproduction: In this study, survival of *C. tentans* larvae was not affected even though significant larval growth retardations relative to the control were found at U concentrations ≥ 157 μg/L. When organisms face a trade-off between growth and survival rate imposed by a toxicant, in this case U exposure, they may reallocate resources

into detoxification instead of growth and so achieve relatively low mortality, but a longer developmental period (Holloway *et al*, 1990). The exposure of *C. tentans* larvae to U may have triggered mechanisms to resist its toxic effects (e.g., detoxification mechanisms, protein synthesis), but these are likely to be energy expensive and hence take place only at the expense of somatic growth (Sibly and Calow, 1989)

Larval growth appears to be a sensitive and biologically relevant endpoint for chironomids. A reduction in larval growth, such as that recorded in this study after U exposure, has also been reported in previous works using other metals. For example, Pascoe *et al.* (1989) recorded significant reductions in *C. riparius* larval growth and development at cadmium concentrations $\geq$ 150 μg/L. Hatakeyama (1988) reported impaired larvae growth in the chironomid *Polypedilum nubifer* exposed to cadmium concentrations of 10 and 20 μg/L. Also, Krantzberg and Stokes (1989) observed severe growth retardations in larvae of the genus *Chironomus* exposed to metal contaminated sediments (copper, zinc, nickel, lead and cadmium). More recently, Timmermans *et al.* (1992) found that growth of *C. riparius* larvae was significantly retarded when exposed to zinc concentrations of 100 and 1000 μg/L.

In the present study, equal numbers of females and males emerged among treatments ($p = 0.065$), but overall adult emergence was significantly affected at U concentrations $\geq$ 175 μg/L ($p < 0.05$). Emergence has been used in numerous studies with Chironomidae as a sensitive endpoint for life cycle test evaluations. Liber *et al.* (1996) reported a 35 to 50% reduction in cumulative adult

emergence with a comparable decrease in growth of *C. tentans* larvae. Sibley *et al.* (1997) also reported a reduction in emergence with decreased food supply for *C. tentans*. Hatakeyama (1987) reported a decrease of 46% in the emergence success of *P. nubifer* exposed to 40 μg/L of cadmium, while other reproductive endpoints (e.g., adult sex ratio, egg hatchability) were not affected. In another study, Pascoe *et al.* (1989) found a decreased number of adults and delay in time to emergence in *C. riparius* exposed to a cadmium concentration of 150 μg/L.

In this study, there was a strong relationship between larvae dry weight and adult emergence (r^2 = 0.88). Growth retardations at U exposure concentrations ≥ 157 μg/L of 30 to 40% lead to a reduction in adult emergence of 40 to 60%, and significant delays in time to adult emergence. Furthermore, if a 50% emergence reduction is considered to be a biological/ecological adverse event, then a growth retardation of 34% in a 10-d test with second to third instar *C. tentans* larvae could be considered a significant adverse effect. Liber *et al.* (1996) found that a growth retardation of 47 % in a 10-d test with *C. tentans* larvae led to a 50 % emergence reduction, which may consequently lead to adverse ecological effects. A substantial increase in time to emergence and a reduction in adult emergence can lead to a reduced reproductive success and subsequent reduction in abundance of future populations (Liber *et al*, 1996).

The general absence of differences among the mean dry weight of adults emerging from U and control treatments (p = 0.18) demonstrated that adult dry body weight was not a sensitive indicator of U exposure in this study. However,

the number of samples analyzed was low and consequently the statistical power of the performed test was lower than 0.8. Differences between female and male adult dry weight were observed ($p < 0.05$). Female dry weight was significantly greater than male dry weight, most likely because females had not yet deposited their egg cases.

Growth reductions in immature stages in response to metal exposure have been show to be accompanied by a constant reproductive output (e.g., number of eggs; number of hatched eggs) during the adult stage. In these situations, commitment of energy to growth is sacrificed to ensure that reproductive success (e.g., number of eggs) does not decline (Benoit *et al*, 1997; Gullan and Cranston, 2000). Our data resembled this scenario, since larval growth reductions did not cause any significant decline in the production and hatchability of eggs at any of the evaluated U exposure levels ($p = 0.66$ and 0.38, respectively). Therefore, based on these evaluations, production and hatchability of eggs in *C. tentans* appear to be relatively insensitive assessment endpoints.

Embryo viability: Environmental challenges during the paternal generation can significantly influence the growth and reproductive potential of the next generation (Mousseau and Dingle, 1991; Rossiter, 1991; Lacey, 1998). For example, environmental stress can lead to a variation in maternal growth, condition and physiological state, which could be transmitted via cytoplasmatic factors (e.g., yolk amount and quality, hormones) in the egg, thus influencing offspring development (Mousseau and Fox, 1998). In our study, F_1 generation

C. tentans larvae that were never directly exposed to U, but originated from adult females exposed to U during their immature life stages, displayed a significant decrease ($p < 0.05$) in 10-d growth that was similar to that observed for the F_0 larvae.

The number of offspring produced by a female and the amount of energy invested in each offspring cannot be simultaneously maximized if the quantity of resources dedicated to reproduction is limited (Bridges and Heppell, 1996). In this study, oviposition in *C. tentans* adults was not affected by the reduced growth experienced by the larvae exposed to U. Thus, female *C. tentans* may decrease the per-offspring investment (e.g., yolk's quality) in order to maximize the number of offspring produced (as number of eggs) (Bridges and Heppell, 1996). Other studies have shown that the amount and quality of the resources allocated in the eggs by females influenced the growth of their progeny (Chambers and Leggett, 1996; Rossiter, 1991, 1996; Barnes, 1984; Wolf *et al*, 1997). Therefore, the nutritional properties of the yolk deposited into the eggs by *C. tentans* females, and the quality of the gelatinous component of the egg mass which constitutes the first food for the newly hatched *C. tentans* larvae, might influence offspring fitness.

Although no significant differences were observed in the dry weight of non-exposed F_1 generation larvae originating from exposed *C. tentans* males ($p = 0.133$), there was an obvious trend of decreased growth that was comparable to that observed for the exposed F_0 larvae. Nutrients donated by males to females at mating represent investment by the male in reproduction. *C. tentans* males

use the reserves gained during larval stages to transfer nutrients with the spermatophore during mating (Sibley *et al*, 2001). Thus, the quality of the resources donated by *C. tentans* males could be affected by environmental conditions (e.g., metal exposure) experienced during their larval stage and therefore have a direct consequence on offspring development. Such effects derived from differences in quality of the resources donated by males have been postulated for other insects (Wolf *et al*, 1997; Thornhill and Sauer, 1992; Boggs, 1990). Decreased *C. tentans* larval growth due to U exposure might therefore affect the larval resources allocated for reproduction. However, the effect of male nutrient donations on offspring fitness is still not well understood (Boggs, 1990).

Uranium accumulation in C. tentans larvae: All aquatic invertebrates accumulate metals in their tissues, whether or not these metals are essential to metabolism. Once incorporated into the insect body, the fate of the metal depends on the physiology of the invertebrate and on whether the metal is used for an essential metabolic purpose, excreted, and/or stored (e.g., metallothioneins, granules) in the body. However, non-essential metals such as U, would have no requirements for a minimum concentration for metabolic purposes and would need to be detoxified or excreted forthwith (Rainbow, 2002). Therefore, toxicity occurs when the rate of metal uptake into the body exceeds detoxification and/or excretion rates, and the critical body burden is reached.

Active regulators are organisms that maintain stable tissue concentrations by excreting metal at rates comparable to the intake rate (Rainbow and Dallinger, 1993; Adams *et al*, 2000). Thus, if an organism actively regulates a metal (e.g., limits uptake or excretion), the slope of the relationship between the sublethal water concentration and the corresponding BAF is expected to be negative. The slope is expected to be near 0 if an organism accumulates a metal in proportion to the metal concentration in the water (e.g., no active regulation mechanism) (Muyssen and Janssen, 2002; Adams *et al*, 2000). Our findings indicate that U might not be well regulated, as demonstrated by the shallow, insignificant slope of BAFs versus exposure concentration, and the significant increase in the U tissue concentration of *C. tentans* larvae with increasing U exposure level. Moreover, there are no confirmed reports of body content regulations of non-essential metals by aquatic invertebrates (Rainbow, 1996; Rainbow and Dallinger, 1993; Hare, 1992).

Uranium fate during metamorphosis: Immature *C. tentans* life stages accumulate U from the water, but subsequently eliminate much of this metal during metamorphosis to the adult stage. Adult midges contained substantially less U than did larvae. The process of U elimination can be explained both by physiological changes taking place during metamorphosis, as well as by the shedding of the exoskeleton when molting from the pupal to the adult stage. Physiological changes may be attributed to the fact that in Diptera most larval cells disintegrate during metamorphosis and the adult midge develops from small groups of undifferentiated cells which are present in the larvae but are not

functional (Timmermans and Walker, 1989). The disintegration of these larval cells (possible contaminated cells) could be an important method of U elimination and thus result in a significant loss of accumulated U.

Other authors have also reported the importance of the exoskeleton in metal accumulation and elimination in chironomids (Timmermans and Walker, 1989; Groenendijk *et al*, 1999; Hare, 1992; Kranztberg and Stokes, 1988; Timmermans *et al*, 1992). The shedding of the exoskeleton often leads to a general reduction in metal concentrations from larvae to adult midges. In the present study, U losses through the exuvia during metamorphosis accounted for approximately 50% of the U accumulated in *C. tentans* larvae.

Although U levels in the exuvia increased significantly with increasing U exposure concentration, and although there were notable differences between larvae and adult U tissue concentrations, the elimination of U was not complete. As a result, transfer of U to adult midges was observed. Therefore, transfer of U from the aquatic to the terrestrial environment could take place after adult emergence.

2.4.2. Gut clearance test

The gut content can represent a significant fraction of the whole-animal metal concentration in some aquatic insects. Gut content can therefore introduce a substantial error in studies that analyze whole organisms to assess the assimilation of metal into tissues. Thus, contaminant concentrations in whole animals, including gut contents, should be measured and an estimate of

the proportion contributed by contaminants in gut content subtracted (Hare, 1992). Our results suggested that gut clearance had an insignificant effect on U concentration in the insect body. This could be explained by an efficient uptake and subsequent removal of U from the gut to the insect body. However, reports indicate that U has a very low rate of uptake (< 5%) through the gut of most organisms (Environment Canada and Health Canada, 2001; Ribera *et al*, 1996; Wrenn *et al*, 1985). Thus, U uptake in *C. tentans* in the present study was likely directly from water, while absorption from food appeared to be minimal (e.g., due to the highly soluble form of U used). As a result, correction to minimize the effect of gut content in whole-animal metal analyses was not necessary in this case.

2.5. Conclusions

Life cycle test: In this study, larval growth was a sensitive and biologically relevant endpoint. Larval growth decreased significantly with increased U exposure concentrations, showing a strong linear relationship ($r^2 = 0.96$). The NOEC and LOEC values for growth in U exposed *C. tentans* larvae were 39 and 157 μg/L, respectively. These concentrations are representative of what may be found downstream of some U mining and milling facilities in northern Saskatchewan (e.g., Rabbit Lake; Liber, unpublished data).

Non-essential metals such as U have no known metabolic purpose and therefore need to be detoxified or excreted (Rainbow, 2002). Thus, under elevated U exposure conditions, *C. tentans* larvae must allocate energy to

detoxification mechanisms at the expense of somatic growth in order to prevent toxic effects (Sibly and Calow, 1989; Holloway *et al*, 1990).

Emergence has been used in numerous studies with chironomids as a measurement endpoint for sublethal toxicity (Hatakeyama, 1987, 1988; Pascoe *et al*, 1989; Taylor *et al*, 1993). In the present study, emergence was also a sensitive endpoint with reductions of 40 to 60% relative to the control at U concentrations $\geq$ 175 μg/L.

Reductions in adult *C. tentans* emergence under laboratory conditions can be predicted from 10-d growth retardation data (Liber *et al*, 1996). Moreover, at growth retardations of 30 to 40%, similar percent reductions in adult emergence can be expected. Among the numerous factors that have been shown to regulate emergence patterns in aquatic insects, a minimum larval weight may be crucial to emergence success (Sibley *et al*, 1997). Under such circumstances, fewer emerging adults, and/or a delay in time to emergence, could reduce the number of adults available to participate in reproduction, leading to a decline in mating success and consequently alter population dynamics.

Uranium effects on larval growth can be observed not only in the directly exposed larvae, but also in the unexposed F_1 generation. Thus, the environment of the parental generation can significantly influence the development of the next generation through environmentally induced parental effects (Mousseau and Dingle, 1991; Rossiter, 1991, 1996; Lacey, 1998; Wolf *et al*, 1997). It is clear that these effects have the potential to make significant

contributions to a number of life history parameters (e.g., growth), which may influence population dynamics and consequently led to long-term population declines. Further research is needed to clarify the physiological and developmental mechanisms by which these effects are transmitted from parents to their progeny.

Although U that accumulated in *C. tentans* immature stages was partially excreted during metamorphosis to the adult stage, metal elimination was not complete. Therefore, significant amounts of U could be transferred from the aquatic to the terrestrial environment by *C. tentans.* Since the transport of U to terrestrial food webs could be significant, the ecological fate of U transported from the aquatic to the terrestrial system could be of environmental importance. Moreover, movement of metals across the aquatic-terrestrial barrier has been a much ignored source of poisoning for insectivorous predators, both aquatic and terrestrial, and deserves more attention (Timmermans and Walker, 1989).

Gut clearance test: The accurate measurement of metal concentrations in the whole body of benthic invertebrates is occasionally confounded by the presence of gut metal content. However, due to the low rate of U uptake through the gut of most organisms, and the U exposure method (via water) used in this study, corrections for the contribution of gut contents to the whole-animal U concentrations were not necessary.

Chapter 3. Uranium accumulation in *Chironomus tentans* larvae and tissue distribution assessment using X-ray microprobe analysis.

3.1. Introduction

All aquatic invertebrates accumulate metals in their tissues, whether or not those metals are essential to metabolism. Once a metal has entered the body of an aquatic invertebrate, it will remain metabolically available until the physiology of the invertebrate interacts to excrete it (e.g., via the exuvia), or to detoxify it (e.g., by binding to metallothioneins and/or insoluble metaliferous granules) (Rainbow, 1996, 2002; Hare, 1992).

Metal localization has been studied in several invertebrates and metal containing granules have been found in Crustacea, Mollusca and Insecta (Brown, 1982). In isopods, Brown (1978) noted that metal storage occurred in the hepatopancreas, an organ important in metal detoxification. Lyon *et al.* (1983), also working with isopods, reported intracellular inclusions of copper, lead and iron. In another study, Walker *et al.* (1975) detected the presence of magnesium, potassium, phosphorous, calcium, iron, copper and zinc granule-rich structures in the midgut of the barnacle, *Balanus balanoides*. Copper and zinc have also been found in granular amoebocytes of mollusks, particularly in

the gut and kidney (George *et al*, 1984). Back (1983) reported zinc and lead storage in the hindgut of oligochaetes.

Metal-rich zones (e.g., metallothioneins, granules) have been reported to occur in numerous orders of aquatic insects, including dipterans of the genus *Chironomus.* Yamamura *et al.* (1983) described the presence of high molecular weight proteins in *Chironomus yoshimatsui* exposed to cadmium (10 μg/L) for only 2 d. In addition, Seidman *et al.* (1986) reported low and high molecular weight cadmium binding proteins in *Chironomus thummi* cells after 4 d of exposure to cadmium concentrations of 10, 100, and 250 μg/L. Cadmium was accumulated primarily in membrane-bound crystalline granules in the posterior midgut epithelium. In a more recent study, Craig *et al.* (1998) found that the digestive tract was the principal organ of cadmium accumulation in *Chironomus staegeri* larvae. Kranztberg and Stokes (1990) used an X-ray microprobe technique to assess metal-rich zones of lead, iron, cadmium, nickel, copper and zinc in *Chironomus* larvae. Metal accumulations were found in the anal papillae, Malphigian tubules and midgut.

Patterns of metal distribution can provide critical information on mechanisms of toxicity and metal exchange between an animal and its environment. Moreover, localization of metal accumulation sites can be a useful means of determining the organs in which patho-physiological effects might be observed (Craig *et al*, 1998).

In this study, whole-body measurements of uranium (U) concentration were coupled with X-ray microprobe analysis to evaluate the differences in U accumulation in *C. tentans* larvae organs/tissues.

3.2. Materials and methods

3.2.1. Study design

Tests were conducted in a controlled environment chamber set at 23 ± 1 °C with a photoperiod of 16:8 h light:dark and an illumination intensity of 800 -1300 lux.

To evaluate U tissue concentration and distribution in *C. tentans,* animals were exposed to two U concentrations (1000 and 2000 μg/L) plus an untreated control. Each treatment had nine replicates with 10 second-instar *C. tentans* larvae per replicate (see Sections 2.2.1.2 and 2.2.1.3, Chapter 2, for details on culturing procedures and acquiring test organisms). Animals that died due to the stress of handling were replaced after 4 h of being transferred to the test beakers. A total of five *C. tentans* larvae were replaced for this test.

After 10 d of exposure, larval survival, growth (as dry weight) and U tissue concentrations were evaluated in three randomly selected beakers from each treatment. The rest of the beakers (six per treatment) continued with the U exposure. When the animals reached the pupal stage they were collected from two beakers from each treatment for determination of dry weight and U tissue concentrations. On day 15, emergence traps were placed over the four

remaining beakers in each treatment. These replicates were used to evaluate adult U tissue concentrations, dry weight and adult emergence.

3.2.2. Sampling procedures and processing

For descriptions on cleaning test materials, processing of animal samples for dry weight evaluation, and obtaining water samples for water quality and U determinations refer to Section 2.2.5, Chapter 2.

3.2.2.1. Collection of samples for X-ray microprobe analysis

Uranium distribution and the presence of U-rich zones in *C. tentans* larvae were measured using an X-ray microprobe. The microprobe technique is a non-destructive, *in situ* analytical procedure that works by bombarding the sample surface with high-energy electrons. When the electrons strike the sample some of them penetrate and cause its constituent atoms to emit characteristic X-rays. Characteristic X-rays are X-rays with energies specific to the elements from which they are emitted. Thus, the number of emitted X-rays is proportional to the concentration of the element in each sample.

Prior to X-rays microprobe analysis, collected organisms were placed in 8-ml pre-cleaned Nalgene® bottles containing a solution of 50% methanol (95%) and 50% deionized water (Milli-Q®) to stop U diffusion processes as rapidly as possibly. Subsequently, the specimens were mounted using an epoxy resin and the resin surface carefully polished until a flat unscratched surface was obtained. Residual contamination adhering to the sample surface from the

polishing procedure was cleaned using pure ethanol, before coating the samples with a carbon layer to make them electrically conductive. The samples were analyzed at the Department of Geological Sciences, University of Saskatchewan, using a JEOL JXE 8600 superprobe (Soquelec Ltd., Montreal, QC).

3.2.3. Statistical analysis

Statistical analyses were performed using the computer program SigmaStat®, version 2.03 (SPSS Inc., Chicago, IL, USA), with a 95% (α = 0.05) level of confidence. Significant differences among treatments in survival, dry weight and U tissue concentrations were assessed using one-way ANOVA. Comparison of means was conducted using Dunnett's test. Uranium tissue accumulation in pupae and exuvia, as well as dry weight data, failed tests for homogeneity of variance. A Kruskal-Wallis one-way ANOVA on ranks was, therefore, used to evaluate differences among treatments. Multiple pairwise comparisons were conducted using Dunn's Test.

Since an inadequate number of adults were recovered from the 2000 μg U/L treatment for dry weight and U tissue concentration measurements, the differences in these measures between the control and 1000 μg U/L treatment were evaluated using a *t*-test. Uranium tissue accumulation data for adult midges failed the test for normality so data were transformed using the $\log_{(10)}$ transformation.

3.3. Results

Uranium exposure concentrations and water quality variables for the control and U treatments are shown in Table 3.1. Measured U concentrations were close to nominal values and water quality remained relatively constant throughout the experiment.

Survival was higher in the control group (85 %) than in the two U treatments (73 and 53% for the 1055 and 2159 μg/L mean exposure concentrations, respectively), but only the highest concentration was significantly different from control ($p < 0.05$; Figure 3.1). Significant differences ($p < 0.05$; 45 and 55% reductions) were found in larvae dry weight between the control and each of the two U treatments (Table 3.2). The decrease in larval growth was best explained by the equation $Y = -0.073X + 0.52$ ($r^2 = 0.96$). Pupae and exuvia dry weights were not affected by U exposure ($p = 0.33$ and 0.87, respectively). Mean pupae dry weight ranged from 0.54 to 0.71 mg, whereas for exuvia the mean weight was approximately 0.06 mg for all treatments (Table 3.2). Adult dry weight was not affected by exposure to both 1055 μg U/L ($p = 0.48$; Table 3.2). Contrarily, adult emergence was significantly affected ($p < 0.05$) by exposure to 1055 and 2159 μg U/L with reductions of 76-90% in the total number of adult midges emerging from the two U treatments in comparison to the control (Figure 3.2).

Chironomus tentans larvae exposed to U for 10 d accumulated a considerable amount of the metal at both concentrations tested, with U tissue concentration increasing significantly with increased level of U exposure ($p < 0.05$; Figure 3.3). Although U accumulation in the pupae and exuvia also

increased with increasing U concentration, significant differences were only observed for the exuvia ($p = 0.07$ and $p < 0.05$, respectively; Figure 3.4). There was a decrease in the amount of U accumulated by *C. tentans* adults compare to larvae, however, U concentrations in adult midges exposed to 1055 μg U/L were still significantly greater (mean ± SD = 6.21 ± 3.7 mg/kg; $p = 0.002$) than those found in control organisms (mean ± SD = 0.114 ± 0.07 mg/kg).

Only *C. tentans* larvae sampled after 10-d of exposure to 960 μg U/L were analyzed using the X-ray microprobe technique, as a consequence of the method selected for sample fixation. Based on X-ray metal detection, U was found within the entire body of *C. tentans* larvae, although areas of high U concentration were generally limited to the central and posterior sections of the larvae (Figure 3.5).

Table 3.1. Water quality measurements from the control and two uranium treatments calculated over the duration of the test. Data represent the mean ± SD of three replicate samples.

Variable	Unit	DL[e]	Control	1000 µg/L	2000 µg/L
U (10 d)[a]	µg/L	0.04 - 0.1	0.55 ± 0.15	960 ± 94	1937 ± 94
U (entire test)[b]	µg/L	0.04 - 0.1	1.07 ± 0.52	1055 ± 76	2159 ± 97
pH	pH	0.05	7.96 ± 0.34	8.01 ± 0.25	7.93 ± 0.24
Total-Hardness	mg/L[c]	10	155 ± 23	152 ± 19	149 ± 19
Alkalinity	mg/L[c]	10	92 ± 17	87 ± 10	84 ± 10
DO[d]	mg/L	0.1	7.2 ± 0.2	7.3 ± 0.3	7.3 ± 0.3
Temperature	°C	0.1	22.7 ± 0.7	22.6 ± 0.5	22.6 ± 0.6
Ammonia	mg/L	0.005	0.73 ± 0.35	0.85 ± 0.42	0.78 ± 0.33

[a] Mean U concentration calculated for the first 10 d of exposure.
[b] Mean U concentration measured over the entire test.
[c] mg/L as $CaCO_3$.
[d] Dissolved Oxygen.
[e] Analytical Detection Limit.

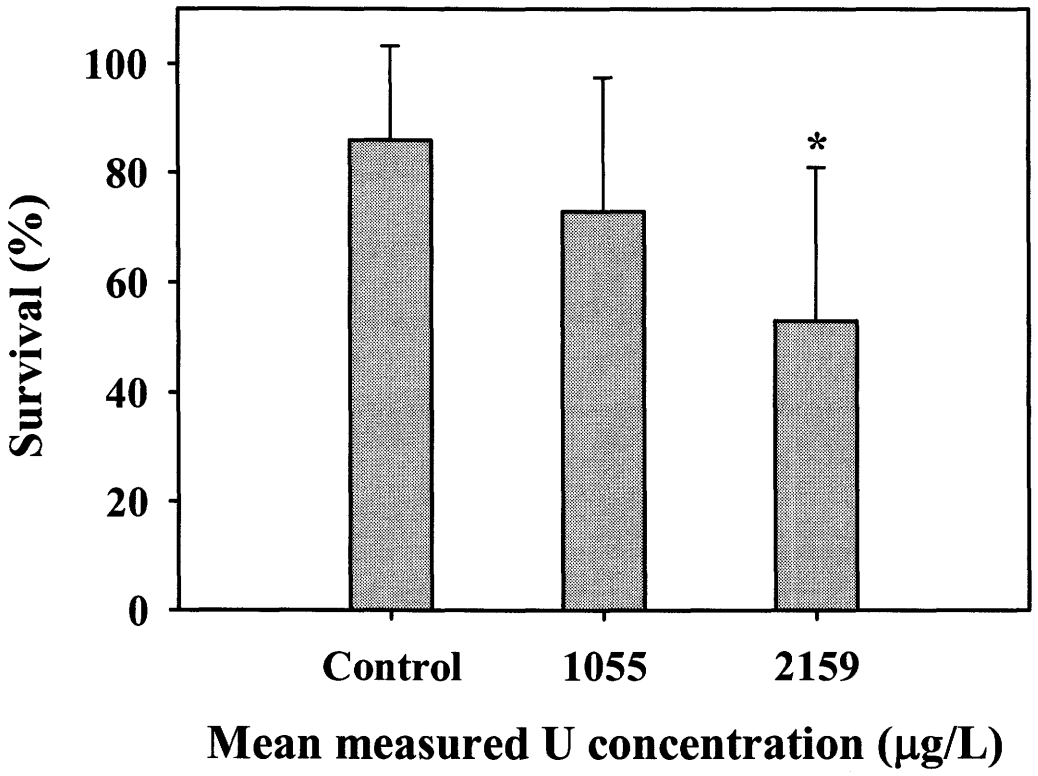

Figure 3.1. Percent survival (mean ± SD) of *Chironomus tentans* in control and uranium treatments for the entire duration of the test. * Significantly different from control ($p < 0.05$).

Table 3.2. Dry weight (mean ± SD) of different *Chironomus tentans* life stages expressed in mg, and corresponding *p*-value. The uranium exposure concentration during the larval stage is the calculated mean after 10 d of exposure.

Life stage	Control	960 µg/L	1937 µg/L
Larvae	0.55 ± 0.08	0.30 ± 0.13* ($p < 0.05$)	0.25 ± 0.13* ($p < 0.05$)
	Control	**1055 µg/L**	**2159 µg/L**
Pupae	0.68 ± 0.02	0.71 ± 0.16 ($p = 0.33$)	0.54 ± 0.05 ($p = 0.33$)
Exuvia	0.065 ± 0.004	0.063 ± 0.002 ($p = 0.87$)	0.065 ± 0.01 ($p = 0.87$)
Adult	0.57 ± 0.05	0.54 ± 0.04 ($p = 0.48$)	NA[a]

[a] NA = not available (insufficient number of animals available for dry weight determination).
* Significantly different from the corresponding control.

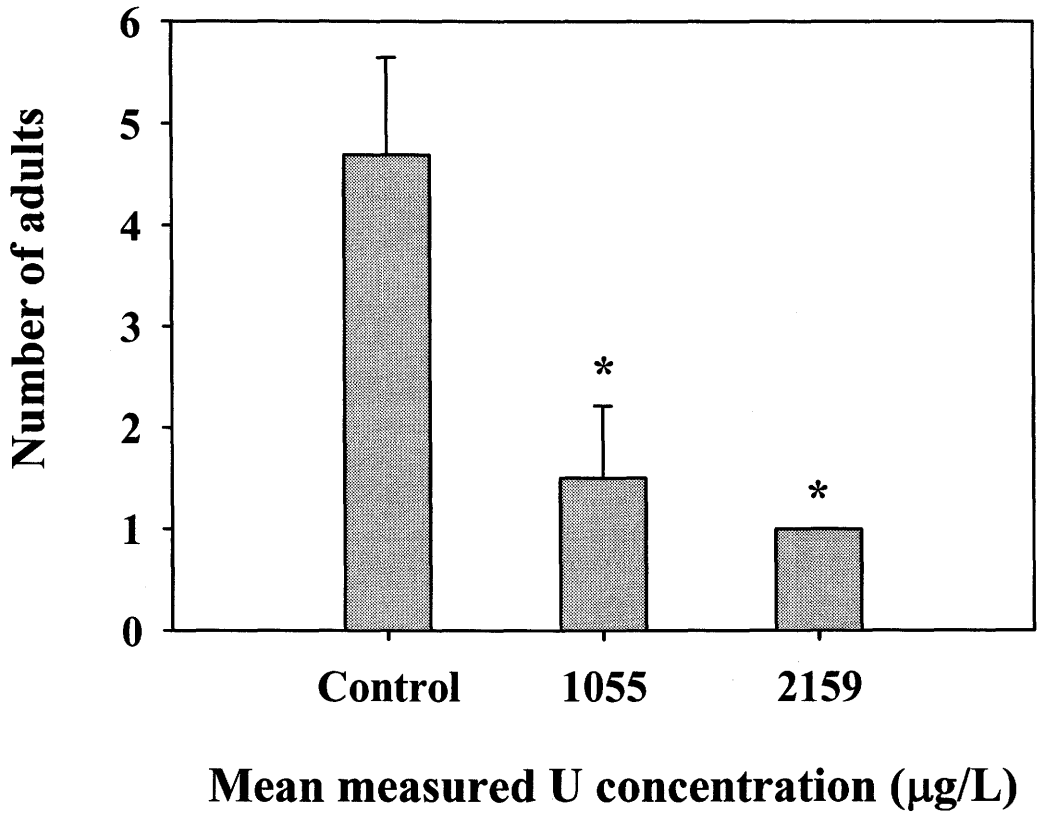

Figure 3.2. Number of successfully emerged adult *Chironomus tentans* among treatments. Data represent mean (± SD) of four replicate samples. * Significantly different from control ($p < 0.05$).

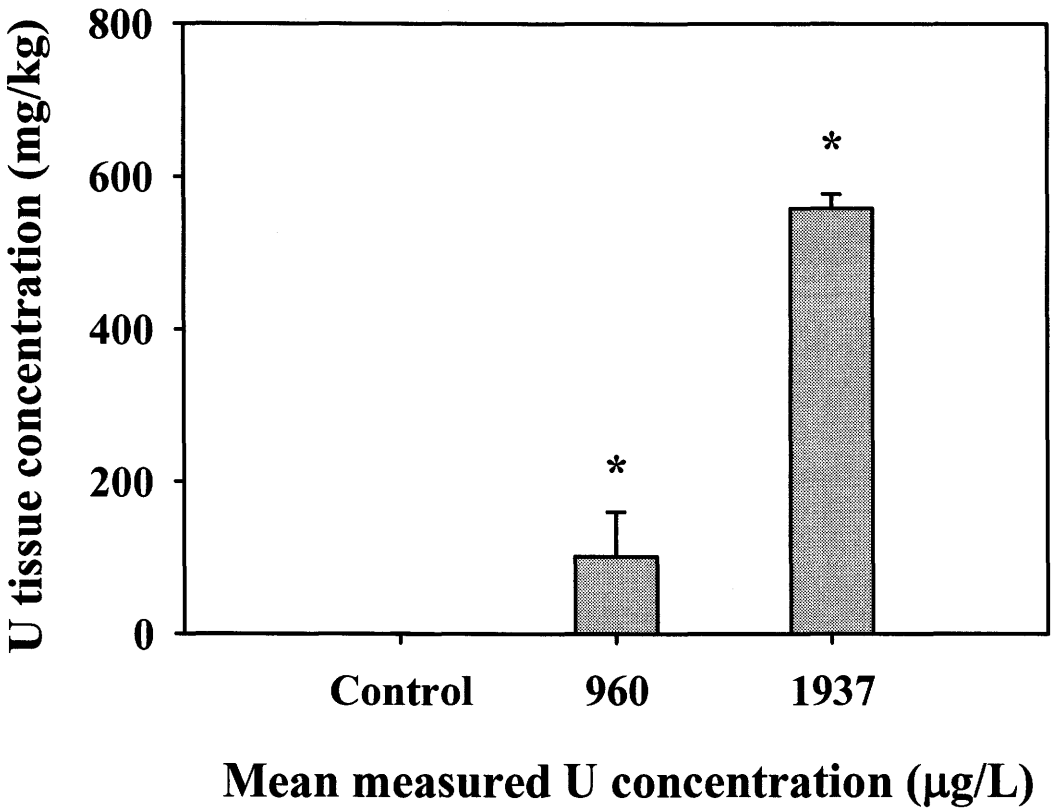

Figure 3.3. Uranium (U) concentration in *Chironomus tentans* larvae after 10 d of U exposure. Data represent mean (± SD) of three replicate samples. * Significantly different from control ($p < 0.05$).

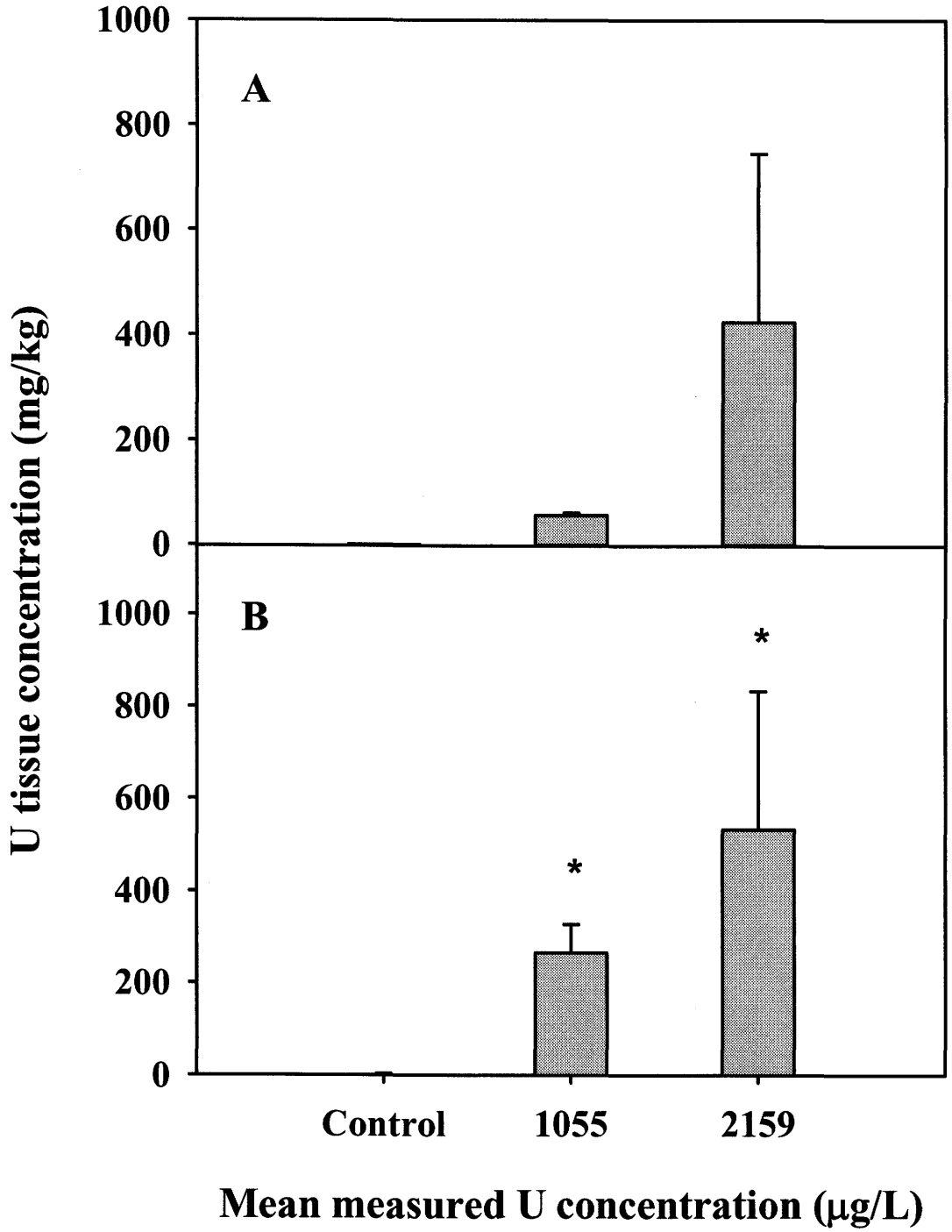

Figure 3.4. Accumulation of uranium in *Chironomus tentans* pupae (A) and exuvia (B). Data represent the mean (± SD) of two and four replicate samples, respectively.
* Significantly different from control ($p < 0.05$).

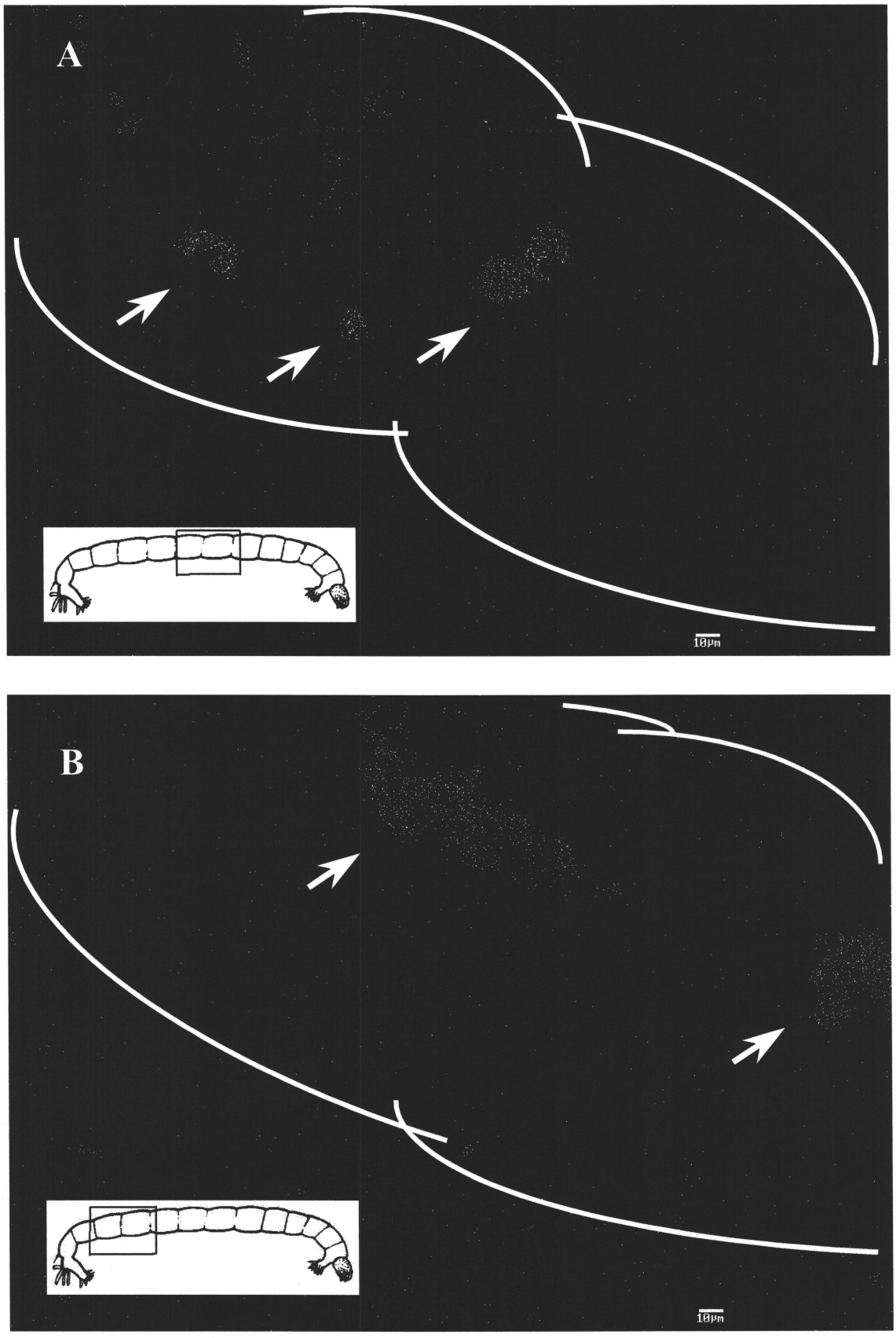

Figure 3.5. Uranium (U) localization in central (A) and posterior (B) sections of *Chironomus tentans* larvae exposed to a mean U concentration of 960 µg/L for 10 d.

3.4. Discussion

Organisms stressed by exposure to contaminants, such as U, will likely have less energy available for growth, due to the reallocation of resources into detoxification processes instead of somatic growth (Holloway *et al*, 1990; Sibly and Calow, 1989). Continued exposure to a stressor can lead to the eventual death of the organism and therefore result in changes in population dynamics.

Growth of *C. tentans* larvae was significantly reduced after 10 d of exposure to U concentrations ≥ 960 μg/L. The relationship between larval growth and U exposure concentration observed in this study ($Y = -0.073X + 0.52$; $r^2 = 0.96$) was similar to that seen in a previous U study (Refer to Section 2.3.1, Chapter 2).

In addition to reductions in growth and survival, exposure of *C. tentans* to U resulted in an overall reduction in the emergence of adult midges. Such a reduction in adult emergence caused by metal exposure has been noted in other studies (Hatakeyama, 1987, 1988; Pascoe *et al*, 1989; Williams et al, 1986). In short-lived insects, lowered emergence rates will reduce the number of adults available to participate in reproduction, which could lead to a decline in mating success and consequently to a change in population dynamics (Sibley *et al*, 1997).

The differences in U tissue accumulation among *C. tentans* life stages might be due to shedding of the exoskeleton and the physiological changes taking place during metamorphosis to the adult stage. The lack of a statistical difference in U tissue accumulation among treatments in *C. tentans* pupae

might have been due to the use of a non-parametric test associated with a low number of samples. Consequently, the probability of detecting statistical differences among the treatments was low.

The gut of aquatic insects can be divided into three regions: foregut, midgut, and hindgut. The midgut and hindgut are implicated in nutrient absorption, as well as in reabsorption of salt, water and other substances. These regions are also thought to be the main areas for metal accumulation in aquatic insects, whereas, the malphigian tubules and respiratory structures appear to store only small quantities of metal (Hare, 1992).

Other authors have also reported metal storage in chironomid tissues. Kranztberg and Stokes (1990) found lead, iron, cadmium, copper, nickel and zinc in the gut, and to a lesser extent in the anal papillae, of *Chironomus* larvae. Craig *et al.* (1998) reported cadmium accumulation in a small section of the midgut in exposed *C. staegeri* larvae. Seidman *et al.* (1986) observed that accumulation of cadmium was confined to the posterior midgut ephitelium of *C. thummi* larvae.

In this study, areas of high U accumulation were observed in the central and posterior sections of *C. tentans* larvae. These sections are consistent with midgut and hindgut localizations. However, the present results are inconclusive due to the low number of samples analyzed (n = 4). Sample preparation has been shown to be an important and sometimes limiting factor in microprobe analysis of biological tissues (Luckey and Venugopal, 1978). Interference with the electron beam from the materials used to fix the samples, leaching and

displacement of elements of interest during sample preparation, and the presence of water in the sample can lead to wrong or misleading conclusions. Thus, special attention should be paid to specific sample preparation requirements, in order to better quantify the elements under study. In addition, the electron beam used could damage the specimens, leading to the loss of samples under study. Kranztberg and Stoke (1990) for example, suggested that samples should be fast-frozen in an ethanol-dry ice bath and then freeze dried at –120 °C before fixation to possibly prevent damage of the samples under study.

3.5. Conclusions

Reductions in larval growth of 45 to 55% of controls were observed at mean U concentrations $\geq$ 960 μg/L. Uranium that accumulated in *C. tentans* immature stages was partially excreted during metamorphosis to the adult stage. The processes of U elimination, as observed here, can be explained both by physiological changes taking place during metamorphosis, as well as by shedding of the exoskeleton when molting from pupae to adult.

Although, the results from this study show that U accumulation sites can be found in the central and posterior sections of *C. tentans* larvae exposed to 960 μg U/L, the results were not conclusive due to the method selected for sample fixation. Despite the fact that X-ray microprobe analyzes is a well established technique for the study of elemental concentrations in biological samples, many problems and difficulties have been documented. In our experience, selection of

other techniques that require minimal sample preparation, improved resolution without damaging the analyzed samples, and enhanced quantification and distribution assessment of the element under study is recommended. In this regard, the used of synchrotron radiation analysis may offer a more reliable method for describing and quantifying U accumulation in aquatic invertebrates, such as *C. tentans*.

Chapter 4. Uranium uptake and depuration in *Chironomus tentans* larvae.

4.1. Introduction

Mechanisms by which essential metals are assimilated by aquatic organisms are not always sufficiently selective. Metals can therefore be accumulated by organisms whether or not those metals are essential for metabolism. As a result, non-essential metals may enter cells by transport mechanisms usually used by essential metals (Beeby, 1991; Hare, 1992).

Metal entering an insect body must first cross the membrane which separates the animal from its environment and which constitutes an important barrier to the entry of metals (Hare, 1992). Both active and passive transport mechanisms have been proposed to explain the uptake of metal from solution into aquatic invertebrates. The active transport of metals across membranes requires the expenditure of energy and can be protein carrier-mediated (e.g., copper, zinc), or occur via ion pump (e.g., cadmium, calcium), or by endocytosis (e.g., iron, lead) (Hare, 1992). Conversely, passive transport does not make use of energy and can occur by facilitated diffusion (down a concentration gradient), through protein channels and/or protein carriers (e.g., cadmium), or by passive diffusion where neutral metal species dissolve in the membrane's lipid bilayer and cross it rapidly (e.g., mercury).

Once a metal has entered the body of an aquatic invertebrate it can remain metabolically available until the physiology of the invertebrate interacts to excrete it (e.g., in feces or through the exuvia), or to detoxify it (e.g., via metallothioneins and/or insoluble metaliferous granules) (Rainbow, 1996, 2002; Hare, 1992).

An animal's regulation of internal metal concentrations can be achieved through the control of metal uptake and excretion, or through internal storage of metals in forms that are not available (e.g., granules) (Hare, 1992). Metal excretion can be increased to match the uptake rate, but ultimately the uptake rate may be so high that the excretion rate fails to match it (Rainbow, 1996). Therefore, toxicity generally occurs when the rate of metal uptake into the body exceeds the rates of physiological/biochemical detoxification and/or excretion (Rainbow, 1996).

Numerous studies on metal uptake and effects in chironomid larvae have focused on particular aspects, such as acute (e.g., survival) and chronic (e.g., growth, emergence) toxicity (Nebeker *et al*, 1984; Gauss *et al*, 1985; Kranztberg and Stokes, 1989; Anderson *et al*, 1990; Heinis *et al*, 1990; Ingersoll *et al*, 1990; Timmermans *et al*, 1992; Watts and Pascoe, 2000), metal localization (Seidman 1986; Yamamura *et al*, 1983; Kranztberg and Stokes, 1990), uptake through food and water (Hatakeyama, 1987, 1988), and partial and total life cycle experiments (Pascoe *et al*, 1989; Groenendijk *et al*, 1999; McCahon and Pascoe, 1991).

There is a particular need for more data on metal uptake, regulation and depuration in freshwater invertebrates, specifically for less commonly tested elements, such as uranium (U). Therefore, in this study, uptake and depuration rates in *Chironomus tentans* larvae exposed to U were combined with an assessment of larval growth to better understand the rates of U uptake and depuration, as well as U effects in the exposed *C. tentans* larvae. In addition, an evaluation of whether U uptake occurred via an active or passive mechanism was performed.

4.1. Materials and methods

4.2.1. General experimental procedures

Tests were conducted in a controlled environment chamber set at 23 ± 1 °C with a photoperiod of 16:8 h light:dark and an illumination intensity of 800 -1300 lux. Stock solutions of U, as $UO_2(NO_3)_2 \cdot 6.H_2O$ (Strem Chemicals Inc., MA, USA), with a concentration of 100 mg/L, were prepared with deionized water (Milli-Q®). To generate the desired U test concentration (work solution), the stock solution was diluted using test water. The test water used in these tests was Saskatoon carbon-filtered municipal water, the same as that used for culturing the test organisms (for test water quality characteristics refer to Section 2.2.1.2, Chapter 2).

Each 250-ml test beaker contained test water, gentle aeration to maintain an adequate dissolved oxygen (DO) level and a thin layer of sand (particle size 250-425 μm). Aeration was initiated 2 h after the larvae were placed in the test

beakers to allow larvae time to settle into the sand. Water quality variables such as temperature, DO, pH, alkalinity, hardness, and ammonia were evaluated throughout the tests.

4.2.2. Uranium uptake and depuration test

4.2.2.1. Study design

Uranium uptake and depuration was evaluated using second-instar larvae (10-d old) obtained from an in-house laboratory culture (see Section 2.2.1, Chapter 2). During the experimental period, larvae developed to their early fourth-instar stage. A total of 24 replicate treatment beakers plus 6 untreated controls with 10 larvae per replicate was used for this static-renewal test. Water in the test beakers was renewed every 3 d to maintain adequate water quality and relatively constant U concentrations. Samples for water quality and U concentration evaluations were taken from three beakers every 3 d prior to water renewals. The test organisms were fed daily using 1 ml of Tetramin® fish food slurry at a concentration of 6 mg/L. Food was added after animal sampling to ensure that organisms with relatively empty guts were sampled (Timmermans *et al*, 1992).

During the uptake phase of the experiment, larvae were exposed to a sublethal concentration of U (300 μg/L). At t_u = 0, 1, 3, 6 and 9 d, animals were removed from three randomly selected beakers for measurements of dry weight (dw) and U tissue concentration. After 9 d of exposure, animals from all but three of the remaining beakers were transferred to beakers containing clean

culture water and a thin layer of clean sand (250-425 μm). Aeration was again started 2 h after the larvae were added to the beakers. The three remaining beakers from the exposure phase continued with U exposure for an additional 2 d to confirm the presence of steady-state conditions at t_u = 11 d. During the depuration phase, animals were taken from three selected beakers at t_d = 3, 6 and 9 d after the animals had been transferred to clean water to evaluate U elimination and organisms dry weight.

Uranium uptake and depuration were evaluated using a first-order, one-compartment model (Rand and Petrocelli, 1985). The constant for depuration (K_d) was directly calculated from a plot of the decline (slope of the line) in U tissue concentration in *C. tentans* larvae against depuration time. Once the K_d was determined, the uptake constant (K_u) was calculated from the following equation

$$\frac{dC}{dt} = \text{uptake} - \text{loss} = K_u\,\text{Cw} - K_d\,\text{C} \qquad (4.1)$$

where, Cw = mean concentration of U in the water (mg/L), t = time in days, and C = concentration of U in the animal (mg/kg dw). Steady state occurs when the rate of uptake balances the rate of loss, so that

$$\frac{dC}{dt} = 0 = K_u\,\text{Cw} - K_d\,\text{Css} \qquad (4.2)$$

where Css = mean concentration of U in the animal at steady state (mg/kg dw). Therefore, at steady state conditions

$$\frac{K_u}{K_d} = \frac{\text{Css}}{\text{Cw}} \qquad (4.3)$$

and the bioaccumulation factor (BAF) can be defined as

$$BAF = \frac{Css}{Cw} \quad (4.4)$$

Thus, K_u was conveniently calculated from

$$BAF = \frac{K_u}{K_d} \quad (4.5)$$

The time required to reduce by one-half the U concentration in *C. tentans* tissue ($t_{1/2}$) is determined solely by K_d and was calculated using the following equation (Rand and Petrocelli, 1985)

$$t_{1/2} = \frac{\ln 0.5}{K_d} = \frac{0.693}{K_d} \quad (4.6)$$

4.2.3. Uranium mechanism of uptake test

4.2.3.1. Study design

In order to investigate whether U uptake was primarily active or passive, live and freshly killed 10-d old *C. tentans* larvae were exposed to 300 μg U/L. Animals were separated into three groups with 12 replicates each: live larvae (non-fed), live larvae (fed), and dead larvae. A total of 10 *C. tentans* larvae were added to each replicate (refer to Sections 2.2.1.2, Chapter 2 for information on test organisms). Larvae dying due to stress of handling were replaced after 4 h of being transferred to the test beakers. A total of three animals were replaced for this test.

To obtain dead organisms for the test, some larvae were euthanised using a 3-aminobenzoic acid ethyl ester solution (MS-222; SIGMA®, ON, Canada)

which anesthetized them and subsequently killed them within 2 h without any visible changes in colour, rigidity, and body shape. The use of MS-222 ensured that membrane integrity was maintained. Following exposure to MS-222, larvae were thoroughly rinsed with Milli-Q® water before being transferred to the test beakers. Animals were removed from three randomly selected beakers from each group at t = 0, 12, 24 and 48 h for measurement of U tissue concentration and larvae dry weight.

The test was a static test performed in 250-ml glass beakers. Water samples for measurement of general water quality were taken from three randomly selected beakers at 0, 12, 24 and 48 h. Uranium water concentration was only measured after 12 and 24 h. Only animals in the live (fed) group received food during the experiment. These organisms were fed daily with 1 ml of Tetramin® fish food slurry (6 mg/L).

4.2.4. Sampling procedures and processing

For details on cleaning sample vials, processing of animal samples for U and dry weight determinations, and obtaining water samples for water quality and U measurements, refer to Section 2.2.5, Chapter 2.

4.2.5. Statistical analysis

Statistical analyses were performed using the computer program SigmaStat®, version 2.03 (SPSS Inc., Chicago, IL, USA), with a 95% (α= 0.05) level of confidence. Significant differences among dead, live (fed) and live (non-

fed) larvae were assessed using two-way ANOVA with sampling time and test group as the two factors. Individual comparison of means was conducted using Tukey's Post-hoc Test.

4.3. Results

4.3.1. Uranium uptake and depuration test

Uranium concentrations in the exposure water, and routine water quality measured during the experiment, are presented in Table 4.1. Generally, U concentrations in water were in good agreement with the nominal concentration (300 μg/L) and general water quality varied only slightly throughout the test.

Survival of *C. tentans* larvae was greater than 90% in the control and in the U treatment group throughout the test. Results from the U uptake phase revealed that *C. tentans* larvae had negligible U in their tissues at the beginning of the experiment (Figure 4.1A). Upon exposure to 300 μg/L, a significant increase in the U body concentration was observed which continued throughout the exposure period. Steady state conditions appeared to have been reached after approximately 9 to 11 d of exposure.

Accumulated U was rapidly depurated after the larvae were transferred to clean water. Most of the U was eliminated within 3 d and U was almost completely depurated within 6 d (Figure 4.1A). Growth was monitored as an additional endpoint during the uptake and depuration test. It is clearly seen that an increase in U accumulation by *C. tentans* larvae was accompanied by an

impairment in larval growth. Growth was restored once larvae were transferred to clean water (Figure 4.1B).

Uranium depuration data were described by a linear regression function (r^2 = 0.82) with a significant slope (K_d), indicating a good fit to a one-compartment model (Figure 4.2). Calculated uptake and elimination parameters are presented in Table 4.2.

4.3.2. Uranium mechanism of uptake test

Measured U concentrations in water and other water quality variables are presented in Table 4.3. Measured U concentrations were in good agreement with the nominal concentration (300 μg/L). General water quality variables during the test were within expected and acceptable ranges.

No mortality was registered during the experiment, but there were significant differences in U accumulation between live (both fed and non-fed) and killed larvae (p< 0.001; Figure 4.3). After 48 h of exposure to a mean U concentration of 263 μg/L, dead larvae contained approximately 86 times more U than live larvae (non-fed and fed). The BAFs calculated for dead, live (non-fed) and live (fed) larvae were 97, 9, 13 respectively. Despite the fact that live (fed) larvae received food during the experiment, their mean U body concentration was similar to that for non-fed larvae. Accumulated U did not differ significantly between these two groups (p = 0.63).

Table 4.1. Water quality measurements at each phase of the uranium (U) uptake/depuration test. Data represent mean values ± SD of three replicate samples.

Variable	Unit	DL[c]	Control	Uptake phase	Depuration phase
U	µg/L	0.01	0.25 ± 0.03	328 ± 39.7	0.25 ± 0.03
pH	pH	0.05	7.5 ± 0.3	7.72 ± 0.22	7.55 ± 0.14
Total-Hardness	mg/L[a]	10	136 ± 4	144 ± 11	138 ± 10
Alkalinity	mg/L[a]	10	94 ± 6	90 ± 9	95 ± 11
DO[b]	mg/L	0.1	7.8 ± 0.3	7.5 ± 0.3	7.5 ± 0.1
Temperature	°C	0.1	23.7 ± 0.6	22.9 ± 0.5	22.9 ± 0.5
Ammonia	mg/L	0.005	0.84 ± 0.35	0.28 ± 0.15	0.55 ± 0.04

[a] mg/L as $CaCO_3$.
[b] Dissolved Oxygen.
[c] Analytical detection limit.

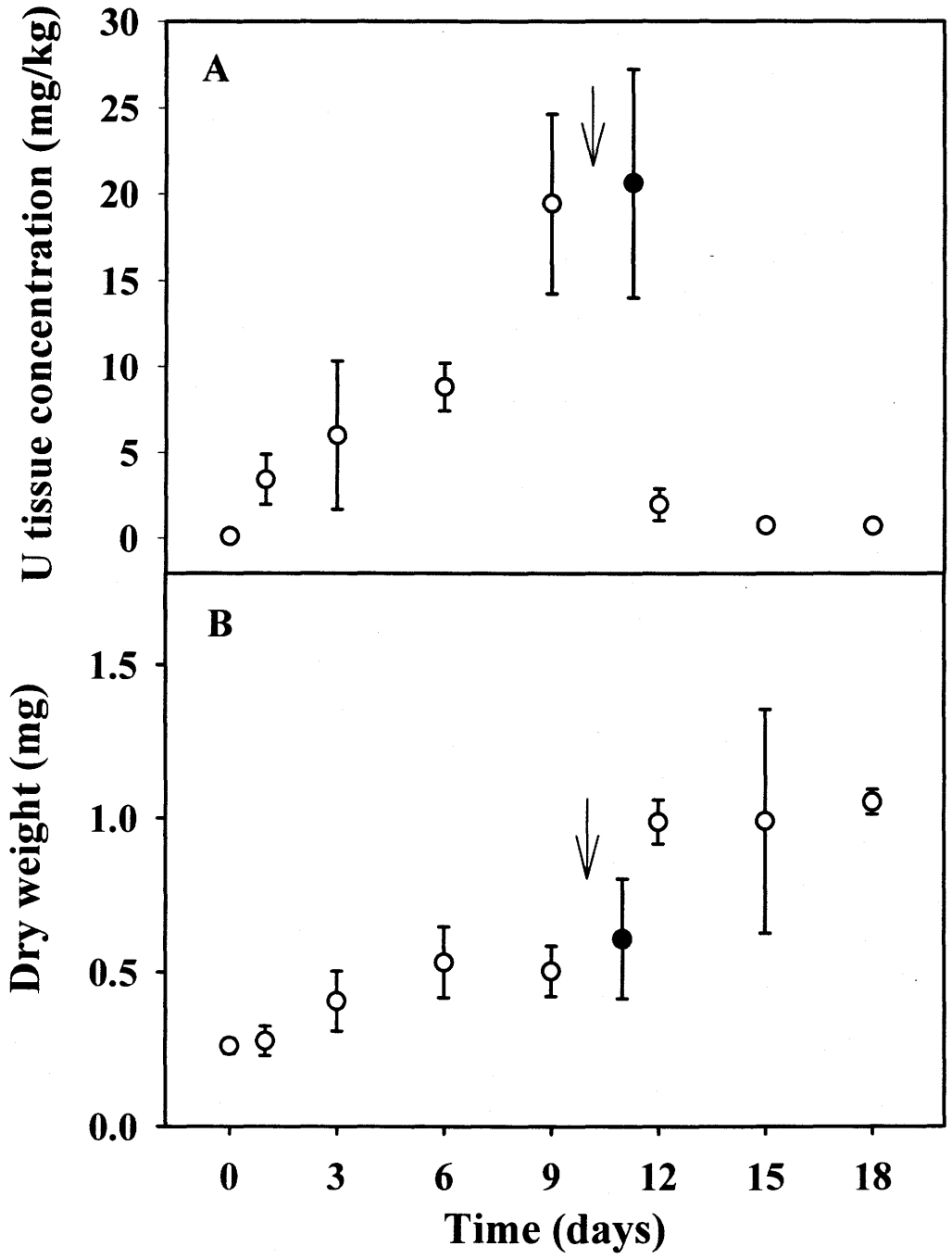

Figure 4.1. Uranium (U) uptake and depuration in *Chironomus tentans* larvae exposed to a 300 μg/L U solution. (A) Measured U concentration in test organisms, and (B) growth of *C. tentans* larvae expressed as dry weight. All data are the mean ± SD. Arrows indicate transfer of animals to clean water (depuration phase). Solid circles represent additional U exposure data at t = 11 d.

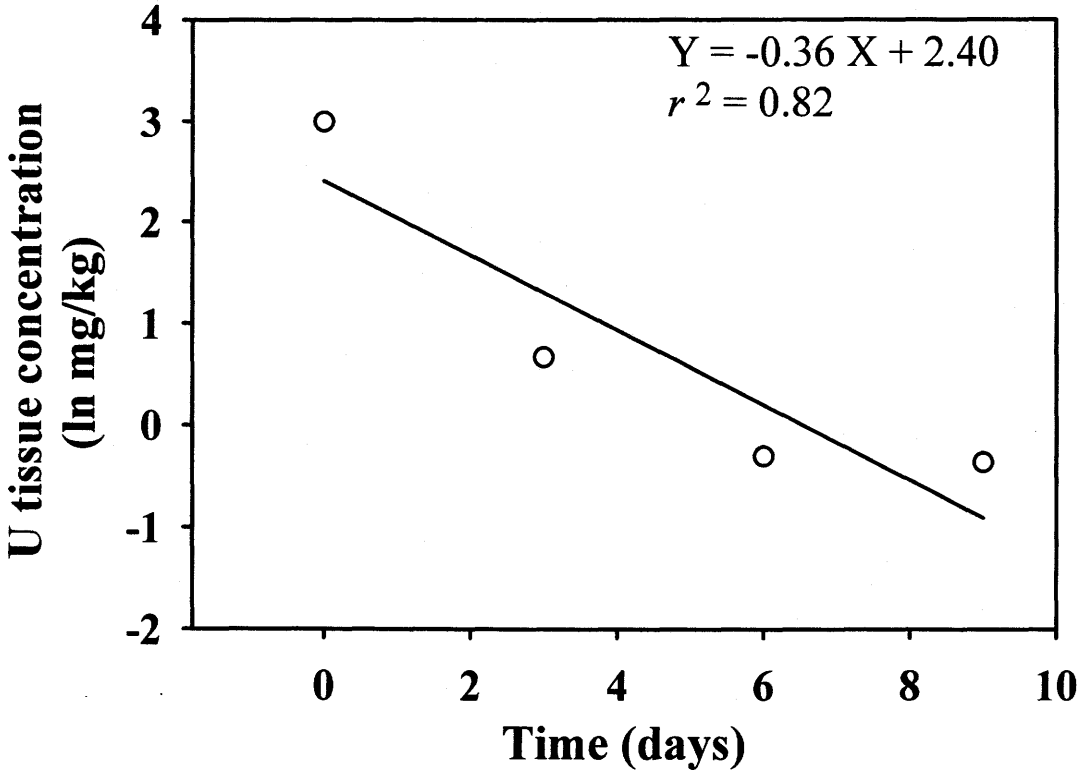

Figure 4.2. Depuration of uranium (U) from *Chironomus tentans* larvae transferred to clean water after 9 days of exposure to a nominal U concentration of 300 μg/L.

Table 4.2. Uranium uptake and depuration rate constants and associated parameters estimated with a first-order, one-compartment model (see Section 4.2.2.1 for definition of parameters).

	Uranium
K_u	20.3
K_d	0.36
Css (mg/kg dw)	20
$t_{1/2}$ (d)	1.9
BAF	56

Table 4.3. Measurements of water quality from each test group in the mechanism of uranium (U) uptake test. The data represent mean values ± SD of two replicate samples.

Variable	Unit	DL[c]	Control	Live (non-fed)	Live (fed)	Dead
U	µg/L	0.01	ND[d]	295 ± 5.7	255 ± 10	263 ± 33
pH	pH	0.05	8.32 ± 0.03	8.30 ± 0.09	8.15 ± 0.11	8.26 ± 0.12
Total-Hardness	mg/L[a]	10	179 ± 6	184 ± 8	183 ± 7	175 ± 20
Alkalinity	mg/L[a]	10	89 ± 6	89 ± 6	93 ± 2	88 ± 7
DO[b]	mg/L	0.1	8.8 ± 0.5	8.1 ± 0.5	7.5 ± 0.2	7.6 ± 0.24
Temperature	°C	0.1	23 ± 0.3	22.8 ± 0.9	22.9 ± 0.5	22.6 ± 0.6
Ammonia	mg/L	0.005	0.65 ± 0.49	0.25 ± 0.11	0.53 ± 0.24	0.26 ± 0.15

[a] mg/L as $CaCO_3$.
[b] Dissolved Oxygen.
[c] Analytical detection limit.
[d] Non detectable.

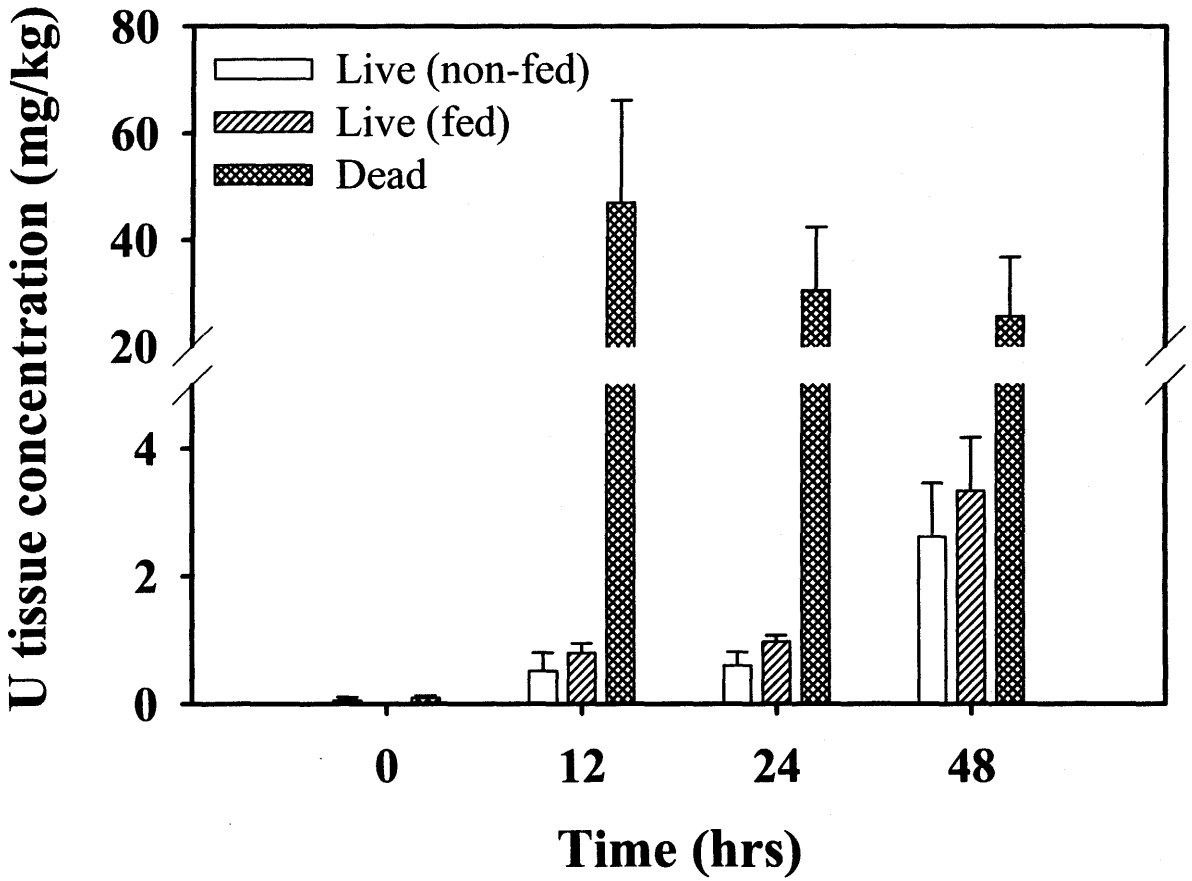

Figure 4.3. Uranium (U) accumulation in live and dead *Chironomus tentans* larvae after exposure to 300 μg U/L for 48 h. Bars represent the mean ± SD of three replicate samples.

4.4. Discussion

Evaluation of several aspects of U uptake/depuration processes and effects on larvae of *C. tentans* was made in this study. Results of the U uptake and depuration test, as well as the mechanism of U uptake test, are discussed separately below.

Uranium uptake and elimination. Uranium accumulation in exposed *C. tentans* larvae increased substantially over time with steady state conditions reached in about 9 to 11 d. Organisms transferred to uranium-free water showed a rapid decline in U tissue concentration during the first 3 d of depuration, followed by much slower losses over the next 6 d. This change in the slope of the depuration curve is consistent with the expected pattern for a two-compartment model, where U is lost quickly from the "fast" compartment (e.g., haemolymph) and slowly from the "slow" compartment (e.g., metal bound to tissues and metallothineins) (Dawson *et al*, 2003; Yamamura *et al*, 1983). Consequently, a short half-life (1.9 d) was calculated for U depuration in *C. tentans* larvae.

In the present study, a first-order, one-compartment model did not always provide a good description of the experimental data. However, in the special case where the peripheral compartment (slower compartment) depurates rapidly (depuration from the slower compartment > uptake from the faster compartment), the model reduces to a one-compartment model (Rand and Petrocelli, 1985). This may be consistent with the hypothesis that most of the U remains un-bound in the haemolymph and that U incorporation into the tissues is a slow process.

Some authors have suggested that rapid metal elimination may be explained by the fact that larval growth contributed to an overall decline of metal concentrations in the insect's tissues (Timmermans *et al*, 1992, Hare, 1992). However, such growth dilution clearly did not explain the rapid loss of U observed in this study. In addition, the concentration of U in *C. tentans* tissues in this test was measured taking into consideration the influence of body weight.

Metal accumulation can occur only if the rate of metal uptake by an organism exceeds the rate of elimination (Rand and Petrocelli, 1985). Regulation at the whole-body level is not a common strategy for invertebrates, apparently being restricted to some essential metals (e.g., zinc). In the present study, U appeared not to be well regulated in *C. tentans* larvae, as evidenced by the continued increase in U body concentration in larvae during the exposure period (9-11 d) and the insignificant relationship between BAFs and U exposure concentration observed in a previous experiment (see Chapter 2, Section 2.3.1.1)

Larval growth ceased during the end of the U exposure period, reaching a plateau within 6 to 9 d of exposure. Steady growth was again observed during the depuration period in clean water. Exposure of *C. tentans* larvae to U could trigger detoxification mechanisms to resist toxic effects, but such mechanisms are likely to be energy consuming and hence take place at the expense of somatic growth. Conversely, in the absence of U the reallocation of resources to detoxification mechanisms is not required and an increase in growth rate would again be observed.

Uranium mechanism of uptake. Use of live and killed insect larvae to obtain more insight into the possible mechanisms of metal uptake is well documented (Dodge and Theis, 1979; Hare, 1992; Timmermans *et al*, 1992). Accumulation of metals in an insect's body may result in metal concentrations in live larvae being the same as in dead larvae, indicating that active uptake is of minor importance in metal accumulation. Conversely, accumulation in live larvae may be greater than in dead larvae indicating an active uptake process. Another option in which metal concentrations in dead larvae may exceed live larvae is also possible (Timmermans *et al*, 1992). This was the case for *C. tentans* larvae exposed to U in this study. Dead larvae contained approximately 86-90 times more U than live larvae, with significant differences between these groups ($p < 0.001$). Timmermans *et al.* (1992) found similar results for *C. riparius* exposed to cadmium and lead for 24 h. In their study, dead larvae generally contained significantly more cadmium and lead than live larvae. However, distinct differences between essential (zinc and copper) and non-essential (cadmium and lead) metals were noted. When larvae were exposed to zinc, dead larvae accumulated limited amounts of this metal compare to living larvae.

It can be hypothesized that this greater accumulation in dead larvae might result from one or a combination of the following factors: increased permeation of metals throughout membranes in dead animals due to membrane disruption (e.g., alterations in membrane electrical potential, loss of membrane integrity); lack of the protective nature of the *C. tentans* larval tube against chemical toxicants (e.g., dead larvae did not have tubes) (Halpern *et al*, 2002a, 2002b);

and/or a passive process of metal uptake associated with an active mechanism of metal elimination (e.g., dead larvae lack the required metabolism and energy for the active processes of metal elimination). The lack of significant differences in U accumulation between fed and non-fed live larvae indicated that food was an unimportant source of U uptake for *C. tentans* larvae. Furthermore, U binding to food particles seemed to be negligible in this test with a dissolved U water concentration similar to its nominal value in all treatments.

4.5. Conclusion

Uranium concentrations in *C. tentans* larvae increased during the time of exposure (9-11 d), indicating that this non-essential metal might not be well regulated. Transfer of exposed larvae to clean water, resulted in a rapid decline in U tissue concentration ($t_{1/2}$ = 1.9 d). This may be attributed to the hypothesis that the bulk of this metal was not tissue-bound and consequently was rapidly eliminated when larvae were transferred to U-free water. In addition, exposure of *C. tentans* larvae to U (300 μg/L) resulted in reduced growth. This suggested that U might reduce the growth rate of exposed *C. tentans* larvae by relocation of resources into detoxification mechanisms at the expense of somatic growth.

Although U accumulation occurred in both live and dead larvae during these experiments, the amount of U accumulated by dead *C. tentans* larvae was significantly higher than that accumulated by live organisms. A possible explanation for this could be a passive mechanism of uptake coupled with an active mechanism of U depuration. However, from the data collected here no

conclusive evidence regarding specific U uptake and depuration mechanisms in *C. tentans* larvae can be provided.

Chapter 5. General discussion

5.1. Project summary

Canada is the largest uranium (U) producer in the world, generating one third of the world's U output (World Nuclear Association, 2003; Cameco, 2001), with all of the country's production coming from Saskatchewan. Mining of U ore can result in its release to natural bodies of water. In aquatic systems, benthic invertebrates are often the most highly exposed organisms, due to the propensity of sediments for accumulating U. One of the most commonly used invertebrate species in water and especially sediment toxicity testing is the midge, *Chironomus tentans*. This freshwater macroinvertebrate has been extensively used as a test species because of its short life cycle, the variety of developmental endpoints that can be monitored, its wide distribution and ecological relevance, the ease with which it can be maintained and cultured in the laboratory, and its sensitivity to a broad range of contaminants (U.S EPA, 2000; Sibley *et al*, 1997, Environment Canada, 1997; Craig *et al*, 1998; Armitage *et al*, 1995; Benoit *et al*, 1997).

The primary objective of the research presented in this thesis was to determine the effects of U on the aquatic invertebrate *Chironomus tentans*. The

first approach was to assess the effects of U over the majority of the life cycle of *C. tentans*, to evaluate the concentration of U in different life stages, and measure the distribution of this metal in larval tissues. The second approach was to evaluate the rates and possible mechanisms of U uptake and depuration in order to better understand the relationship between U accumulation and its toxic effects.

5.1.1. Life cycle test

Chronic U toxicity was assessed by conducting laboratory studies using a static-renewal test design. Organisms were exposed to U from 10-d old until adult emergence. During this study, U significantly affected growth of the exposed organisms at U concentrations $\geq$157 μg/L. It is hypothesized that these organisms had less energy available for growth than un-exposed organisms, due to the relocation of resources into detoxification mechanisms.

Effects on growth in *C. tentans* larvae significantly altered adult emergence success. Emergence reductions and delay in time to emergence were observed even at the lowest U concentration (31 μg/L). However, statistically significant effects on emergence were only observed at U concentrations $\geq$175 μg/L. It is clear that changes in larval growth can have significant effects on population size by reducing the number of adults available to participate in reproduction (Sibley *et al*, 1997). Studies assessing the toxicity of metals therefore often incorporate growth as a primary effects endpoint. However, the nature of the relationship between growth and reproduction is poorly understood in *C.*

tentans (Sibley *et al*, 1997). In this study, the ecological relevance of stress-induced changes in growth of *C. tentans* was assessed by evaluating the relationship between growth and reproductive endpoints.

Exposure to U did not affect the reproductive success of *C. tentans* adults (e.g., no effects were observed in the number of produced and hatched eggs in successfully emerged *C. tentans* adults among treatments). However, exposure of *C. tentans* immature stages (F_0) to U led to a significant decrease in 10-d growth in F_1 generation larvae that was similar to that observed for the directly exposed F_0 generation. The environment of the parental generation apparently had an effect on the development of the next generation through environmentally induced parental effects. Changes in future generations can therefore be expected as soon as a single generation is exposed to unsafe levels of U. In this study, U had significant effects on the F_1 generation at a mean concentration of 856 μg U/L.

With respect to bioaccumulation, U that accumulated during early *C. tentans* life stages was partially excreted during metamorphosis to the adult stage. However, U depuration was not complete, suggesting that U transfer from aquatic to terrestrial environments could take place after adult emergence.

5.1.2. Uranium distribution, uptake and depuration

5.1.2.1. Uranium tissue distribution

A better understanding of the patterns of U distribution should assist in identifying possible mechanisms of U exchange between the animal and its

environment, and identify the organs/tissues in which primary effects from U exposure might be observed. To this end, whole-body measurements of U combined with X-ray microprobe analysis were used in an effort to identify areas of high U accumulation in *C. tentans*. Although the X-ray microprobe analysis was only marginally successful and only limited data were obtained, the principal sites of U accumulation did appear to be the central and posterior portion of larvae, consistent with the midgut and hindgut regions. Thus, these sections of the digestive tract seem to be important sites for U accumulation in this invertebrate, consistent with their function as absorption sites.

5.1.2.2. Uranium uptake and depuration

Uranium uptake and depuration studies, combined with chemical analysis and effects on growth, were conducted to better understand the possible mechanisms of U uptake and depuration in *C. tentans* larvae, as well as to investigate the relationship between metal uptake/depuration and its biological effects (e.g., growth reductions). In this study, U accumulation in dead organisms was observed to be greater than in live larvae, suggesting a passive mechanism of U uptake and/or an active mechanism of U depuration. In addition, U appears not to be actively or well regulated by *C. tentans* larvae, since the concentration of this metal in larval tissues increased over time, approximating steady states conditions after 9 to 11 d of exposure. Accumulated U was rapidly depurated within 3 d of the larvae being placed in clean water, indicating that the bulk of this metal might not be tissue-bound or otherwise strongly sequestered in *C. tentans*.

The reallocation of resources into detoxification mechanisms generally occurs at the expense of somatic growth. For example, a plateau was observed in *C. tentans* growth after 6 to 9 d of exposure to U. This effect was reversible in the absence of U, with an increase in *C. tentans* growth again noticeable after 3 d of being transferred to clean water.

5.1.3. Integration of results

Uranium uptake in *C. tentans* from aqueous solution could be explained by a passive transport mechanism. This process does not require the expenditure of energy and could be passive diffusion (where neutral species of metals dissolve in the membrane), or facilitated diffusion (down a concentration gradient, through protein channels and/or protein carriers). The later is the most likely mechanism to explain U uptake in *C. tentans*. Once inside the insect body, U has to be detoxified or excreted rapidly to avoid toxicity. Reallocation of resources into detoxification mechanisms generally takes place at the expense of somatic growth. The observed growth reduction in directly-exposed *C. tentans* larvae reduced not only the number of adults available to participate in reproduction, but also reduced the growth rate of the next generation larvae through environmentally induced parental effects.

The chronic toxicity of U and the ecological implications of U exposure to aquatic invertebrates have not been extensively investigated. These laboratory studies give a more insightful picture of the potential toxic effects of U to aquatic invertebrates. Downstream of U mines, the U concentration in water could

range from 100 to approximately 500 μg U/L (Environment Canada and Health Canada, 2001; Hynes *et al*, 1987; Melville, 1995). In our study, the LOEC (growth) for *C. tentans* exposed to U was 157 μg/L, therefore it is possible that aquatic invertebrates living downstream U mines could experience effects on growth and consequently on adult emergence. Reduction in the number of adults by approximately 40 to 50%, and a delay in time to emergence, could also be expected at the observed U concentrations in lakes and rivers impacted by U mining, possible leading to a decline in mating success and consequently alteration in population dynamics.

In addition, high concentrations of U in tissues of *C. tentans*, and by extension in other midge species, may occur as a result of U exposure at contaminated sites. Furthermore, U will not be completely depurated during immature stages and may enter into the terrestrial food webs after adult emergence. When the *C. tentans* larvae were transferred to U-free water, the accumulated U was almost completely depurated and growth increased noticeably. Thus, the potential growth effect of U in aquatic invertebrates could be rapidly reversed in the absence of U. Hence efforts should be made to reduce the concentration of U in mining effluents and tailings. However, U effects on the parental generation may lead to effects on future generations, indicating that even if the U concentration in the effluents was diminished, changes in future generations can be expected as soon as a single generation is exposed to unsafe levels of U.

The release of effluents from U mines not only increases the concentrations of U in the receiving environment, but also favors the occurrence of significant concentrations of the toxic species of U (UO^{+2}, UO_2OH^+) due to the release of elevated concentrations of sulphate (UO_2SO_4). The presence of elevated concentrations of sulphate not only decreases the concentration of carbonates species of U but, also increases the occurrence of its toxic forms. The evidence presented in this thesis supports the conclusion that releases of U at operating U mines are potentially harmful to aquatic biota, especially at concentrations $\geq$157 μg U/L.

5.2. Future research considerations

This thesis documents the effects of U in the aquatic invertebrate *C. tentans* and shows that this non-essential metal poses a potential hazard to aquatic organisms in environments receiving U mine effluent. Also, novel information regarding the possible mechanisms of U uptake and depuration, as well as tissue distribution, is provided. However, there are prospects for additional research to be explored.

Investigations on the uptake and depuration of metals by freshwater invertebrates, in relation to metal exposures, are among the first step in assessing the significance of metals in aquatic systems. Although in this thesis I postulated some hypotheses about the possible mechanisms of U uptake and depuration, the experimental proof of actual uptake and elimination strategies in invertebrates is often lacking. Additional research is required to fully understand

uptake and depuration mechanisms, and metal kinetics, considering the important of these processes in both, metal toxicity and accumulation in freshwater invertebrates. Additional research is therefore required to address issues raised in this study such as, the fundamental mechanism(s) of U toxicity in *C. tentans,* the possible target organs of toxicity, the remobilization of U within tissues, and major sites of U accumulation in this invertebrate.

It was reported in this thesis that exposure of the parental generation (F_0) to U led to effects in F_1 *C. tentans* larvae that were never directly exposed to U. Therefore, further studies that address issues regarding U transgenerational effects are needed. Consecutive generation studies evaluating changes in life cycle, tolerance caused by prior U exposure, and offspring viability are required in order to better understand long term effects of U on invertebrate development.

A major difficulty in establishing the effects of contaminants on benthic organisms is in differentiating between those impacts caused by contaminated sediments and those by contaminated water. This study focused primarily on U exposure from water, however, it will be important to evaluate different routes of U exposure, such as food as a potential metal source. Such information would be crucial in evaluating the potential toxic effects of U within food webs. Also, U exposure through sediment and pore-water contact should be considered in order to provide a more complete and realistic assessment of the risk caused by this metal in aquatic systems.

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