Uptake of waterborne selenite, and its toxic effects, in the water flea (*Daphnia magna*), Westslope cutthroat trout (*Oncorhynchus clarkii lewisi*) and rainbow trout (*Oncorhynchus mykiss*)

by

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Abstract

Selenium is an essential element, playing an important role in many physiological processes. However, it possesses a narrow margin between essentiality and toxicity. In aquatic systems, selenium is increasingly identified as a trace element of major concern with levels exceeding regulatory guidelines detected. The major sources of anthropogenic selenium are the coal mining and agricultural industries. Selenium exposure in aquatic biota results in bioaccumulation and is associated with toxic effects such as teratogenesis, tissue pathologies, oxidative stress, ionoregulatory and enzymatic disruption, reproductive alterations and even death. Although the diet is the main route of selenium exposure to higher organisms, aqueous exposures can still contribute to accumulation and subsequent toxicity. There is, however, little mechanistic understanding of how waterborne selenium is taken up and accumulated in aquatic organisms. There are also limited data regarding how water chemistry can affect these processes, and how accumulation relates to toxic effects. The relationship between accumulation and toxicity is an important consideration for the environmental risk assessment of selenium. The overall aim of this thesis is to advance our mechanistic understanding of waterborne selenite uptake and how water chemistry may affect accumulation in aquatic species.

To assess uptake mechanisms of waterborne selenite in a primary consumer, *Daphnia magna* were exposed to increasing aqueous selenite concentrations with varied water chemistries. At concentrations found in heavily contaminated areas, selenite uptake was mediated by a phosphate transporter while at higher, less environmentally-relevant concentrations, selenite uptake was likely mediated by a bicarbonate transporter.

To investigate selenite handling in fish, and to determine if patterns of selenite accumulation are conserved in fishes, Westslope cutthroat trout (*Oncorhynchus clarkii lewisi*) and

rainbow trout (*Oncorhynchus mykiss*) were exposed to increasing aqueous selenite with varied water chemistries as per the *D. magna* study. Increasing phosphate in the water mediated an inhibition of selenite accumulation in the gills and liver of Westslope cutthroat trout, an outcome that contrasted with the lack of effect of phosphate in rainbow trout.

The sensitivity of Westslope cutthroat trout to selenium was assessed via exposure to a graded concentration series of waterborne selenite. A median lethal (LC_{50}) value of 15.55 mg L^{-1} was determined. Sub-lethal biochemical effects were also examined. Protein carbonylation, a marker of oxidative damage, was significantly reduced in the gill and liver indicating an improved oxidative status associated with sub-lethal selenium exposure. This effect was not mediated by changes in glutathione peroxidase, as activity of this important antioxidant enzyme remained unchanged in gill and liver. Activities of the key ionoregulatory enzymes Na^+/K^+-ATPase and H^+-ATPase activity were upregulated in the gills, but there was no effect of waterborne selenite on lactate dehydrogenase, a key marker of metabolic status.

Uptake and accumulation of waterborne selenite in *D. magna* and *O. clarkii lewisi* were consistent with the original hypothesis of anionic-mediated transport. However, accumulation in *O. mykiss* was not consistent with anion-dependence. Furthermore, the relative sensitivity of Westslope cutthroat trout was lower than expected. However, sub-lethal toxic endpoints were still altered, suggesting aqueous exposures may still be of relevance and concern to this species.

Overall, this thesis contributes to the knowledge of the mechanisms of uptake and accumulation of waterborne selenite in aquatic species and provides a better understanding of the sensitivity of a previously unstudied fish species to a prominent, ubiquitous contaminant. The findings of this research suggest that specific water chemistries could alter the toxicological impacts of selenite in contaminated waters. Furthermore, this thesis provides support that

waterborne exposure results in significant accumulation and this remains a concern for the health of local aquatic species. These results also provide insight into potential protective measures and can help inform water quality management plans to help conserve water bodies susceptible to selenium contamination.

Preface

Chantelle Klaczek is the principal researcher for all work within this thesis. All experimental work was conducted at the University of Alberta, Edmonton, Canada, in the Department of Biological Sciences. The entirety of this thesis was supervised by Dr. Greg Goss and Dr. Chris Glover. All experiments conducted on live organisms (*Oncorhynchus clarkii lewisi* and *Oncorhynchus mykiss*) were approved by the University of Alberta Animal Care Committee through AUP00004068.

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The selenium water sample analysis for the 96-h acute exposures outlined in Chapter 4 were done in collaboration with the Biogeochemical Analytical Service Laboratory within the Department of Biological Sciences at the University of Alberta. Sample analysis was conducted via Inductively Coupled Argon Plasma- Optical Emission Spectrometer (ICP-OES) by Alvin Kwan and Dr. Mingsheng Ma.

Chapter 2 has been previously published in Conservation Physiology where the biographical details of this chapter are listed below and indicated at the beginning of the chapter. The roles of all authors for this chapter along with specific contributions are described below. Chapter 3 and 4 have not yet been published as of the submission of this work to the University of Alberta.

Chapter 2:

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This work was conceived and designed by CEK, GGG, CNG. Roles and authorship contributions for each author are explained as follows: CEK- Conceptualization, Investigation, Writing- original draft, Writing- review and editing. GGG- Conceptualization, Supervision, Resources, Writing- review and editing. CNG- Conceptualization, Investigation, Supervision, Resources, Writing- review and editing.

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List of Abbreviations

%	percent
~	approximately
°C	degrees Celsius
μC_i	microcurie
$\mu g\ g^{-1}$	microgram per gram
$\mu g\ L^{-1}$	microgram per litre
µL	microliter
µM	micromolar
AChE	acetylcholinesterase
ACTH	adrenocorticotropic hormone
AE1	anion exchanger 1
AspAT/AlaAT	aspartate/alanine aminotransferase
ATP	adenosine triphosphate
ATPase	adenosine triphosphatase
BCF(s)	bioconcentration factor(s)
$C_6H_{11}KO_7$	potassium gluconate
$C_6H_{11}NaO_7$	sodium gluconate
Ca	calcium
$CaCl_2 \cdot 2H_2O$	calcium chloride dihydrate
$CaSeO_4$	calcium selenate
CAT	catalase

CCME	Canadian Council of Ministers of the Environment
CI	confidence interval
Cl^-	chloride ion
COSEWIC	Committee on the Status of Endangered Wildlife in Canada
cpm $pmol^{-1}$	counts per minute per picomole
cpm	counts per minute
CRH	corticotropin releasing hormone
d	days
DIDS	4,4’-diisothiocyano-2,2’-stilbenedisulfonic acid
DMSO	dimethyl sulfoxide
DNPH	2,4-dinitrophenylhydrazine
DO	dissolved oxygen
DTT	dithiothreitol
EC_{50}	median effect concentration
EDTA	ethylenediamine tetraacetic acid
EGTA	ethylene glycol tetraacetic acid
g L^{-1}	gram per litre
g	gram
g	gravitational force
GOT	glutamic oxalate transaminase
GPT	glutamic pyruvate transaminase

GPx	glutathione peroxidase
GRs	glucocorticoid receptors
GSH	glutathione
GST	glutathione-s-transferase
h	hours
H^+	hydrogen ion
H_2O_2	hydrogen peroxide
HB	hemoglobin
HCl	hydrochloric acid
HCO_3^-	bicarbonate
Hct	hematocrit
HEPES	4-(2-Hydroxyethyl)-1-piperazine ethanesulfonic acid
$HSeO_3^-$	hydrogen selenite
HSP70	heat shock protein 70
ICP-OES	Inductively Coupled Argon Plasma- Optical Emission Spectrometer
K	potassium
K^+	potassium ion
KCl	potassium chloride
L	litre
LC_{50}	median lethal concentration
LDH	lactate dehydrogenase

LOAEC	lowest observed adverse effect concentration
LOEC	lowest observed effect concentration
M	molar
M kg y^{-1}	million kilogram per year
MDA	malondialdehyde
MDH	malate dehydrogenase
MES	2-(N-morpholino)ethanesulfonic acid
mg kg^{-1}	milligram per kilogram
mg L^{-1}	milligram per litre
Mg	magnesium
mg	milligram
$MgCl_2 \cdot 6H_2O$	magnesium chloride hexahydrate
$MgSO_4 \cdot 7H_2O$	magnesium sulfate heptahydrate
min	minutes
mL	millilitre
mL^{-1}	per milliliter
mm	millimetre
mM	millimolar
MΩ	megaohm
n	sample size
Na	sodium
Na^+	sodium ion
Na_3PO_4	trisodium phosphate

NaCl	sodium chloride
NAD^+	nicotinamide adenine dinucleotide
NADH	reduced nicotinamide adenine dinucleotide
NADPH	reduced nicotinamide adenine dinucleotide phosphate
$NaHCO_3$	sodium bicarbonate
NCBI	National Center for Biotechnology Information
NKA	Na^+/K^+-ATPase
nm	nanometer
nM	nanomolar
NRCSS	National Research Council (US) Subcommittee on Selenium
OECD	Organization for Economic Cooperation and Development
p	probability
PC	protein carbonyl(ation)
PEP	phosphoenolpyruvate
PFA	phosphonoformate
PK	pyruvate kinase
pmol g^{-1}	picomole per gram
pmol kg^{-1} h^{-1}	picomole per kilogram per hour
pmol kg^{-1}	picomole per kilogram
pmol mg^{-1}	picomole per milligram

pmol	picomole
PO_4	phosphate
ppm	parts per million
PVC	polyvinyl chloride
RBC	red blood cells
RBT	rainbow trout
ROS	reactive oxygen species
s	seconds
Se(IV)	selenite
Se(VI)	selenate
Se^{75}	selenium-75
SeCys	selenocysteine
SEID	sucrose, EGTA, imidazole buffer + sodium deoxycholate
SeMet	selenomethionine
SeNP	selenium nanoparticles
SeO_3^{2-}	selenite ion
SeO_4^{2-}	selenate ion
SLC26A6	solute carrier family 26 member 6
SLC34	solute carrier family 34
SLC39A8	solute carrier family 39 member 8
SLC4	solute carrier family 4
SLC4A1	solute carrier family 4 member 1

SOD	superoxide dismutase
TBARS	thiobarbituric acid-reactive substances
TCA	trichloroacetic acid
TMS	tricaine methanesulfonate
U mL^{-1}	units per millilitre
USEPA	United States Environmental Protection Agency
WBC	white blood cells
WCT	Westslope cutthroat trout
x	times
YCT	yeast, cereal leaf, trout chow mix
ZIP8	zinc-regulated transporters, iron-regulated transporter-like proteins member 8

Chapter 1
General introduction

1.1 Selenium

Selenium (Se) is the 34th element on the periodic table and was discovered in 1817 by Jöns Jacob Berzelius (Hatfield et al., 2012). It is one of 5 elements that make up group 16 on the periodic table, also termed the chalcogen group, which includes oxygen, sulfur, selenium, tellurium, and polonium. Unlike a true metal, selenium forms oxyanions in aqueous solutions rather than cations, resulting in it displaying relatively high solubility (Young et al., 2010). However, it can display conductive properties which is more similar to true metals than non-metals (Henkels, 1951). As selenium has properties that resemble both metals and non-metals (Young et al., 2010), it is often classified as a metalloid (National Research Council (US) Subcommittee on Selenium (NRCSS), 1983; Young et al., 2010).

Selenium has the unique property of photoconductivity making it a valuable element for industrial use (Qamhieh et al., 2005). Specifically, there has been increased use and interest in the application of selenium for medical diagnostic imaging (e.g., Kasap & Rowlands, 2000). It is also used in photocopiers, solar cells and rectifiers due to its catalytic properties (Hatfield et al., 2012). Selenium also exhibits many biological roles, being an essential nutrient for organisms. Indeed, many enzymes and proteins rely on selenium for proper functionality (Frost & Lish, 1975; Reddy & Massaro, 1983). For example, there are selenoproteins and a selenium dependent glutathione peroxidase (GPx) that are vital for homeostasis and can play protective roles against oxidative damage in organisms (Frost & Lish, 1975; Reddy & Massaro, 1983). Research investigating selenium has been on a steep incline since the 1990's due to its essential element properties. However, there is a growing recognition of its toxicological risk to aquatic organisms at higher environmental concentrations. The threats of selenium to aquatic biota is largely a consequence of anthropogenic activities, a factor that also drives research (Hatfield et al., 2012).

1.1.1 Sources and concentrations of selenium in the environment

Selenium is a naturally occurring trace element. It is an important component in geological material including igneous rocks, shales, sandstones and limestones (NRCSS, 1983; Wilber, 1980). Specifically, selenium concentrations are highest in minerals such as chalcopyrite, borite and pyrite (Cooper et al., 1970; NRCSS, 1983). For example, concentrations of selenium ranging from 500-2,100 mg kg^{-1} have been found in these minerals (NRCSS, 1983). However, in rocks themselves selenium is less concentrated and ranges of 0.24-277 and 2-130 mg kg^{-1} have been documented in global shales and sandstones respectively (NRCSS, 1983).

Selenium is naturally found in water bodies when weathering processes cause it to leach from these rocks and minerals. Concentrations of selenium in natural waters are well below those found in the rocks and minerals themselves. Typically, total selenium levels in environmental waters range from 0.1-400 µg L^{-1} (Table 1.1). However, elevated levels of selenium have been found in waters downstream of anthropogenic activities and industries (Table 1.1). For example, practices such as agriculture, oil refining, and mining activities have all been documented to release an estimated 6.4 M kg y^{-1} selenium, leading to increased concentrations in natural waters (USEPA, 2016; Young et al., 2010). One of the two largest contributors to selenium mobilization and introduction to the water environment is irrigation of selenium rich soils, which results in selenium run-off into neighbouring waters (El-Ramady et al., 2015; USEPA, 2016). For example, selenium concentrations of 140-1400 µg L^{-1} have been documented in agricultural drainage waters in the United States (Table 1.1; Chapman et al., 2010; Santos et al., 2015; Zhang et al., 2005). Surface mining of fossil fuels and metals is another of the largest contributors to selenium accumulation in aquatic systems (Selinus, 2013; USEPA, 2016). Concentrations ranging from 3 µg L^{-1} to 2,700 µg L^{-1} have been documented in various mine wastewaters (Table 1.1). Specifically, some have argued

that coal mining and combustion is the leading source of selenium contamination in natural aquatic ecosystems (Coleman et al., 1993; Selinus, 2013). Indeed, mining inevitably brings underground deposits to the surface which are commonly enriched with selenium. These rocks are crushed, increasing the surface area to volume ratio, allowing increased weathering processes, ultimately causing selenium to be released. Furthermore, the combustion of coal results in large quantities of fly ash which is usually disposed of as a water-based slurry into specific ponds which are commonly drained into natural rivers (Fulekar & Dave, 1986; Lokeshappa & Dikshit, 2011; Selinus, 2013; Sharma et al., 1989). Fly ash particulate also has a very high surface area to volume ratio resulting in high adsorption of selenium, concentrating selenium to levels that are 4-10 times those of the parent material (Young et al., 2010). Thus, given that selenium is present in large quantities and has a high leaching rate, draining and runoff from these fly ash ponds often results in it entering the environment at high and concerning concentrations (Querol et al., 2001; USEPA, 2016).

Given that selenium is an essential nutrient for all biota, its occurrence in aquatic systems is important for the nutrition of aquatic organisms. Specifically, it plays a key role as a co-factor for multiple enzymes and proteins (Conde & Alaejos, 1997). For example, there are selenocysteine (SeCys) containing selenoproteins such as thioredoxin reductase and selenoprotein P which are important for antioxidant defense mechanisms (Hatfield et al., 2012; Holben & Smith, 1999). Furthermore, there are selenium dependent enzymes like GPx and thyroid hormone deiodinases, which are important for antioxidant processes, growth, development and metabolism (Holben & Smith, 1999). If an organism experiences selenium deficiency, it can result in a decrease of the activity of these proteins and enzymes. However, the concentration for optimal physiological health in fish can vary greatly. For example, previous studies have demonstrated that dietary levels

between 0.1-2 mg kg^{-1} are recommended to avoid negative consequences in fish species whereas even moderate excesses in selenium are known to elicit toxicity to biota (Prabhu et al., 2016; see section 1.3).

Table 1.1 Global concentrations of selenium in water and wastewater.

Region	Sampling site	Total Se Concentration ($\mu g\ L^{-1}$)	Reference
Africa	Lake Ziway water	0.83-10.4	Merga et al., 2020
Canada	Sudbury, Ontario lakes	0.1-0.4	Nriagu & Wong, 1983
	Mine effluent, Quebec	65	Etteieb et al., 2021
	Alberta unimpacted surface waters	0.3-0.7	Beatty & Russo, 2014; Environment and Climate Change Canada, 2022
	Atlantic provinces unimpacted surface waters	0.01-1.0	
	Elk River, British Columbia, wastewater	>300	
	Elk River, British Columbia, surface water	50-80[a]	Chapman et al., 2010
	Treated mine effluent	4.9-110	BEAK, 2002
	Luscar Creek, Alberta	17	Neufeld & Christensen, 2021
China	Taihu Lake	1.54	Li et al., 2024
	Yellow River estuary	1.07	
	Wanshan mining area, stream water	1.01-30.62	Zhang et al., 2014a
Belgium France Italy	River water	0.05-2.0 0.08-10 0.169-83	Conde & Alaejos, 1997
USA	St. Charles River, Colorado	23.5-30[b]	Mueller et al., 1991
	Solomon River Basin, Kansas	0.6-25	May et al., 2008
	Salton Sea Region, California	0.001-46	Rosen et al., 2023
	Kesterson Reservoir, California, agricultural drainage	140-1,400	Chapman et al., 2010

	Belows Lake, North Carolina, wastewater	100-200	Chapman et al., 2010
	Hyco Lake, North Carolina, ash pond effluent	7-14	
	Savannah River, South Carolina	100-110[a]	
	Martin Creek, Texas, coal effluent	<2,700	
	Appalachian Mountains	<42	
	Blackfoot River Watershed, Idaho, mine wastewater	>1000	
	Blackfoot Rover Watershed, Idaho, streams	<400	

[a]: Dissolved concentration
[b]: Median concentration

1.1.2 Selenium in aquatic systems

Waterborne selenium can occur in various forms determined by environmental factors and its source. The most common chemical species in aquatic ecosystems are the inorganic anions, selenite (SeO_3^{2-}; Se(IV)) and selenate (SeO_4^{2-}; Se(VI)) (Young et al., 2010). It has been suggested that Se(IV) is preferentially taken up by organisms (Vandermeulen & Foda, 1988). However, both of these anions can be taken up by primary producers and converted to organic selenium compounds such as selenomethionine (SeMet) which can subsequently be accumulated in higher trophic levels (Young et al., 2010). For example, it has been documented that diet is the main source of selenium uptake and accumulation in invertebrates and fish (Besser et al., 1993; Lemly, 2004; Presser & Luoma, 2010). However, abiotic factors, such as the physical characteristics of aquatic systems can influence selenium speciation in the water and its subsequent accumulation into organisms.

Factors such as water circulation, water mixing, salinity, redox potential and ion composition are major contributors affecting selenium speciation in aquatic systems (Conde & Alaejos, 1997; Simmons & Wallschläger, 2005). However, it is generally considered that pH is the most important physicochemical factor affecting selenium speciation in water. Selenite is dominant at circumneutral pHs (i.e., pH 5-9), whereas Se(VI) occurs in either highly acidic or alkaline conditions (i.e., pH <5 and >9; Selinus, 2013; Sharma et al., 2015; Torres et al., 2011). It has also been documented that selenium speciation, bioavailability and thus bioaccumulation, changes between lentic and lotic systems due to their respective chemistry and hydrology (Simmons & Wallschläger, 2005). For example, when there is increased flow, this results in higher redox potentials and thus Se(VI) is the dominant form in lotic systems (Simmons & Wallschläger, 2005). Furthermore, stagnant, lentic systems can tolerate lower loads of selenium contamination

than lotic systems (Table 1.2), where Se(IV) is often predominant due to comparably lower redox potentials (Simmons & Wallschläger, 2005).

Owing to its increased prevalence and toxicological implications, water quality guidelines have been issued for selenium. Where possible, these guidelines are derived by plotting a species-sensitivity distribution. For any given endpoint (although usually mortality) responses of a range of species from distinct taxa are plotted, and from these data a hazard co-efficient is calculated, usually representing the value of the toxicant where 95% of the species would be protected. While chronic data are preferred, in the absence of such data an acute-to-chronic ratio can be used to convert acute data to the more environmentally-relevant chronic value. Based on this general approach, water quality guidelines for selenium for aquatic life protection have been developed for North American waters, and range between 1 and 3.1 $\mu g\ L^{-1}$ (Table 1.2). Canada takes a more conservative approach due to requiring further validation of toxicity, however this could lead to over- or under-protection of species (Canadian Council of Ministries of the Environment (CCME), 2007). These guideline values are designed to be relatively simple to enhance understanding and to be applicable to a wide range of water bodies. However, because water chemistry may not be integrated into these values, the guidelines may not always be protective of species, particularly in waters with unusual chemistry. Although, in general, this regulatory approach is similar worldwide, different jurisdictions have different guideline values for selenium (see Table 1.2), often depending on the degree of protection that is considered appropriate (i.e., anywhere from 80 to 99% of species protected), and whether water chemistry is accounted for.

Table 1.2 Freshwater selenium guidelines for aquatic life protection in different jurisdictions.

Jurisdiction	Water quality guideline ($\mu g\ L^{-1}$)	Reference
Australia[a] New Zealand[a]	5-34[b]	ANZECC/ARMCANZ, 2000
Alberta, Canada British Columbia, Canada	2	Government of Alberta, 2018 Ministry of Environment Province of British Columbia, 2014
Saskatchewan, Canada Manitoba, Canada	1	Water Security Agency, 2015 Manitoba Water Stewardship, 2011
United States[a]	1.5[c] 3.1[d]	USEPA, 2016

[a]: Federal jurisdiction
[b]: 80-99% level of protection
[c]: Lentic aquatic systems
[d]: Lotic aquatic systems
ANZECC/ARMCANZ: Australian and New Zealand Environment and Conservation Council/ Agriculture and Resource Management Council of Australia and New Zealand

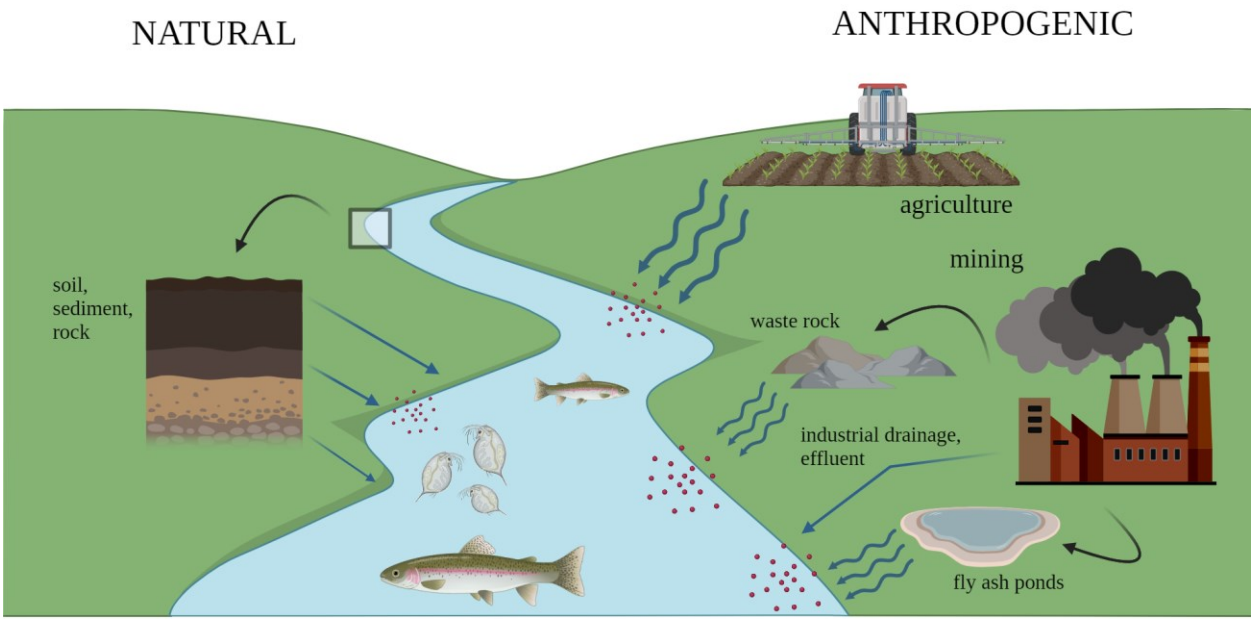

Figure 1.1 Schematic of sources of natural and anthropogenic selenium into adjacent waterways. Natural selenium is released from the earth's crust, whereas anthropogenic selenium comes from agriculture and mining activities. Invertebrates and salmonids are often located in areas affected by elevated selenium.

1.2 Uptake pathways and accumulation of selenium in freshwater species

Selenium has many putative uptake routes varying across different species (see sections 2.1 and 3.1). In aquatic animals, however, the limited data to date supports selenium uptake via anionic transporters (Araie et al., 2011; Misra et al., 2012; Yu & Wang, 2002). Of the two main oxidation states of selenium, Se(IV) is more bioavailable to aquatic species than Se(VI), resulting in relatively higher uptake and accumulation in tissues (Brasher & Ogle, 1993; Franz et al., 2011; Ma et al., 2018; Simmons & Wallschläger, 2005; Vandermeulen & Foda, 1988).

In primary producers and consumers, the bulk of selenium accumulation occurs via direct assimilation from the water (Besser et al., 1993; Presser & Luoma, 2010). The selenium taken up by algae is subsequently transformed into organic forms which are consumed and accumulated in higher trophic levels (Presser & Luoma, 2010). Bioconcentration factors (BCFs) show a strong capacity for the accumulation of selenium in aquatic biota. For example, bioconcentration factors (BCFs) of up to 16,000 and 200,000 have been documented in algae and daphnids respectively, whereas a BCF of up to 5,000 was documented in fish (Besser et al., 1993).

As noted above, once absorbed from the water inorganic selenium is converted into organic forms. This process involves the reduction of selenium to selenide with subsequent conversion to organic selenium by microorganisms and microalgae (Mehdi et al., 2013). A number of organic selenium compounds have been identified, such as SeCys, Se-methylselenocysteine, SeMet and dimethyl diselenide (Shrift, 1969). However, the predominant organic forms in aquatic systems are SeMet and SeCys (Mehdi et al., 2013). These organic forms of selenium have high bioavailability, in part because they can be taken up via amino acid transporters in the gut (e.g., McConnell & Cho, 1965). This contributes to the importance of the dietary pathway of uptake in invertebrates and fish, and the general partitioning of inorganic selenium uptake via the water and

organic selenium uptake via the diet. For example, Besser et al. (1993) demonstrated that fish took up more organic selenium from their food than aqueous exposures and more inorganic selenium from aqueous exposures than food. A study by Berntssen et al. (2017) demonstrated a similar finding in Atlantic salmon, where SeMet had higher dietary uptake than Se(IV). Importantly, as seleno-amino acids non-specifically replace cysteine and methionine in proteins, SeCys and SeMet can have distinct toxicological implications compared to inorganic selenium (USEPA, 2021). However, it is important to emphasise that higher trophic levels can still accumulate waterborne inorganic selenium, and that this can exert toxic effects (Table 1.3 and 1.4).

The route of selenium uptake is also likely to impact patterns of selenium accumulation. Specifically, it has been suggested that waterborne inorganic selenium taken up via the gills has a more generalized tissue distribution, whereas dietary selenium passes through the liver first via hepatic portal circulation (Hodson & Hilton, 1983; Misra et al., 2012). Consequently, this may influence selenium burdens in different tissues, selenium excretion rates, and ultimately, selenium toxicity.

The specific uptake pathways of selenium species remain understudied (see sections 2.1 and 3.1 for more extensive overview), especially in aquatic organisms. Misra et al., (2012) suggested that isolated fish intestinal cells may take up selenium via Cl^-/HCO_3^- exchangers. In contrast, a study done by Yu and Wang (2002) demonstrated that waterborne selenium uptake in *Daphnia magna* is affected by calcium suggesting a possible uptake route related, directly or indirectly, to this important cation. To my knowledge, no studies have examined selenium uptake directly via the gill, which is the main uptake route of waterborne selenium for aquatic macrofauna. Indeed, understanding the uptake mechanism of a pollutant can allow us to better predict how

much is accumulating in the organism, how water chemistry may affect uptake and thus potential routes of toxicity, providing valuable insight into potential protective measures (Glover, 2018).

1.3 Toxicity of selenium

Although selenium is considered an essential nutrient, it possesses a narrow margin between essentiality and toxicity in biota (Lemly, 2004; Selinus, 2013). However, studies have demonstrated that there is a considerable range in species sensitivities (Table 1.3). For example, within fish the 96 h LC_{50} (median lethal concentration) ranges from 7.8-34 mg L^{-1} (Buhl & Hamilton, 1991). Indeed, even species of the same genus such as *Oncorhynchus kisutch* and *Oncorhynchus mykiss* have considerable variation in their lethal sensitivities (Table 1.3). Although, these studies could be done on fish of different sizes and ages, or in different water chemistries, potentially affecting accumulation and explaining the differences in observed lethal limits. However, it is also possible that the observed differences in lethal sensitivities could represent differences in the way fish species take up and accumulate selenium or variation in the sensitivity of pathways impacted by selenium. Furthermore, it has been documented that for inorganic selenium species, Se(IV) induces lethality at lower concentrations than Se(VI). For example, *D. magna* had a 48 h LC_{50} of 550 µg L^{-1} for Se(IV) and 2,840 µg L^{-1} for Se(VI) (Maier et al., 1993). Elevated selenium concentrations have also been linked to detrimental effects on reproductive success, ultimately leading to extirpation of fish species, particularly in North American waters (Chapman et al., 2010). For example, in the late 1970s, high selenium contamination in Belews Lake, North Carolina led to the extirpation of 16 fish species (Chapman et al., 2010).

Many toxic effects have been observed in aquatic biota in response to elevated selenium exposures (Table 1.4). For example, Mo et al. (2021) demonstrated that inorganic Se(IV) causes reduced reproductive function in female zebrafish; whereas Zhao et al. (2022) demonstrated that

the organic SeMet induces cardiovascular defects in zebrafish. Furthermore, changes in metallothionein, glucose, cortisol, various enzymatic activities, and gene expression have been observed in various aquatic species exposed to excess selenium (see Table 1.4 for details). Additionally, histological alterations, altered neurotransmission and behaviour, deformities and developmental effects have all been linked to elevated selenium in aquatic systems (see Table 1.4 for details). Selected endpoints relevant to this thesis are further discussed below and in Chapters 2-4.

Antioxidant defense systems are often used as a proxy for evaluating oxidative stress in teleost species. Specifically, enzymes such as catalase (CAT), GPx, superoxide dismutase (SOD) and glutathione-S-transferase (GST) are all commonly used as indicators of antioxidant responses and thus oxidative stress (Figure 1.3). Indeed, these oxidative stress proxies have all been documented to respond to elevated selenium exposure in fish (for more detail please refer to Chapter 4). For example, altered activity of CAT, GPx, SOD and GST in response to Se(IV) concentrations as low as 5 μg L^{-1} and up to 400 μg L^{-1} have been documented in tilapia, *Oreochromis mossambicus*, and northern snakehead fish, *Channa argus* (Gobi et al., 2018; Li et al., 2019). Furthermore, protein carbonyl (PC) content, a biomarker for oxidative damage, was upregulated in *O. mossambicus* in a dose-dependent manner starting at 5 μg L^{-1} of waterborne Se(IV) (Gobi et al., 2018).

Enzymes vital for maintaining homeostasis and proper cell functioning in organisms have also been shown to respond to elevated selenium levels. For example, in response to 2.38 mg L^{-1} waterborne Se(IV), the teleost fish rohu, *Labeo rohita*, displayed significantly increased lactate dehydrogenase (LDH) activity after 7 days with a continued increase over 35 days (Ramesh et al., 2014). This enzyme serves as a biomarker for tissue damage induced by stressful conditions and

anaerobic metabolism (Farhana & Lappin, 2023). Furthermore, after 14 days the California blackworm, *Lumbriculus variegatus*, had decreased Na^+/K^+-ATPase (NKA) activity in response to 15 μg L^{-1} waterborne Se(IV) (Xie et al., 2016b). This enzyme is a pump found on the cell membrane responsible for the creation and maintenance of sodium and potassium gradients, helping maintain membrane potential, and thereby influencing a variety of physiological processes (Suhail, 2010).

The length of exposure to selenium also has the potential to influence its toxic effects on aquatic biota. Acute exposures may be of reduced environmental relevance; however, they are easier to complete in a laboratory setting as there are logistical difficulties in conducting chronic studies. Although acute exposures to high selenium at a drainage site is environmentally plausible, most aquatic species will experience a chronic exposure to comparably lower concentrations in natural aquatic systems (Davis et al., 1988). In this regard, numerous studies have detailed chronic or sub-chronic effects of elevated selenium exposure in aquatic biota (Table 1.4) with chronic data suggesting similar toxicological impacts as acute studies. For example, Attaran et al. (2019) demonstrated that after 60 days zebrafish experienced altered social and antipredator behaviour in response to dietary SeMet. Furthermore, in a sub-chronic study, alterations in stress proteins and immune related genes were associated with high selenium concentrations in tilapia (Chen et al., 2020). Similarly, immune-related genes were generally upregulated in snakehead fish after 8 weeks (Li et al., 2020). Plasma cortisol, triiodothyronine and thyroxine levels were also increased in rainbow trout (RBT) after a 30-day sub-chronic exposure to Se(IV) (Miller et al., 2007). Indeed, it is clear that sub-chronic and acute exposures can elicit similar toxicological affects albeit at different exposure concentrations (see Table 1.4 for more extensive overview).

Selenium is well known to be toxic to aquatic organisms, however effect concentrations can greatly vary between species (Table 1.3 and 1.4). Therefore, knowledge surrounding the potential sub-lethal effects of waterborne selenium on non-model organisms and local species is an important component for insight into protective measures.

Table 1.3 Effect and lethal concentrations of selenite to freshwater algae, protozoan, invertebrate, and fish organisms.

Group	Latin name	Endpoint	Exposure length	Se(IV) concentration ($\mu g\ L^{-1}$)	Reference
Algae	*Scenedesmus quadricauda*	Incipient inhibition	96 h	2,500	USEPA, 2021
Protozoans	*Entosiphon sulcatum*	Incipient inhibition	72 h	1.8	USEPA, 2021
	Microreqma heterostoma	Incipient inhibition	28 h	183,000	USEPA, 2021
	Chilomonas paramecium	Incipient inhibition	48 h	62	USEPA, 2021
	Uronema parduezi	Incipient inhibition	20 h	118	USEPA, 2021
Invertebrate	*Daphnia magna*	LC_{50}	24 h 48 h	16,000 550	USEPA, 2021; Maier et al., 1993
		EC_{50}	48 h	2,500	USEPA, 2021
		LC_{50}[a]	48 h	680	Johnston, 1987
		EC_{50}[a]	48 h 96 h	710 430	Halter et al., 1980
	Hyalella azteca	LC_{50}	48 h	623	Brasher & Ogle, 1993
		LC_{50}[a]	14 d	70	Halter et al., 1980
		LOEC	24 d	200	Brasher & Ogle, 1993
	Chironomus riparius	LC_{50}	48 h	7,950-14,600	Ingersoll et al., 1990
Fish	*Oncorhynchus kisutch*[b]	LC_{50}	96 h 43 d	7,830 160	Adams, 1976; Buhl & Hamilton, 1991
	Oncorhynchus mykiss[c]	LC_{50}	96 h 5 d 21 d 96 d	9,000 2,700 460 280	Adams, 1976; Buhl & Hamilton, 1991

		LC_{50}[a]	96 h 9 d	7,200 5,410	Hodson et al., 1980
		LOAEC	41 d	26	Hodson et al., 1980
	Esox lucius[d]	LC_{50}	76 h	11,100	Klaverkamp et al., 1983a
	Pimephales promelas[e]	LC_{50}[a]	96 h	1,000	Halter et al., 1980
	Lepomis macrochirus[f]	LC_{50}	48 d	400	Adams, 1976
	Perca flavescens[g]	LC_{50}	10 d	4,800	Klaverkamp et al., 1983a,b
	Thymallus arcticus[h]	LC_{50}	96 h	34,300	Buhl & Hamilton, 1991

[a]: Dietary
[b]: Coho salmon
[c]: Rainbow trout
[d]: Northern pike
[e]: Fathead minnow
[f]: Bluegill
[g]: Yellow perch
[h]: Arctic grayling
Incipient inhibition: concentration resulting in a 3% reduction in growth
LC_{50}: Concentration where 50% of the exposed population would experience mortality.
EC_{50}: Concentration where 50% of the exposed population would experience an effect.
LOEC: Lowest concentration where an effect is observed.
LOAEC: Lowest concentration where an adverse effect is observed.

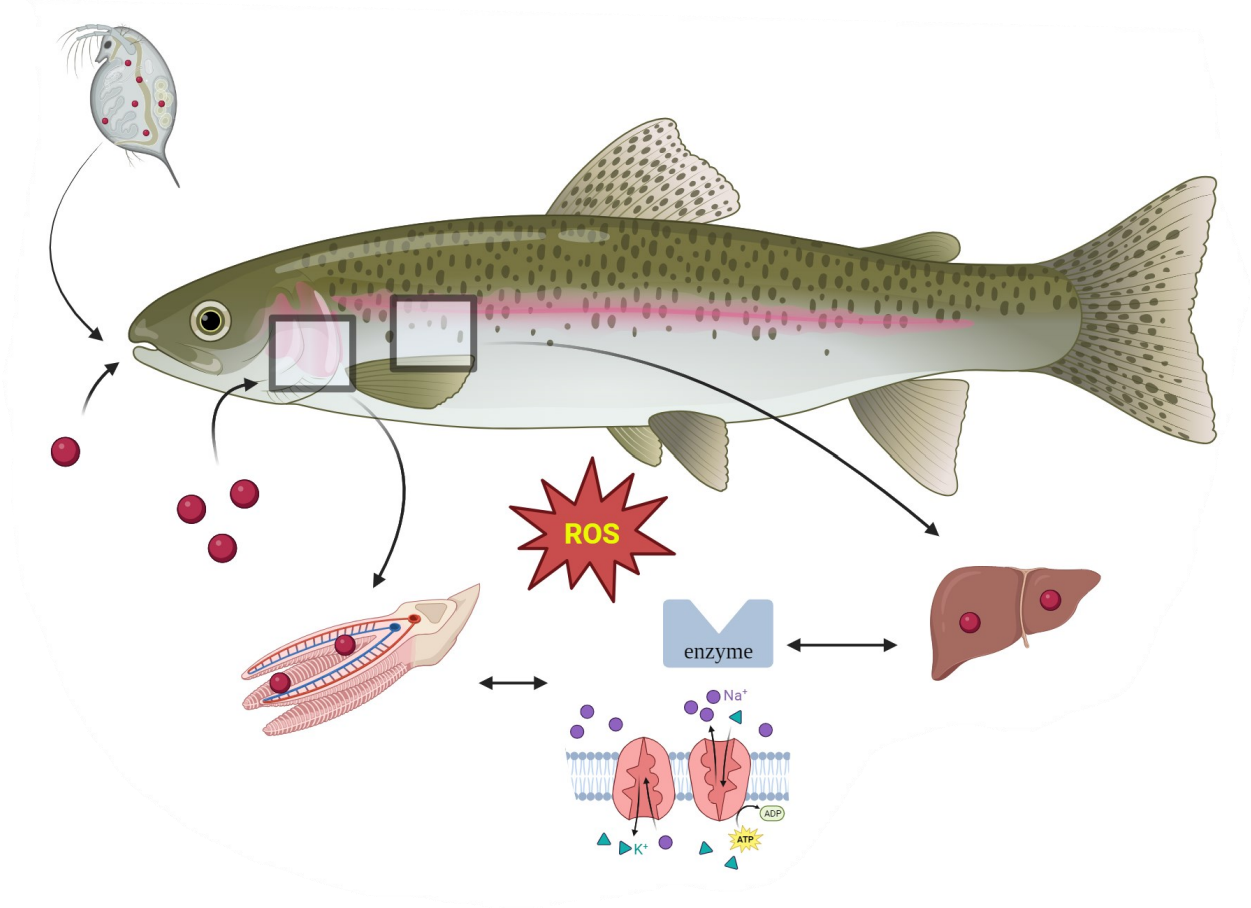

Figure 1.2 Schematic of sources of uptake and accumulation of selenium into a teleost and endpoints of its elicited toxicity. Fish can take up selenium via their diet across the gut, or via the water across the gill. Accumulation can then occur in various tissues affecting oxidative stress (i.e., reaction oxygen species (ROS)), enzymatic activity and ionoregulation.

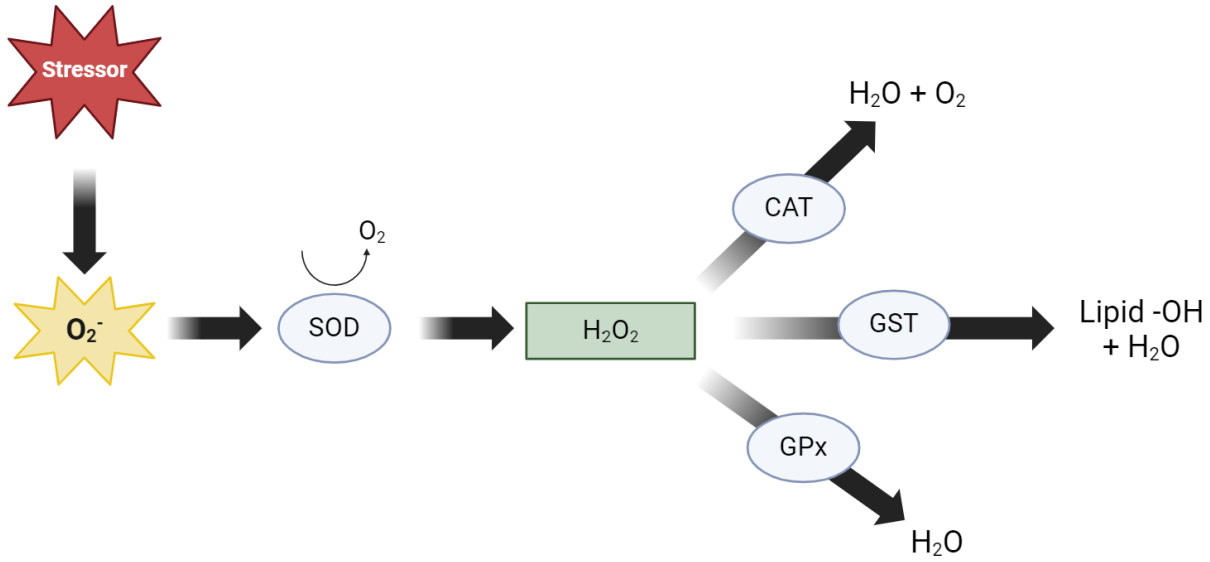

Figure 1.3 Schematic of key enzymatic antioxidant defense mechanisms in fish. SOD: superoxide dismutase; CAT: catalase; GST: glutathione-S-transferase; GPx: glutathione peroxidase.

Table 1.4 Toxicity of selenium exposure in aquatic organisms.

Phylum	Organism species	Latin name	Selenium species	Selenium concentration	Acute vs Chronic	Endpoint	Effect	Reference
Arthropoda	Water flea	*Daphnia magna*	Se(IV)	>200 µg L^{-1}	Chronic	Reproduction	Reduced	Brasher & Ogle, 1993
			SeMet SeCys	0.045 mg L^{-1} 0.52 mg L^{-1}	Acute	Immobilisation	Increased	Maier et al., 1993
	Midge	*Chironomus dilutus*	SeNPs	1000 µg L^{-1}	Acute	Growth	Reduced	Gallego-Gallegos et al., 2013
Annelida	Blackworm	*Lumbriculus variegatus*	Se(IV) Se(VI) SeMet	20 µg g^{-1} **b,c** 15 µg L^{-1}	Acute	Mortality Lipid peroxidation	Increased	Xie et al., 2016b
						NKA	Reduced	
Chordata	Fathead minnow	*Pimephales promelas*	Se(IV)	>15 mg L^{-1}	Acute	Incubation time of eggs	Reduced	Adams, 1976
				>1 mg L^{-1}		Post-hatch survival time		
	Rainbow trout	*Oncorhynchus mykiss*	Se(IV)	>0.25 mg L^{-1}	Acute	Growth	Reduced	
			Se^{ns}	6-32 µg L^{-1} **a**	Chronic	Craniofacial defects Edema Skeletal defects	Increased	Holm et al., 2005
			Se(IV)	0.72-3.6 mg L^{-1}	Acute	Cortisol Glucose	Increased	Miller et al., 2007
						GSH Lipid peroxidation GPx	Reduced	
				0.07-0.36 mg L^{-1}	Sub-chronic	Cortisol Thyroxine Triiodothyronine	Increased	

				3.95-15.8 mg L^{-1}	Acute	CAT SOD GPx Apoptosis	Increased	Misra & Niyogi, 2009
			Se^{ns} (organic)	20-40 mg kg^{-1}**b,c**	Sub-chronic	Growth	Reduced	Knight et al., 2016
Chordata cont.	Green sunfish	*Lepomis cyanellus*	Se^{ns}	6.6-10.3 mg kg^{-1}**b**	Chronic	Jaw deformities	Shortened maxillae	Arnold et al., 2014
	Creek chub	*Semotilus* sp.	Se^{ns}	2 mg kg^{-1}**b**	Chronic	Gill aneurysms	Increased	
	Zebrafish	*Danio rerio*	SeMet	2.1-31.5 µg g^{-1}**b,c**	Chronic	Social behaviour	Increased fear, less time in groups	Attaran et al., 2019; Li et al., 2021
			SeMet Se(IV)	2.1-31.5 µg g^{-1}**b,c**	Chronic	Serotonergic neurotransmission	Altered gene expression	
			Se(IV)	1 mg L^{-1}	Acute	GSH	Increased	Hauser-Davis et al., 2016
						Metallothionein	Increased (brain), decreased (liver)	
				7.98-79.6 µg L^{-1}	Chronic	Reproductive function	Decreased	Mo et al., 2021
			SeMet	19.75-158 µg L^{-1}	Acute	Cardiovascular defects	Increased	Zhao et al., 2022
	Atlantic salmon	*Salmo salar*	Se(IV)	15 mg L^{-1}**c**	Sub-chronic	Growth	Reduced	Berntssen et al., 2017
						Vitamin E	Reduced	
						TBARS	Increased	
			Se(IV) SeMet	1-15 mg L^{-1}**c**	Sub-chronic	Endocannabinoids Antioxidant Metabolism	Reduced	

Chordata cont.	Nile tilapia	*Oreochromis niloticus*	Se(IV) Se^{ns} (organic)	3-12 µg g^{-1} [b]	Sub-chronic	Immune genes Stress proteins Lipid peroxidation Antioxidant	Increased	Chen et al., 2020
						AChE Digestive enzyme	Reduced	
			Se^{ns}	2-8 mg kg^{-1} [c]	Sub-chronic	Histopathological abnormalities	Increased	Iqbal et al., 2020
	Mozambique tilapia	*Oreochromis mossambicus*	Se(IV)	5-100 µg L^{-1}	Acute	Metallothionein SOD CAT (5-25 µg L^{-1}) GPx GST Glutathione Lipid peroxidation Protein carbonyl	Increased	Gobi et al., 2018
				25-100 µg L^{-1} 50-100 µg L^{-1}		CAT AChE	Reduced	
				10-100 µg L^{-1}		NKA	Reduced	Gopi et al., 2021
	Goldfish	*Carassius auratus*	Se(IV)	2-4 mg L^{-1}	Acute	Metallothionein AspAT/AlaAT NKAα CRH ACTH GRs Glucose Cortisol Oxidative stress	Increased	Choi et al., 2015
	Flathead gudgeon	*Philypnodon grandiceps*	Se^{ns}	4.6-9 µg g^{-1} [a]	Chronic	Spinal deformities	Increased	Jasonsmith et al., 2008
			Se(IV)		Acute	MDA	Increased	

	Topmouth gudgeon	*Pseudorasbora parva*	Se(VI)	10-1000 µg L^{-1}		AChE		Ma et al., 2018
						GST GSH SOD	Decreased	
Chordata cont.	Red sea bream	*Pagrus major*	Se(IV)	>100 µg L^{-1}	Sub-chronic	Growth RBC Hct HB	Reduced	Kim & Kang, 2014
						Glucose GOT GPT	Increased	
				50-400 µg L^{-1}		SOD GST GSH Lysozyme	Increased	Kim & Kang, 2015
						AChE Peroxidase	Reduced	
	Black sea bream	*Acanthopagrus schlegelii*	Polysac-charide	0.34-3.06 mg L^{-1}[c]	Sub-chronic	Growth SOD GPx CAT	Increased	Wang et al., 2019a
	Iridescent shark (catfish)	*Pangasius hypophthalmus*	SeNP Se^{ns}	2.5-4 mg L^{-1} 4.5-6 mg L^{-1}	Acute	CAT SOD GST LDH MDH Cortisol HSP70 Histopathological abnormalities	Increased	Kumar et al., 2018
						AChE	Reduced	
	Bluegill	*Lepomis macrochirus*	Se(IV) Se(VI)	5 µg L^{-1}[d]	Chronic	Respiratory demand	Increased	

			SeMet			Oxygen consumption Gill pathology Teratogenesis		Lemly, 1993a, 2014
						Hct HB	Reduced	
	Largemouth bass	*Micropterus salmoides*	Se^{ns}	5.9-8.5 mg kg^{-1}[a]	Chronic	Teratogenesis	Increased	Lemly, 2018
	Northern snakehead	*Channa argus*	Se^{ns}	100-200 µg L^{-1}	Sub-chronic	SOD CAT GPx GST	Reduced	Li et al., 2020
						MDA PC Immune genes	Increased	
Chordata cont.	Chu's croaker	*Nibea coibor*	Se(IV)	0.53-1.72 mg kg^{-1}[c]	Sub-chronic	GPx	Increased	Lin et al., 2021
				0.79-1.45 mg kg^{-1}[c]		Growth SOD CAT		
	Indian carp	*Labeo rohita*	Se(IV)	2.38 mg L^{-1}	Sub-chronic	HB Hct RBC	Reduced	Ramesh et al., 2014
						WBC Glucose GOT GPT LDH	Increased	
	Meagre	*Argyrosomus regius*	Se^{ns} (organic)	1-3 mg kg^{-1}[c]	Sub-chronic	Growth CAT SOD	Increased	Mansour et al., 2017
						TBARS	Reduced	

[a]: Organisms collected from the field

[b]: dry weight
[c]: dietary
[d]: combined waterborne and dietary (5 µg L^{-1} each)
[ns]: not specified
Polysaccharide: organic selenium compound formed by the combination of polysaccharide and selenium
SeNP: selenium nanoparticles
SOD: superoxide dismutase
CAT: catalase
AChE: acetylcholinesterase
GPx: glutathione peroxidase
GSH: glutathione
AspAT/AlaAT: aspartate/alanine aminotransferase
NKA(α): Na^+/K^+-ATPase (alpha subunit)
CRH: corticotropin releasing hormone
ACTH: adrenocorticotropic hormone
GRs: glucocorticoid receptors
RBC: red blood cells
Hct: hematocrit
HB: hemoglobin
GOT: glutamic oxalate transaminase
GPT: glutamic pyruvate transaminase
GST: glutathione-S-transferase
LDH: lactate dehydrogenase
MDA: malondialdehyde
MDH: malate dehydrogenase
HSP70: heat shock protein 70
PC: protein carbonylation
WBC: white blood cells
TBARS: thiobarbituric acid-reactive substances

1.4 Model Species

1.4.1 Daphnia magna

Daphnia magna, commonly known as the water flea, is a globally distributed freshwater invertebrate particularly widespread across the Northern Hemisphere. They are considered to be a strong ecological interactor due to being a primary consumer, and form the basis of the food chain for higher trophic levels (e.g., predatory invertebrates, fish) in aquatic systems (Miner et al., 2012). *Daphnia* possess several behavioural and physiological traits that make them an important model organism for toxicity testing (Tkaczyk et al., 2021). For example, they have a short life cycle, high reproductive output and a small body size allowing for rapid, simultaneous testing (Tkaczyk et al., 2021). Furthermore, they have well characterised behaviours that are easily observable and their clear carapace can allow for non-invasive measurements (Tkaczyk et al., 2021). Indeed, the use of *D. magna* for toxicity testing dates back to 1944, where they were used to assess toxicity of industrial wastewater (Anderson, 1944). Today they are used as a model organism for aquatic biology and toxicology, and standardized tests have been developed by the Organization for Economic Cooperation and Development (OECD), Canadian Council of Ministers of the Environment (CCME) and United States Environmental Protection Agency (USEPA) (Biesinger et al., 1987; Environment Canada, 2000; OECD, 2004).

A more thorough examination of selenium research in *Daphnia* is presented in Chapter 2 of this thesis. Briefly, however, the sensitivity of *Daphnia* to waterborne selenium has already been characterised (Table 1.3 and 1.4), and it is known that the accumulation of selenium in this group can lead to biomagnification of selenium through the aquatic food chain. However, knowledge on how waterborne selenium is being transported across daphnid epithelia is lacking. The second

chapter in this thesis will examine the mechanistic uptake of waterborne Se(IV) in *D. magna* and how various water chemistries can affect its toxicity.

1.4.2 Oncorhynchus clarkii lewisi

Commonly known as Westslope cutthroat trout (WCT), *Oncorhynchus clarkii lewisi* are a salmonid species native to North America, with a distribution primarily focused in Montana, Idaho, southeastern British Columbia (B.C.) and southwestern Alberta (Costello & Rubidge, 2006; McIntyre & Rieman, 1995). Populations of *O. clarkii lewisi* can be adfluvial, fluvial or resident, where they tend to inhabit cold and nutrient-poor waters (Cleator et al., 2009; Costello & Rubidge, 2006; McIntyre & Rieman, 1995). To sustain a healthy population they require clean water with various forms of cover (Costello & Rubidge, 2006). Indeed, due to their specific habitat needs, they are often viewed as an indicator species for overall freshwater system health (Cleator et al., 2009; Costello & Rubidge, 2006). Furthermore, they are opportunistic eaters tending to consume whatever is most abundant, which commonly consists of invertebrates and zooplankton. Consequently, they are susceptible to biomagnification and bioaccumulation of anthropogenic contaminants.

Unfortunately, the loss of suitable habitats due to fragmentation and degradation has caused global populations of WCT to decline drastically. Specifically, the Committee on the Status of Endangered Wildlife in Canada (COSEWIC) has designated *O. clarkii lewisi* populations as *Special Concern* and *Threatened* as of May 2005 in B.C. and Alberta respectively (Costello & Rubidge, 2006). Indeed, their primary habitat range overlaps with the locations of leading sources of waterborne selenium pollution, such as mining activities and agricultural runoff. However, there is a general lack of knowledge surrounding how WCT handle waterborne selenium and the subsequent toxicity that may ensue. Furthermore, it is unknown how, and if, their selenium

handling and sensitivity differs from better-studied salmonids, such as RBT (see below). It is known that these two species can differ in their tolerances to environmental stressors. For example, RBT have a higher thermal tolerance, withstanding temperatures 4.7°C higher than WCT (Bear et al., 2007). Furthermore, RBT can withstand higher concentrations of metal toxicants than cutthroat trout, having a LC_{50} 1.5 and 9.8 times higher for zinc and cadmium respectively (Harper et al., 2008; Naddy et al., 2015). Comparison of toxic responses between WCT and RBT will enable extrapolation of studies between the better-studied fish (RBT) to the species more likely to be exposed to selenium in Alberta's waters (WCT). It is therefore important to establish fundamental characteristics of selenium accumulation, lethality, and sub-lethal effects in WCT, which is the focus of Chapters 3 and 4 of this thesis.

1.4.3 Oncorhynchus mykiss

Commonly known as RBT, *Oncorhynchus mykiss* are a salmonid fish that have an extensive global distribution. Native to North America and Russia, their non-native distribution extends throughout South America, Asia and Europe (MacCrimmon, 1971; Muhlfeld et al., 2019). They primarily inhabit freshwater ecosystems; however, some populations are known to be anadromous inhabiting seawater only returning to freshwater to spawn (Hardy, 2002). Indeed, this species can tolerate a wide range of environmental conditions, including variations in water temperature and water quality. In general, they prefer cold, highly oxygenated, clean lakes, streams, and rivers. Commonly considered secondary consumers, they have a carnivorous diet, feeding on insects and other small fish, making them susceptible to contaminant biomagnification.

Rainbow trout are extensively used as a model organism in many biological disciplines (Thorgaard et al., 2002; USEPA, 2002). Specifically, as they are sensitive to various chemical stressors (Teather & Parrott, 2006), and can be used as a surrogate species for other cold water

teleosts (Dwyer et al., 1995), they are a valuable species for toxicology research. Indeed, standardized toxicity protocols have been developed for RBT (OECD, 2019; USEPA, 2002).

As briefly described later in this thesis (section 3.1), exhibiting such an extensive habitat range (as outlined above) results in large overlap with locations of anthropogenic sources of selenium contamination (Pearce et al., 2011; U.S. Energy Information Administration, 2023). Indeed, their relative sensitivity to waterborne selenium has been characterised (see Table 1.3 and 1.4), however knowledge of how various water chemistries may affect selenium handling is lacking. The effects of varying water composition on *O. mykiss* Se(IV) accumulation are discussed in Chapter 3 of this thesis.

1.5 Thesis aims

Given that elevated waterborne selenium levels are commonly associated with anthropogenic activities, such as extractive coal mining, which directly overlap in space with WCT populations, understanding the relative sensitivities of local populations will be critical for species protection. Knowledge of how waterborne Se(IV) is taken up across epithelia and accumulates in tissues is limited in aquatic organisms and how this may affect selenium toxicity in populations present in receiving environments is not well understood. Thus, my research aims to identify pathways responsible for Se(IV) handling and subsequent toxicity in local aquatic species, where specific objectives are outlined below.

Objective 1: Identify the mechanism of waterborne Se(IV) uptake in *Daphnia magna*

The first objective of my thesis research was to determine the pathways involved in the uptake of waterborne Se(IV) across daphnid epithelia. I hypothesized that as Se(IV) forms an anion in water, uptake into *Daphnia* will occur via an anion transporter. If the mechanistic uptake of

Se(IV) is specifically anion dependent, then the increased presence of specific anions in the water should competitively inhibit Se(IV) uptake into the organism. To accomplish this objective, radiolabelled selenite (Se^{75}) was used to quantify uptake in the organism. Individual *D. magna* were exposed to multiple water chemistries varying in specific anion composition and concentration. To further probe the uptake mechanism, putative transporter inhibitors were also utilized. Through analysis of whole-body radioactivity, I examined if Se(IV) uptake was indeed anion dependent and if so, which anions were competing with Se(IV) for uptake and thus which transporters were responsible for Se(IV) uptake into the organism.

Objective 2: Identify the pathways involved in waterborne Se(IV) accumulation in WCT (*Oncorhynchus clarkii lewisi*) and RBT (*Oncorhynchus mykiss*)

The second objective of my thesis research was to determine the pathways involved in waterborne Se(IV) tissue accumulation in *O. clarkii lewisi* and *O. mykiss*. I wanted to examine if the patterns of Se(IV) accumulation were conserved across genera (compared with *Daphnia*; Objective 1), and within genera (*Oncorhynchus* spp.). Understanding factors that influence Se(IV) accumulation will give valuable insight into potential routes of toxicity and also provide information on possible protective measures. I hypothesized that pathways involved in waterborne Se(IV) accumulation would be conserved across and within genera, and as Se(IV) is present as an anion, anionic water composition would affect tissue accumulation. To accomplish this objective, radiolabelled selenite (Se^{75}) was used to quantify accumulation in various fish tissues, upon exposure to various water chemistries. Gill, liver, gut, and muscle tissues were analyzed for radioactivity to examine if Se(IV) accumulation was affected by water treatment.

Objective 3: Examine the relative sensitivity and assess sub-lethal effects of *O. clarkii lewisi* to waterborne Se(IV)

The third objective of my thesis research was to determine the 96-h LC_{50} and sub-lethal effects of waterborne Se(IV) on *O. clarkii lewisi*. I hypothesized that *O. clarkii lewisi* would be sensitive to waterborne Se(IV), demonstrating a 96-h LC_{50} lower than that observed in *O. mykiss,* given the two species are known to differ in metal toxicity tolerances (see section 1.4 above; Harper et al., 2008; Naddy et al., 2015). Additionally, elevated waterborne Se(IV) was predicted to induce changes in reactive oxygen species, ionoregulation and enzymatic activity in *O. clarkii lewisi* tissues. To accomplish this objective, WCT were exposed to various concentrations of Se(IV) for 96-h and lethality was assessed. At concentrations where no lethality was observed, fish tissues were collected for subsequent analysis of sub-lethal endpoints. Branchial and hepatic PC and GPx, and branchial NKA, proton ATPase (H^+-ATPase) and LDH were examined. This analysis was conducted to determine if elevated Se(IV) would induce lethality and if it would disrupt homeostatic processes in WCT.

Chapter 2

Mechanistic characterisation of waterborne selenite uptake in the water flea, *Daphnia magna*, indicates water chemistry affects toxicity in coal mine-impacted waters

Klaczek, C.E., Goss, G.G., & Glover, C.N. (2024). Mechanistic characterization of waterborne selenite uptake in the water flea, *Daphnia magna*, indicates water chemistry affects toxicity in coal mine-impacted waters. *Conservation Physiology, 12*(1), coad108.

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Abstract

Concentrations of selenium that exceed regulatory guidelines have been associated with coal mining activities and have been linked to detrimental effects on aquatic ecosystems and the organisms therein. Although the major route of selenium uptake in macroinvertebrates is via the diet, the uptake of waterborne selenite ($HSeO_3^-$), the prominent form at circumneutral pH, can be an important contributor to selenium body burden, and thus selenium toxicity. In the current study, radiolabelled selenite (Se^{75}) was used to characterise the mechanism of selenite uptake in the water flea, *Daphnia magna*. The concentration-dependence (1 to 32 μM) of selenite uptake was determined in one-hour uptake assays in artificial waters that independently varied in bicarbonate, chloride, sulphate, phosphate, and Se(VI) concentrations. At concentrations representative of those found in highly contaminated waters, selenite uptake was phosphate-dependent and inhibited by foscarnet, a phosphate transport inhibitor. At higher concentrations, selenite uptake was dependent on waterborne bicarbonate concentration, and inhibited by the bicarbonate transporter inhibitor DIDS (4,4′-diisothiocyano-2,2′-stilbenedisulfonic acid). These findings suggest that concentrations of phosphate in coal mining-affected waters could alter selenite uptake in aquatic organisms and could ultimately affect the toxic impacts of selenium in such waters.

2.1 Introduction

One of the key environmental concerns associated with coal mining is its contribution to elevated water selenium, and the resulting toxic effects of selenium on aquatic biota (Etteieb et al., 2020; Lemly, 2004). Indeed, tissue pathologies and teratogenesis have been observed in several field studies of fish species inhabiting waters impacted by coal mining effluents worldwide (e.g., Holm et al., 2005; Jasonsmith et al., 2008; Lemly, 1993b, 2014, 2018). For fish, exposure to selenium through the diet is considered to be the route of greatest toxicological significance (e.g., Besser et al., 1993), but studies have shown that the invertebrate prey items that constitute fish diets may exhibit toxicity at selenium body burdens lower than those considered to protect consumer organisms at higher trophic levels (deBruyn & Chapman, 2007). Sub-lethal effects in freshwater invertebrates include the generation of oxidative stress and the disruption of the important ionoregulatory enzyme sodium/potassium ATPase, with the magnitude of these responses generally corresponding to selenium body burden (Xie et al., 2016b).

In most natural freshwaters selenium exists mainly as selenite (Se(IV)), although in waters of elevated pH and under oxidizing conditions Se(VI) predominates (Conde & Sanz Alaejos, 1997; Torres et al., 2011). The predominant selenium oxidative state also depends on the contributing source. For example, Se(IV) is more prominent in waters receiving discharges from coal ash or oil refineries, whereas Se(VI) dominates in waters receiving discharges from agriculture and copper mining (USEPA, 2016). In micro-organisms, Se(IV) appears to have a higher bioavailability than Se(VI) and once taken up, selenium is accumulated in an organic form, mostly complexed to amino acids (Brasher & Ogle, 1993; Franz et al., 2011; Vandermeulen & Foda, 1988). This organic selenium is therefore the form considered of greatest relevance to subsequent trophic transfer. However, it is known that freshwater invertebrates can absorb inorganic selenium directly from

the water. For example, significant bioconcentration has been shown in studies exposing larvae of the midge *Chironomus dilutus* to waterborne Se(IV) (Franz et al., 2011; Gallego-Gallegos et al., 2013). In the aquatic oligochaete *Lumbriculus variegatus*, a 14-d waterborne exposure to 15 µg L^{-1} Se(IV) led to a significantly higher body burden than exposure to the same concentration of Se(VI) (Xie et al., 2016b). However, this was more than an order of magnitude less than the burdens observed for sediment exposure to 20 µg g^{-1} Se(IV), representing a dietary route of uptake (Xie et al., 2016b). Indeed, it is well-recognised that the diet is the dominant route of selenium uptake, accounting for up to 95% of total selenium accumulation (e.g., Fowler & Benayoun, 1976; Lee et al., 2006; Presser & Luoma, 2010; Roditi & Fisher, 1999; Schlekat et al., 2004), with the relative importance of the waterborne pathway being species-specific, driven by organismal physiology (Presser & Luoma, 2010). Nevertheless, evidence to date therefore indicates that waterborne selenium may contribute to selenium burden, which in turn has toxicological implications for the invertebrate itself, and for the organisms that consume it. Consequently, knowledge of the pathways of waterborne Se(IV) uptake are important for characterising bioavailability and for interpreting how changes in water chemistry may affect uptake and toxicity.

In most natural waters, Se(IV) is present as an anion (i.e., $HSeO_3^-$). As such, Se(IV) uptake in aquatic biota is likely to proceed via anionic transport pathways. Indeed, anionic pathways of uptake have been observed in other studied systems. For example, in human erythrocytes, Se(IV) uptake is likely achieved by the bicarbonate/chloride anion exchanger (AE1 or Band 3 or SLC4A1; Kaur et al., 2020) while McDermott and colleagues (2016) suggested Se(IV) uptake was associated with ZIP8 (SLC39A8), and was transported into a variety of mammalian cells along with zinc and bicarbonate as part of an electroneutral transport process. In plants, considerable research links Se(IV) uptake to phosphate transporters (Bai et al., 2022; Wang et al., 2019b; Zhang et al., 2014b),

and evidence also exists for phosphate transporter-mediated Se(IV) uptake in yeast (Lazard et al., 2010), and in phytoplankton (Araie et al., 2011), although in both groups alternative pathways have also been suggested (monocarboxylate transporters in yeast; McDermott et al., 2010; sulphate and nitrate transporters in phytoplankton; Morlon et al., 2006). In the bacterium *Escherichia coli*, Se(IV) uptake is proposed to be achieved by a sulphate transporter (Lindblow-Kull et al., 1985). In one of the few studies to examine Se(IV) transport in an aquatic eukaryote, Misra et al. (2012) showed that Se(IV) uptake in RBT hepatocytes and enterocytes was affected by the presence of sulphite and the AE1 inhibitor 4,4′-diisothiocyano-2,2′-stilbenedisulfonic acid (DIDS). Consequently, a wide range of putative Se(IV) uptake pathways have been described, varying across organisms and tissues.

In the current study the mechanism of waterborne Se(IV) uptake was characterised in the water flea *Daphnia magna*. *Daphnia* perform a critical role in freshwater food chains, being both a primary consumer and a prey item for fish (Miner et al., 2012), and they are a valuable model for studying trace metal handling in aquatic biota (Tsui & Wang, 2007). The sensitivity of *D. magna* to waterborne selenium has been previously characterised, and Se(IV) has been shown to be more toxic than Se(VI), with 48-h median lethal concentrations of 0.55 and 2.84 mg L^{-1}, respectively (Maier et al., 1993). These data reflect the higher bioavailability of waterborne Se(IV) relative to waterborne Se(VI) in this species (Besser et al., 1993). To date, however, there is little mechanistic understanding of how waterborne Se(IV) is taken up across daphnid epithelia. The exception is a single study that showed Se(IV) uptake could be affected by pH and waterborne calcium but remained unimpacted by the presence of waterborne sulphate (Yu & Wang, 2002). To test the hypothesis that, by analogy with other biota, Se(IV) uptake in *D. magna* is achieved via an anion transporter, we employed radiolabelled Se^{75} and a variety of water chemistries to examine

short-term Se(IV) uptake. Specifically, using an artificial water as a basal medium, all permeant anions were replaced with gluconate salts, and thereafter concentrations of bicarbonate, chloride, sulphate, and phosphate were altered to examine their impacts on Se(IV) uptake. Where effects were shown, inhibitors of specific transporters were used to further probe the mechanism of uptake. The effect of waterborne Se(VI) on Se(IV) uptake was also examined to assess the possibility that the two selenium oxidation states were absorbed through similar mechanisms. Uptake was examined over a range of Se(IV) concentrations (1 to 32 μM = 79 to 2527 μg L^{-1}) to determine its concentration-dependence. Ultimately, knowledge of the mechanism of Se(IV) uptake in daphnids will facilitate an understanding of how water chemistry may affect uptake and toxicity of this important aquatic toxicant and may provide insight into conservation approaches for aquatic environments impacted by selenium-rich effluents, such as those associated with coal mining activity.

2.2 Materials and Methods

2.2.1 Daphnia maintenance

Adult *Daphnia magna* (between 20 and 23 d old; mean (± standard deviation) wet mass = 3.30 ± 0.56 mg) were used for all experiments. These animals were sourced from an established laboratory culture maintained in the Department of Biological Sciences at the University of Alberta. This culture was subjected to a 16h:8h light/dark photoperiod at 22°C and cultured in 1L glass beakers following Organization for Economic Cooperation and Development (OECD) guidelines (OECD, 2004), under the following water chemistry: 2 mM $CaCl_2 \cdot 2H_2O$; 0.5 mM $MgSO_4 \cdot 7H_2O$; 0.77 mM $NaHCO_3$; 0.08 mM KCl; pH ~7.5. Animals were fed once daily with YCT (a yeast, cereal leaf, trout chow mix) and algae (*Raphidocelis subcapitata*).

2.2.2 Selenium uptake experiments

The uptake of Se(IV) was examined in a variety of water chemistries (detailed below). Except in the case of testing the effects of inhibitors (see below), in each water chemistry, uptake was determined as a function of Se(IV) concentration (1, 2, 4, 8, 16, 32 μM; as sodium selenite from a 1 g L^{-1} stock) over a 1-h exposure period. The concentration range and exposure duration were chosen following preliminary studies that showed uptake was in the linear phase at these concentrations and at this exposure duration. Exposures were conducted in 2-mL microcentrifuge tubes, each containing an individual daphnid (n = 8) and 1.5-mL of test water. Uptake was determined using radiolabelled selenite (Se^{75}; University of Missouri Research Reactor Centre; 0.1-0.25 μCi per test chamber, varying with 'cold' Se concentration). After 1-h of exposure, daphnids were carefully removed from the exposure chambers using a plastic pipette, and individually rinsed through a series of three solutions to remove adsorbed, but not absorbed, isotope and to exchange radiolabelled water trapped in the daphnid carapace (2 x 10 s in unlabelled water equivalent to the test water chemistry albeit without selenium, followed by 10 s in 1 g L^{-1} Se(IV) (as sodium selenite)). After rinsing, daphnids were gently blotted dry with tissue paper and weighed using a microbalance (Orion Cahn C-35; Thermo Electron Corporation),

The following water chemistries were examined: 1. OECD culture water (control treatment; composition as above); 2. 'Gluconate' (water with no permeant anions; 0.77 mM $C_6H_{11}NaO_7$ and 0.08 mM $C_6H_{11}KO_7$ as the only salts); 3. 'Bicarbonate' (OECD water without $NaHCO_3$); 4. 'Chloride' (two chemistries, one with chloride salts removed from OECD water and one where chloride salts were doubled in concentration relative to OECD water); 5. 'Sulphate' (OECD water without $MgSO_4 \cdot 7H_2O$); 6. 'Phosphate' (OECD water supplemented with 10, 100 or 1000 μM Na_3PO_4). All test chemistries used ultrapure (>18 MΩ) water as a base, and all had pH

adjusted to pH 7.5 using potassium hydroxide, hydrochloric acid (all treatments except "chloride") and/or sulphuric acid ("chloride" water chemistries). No attempts were made to correct for ionic strength or to hold cation concentrations identical across all treatments. Preliminary studies indicated that there were minor fluctuations of Se(IV) uptake rates over time, and thus all test water chemistries were run concurrently with a time-matched OECD water control. Selenium speciation in all experimental waters was modelled using Visual MINTEQ.

After weighing, daphnids were then individually placed in 12 x 75mm borosilicate culture tubes and radioactivity (i.e., counts per minute) was measured using a Cobra Quantum gamma counter. All radioactivity counts were transformed to pmol of Se(IV) by dividing counts per minute (cpm) by water specific activity (cpm $pmol^{-1}$). This value was then divided by daphnid wet mass to give pmol mg wet $weight^{-1}$.

2.2.3 Inhibitor experiments

These studies were all conducted using OECD water as the base medium, to which Se(IV) was added from 1g L^{-1} Se(IV) (as sodium selenite) solution. All studies were 1-h in length and proceeded as described above, unless specifically noted otherwise below.

The effects of 4-4'-diisothiocyano-2,2'-stilbenedisulfonic acid (DIDS; ≥85%, Sigma-Aldrich, St. Louis, MO), a bicarbonate transport inhibitor, was tested at Se(IV) concentrations of 1, 8 and 32 µM, chosen based on the outcomes of the 'bicarbonate' test series. Stock solutions of DIDS at 1000x working concentrations suspended in dimethyl sulfoxide (DMSO) were made immediately prior to exposures and added to test solutions immediately prior to daphnid addition for final concentrations of 200, 500 and 1000 µM. This highest test concentration has previously

been applied in adult *D. magna* (Bianchini & Wood, 2008). The time-matched controls were inoculated with an equivalent concentration (0.001%) of DMSO.

Nicotinamide adenine dinucleotide (NAD^+; ≥95%, Sigma-Aldrich, St. Louis, MO) was added to 2 μM Se(IV) at a concentration of 300 μM, immediately prior to daphnid addition. NAD^+ is a putative phosphate transporter inhibitor, and 300 μM has been shown to effectively inhibit phosphate transport in rat membrane vesicle studies (Kempson et al., 1981). Sodium phosphonoformate (PFA, aka Foscarnet; Sigma-Aldrich St. Louis, MO), is another putative phosphate transporter inhibitor, and was added to 2 μM Se(IV) at concentrations of 1, 10 or 50 mM immediately prior to daphnid addition. These concentrations are in the range where effects on phosphate transport have been previously observed in RBT and hagfish (Avila et al., 2000; Schultz et al., 2014).

To test whether Se(VI) and Se(IV) were using the same transporter for uptake in *D. magna*, the effects of waterborne Se(VI) on Se(IV) uptake were examined. To maximise the capacity for Se(VI) inhibition, effects of 4 and 32 μM Se(VI) (from a 1 g L^{-1} sodium selenate stock) were examined in the presence of 0.75 nM Se(IV) (representing only Se(IV) added with 0.1 μCi of the radiolabelled stock and no 'cold' Se(IV) addition). Because of the low Se(IV) concentration examined, the final units for Se(IV) uptake in this study were expressed in pmol g wet $weight^{-1}$.

2.2.4 Statistics

Significant differences between treatments and time-matched controls were assessed within each tested Se(IV) concentration using a one-way ANOVA, with a Holm-Sidak post hoc test. Since this resulted in multiple analyses within each dataset, a Bonferroni multiple comparisons correction was applied, leading to different alpha levels for each analysis. A two-way

ANOVA, where effects of treatment and concentration were simultaneously assessed could not be used owing to lack of normality and inequality of variance, as determined by Shapiro-Wilk and Brown-Forsythe tests, respectively. Data transformations failed to render these data appropriate for parametric analysis. Shapiro-Wilk and Brown-Forsythe analyses were also used to interrogate the data prior to conducting one-way ANOVAs, and where data failed either test, a transformation was performed until the assumptions of normality and equality of variance were met, or a non-parametric Kruskal-Wallis ANOVA was conducted. The specific test and/or transformation used and the alpha value for each dataset is reported in Table A1. All statistical analyses were performed using SigmaPlot (ver. 14.5; Systat Software Inc.).

2.3 Results

Varying the exposure water chemistry did not affect Se(IV) speciation at pH 7.5 as modeled by Visual MINTEQ. Exposures with Se(IV) resulted in $HSeO_3^-$ (84%), and SeO_3^{2-} (16%) being the dominant species. In the study with added Se(VI), SeO_4^{2-} (96%), and $CaSeO_4$ (4%) were the dominant species. Selenium speciation did not vary significantly as a function of exposure concentration over the range of concentrations tested.

To confirm whether Se(IV) uptake was anion-dependent, we exposed daphnids to a water chemistry where all permeant anions were replaced by gluconate. These data showed elevated Se(IV) uptake rates in the gluconate water chemistry relative to the OECD water at Se(IV) concentrations of 2, 8, 16 and 32 μM (Figure 2.1; see Table A1 for statistical outcomes).

To identify which anions may have been responsible for limiting Se(IV) uptake in the gluconate experiment, a series of tests were conducted where the anion concentrations in the test water chemistry were varied. In the absence of sodium bicarbonate, there was no significant effect

on Se(IV) uptake relative to the OECD water control containing 0.77 mM $NaHCO_3$ at low Se(IV) concentrations (1, 2 and 4 μM). However, at high Se(IV) concentrations (8, 16 and 32 μM), the presence of bicarbonate significantly reduced Se(IV) uptake, to values between 32 and 50% of the bicarbonate-free treatment (Figure 2.2A; Table A1). The addition of 200 μM and 500 μM DIDS significantly reduced Se(IV) uptake at 8 μM and 32 μM, while only the former significantly reduced Se(IV) uptake at 1 μM (Figure 2.2B; Table A1).

In contrast to the effect of water bicarbonate, altering the chloride concentration of the water had no effect on Se(IV) uptake (Figure 2.3). A similar lack of effect was observed for sulphate, where removing magnesium sulphate from the OECD water had no significant impact on waterborne Se(IV) uptake across the tested Se(IV) concentration range (Figure 2.4).

The addition of phosphate to OECD water had a significant effect on Se(IV) uptake. At Se(IV) concentrations of 4 μM or lower, 1000 μM phosphate significantly reduced Se(IV) uptake relative to the phosphate-free control (Figure 2.5A; Table A1). Indeed, at 1 μM Se(IV) uptake was reduced by up to 36% of the control value. Additionally, at 2 and 4 μM, 10 μM phosphate significantly reduced Se(IV) uptake (Figure 2.5A; Table A1). The addition of 300 μM NAD^+ failed to affect Se(IV) uptake when tested at a waterborne Se(IV) concentration of 2 μM (Figure 2.5B), but 10 and 50 mM PFA/foscarnet significantly reduced Se(IV) uptake at this Se(IV) concentration (Figure 2.5C; Table A1). This effect was dose-dependent, with 10 mM PFA reducing Se(IV) uptake by 45%, and 50 mM PFA reducing Se(IV) uptake by 70%.

The addition of waterborne Se(VI) had no effect on Se(IV) uptake (Figure 2.6; Table A1). This effect persisted for both tested waterborne Se(VI) concentrations (4 and 32 μM), and despite

a large molar excess of Se(VI) relative to the very low Se(IV) concentration in the water (0.75 nM).

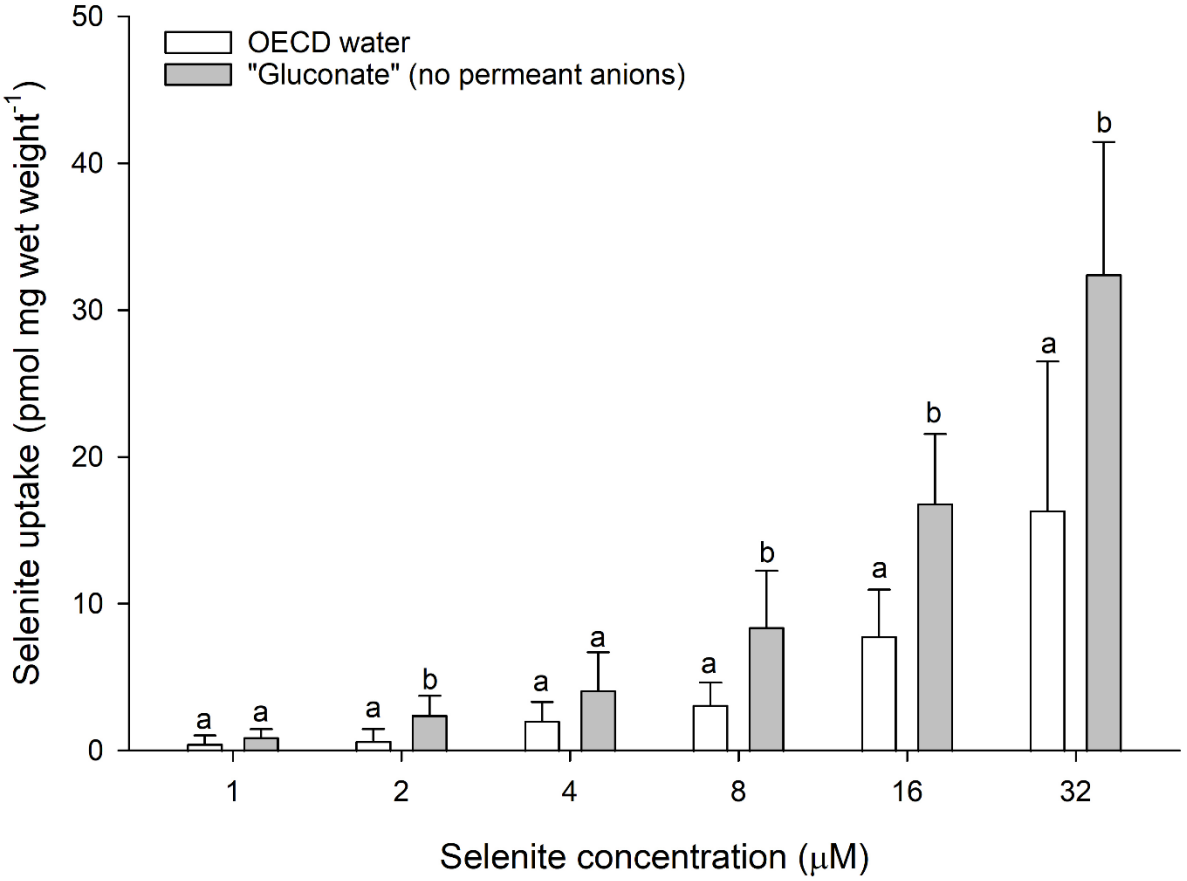

Figure 2.1 Selenite uptake (pmol mg wet weight^{-1}) in daphnids as a function of waterborne selenite concentration following a 1-h exposure to water without any permeant anions ("Gluconate", 0.77 mM $C_6H_{11}NaO_7$ and 0.08 mM $C_6H_{11}KO_7$; grey bars) or OECD water (control, white bars). Plotted points represent the means (± standard deviation) of 8 replicates. Bars sharing letters within each selenite concentration are not statistically significantly different, as determined by a one-way ANOVA followed by a post hoc Holm-Sidak test.

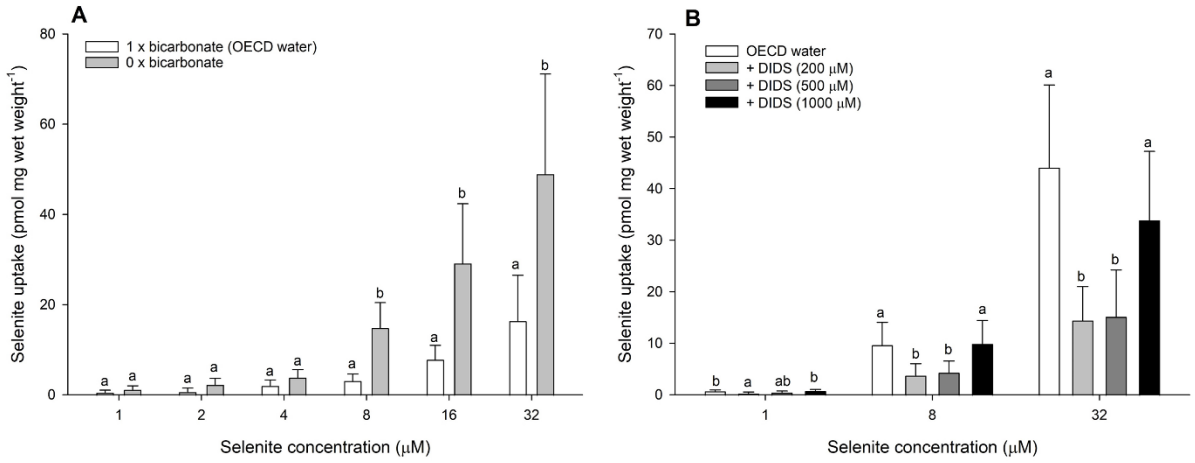

Figure 2.2 Selenite uptake (pmol mg wet weight^{-1}) in daphnids as a function of waterborne selenite concentration following a 1-h exposure to OECD water without (0 mM, grey bars) or with (0.77 mM, white bars) sodium bicarbonate (A), and in OECD water with varying DIDS concentration (0 mM, white bars; 200 μM, light grey bars; 500 μM, dark grey bars; 1000 μM, black bars; B). Plotted points represent the means (± standard deviation) of 8 replicates. Bars sharing letters within each selenite concentration are not statistically significantly different, as determined by a one-way ANOVA followed by a post hoc Holm-Sidak test or Kruskal-Wallis ANOVA (32 μM Se, Panel B).

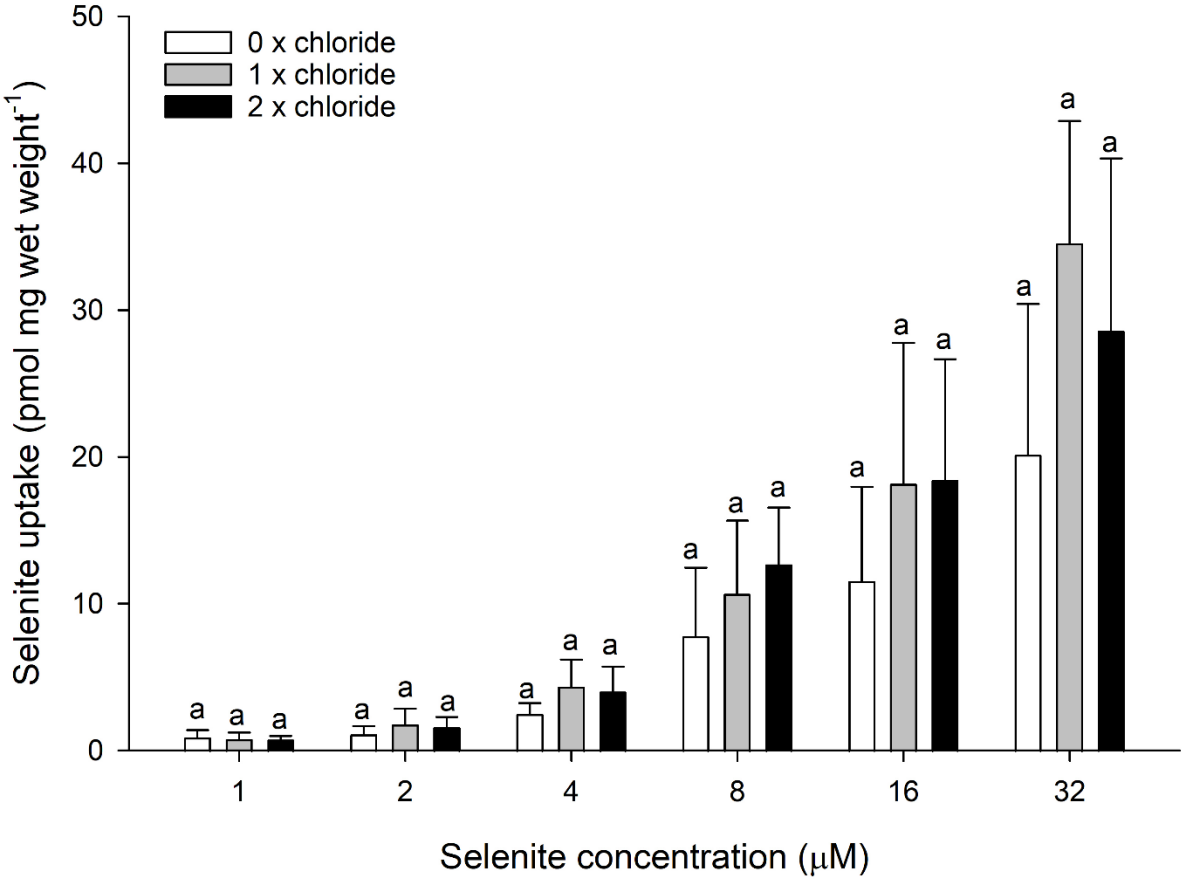

Figure 2.3 Selenite uptake (pmol mg wet weight^{-1}) in daphnids as a function of waterborne selenite concentration following a 1-h exposure to OECD water with varying chloride concentration (0 mM, white bars; 2 mM $CaCl_2 \cdot 2H_2O$ + 0.08 mM KCl, grey bars; 4 mM $CaCl_2 \cdot 2H_2O$ + 0.16 mM KCl, black bars). Plotted points represent the means (± standard deviation) of 8 replicates. Bars sharing letters within each selenite concentration are not statistically significantly different, as determined by a one-way ANOVA followed by a post hoc Holm-Sidak test.

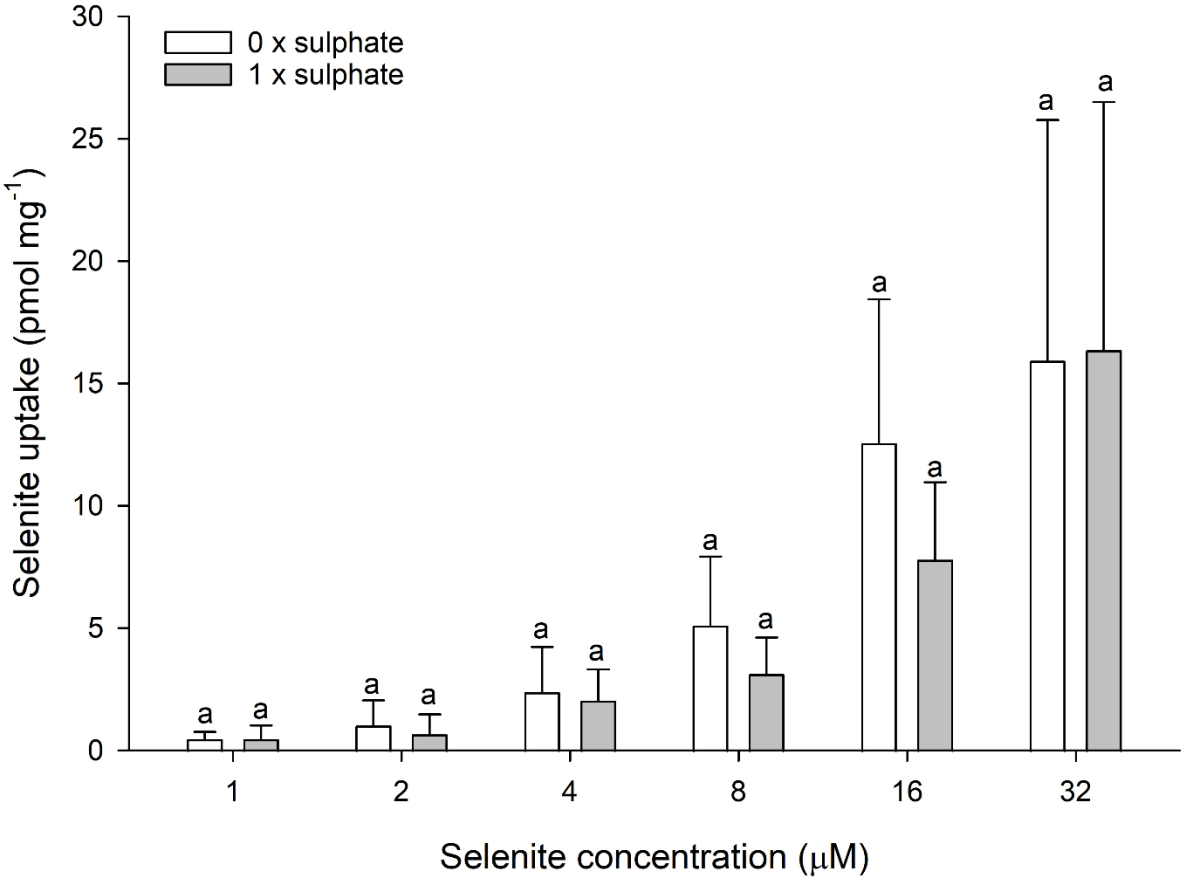

Figure 2.4 Selenite uptake (pmol mg wet weight^{-1}) in daphnids as a function of waterborne selenite concentration following a 1-h exposure to OECD water with varying sulphate concentration (0 mM, white bars; 0.5 mM $MgSO_4 \cdot 7H_2O$, grey bars). Plotted points represent the means (± standard deviation) of 8 replicates. Bars sharing letters within each selenite concentration are not statistically significantly different, as determined by a one-way ANOVA followed by a post hoc Holm-Sidak test.

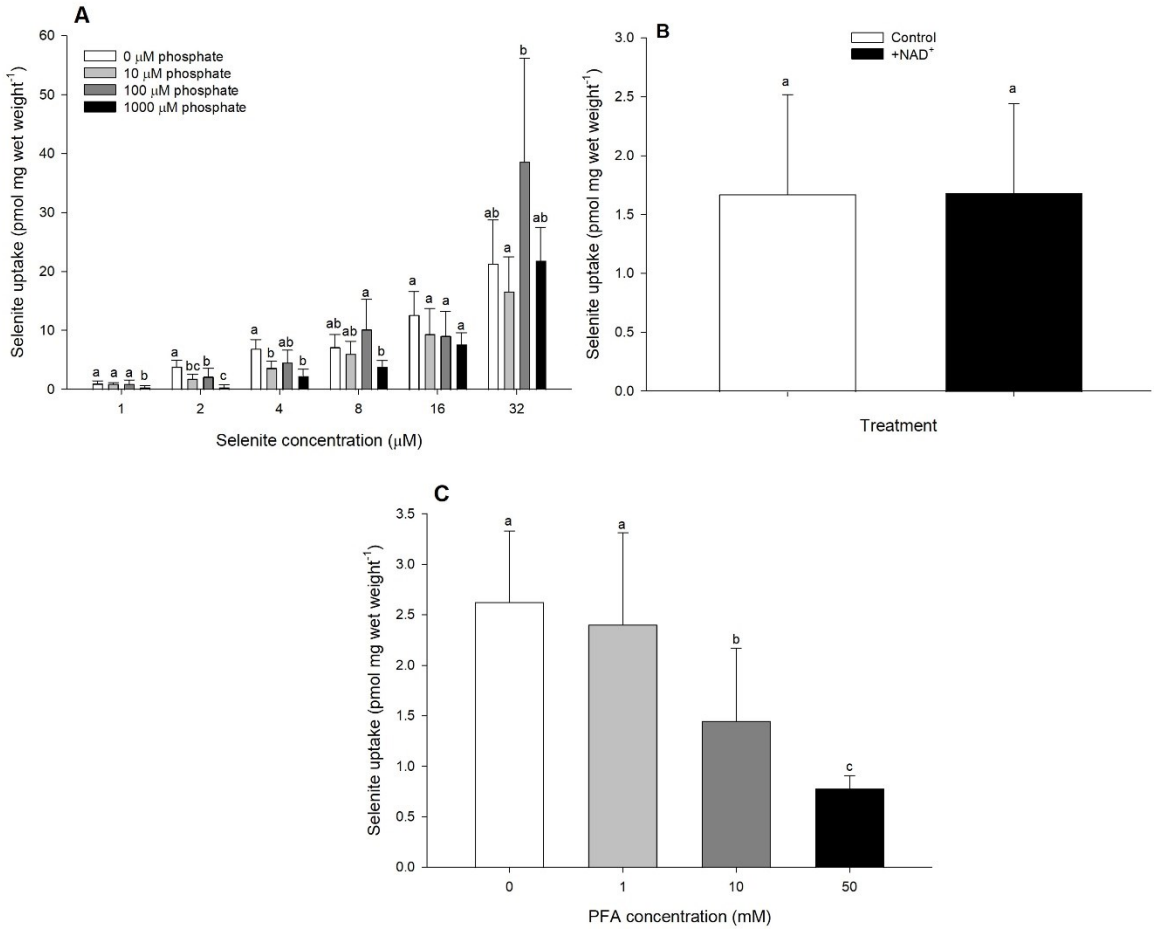

Figure 2.5 Selenite uptake (pmol mg wet weight^{-1}) in daphnids as a function of waterborne selenite concentration following a 1-h exposure to OECD water with varying sodium phosphate concentrations (0 μM, white bars; 10 μM, light gray bars; 100 μM, dark gray bars; 1000 μM, black bars; A), or in the presence of putative phosphate transporter inhibitors NAD^+ (0 μM, white bar; 300 μM, black bar; B) or PFA (0 mM, white bar; 1 mM, light gray bar; 10 mM, dark gray bar; 50 mM black bar; C). Plotted points represent the means (± standard deviation) of 8 replicates. Bars sharing letters within each selenite concentration are not statistically significantly different, as determined by a one-way ANOVA followed by a post hoc Holm-Sidak test.

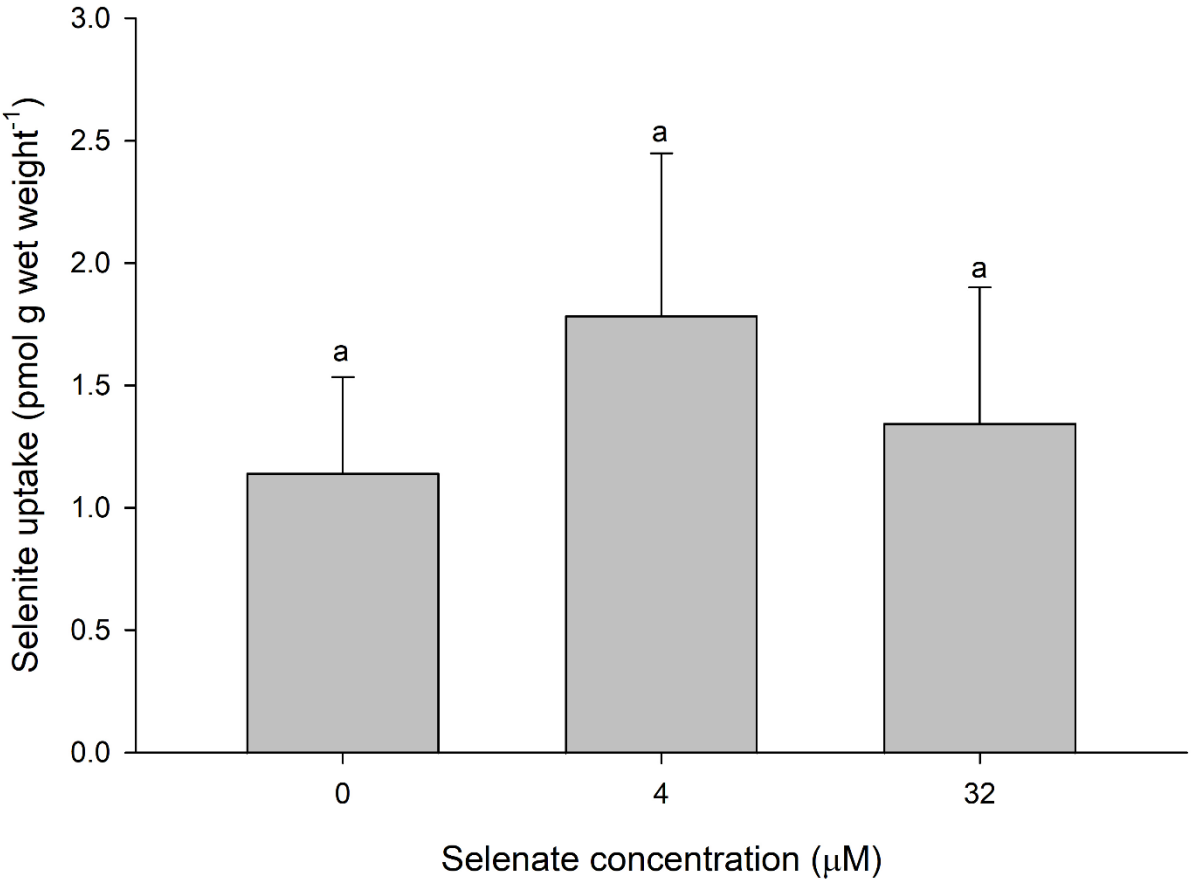

Figure 2.6 Selenite uptake (pmol g wet weight^{-1}) in daphnids as a function of waterborne selenate concentration following a 1-h exposure to OECD water. Plotted points represent the means (± standard deviation) of 8 replicates. Bars sharing letters within each selenite concentration are not statistically significantly different, as determined by a one-way ANOVA followed by a post hoc Holm-Sidak test.

2.4 Discussion

The uptake of waterborne Se(IV) in *Daphnia magna* is anion-dependent. As evidence for this, the removal of all permeant anions from the OECD culture water (i.e., no bicarbonate, chloride, or sulphate) in the gluconate treatment resulted in increased Se(IV) uptake compared to uptake in the OECD medium containing permeant anions. This suggests that at least one of the anions in OECD water likely competes with Se(IV) for uptake. This hypothesis, and the putative identity of competing anions, is discussed further below.

It is important to note that gluconate (added as a replacement for permeant anions in this study) is known to bind cations (Christoffersen & Skibsted, 1975). This is an important observation, because Yu & Wang (2002) showed that Se(IV) uptake in *D. magna* is calcium-dependent. However, at the gluconate concentrations (<1 mM), and the high ratio of calcium: gluconate (~2.3:1), used in the current study, calcium binding by gluconate would have been negligible (Leonhard-Marek et al., 2007). Furthermore, the chloride experiment provides indirect support for a lack of effect of calcium. In this study, the levels of $CaCl_2 \cdot 2H_2O$ ranged from 0 to 4 mM, and although this experiment was designed to test the effect of the anion, it also shows that there was no effect associated with the variation in calcium. This contrast with the study of Yu & Wang (2002) may be a consequence of a much lower Se(IV) concentrations over a much longer exposure period in the previous work than in the current study (0.016-0.643 µM versus 1-32 µM and 12 h versus 1 h), reflecting indirect responses of calcium (e.g., on membrane permeability; Yu & Wang, 2002), rather than effects directly related to interactions at a Se(IV) transporter. Overall, therefore, it is more likely that the effects of gluconate on Se(IV) uptake reflect the impact of permeant anion removal, rather than changes in cation bioavailability.

The inhibition of Se(IV) uptake at low concentrations (≤ 4 µM; 316 µg L^{-1}) by phosphate addition to the test water indicated that this anion was a key competitor. This hypothesis that Se(IV) uptake likely occurs through a phosphate transporter is consistent with studies on Se(IV) uptake in plants and yeast (Araie et al., 2011; Bai et al., 2022; Lazard et al., 2010; Vriens et al., 2016; Wang et al., 2019b; Zhang et al., 2014b). The finding of Se(IV) uptake in *D. magna* via a phosphate transporter is further supported by the effect of application of the phosphate transporter inhibitor PFA/foscarnet, wherein increased levels of this blocker reduced Se(IV) uptake. However, these results were not supported by the outcomes of an experiment with another putative phosphate transporter blocker, NAD^+, which failed to inhibit Se(IV) uptake. These inhibitors both target sodium-phosphate transporters such as those of the SLC34 family, however they have distinct putative modes of action. It is suggested that PFA has a competitive effect directly blocking the uptake site, whereas NAD^+ acts non-competitively by indirectly effecting transporter function through transporter modification (Loghman-Adham, 1996; Lucea et al., 2022; Sorribas et al., 2019). While evidence exists for sodium-dependent phosphate transporters in daphnids (e.g., National Center for Biotechnology Information (NCBI) Accession No. NC_059183.1), the relative efficacy of different inhibitors of the daphnid sodium-dependent phosphate transporters remain uncharacterised.

Our study provides evidence for Se(IV) uptake through a phosphate transporter at Se(IV) concentrations less than 4 µM (316 µg L^{-1}), with our lowest test concentration of 1 µM (79 µg L^{-1}). Although Se(VI) is the predominant oxidation state of selenium in most effluent-impacted waters (Martin et al., 2011), waterborne Se(IV) concentrations approaching our lowest test concentration have been reported. For example, a Se(IV) concentration of 57 µg L^{-1} (Etteieb et al., 2021) has been measured associated with mine effluents in Quebec, Canada. For context, water

quality guidelines for total selenium in North America range between 1 and 3.1 μg L^{-1} depending on jurisdiction and water body type (see Environment and Climate Change Canada, 2022). Consequently, the Se(IV) concentrations tested in the current study are of the same order of magnitude as elevated environmental concentrations. It is also worth highlighting that the phosphate concentrations used in the current study are also elevated relative to most natural waters. For example, our lowest test concentration of 10 μM would be considered at the higher end of the range of naturally-occurring phosphate concentrations of North American lakes (0.03 to 17 μM; Hudson et al., 2000). However, the phosphate concentrations were selected relative to the experimental Se(IV) concentrations, which as noted above, are high. Therefore, the key measure here is the ratio of phosphate to Se(IV). In the current work phosphate inhibition of Se(IV) uptake in *D. magna* occurred at ratios as low as 2.5:1 (10 μM phosphate versus 4 μM Se(IV)). Selenite concentrations in contaminated waters are usually in the order of 0.15 μM (Bujdoš et al., 2005; Lashari et al., 2022), and thus phosphate concentrations in the vicinity of 0.5 μM are likely to have an effect on daphnid Se(IV) uptake, assuming that use of the putative phosphate transporter is conserved at lower Se(IV) concentrations. Phosphate could be even more effective at inhibiting Se(IV) uptake, as lower concentrations of phosphate were not tested. For example, in the yeast *Saccharomyces cerevisiae* phosphate and Se(IV) were shown to share uptake pathways, but the two transporters characterised were 35 to 1260 times more specific for phosphate than for Se(IV), (Lazard et al., 2010). A full kinetic characterisation of phosphate uptake in the presence and absence of Se(IV) (and of Se(IV) uptake in the absence and presence of phosphate) would be required to draw conclusions regarding the relative affinities of the daphnid transporters for these two substrates. In general, therefore, lower, more environmentally relevant Se(IV) and phosphate concentrations were not tested in the current study and analysis of Se(IV) uptake at such

concentrations would be required to further confirm the broader environmental and conservation value of this finding.

Altering water bicarbonate concentration also impacted waterborne Se(IV) uptake in *D. magna*. Our results demonstrated that at relatively high Se(IV) concentrations (8-32 μM; 632 – 2527 μg L^{-1}) bicarbonate can partially block Se(IV) uptake. Bicarbonate-dependence of Se(IV) uptake has been suggested in human erythrocytes where Se(IV) demonstrated affinity for the AE1 (Band 3) bicarbonate/chloride transporter (Galanter et al., 1993; Kaur et al., 2020). Evidence from the current study supports this, with addition of DIDS, an inhibitor of AE1 (Romero et al., 2004), significantly reducing Se(IV) uptake. In our study the effects of DIDS were only seen in the two lower test concentrations (200 and 500 μM) and not at the highest test concentration (1 mM). Previous work has also shown 1 mM DIDS to be ineffective in modifying daphnid sodium transport (Bianchini & Wood, 2008), but lower concentrations were not tested. We speculate that the lack of dose dependency of DIDS may relate to non-specific effects on other transport pathways in daphnids, as has been observed in other species at high DIDS concentrations (Cabantchik & Greger, 1992). Importantly, there is also support for DIDS inhibition of Se(IV) accumulation in RBT (Misra et al., 2012), suggesting that this may be a conserved pathway for Se(IV) uptake. However, at least for daphnids, this pathway is unlikely to be of any environmental relevance, given its presence only at very high Se(IV) concentrations (> 632 μg L^{-1}).

The lack of effect of water chloride manipulation argues against Se(IV) uptake being achieved through a bicarbonate/chloride transporter. However, there are several key points of evidence to consider. First, DIDS does not completely inhibit Se(IV) uptake (application of 200 and 500 μM DIDS drops Se(IV) uptake to between 33 and 67% of the uninhibited control), indicating that a component of Se(IV) transport occurs independently of DIDS-sensitive

transporters. This could even be indicative of the previously noted phosphate transporter that may become more important at high Se(IV) concentrations in the absence of a viable bicarbonate-related pathway. Second, the addition of DIDS is likely to be a more effective intervention than removing water chloride. Removing chloride from the water does not remove chloride from the animal. Therefore, the presence of chloride in daphnid tissues may be sufficient to maintain the actions of a bicarbonate/chloride exchange even in the absence of water chloride. Third, DIDS is known to inhibit other bicarbonate transporters in the SLC4 family, such as sodium/bicarbonate exchangers (Romero et al., 2004). Indeed, evidence in fish suggests that sodium/bicarbonate exchange may be a pathway of Se(IV) uptake in enterocytes and hepatocytes (Misra et al., 2012). Taken together the effects of DIDS and the lack of effect of water chloride, indicate that a bicarbonate exchanger of the SLC4 family may be the mediator of the bicarbonate-dependent Se(IV) uptake in *D. magna*.

Similar to the lack of effect of chloride, changing water sulphate also had no impact on Se(IV) uptake in *D. magna*. This indicates that sulphate transporters are unlikely to be involved in Se(IV) uptake in this species. This is in accordance with multiple other studies that demonstrate Se(IV) is not sulphate dependent. This is in contrast to uptake of Se(VI), which is shown to be sulphate-dependent in a number of different study systems (Brown & Shrift, 1980; Hansen et al., 1993; Ogle & Knight, 1996; Selinus, 2013; Vriens et al., 2016), including *D. magna* (Yu & Wang, 2002).

As Se(VI) uptake occurs via sulphate transporters, and Se(IV) uptake is sulphate-independent, then it is no surprise that there was a lack of effect of Se(VI) on Se(IV) uptake in our study. The lack of effect also suggests that there is no significant conversion of Se(VI) to Se(IV), for example by chemical or enzymatic reduction in the vicinity of the epithelial surface. This is a

phenomenon that has been shown to occur for the uptake of copper and iron in aquatic biota (Bury et al., 2003).

2.5 Conclusion

The current study demonstrated that *D. magna* take up waterborne selenium in the form of Se(IV). Furthermore, at Se(IV) concentrations characteristic of highly contaminated systems, water phosphate addition inhibited Se(IV) uptake. At even higher Se(IV) concentrations, waterborne bicarbonate blocked uptake. The former observation is likely mediated by a sodium-phosphate transporter as blockade of this transporter by the phosphate transporter inhibitor PFA/foscarnet also blocked Se(IV) uptake. The effect of water bicarbonate was blocked by the AE1 inhibitor DIDS, but not affected by water chloride, suggesting that Se(IV) uptake may be mediated by a DIDS-sensitive sodium/bicarbonate transporter. Therefore, further studies are necessary to better clarify the mechanism by which bicarbonate interferes with Se(IV) uptake in daphnids. Further work also needs to examine Se(IV) uptake at more environmentally-realistic concentrations, to determine if the effects of phosphate persist in natural settings. Critically however, the evidence to date suggests that the concentration of phosphate in a receiving water could provide protection against the accumulation of Se(IV) in zooplankton affected by coal mining effluents. Such an effect would likely offset potential selenium toxicity and limit biomagnification in higher trophic levels protecting biodiversity in impacted waterways.

Chapter 3

Water chemistry differentially affects tissue selenite accumulation in two freshwater salmonid fish

3.1 Introduction

Mining and agricultural activities are associated with the introduction of the trace element selenium to aquatic environments (Shan et al., 2019; USEPA, 2016). Selenium in aquatic systems is of major concern as there is a narrow margin between concentrations that are considered essential and those that cause toxicity (Lemly, 2004; Selinus, 2013). For example, at an organismal level toxic effects of selenium include reduced fitness, metabolic stress, and teratogenesis (see Table 1.4). At a population level, selenium has been associated with extirpation of fish in multiple field studies worldwide (Brandt et al., 2017; Skorupa, 1998). The effects of selenium on aquatic biota are strongly correlated to tissue accumulation (DeForest & Adams, 2011). Indeed, selenium accumulation in fish tissue is a better indicator of potential toxicity than environmental concentrations as selenium bioavailability can be species-dependent and impacted by environmental factors (DeForest & Adams, 2011).

Within natural freshwater systems, inorganic selenium occurs in two different forms: Se(IV) and Se(VI), where the dominant form depends upon water chemistry. For example, at pH 5 to 9 Se(IV) dominates and at pH < 5 and > 9 Se(VI) dominates (Sharma et al., 2015; Torres et al., 2011). Under mildly oxidizing conditions Se(IV) dominates whereas at highly oxidizing conditions Se(VI) dominates (Sharma et al., 2015). Furthermore, speciation in water can depend on the source of selenium, where Se(VI) largely comes from agricultural runoff and Se(IV) comes from mining practices (USEPA, 2016). Selenite is generally more bioavailable to aquatic organisms than Se(VI), and as a consequence has a greater bioaccumulation (Ma et al., 2018; Simmons & Wallschläger, 2005). For example, when topmouth gudgeon, *Pseudorasbora parva*, were exposed to 1000 μg L^{-1} of waterborne Se(IV) and Se(VI), total gill selenium burden was 173% higher in the Se(IV) treatment than in the Se(VI) treatment (Ma et al., 2018). Although

waterborne selenium is an important contributor to selenium burden (Besser et al., 1993; Lee et al., 2006; Roditi & Fisher, 1999; Wang et al., 1996; Wang & Fisher, 1999), at higher trophic levels the majority of selenium is sourced from the diet, likely as organic selenium forms such as SeMet (Besser et al., 1993; Lemly, 2004; Presser & Luoma, 2010). Nevertheless, evidence to date suggests that waterborne uptake is still an important contributor to fish selenium body burden and its toxic effects (see Table 1.4). Despite the importance of waterborne selenium to fish health, the pathways involved in waterborne Se(IV) tissue accumulation in fish are poorly characterised.

In most natural systems Se(IV) is present as an anion (i.e., $HSeO_3^-$), and thus uptake and accumulation in fish is likely to be mediated by anionic transporters. This hypothesis is supported by evidence from other studied systems. For example, Se(IV) uptake in *Daphnia magna* is influenced by concentrations of phosphate and bicarbonate in the water and is responsive to phosphate and bicarbonate transporter inhibitors (Chapter 2; Klaczek et al., 2024). In one of the few studies to examine Se(IV) handling in a freshwater teleost, Misra et al. (2012) suggested that there is a competitive interaction between Se(IV) and sulphite in RBT hepatocytes. These authors also demonstrated that Se(IV) accumulation was inhibited by the presence of the anion exchanger 1 inhibitor 4-4'-diisothiocyano-2,2'-stilbenedisulfonic acid in hepatocytes and enterocytes (Misra et al., 2012). To date, however, selenium handling by the fish gill has not been examined. As the gill is the key site mediating exchange between the organism and the environment, it is likely to have an important influence on waterborne Se(IV) handling in fish (Evans et al., 2005).

Westslope cutthroat trout (*Oncorhynchus clarkii lewisi*) and RBT (*Oncorhynchus mykiss*) are salmonid fish native to North America with habitat ranges that overlap with that of coal mine locations (Pearce et al., 2011; U.S. Energy Information Administration, 2023). As such there is concern regarding the exposure of these fish to effluents rich in selenium. Indeed, coal mine

wastewater can contain up to 1000 µg L^{-1} of selenium, and although most studies lack appropriate characterisation of speciation, 57 µg L^{-1} of Se(IV) has been observed downstream of mining activities in Canada (Chapman, 2010; Etteieb et al., 2021). Furthermore, WCT are viewed as an indicator species for overall freshwater system health due to their restricted habitat needs (Cleator et al., 2009), whereas RBT are a model species with a well-characterised sensitivity to waterborne selenium (juvenile 96-h LC_{50} of 9 mg L^{-1}; Buhl & Hamilton, 1991). Although closely related, these species are known to differ in their physiology (Bear et al., 2007; Rasmussen et al., 2012; Robinson, 2007). For example, it has been demonstrated that RBT are more tolerant to thermal stress, having a lethal limit 4.7°C higher than WCT (Bear et al., 2007), and this species also has activities of the enzymes LDH, citrate synthase and acetylcholinesterase that are higher than those of WCT (Rasmussen et al., 2012). In terms of assessing the potential risk of selenium-rich effluents to biota in receiving environments it is important to understand whether the patterns of selenium handling are conserved between even closely related species, and thus whether principles established in one species are more widely applicable.

The current study aimed to examine the mechanism of waterborne Se(IV) accumulation in *O. clarkii lewisi* and *O. mykiss*. To date, there is little mechanistic understanding of how waterborne Se(IV) is handled in freshwater teleosts. We utilized radiolabelled selenite (Se^{75}) and a variety of different water chemistries to evaluate the role of anionic transporters in Se(IV) accumulation. Effects of a putative phosphate transporter inhibitor, PFA, was used to further probe the mechanism of accumulation. The effects of Se(VI) were examined to assess if Se(VI) affects Se(IV) accumulation (i.e., pathways of handling were similar between the two selenium species). Mechanistic understanding of the factors affecting accumulation of waterborne Se(IV) in WCT and RBT will provide valuable insight into species differences in Se(IV) handling, and how water

chemistry may affect Se(IV) tissue burden and subsequent toxicity. Indeed, when detoxification and excretion rates can not match accumulation rates, excess selenium in tissues disrupts normal cellular functions and causes harm to important enzymes and proteins. Often, the level of effect coincides with the level of selenium accumulation in the organism where toxicity occurs as the organism can not regulate vital processes or detoxify accordingly (e.g., Chen et al., 2020; Gobi et al., 2018; Kim & Kang, 2014).

3.2 Materials and Methods

3.2.1 Animals

Westslope cutthroat trout (*Oncorhynchus clarkii lewisi*) of mean (± standard deviation) mass 4.9 ± 1.0 g (n = 30) and RBT (*Oncorhynchus mykiss*) of mean (± standard deviation) mass 48.2 ± 10.8 g (n = 30) were used in the current study. These were sourced from Government of Alberta fish hatcheries and maintained in the aquatic facility at the University of Alberta, in flow-through dechlorinated City of Edmonton water ("facility" water; 1.5 mM Na, 1.2 mM Ca, 0.5 mM Mg, 0.06 mM K, pH 7.5) until experimental exposures. Fish were maintained at temperatures of 10°C, fed twice daily with commercial trout chow and subjected to a 16:8-h light:dark cycle. All procedures were approved by the University of Alberta Biosciences Animal Care and Use Committee (Animal Use Protocol 4068).

3.2.2 Selenium accumulation

The accumulation of Se(IV) was examined in a variety of water chemistries (detailed below), over a 6-h exposure period. This exposure duration was chosen following preliminary studies that demonstrated uptake was in the linear phase at 6 h. Westslope cutthroat trout exposures were conducted in covered, aerated 600-mL PYREX glass beakers, each containing an individual

fish (n = 5) and 400-mL test water. Rainbow trout exposures were conducted in covered, aerated tanks made of black PVC, each containing an individual fish (n = 5) and 600-mL test water. Test water pH was 7.5 and temperature was maintained at ~10°C, by placing exposure chambers in a temperature-controlled wet table. Uptake was determined using radiolabelled selenite (Se^{75}; University of Missouri Research Reactor Centre; 1.4 µCi per test chamber). After 6-h of exposure, fish were carefully removed from the exposure chambers, and individually rinsed through a series of three solutions to remove adsorbed, but not absorbed isotope (2 x 10 s in unlabelled facility water, followed by 10 s in 1 g L^{-1} Se(IV) (as sodium selenite)). After rinsing, fish were euthanized (200 mg L^{-1} tricaine mesylate, buffered to neutral pH with sodium bicarbonate), weighed, measured, and dissected. The following tissues were removed, weighed, and processed as detailed below: gill, muscle, liver, and gut.

The following water chemistries were examined: 1. Artificial freshwater (termed "OECD" from this point forth) (2 mM $CaCl_2 \cdot 2H_2O$; 0.5 mM $MgSO_4 \cdot 7H_2O$; 0.77 mM $NaHCO_3$; 0.08 mM KCl; pH ~7.5); 2. Facility water (composition as above); 3. Phosphate (OECD water supplemented with 1 mM Na_3PO_4); 4. Bicarbonate (OECD water without $NaHCO_3$); 5. Selenate (OECD water supplemented with 32 µM Se(VI) (as sodium selenate)). All artificial water chemistries used ultrapure (>18 MΩ) water as a base, and all had pH adjusted to pH ~7.5 using potassium hydroxide and/or hydrochloric acid. No attempts were made to correct for ionic strength or to hold cation concentrations identical across treatments. To account for potential fluctuations of Se(IV) accumulation rates over times, a time-matched control was run concurrently with all test water chemistries. Selenium speciation in all experimental waters was modelled using Visual MINTEQ.

After weighing, individual tissues were placed in 12 x 75mm borosilicate culture tubes and whole tissue radioactivity (i.e., counts per minute) was measured using a Cobra Quantum gamma

counter. All radioactivity counts were transformed to pmol of Se(IV) by dividing counts per minute (cpm) by water specific activity (cpm $pmol^{-1}$). This value was then divided by tissue mass to give newly accumulated Se(IV) in pmol mg^{-1}, converted to pmol kg^{-1}, and subsequently divided by exposure time in hours to give pmol kg^{-1} h^{-1}. The limit of detection was calculated as the mean + 3 x standard deviation of measured blanks (empty tubes).

3.2.3 Inhibitor experiments

Inhibitor studies were conducted as described above, except for the addition of putative modifiers of Se(IV) accumulation. Phosphonoformate (aka Foscarnet; Sigma-Aldrich St. Louis, MO), a putative phosphate transporter inhibitor, was added to facility water at a concentration of 10 mM immediately prior to fish addition. This concentration is above that previously demonstrated to affect phosphate transport in RBT (Avila et al., 2000).

3.2.4 Statistics

All data were first assessed for normality and homogeneity of variance using Shapiro-Wilk and Brown-Forsythe tests, respectively. Where data did not conform to parametric assumptions, and after transformations failed to make the data normal and homoscedastic, data were analyzed via non-parametric tests. Ultimately, a t-test was used to analyze differences between the facility water and PFA treatment (the two manipulations conducted in natural media), whereas a one-way ANOVA followed by a post hoc Tukey's test was used to analyze the differences between treatments conducted in artificial waters (OECD, phosphate, bicarbonate, and Se(VI)). The exceptions to this were for WCT gut and RBT gills, where a non-parametric Kruskal-Wallis test, with post hoc Dunn's test was performed, and for WCT muscle where a Mann-Whitney U test was utilized to analyze the differences between facility water and PFA. All statistical analyses were performed, and graphs were made using PRISM GraphPad 10. Significance for all data was

assessed at an alpha level of 0.05. Unless otherwise stated, all data are expressed as the mean ± standard deviation.

3.3 Results

Fish survival across all water treatments was 100%. Water quality parameters (pH, temperature) among all exposures were maintained within acceptable standard levels with minimal fluctuations between treatments. Selenite speciation remained consistent across water treatments at pH 7.5 as modeled by Visual MINTEQ: $HSeO_3^-$ (84%), SeO_3^{2-} (16%). In the Se(VI) treatment, Se(VI) speciated as follows: SeO_4^{2-} (96%), $CaSeO_4$ (4%).

Overall, it is important to note that WCT had higher accumulation rates relative to RBT. For example, the gills of WCT demonstrated mean accumulation of 4.1 pmol kg h^{-1} in the facility water control whereas RBT had mean accumulation of 1.1 pmol kg h^{-1} (Figure 3.1).

There was a significant effect of water chemistry on Se(IV) accumulation in the gills of WCT. The addition of the phosphate transporter inhibitor PFA to the exposure water significantly reduced Se(IV) uptake by 54% in the gills when compared to the facility water control (Figure 3.1A). The addition of phosphate to OECD water reduced branchial Se(IV) uptake by 57% in WCT, but this effect narrowly eluded statistical significance (Figure 3.1A; Tukey's test, p = 0.0936). There was no effect of bicarbonate or Se(VI) treatments on branchial Se(IV) burden in WCT (Figure 3.1A). There was no significant effect of any water treatment on Se(IV) accumulation in the gills of RBT (Figure 3.1B).

The addition of phosphate to OECD water significantly reduced Se(IV) burden in the liver by 84% in WCT (Figure 3.2A). However, there was no significant effect of PFA on Se(IV) uptake (Figure3.2A; t-test, p = 0.0507). Bicarbonate and Se(VI) treatments had no significant effect on

hepatic Se(IV) accumulation in this species (Figure 3.2A). No significant effect was observed in RBT Se(IV) liver burden for any water treatment (Figure 3.2B).

A lack of significant effect of Se(IV) accumulation in the gut was observed for all water treatments of both WCT and RBT (Figure 3.3A,B). Similarly, altering the water chemistry had no significant effect on WCT muscle Se(IV) accumulation (Figure 3.4). Muscle tissue from RBT displayed Se(IV) concentrations that were below the limit of detection (0.096 pmol kg^{-1} h^{-1}) and were thus excluded from further analysis.

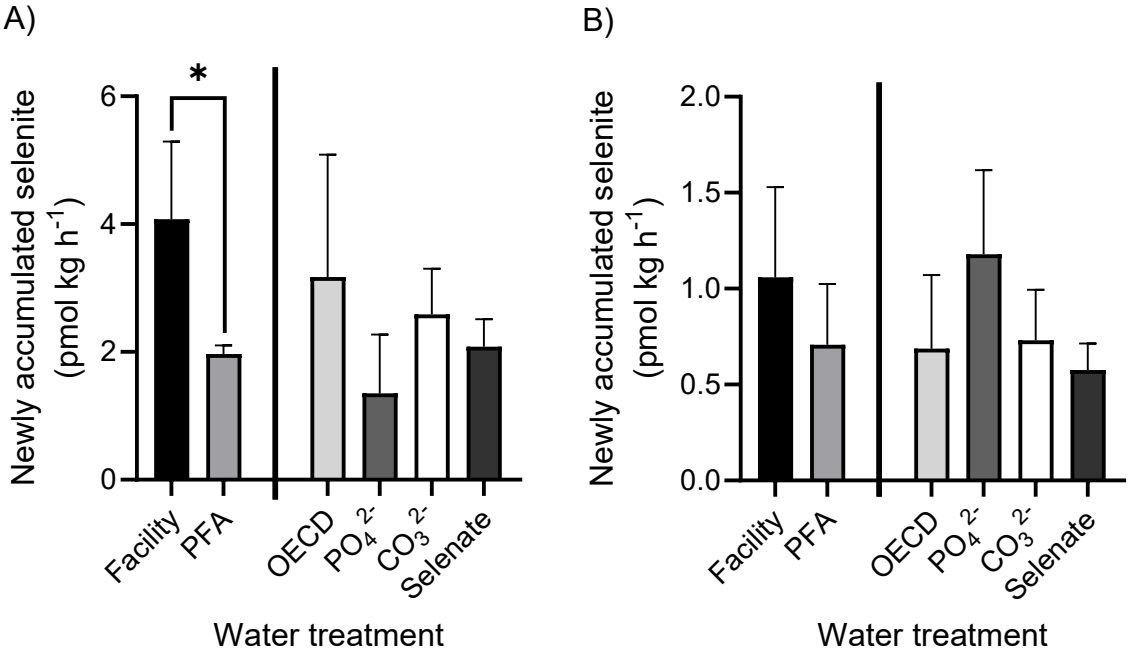

Figure 3.1 Selenite uptake (pmol kg h^{-1}) in A) Westslope cutthroat trout and B) rainbow trout gills following a 6-h exposure in various water chemistries: Facility (1.5 mM Na, 1.2 mM Ca, 0.5 mM Mg, 0.06 mM K; black bars), PFA (10 mM; medium light grey bars), OECD (2 mM $CaCl_2 \cdot 2H_2O$; 0.5 mM $MgSO_4 \cdot 7H_2O$; 0.77 mM $NaHCO_3$; 0.08 mM KCl; light grey bars), PO_4 (OECD water supplemented with 1 mM Na_3PO_4; medium grey bars), CO_3 (OECD water without $NaHCO_3$; white bars) and selenate (OECD water with 32 µM selenate; dark grey bars). Bars represent the means (± standard deviation) of 5 replicates. Asterisk indicates a statistically significant difference, as determined by a t-test.

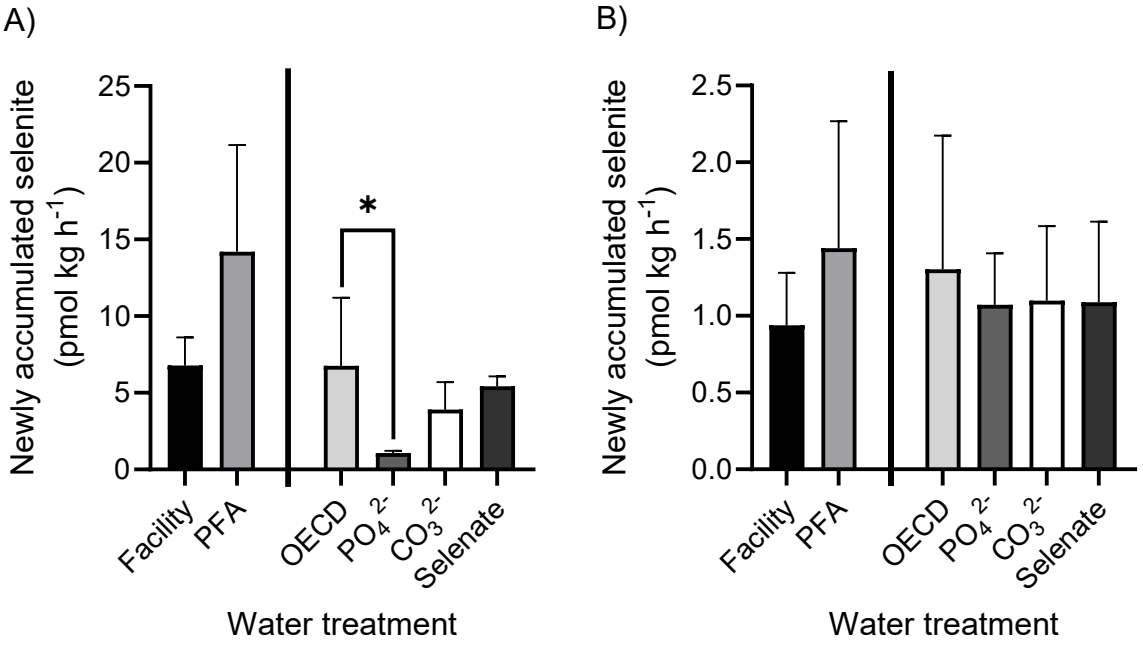

Figure 3.2 Selenite uptake (pmol kg h^{-1}) in A) Westslope cutthroat trout and B) rainbow trout livers following a 6-h exposure in various water chemistries: Facility (1.5 mM Na, 1.2 mM Ca, 0.5 mM Mg, 0.06 mM K; black bars), PFA (10 mM; medium light grey bars), OECD (2 mM $CaCl_2 \cdot 2H_2O$; 0.5 mM $MgSO_4 \cdot 7H_2O$; 0.77 mM $NaHCO_3$; 0.08 mM KCl; light grey bars), PO_4 (OECD water supplemented with 1 mM Na_3PO_4; medium grey bars), CO_3 (OECD water without $NaHCO_3$; white bars) and selenate (OECD water with 32 µM selenate; dark grey bars). Bars represent the means (± standard deviation) of 5 replicates. Asterisk indicates a statistically significant difference, as determined by a one-way ANOVA followed by a post hoc Tukey's test.

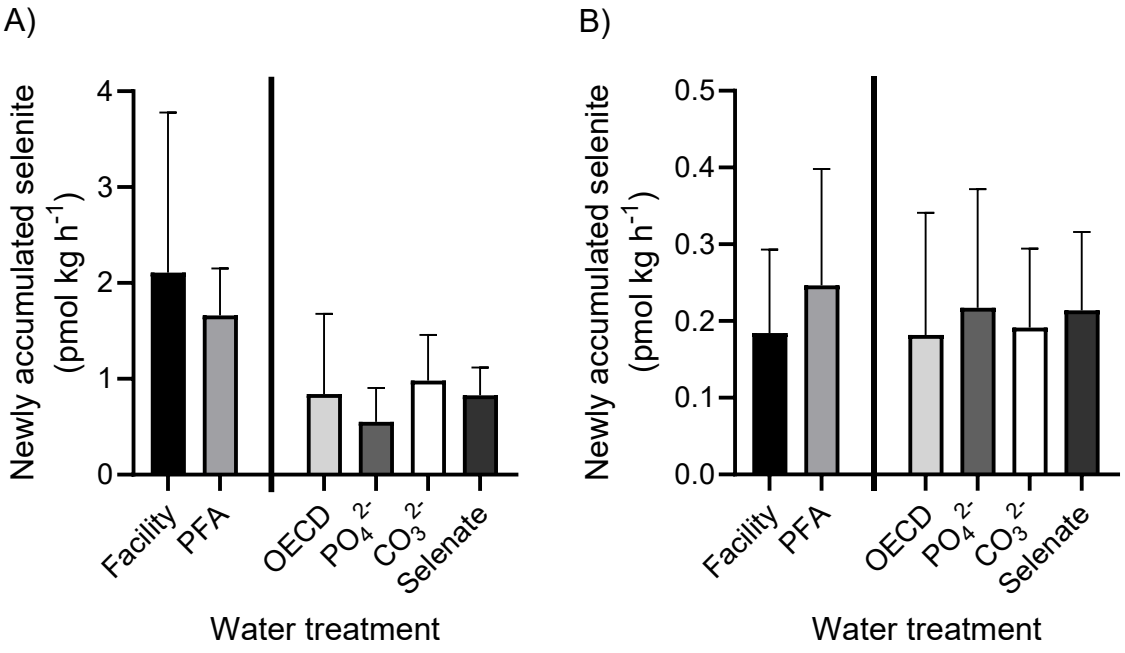

Figure 3.3 Selenite uptake (pmol kg h^{-1}) in A) Westslope cutthroat trout and B) rainbow trout guts following a 6-h exposure in various water chemistries: Facility (1.5 mM Na, 1.2 mM Ca, 0.5 mM Mg, 0.06 mM K; black bars), PFA (10 mM; medium light grey bars), OECD (2 mM $CaCl_2 \cdot 2H_2O$; 0.5 mM $MgSO_4 \cdot 7H_2O$; 0.77 mM $NaHCO_3$; 0.08 mM KCl; light grey bars), PO_4 (OECD water supplemented with 1 mM Na_3PO_4; medium grey bars), CO_3 (OECD water without $NaHCO_3$; white bars) and selenate (OECD water with 32 µM selenate; dark grey bars). Bars represent the means (± standard deviation) of 5 replicates.

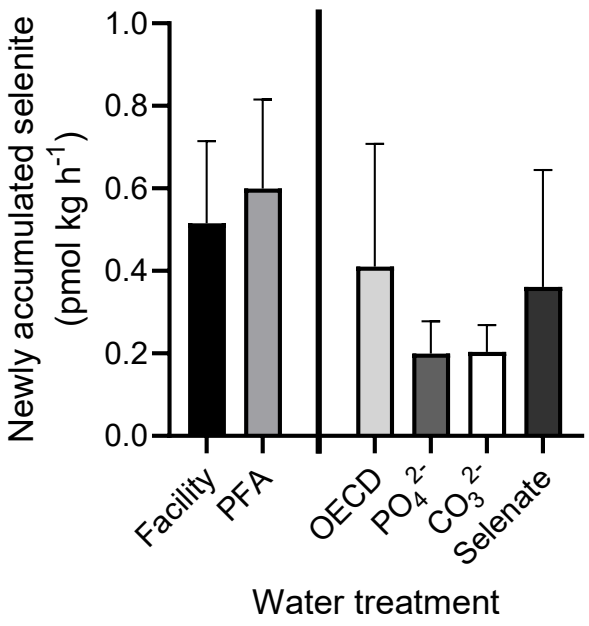

Figure 3.4 Selenite uptake (pmol kg h^{-1}) in Westslope cutthroat trout muscle following a 6-h exposure in various water chemistries: Facility (1.5 mM Na, 1.2 mM Ca, 0.5 mM Mg, 0.06 mM K; black bars), PFA (10 mM; medium light grey bars), OECD (2 mM $CaCl_2 \cdot 2H_2O$; 0.5 mM $MgSO_4 \cdot 7H_2O$; 0.77 mM $NaHCO_3$; 0.08 mM KCl; light grey bars), PO_4 (OECD water supplemented with 1 mM Na_3PO_4; medium grey bars), CO_3 (OECD water without $NaHCO_3$; white bars) and selenate (OECD water with 32 µM selenate; dark grey bars). Muscle selenite concentrations for *O. mykiss* were below levels of detection and were excluded from analysis. Bars represent the means (± standard deviation) of 5 replicates.

3.4 Discussion

The accumulation of waterborne Se(IV) was dependent upon water anion composition in *O. clarkii lewisi* but was anion-independent in *O. mykiss*. Specifically, reduced Se(IV) accumulation in response to variations in water PFA and phosphate was observed in *O. clarkii lewisi* gill and liver, respectively.

The inhibition of waterborne Se(IV) uptake by addition of the phosphate transporter inhibitor PFA, along with the trend of an inhibited branchial Se(IV) burden when phosphate was added to the exposure water, suggests that phosphate may block the uptake of Se(IV) at the gill of *O. clarkii lewisi*. A previous study in our laboratory demonstrated a similar finding in daphnids (Chapter 2; Klaczek et al., 2024). Selenite is also proposed to be taken up via phosphate-transporter-mediated pathways in plants (Araie et al., 2011; Bai et al., 2022; Hopper & Parker, 1999; Li et al., 2008; Wang et al., 2019b; Zhang et al., 2014b; Zhu et al., 2009). These data suggest that this may be a conserved route of Se(IV) uptake across eukaryotic organisms.

The phosphate transporter inhibitor PFA specifically targets sodium-phosphate transporters, such as those of the SLC34 family. Inhibition occurs via competition at the uptake site blocking access of the substrate and thereby preventing ion exchange (Loghman-Adham, 1996; Sorribas et al., 2019). Given that blocking this transporter also reduced Se(IV) accumulation, this suggests that a phosphate transporter is responsible for uptake across the gills in this species. In addition to this functional evidence, there is also structural evidence for sodium-dependent phosphate transporters in *Oncorhynchus* spp. (e.g., National Center for Biotechnology Information (NCBI) Accession No. NC_048575.1, NC_034181.2). While expression levels of SLC34 transporters are not high in fish gills specifically, their expression has been detected in this tissue in at least some teleost fish species (Dai et al., 2021; Verri & Werner, 2019).

In contrast to the phosphate sensitive Se(IV) accumulation in the gill of *O. clarkii lewisi*, there was no effect of either phosphate supplementation or PFA application on Se(IV) gill accumulation in *O. mykiss*. Indeed, no water chemistry manipulation had any significant effect on branchial Se(IV) burden in RBT. Although these two fish are closely related, they are known to possess distinct physiological responses (Bear et al., 2007; Rasmussen et al., 2012; Robinson, 2007), and if these differences exist for Se(IV) handling then this could explain the difference in gill accumulation patterns. It is important to note that a previous study on this species identified alternate pathways of uptake for Se(IV). Using isolated liver and intestinal cells, Misra et al. (2012) demonstrated that Se(IV) uptake was related to sulphite transport and was inhibited by the AE1 inhibitor DIDS. These authors did not, however, test for an interaction between phosphate and Se(IV). In the current study, no effect of bicarbonate water manipulation was observed, which therefore contrasts with the finding of AE1-mediated transport in the previous study. Consequently, distinct outcomes exist between our work and previous findings. In our study, Se(IV) uptake was studied in vivo, where there are multiple confounding factors that could modify epithelial transport processes. For example, the presence of gill mucus and effects of microenvironment pH may modify transport processes (Randall et al., 1991; Toa et al., 2000, 2002). Additionally, Misra and colleagues used isolated liver and intestinal cells, while phosphate-related accumulation of Se(IV) in the current work was identified in the gill. Further work is required to understand the differences between these two RBT studies, and to investigate the differences in branchial Se(IV) handling between the two salmonid species in the current study.

It is also possible that the size difference in *O. clarkii lewisi* (~5 g) and *O. mykiss* (~ 50 g) used in our study may have contributed to differences in branchial Se(IV) accumulation. Size dependent accumulation of trace elements has been suggested in other aquatic organisms. For

example, trace element tissue burden has been correlated to body size in mussels with increased accumulation associated with smaller organisms (Wang & Dei, 1999; Wang & Fisher, 1997). This pattern corresponds to the current study, where *O. clarkii lewisi*, the smaller fish, had higher Se(IV) accumulation rates than *O. mykiss*, the larger fish. This could be due to a greater surface area to volume ratio in the smaller fish, thereby allowing for higher uptake rates across the epithelia. This hypothesis has support for selenium handling in aquatic invertebrates, with studies having shown that as organism size increases, Se(IV) accumulation rate declines (Wang & Dei, 1999; Wang & Fisher, 1997). However, smaller fish generally have lower metabolic rates than larger fish (Urbina & Glover, 2013), and thus they would be predicted to have lower flow rates of Se(IV) over the gill, and therefore lower rates of trace element accumulation (Harley & Glover, 2014). One of the few studies to examine the relationship between body size and selenium accumulation in fish, showed that there was no significant correlation between size and gill selenium concentrations (Tashi et al., 2022). However, this study was performed on field-collected fish where uptake would be dominated by dietary selenium.

One other factor that may be size-dependent and which may affect Se(IV) accumulation, is phosphate metabolism. Sambraus et al. (2020) found that earlier life stages (i.e., smaller) Atlantic salmon have higher phosphorous requirements than later life stages (i.e., larger). This could account for the higher Se(IV) accumulation in *O. clarkii lewisi* as higher phosphate demand could result in increased availability of this pathway, and thus greater Se(IV) uptake. However, in another study, the uptake of dietary phosphorus was only slightly affected by body size in RBT, and this relationship depended on dietary composition (Satoh et al., 2002). Overall, therefore, evidence is equivocal regarding the role of body size on tissue selenium accumulation, and thus whether the

differences observed between the two salmonid fish species in this study is a function of body mass or a true species difference.

Similar to the effect seen in the gills in *O. clarkii lewisi*, phosphate significantly reduced Se(IV) uptake in the liver of this species. As noted above, the only other study that has examined Se(IV) uptake in teleost liver (in that case isolated hepatocytes) suggested Se(IV) is taken up by pathways other than those related to phosphate uptake (i.e., related to sulphite and inhibited by the AE1 inhibitor DIDS). The study of Misra and colleagues used higher Se(IV) concentrations than those in the current study, and because pathways of different transport affinity have been suggested to be involved in Se(IV) uptake (Klaczek et al., 2024; Lazard et al., 2010; Misra et al., 2012), then the DIDS sensitive transporter may be a lower affinity pathway not active at our study concentrations. Furthermore, as gills are the site responsible for waterborne ion exchange in fish, effects of water chemistry on the liver are likely to reflect interactions at the gill. Therefore, because of the trend towards reduced gill Se(IV) in the phosphate treatment, less Se(IV) would be available for the liver, reducing hepatic Se(IV) accumulation.

No significant effect of water chemistry on Se(IV) accumulation was demonstrated in the gut tissue of *O. clarkii lewisi* or *O. mykiss*. Gut Se(IV) will accumulate from one of two routes: via Se(IV) taken up from the gill and subsequently transported to the gut tissue, or direct accumulation from imbibed water. Taking the basal drinking rate of RBT in freshwater (Perrott et al., 1992), it can be calculated that Se(IV) accumulation from drinking accounts for only a small percentage of gut Se(IV) burden (<1-3% varying with species). Therefore, it is likely that accumulation in this tissue is due to Se(IV) absorbed across the gill.

The intestine of fish has a copious blood supply (Barron et al., 1987; Stevens, 1968), and therefore the Se(IV) burden in the gut may be a consequence of selenium transport from the gill.

Supporting this, the general trends of gut Se(IV) burden matched those of gill Se(IV) burden across all water chemistries. It is possible that selenium is being mobilized to the gut for excretion. Although the kidneys are the main pathway for selenium excretion in fish, faecal loss can play a significant role in selenium homeostasis (Janz, 2012).

There was, however, no effect of any water chemistry on gut Se(IV) burden for either studied fish species. The only other study to examine Se(IV) uptake in the gut of fish examined Se(IV) uptake across isolated RBT enterocytes (Misra et al., 2012). These authors demonstrated that Se(IV) uptake in enterocyte cells was DIDS-dependent, implicating AE1 in Se(IV) uptake. In the current study no effect of bicarbonate could be observed. As noted above, differences between studies could be due to the Se(IV) concentrations tested and different pathways for Se(IV) uptake at these different concentrations, as has been shown in other species previously (Klaczek et al., 2024; Lazard et al., 2010).

A lack of effect of water chemistry on Se(IV) accumulation was also seen in the muscle tissue of *O. clarkii lewisi.* As the blood supply to the muscle is less than that to the gut (Stevens, 1968), then this likely explains why accumulation in this tissue was the lowest for both species (and below the limit of detection for RBT). A similar result was demonstrated in a study by Hodson et al. (1986) where minimal waterborne Se(IV) was accumulated in juvenile RBT muscle. Because the muscle is a relatively poor sink for selenium, there is limited scope to see effects of water chemistry on this tissue. A better understanding of muscle Se(IV) accumulation may be derived from a longer exposure to higher concentrations.

As significant effects on selenite tissue accumulation were only observed in the phosphate and PFA treatments of WCT, phosphate transporters may be of greatest interest in terms of environmental applications. Indeed, levels of phosphate in the water may provide protection

against selenium accumulation and subsequent toxicity in this species. However, anionic water chemistries tested in this study did not elicit an effect on any tissue accumulation in RBT. Thus, the potential protective effect of phosphate may be species specific. Further research into how water chemistry affects selenite uptake in other fish species is required. Albeit, the results of this study further suggest the importance of understanding site specific water chemistry for the assessment of accumulation and potential toxic effects of selenium in local aquatic biota.

3.5 Conclusion

The current study demonstrated that patterns of waterborne Se(IV) accumulation may differ even between the closely related salmonid fish species *Oncorhynchus clarkii lewisi* and *Oncorhynchus mykiss.* In the gill and liver of *O. clarkii lewisi* there was evidence for Se(IV) accumulation via a phosphate transport pathway. Alternatively, there was no effect of any water chemistry manipulation in *O. mykiss*, suggesting that Se(IV) accumulation is not achieved by uptake through an anionic transport pathway in this species, at least under the experimental conditions used in the current study.

Chapter 4

Lethal and sub-lethal effects of acute waterborne selenite exposure to Westslope cutthroat trout (*Oncorhynchus clarkii lewisi*)

4.1 Introduction

Westslope cutthroat trout, *Oncorhynchus clarkii lewisi*, are a species of freshwater salmonid fish. They are native to North America and have very specific habitat requirements to sustain a healthy population (Costello & Rubidge, 2006; McIntyre & Rieman, 1995). Indeed, they are commonly viewed as an indicator species for freshwater health due to their restricted requirements. However, their populations have been recently declining, to the extent where the COSEWIC designated them as *Threatened* in 2005 (Costello & Rubidge, 2006). Their primary habitat range includes streams and rivers that are receiving waters for selenium-containing effluents from industrial and agricultural activities. Furthermore, as they are an opportunistic secondary consumer, they are susceptible to biomagnification and bioaccumulation of selenium.

Research surrounding the toxicity of selenium in fish has largely focused on dietary studies with fewer focusing on effects resulting from waterborne exposure (see Table 1.4). However, lethal and sub-lethal effects have been attributed to aqueous exposures. Indeed, elevated waterborne selenium concentrations are known to cause oxidative stress, impaired enzyme activity and ionoregulatory disruptions in freshwater biota (see Table 1.4). For example, inorganic selenium can react with thiols generating a chain reaction that can eventually result in the generation of reactive oxygen species (ROS) (Mézes & Balogh, 2009). The generation of ROS induces the activity of enzymes that seek to scavenge these reactive species and therefore nullify their potential to react with valuable cellular lipids, proteins and DNA (Chen et al., 2007; Kim & Kang, 2015; Stewart et al., 1999). Antioxidant defense enzymes include GPx, which reduces H_2O_2 (a product of earlier antioxidant enzyme activity; refer to Figure 1.3) into water. Intriguingly, selenium is an important cofactor of GPx, and thus while selenium can be a pro-oxidant, it may also contribute to antioxidant activities within a cell (Tappel, 1984). If antioxidant defenses fail to effectively

scavenge ROS, then damage such as PC may occur, and this endpoint is a useful measure of oxidative damage due to its stable nature (Dalle-Donne et al., 2002).

Ionoregulatory mechanisms in aquatic organisms are very sensitive to environmental pollutants (Croke & McDonald, 2002; McDonald et al., 1989). Indeed, the NKA pump, which plays an integral role in maintaining sodium and potassium gradients, which has downstream effects on a variety of other homeostatic processes (Suhail, 2010), has been demonstrated to be affected by various trace elements (e.g., Eroglu & Canli, 2013; Silva & Martinez, 2014; Xie et al., 2016b), including selenium (Choi et al., 2015; Gopi et al., 2021; Miller et al., 2007; Xie et al., 2016a). The proton pump, H^+-ATPase, plays a vital role in regulating intracellular pH and ions, and creates an electrochemical gradient, however altered activity has also been shown in response to metal exposure (Beyenbach & Wieczorek, 2006; Chowdhury et al., 2016). Other enzymes, like LDH, can also serve as good diagnostic tools in aquatic toxicology as they are useful biomarkers for stress and tissue damage induced by pollutants in aquatic organisms (Cohen et al., 2001; Farhana & Lappin, 2023; Lavanya et al., 2011). There is existing evidence that selenium exposure can impact LDH activity in aquatic biota (Kumar et al., 2018; Ramesh et al., 2014). Overall, while effects of selenium on oxidative stress, tissue damage and ionoregulation endpoints have been documented, there is limited information as to how conserved these effects are across different aquatic species. Of particular note, is the lack of data regarding the sensitivity of *Oncorhynchus clarkii lewisi*, a declared species of special concern and a resident of effluent-impacted waters, to selenium.

The current study aimed to examine lethal and sub-lethal effects of acute waterborne Se(IV) exposure to *O. clarkii lewisi*. To date, the sensitivity of *O. clarkii lewisi* to waterborne Se(IV) has not yet been characterised. However, previous research has demonstrated the acute

toxicity and effects of waterborne selenium to the closely related RBT (*Oncorhynchus mykiss*) (see Table 1.3 and Table 1.4). *Oncorhynchus clarkii lewisi* were acutely exposed to seven waterborne Se(IV) concentrations (0, 1, 2, 4, 8, 16 and 32 mg L^{-1}) for 96-h. Mortalities were monitored and used to calculate the 96-h LC_{50}. Two concentrations exhibiting no mortalities (4 and 8 mg L^{-1}) were chosen to assess sub-lethal endpoints related to oxidative stress (PC and GPx), ionoregulation (NKA and H^+-ATPase), and general cellular homeostasis (LDH). As *O. clarkii lewisi* are an important indicator species for freshwater ecosystem health and are listed under COSEWIC, gaining an understanding of their relative sensitivity to waterborne Se(IV) and how it may elicit sub-lethal effects will have implications for potential protective measures in selenium contaminated areas. Indeed, as they are known to have very strict habitat needs for maintenance of a healthy population and fitness, where these factors can deteriorate in non ideal conditions, understanding if they also demonstrate a high sensitivity to a trace metal pollutant can provide valuable insight for these areas (Costello & Rubidge, 2006).

4.2 Materials and Methods

4.2.1 Animals

Westslope cutthroat trout (*Oncorhynchus clarkii lewisi*) of mean (± standard deviation) mass 8.2 ± 2.2 g (n = 168) were used in the current study (Table A3). These were sourced from the Government of Alberta fish hatcheries and maintained in the aquatic facility at the University of Alberta, as described in section 3.2.1.

4.2.2 Toxicity of waterborne selenite (Se(IV))

Acute toxicity was evaluated by determining the 96-h LC_{50} value, following Organization for Economic Cooperation and Development (OECD) test guidelines (OECD, 2019). Briefly,

Se(IV) (as sodium selenite) was added to 20 L of facility water in 21L glass aquaria at concentrations of 0, 1, 2, 4, 8, 16 and 32 mg L^{-1} 24-h before fish addition. After 24 hours, fish (n = 7) were added to exposure waters, which were continuously aerated via an air stone. Each treatment was replicated 4 times, apart from the 1 mg L^{-1} and 32 mg L^{-1} concentrations, which were replicated twice. Water parameters (temperature, dissolved oxygen (DO) and pH) were measured at the start of exposure and every 24 h (i.e., 0, 24, 48, 72 and 96 h; Table A2). Water ammonia levels were also monitored throughout and remained at 0 ppm. Total water Se(IV) concentrations were examined at the start and end of the exposure and verified via Inductively Coupled Argon Plasma- Optical Emission Spectrometer (ICP-OES; Table 4.1). The organisms in each replicate were counted twice daily, and any mortalities were removed. Fish that exhibited loss of equilibrium were also removed and euthanized (TMS; 200 mg L^{-1}, neutral-buffered with sodium bicarbonate). At the end of the exposure period, remaining fish were euthanized by anesthetic overdose. All fish were measured for weight and length (Table A3). Sigmoidal regression in SigmaPlot (ver. 14.5; Systat Software Inc.) was used to determine the 96-h LC_{50} values and the 95% confidence interval (CI).

For analysis of sub-lethal endpoints, fish were taken from the two highest concentrations resulting in no deaths (4 and 8 mg L^{-1}), as well as the control (0 mg L^{-1}) exposure group. Two fish from each treatment replicate (n = 8 in total) were randomly selected and gill and liver tissues were dissected and snap-frozen in liquid nitrogen. All tissue samples were ground to a fine powder using a mortar and pestle over liquid nitrogen, partitioned to one of several cryovials to prevent freeze/thaw cycles, and then stored at -80°C until further analysis.

4.2.3 Branchial and hepatic protein carbonyl content

Branchial and hepatic PC content were assessed using a commercial kit according to the manufacturer's protocol (Protein Carbonyl Colorimetric Assay Kit; Cayman Chemicals). Briefly, 35 ± 14 mg (gill) and 2 ± 9 mg (liver) of tissue was homogenized with a handheld tissue homogenizer (2 x 30 s, 30 s rest in between) in 1 mL of cold homogenization buffer (50 mM MES, 1 mM EDTA, pH 6.7) and centrifuged for 15 minutes at 10,000 x *g* at 4°C. A sample and control tube were prepared, where 200 µL of supernatant was added with 800 µL of 2,4-dinitrophenylhydrazine (DNPH; reacts with the carbonylated protein; sample) or 800 µL 2.5 M HCl (control). All tubes were incubated in the dark for 1 hour. After incubation, 1 mL of 20% trichloroacetic acid (TCA) was added, the tubes were incubated for 5 min on ice and centrifuged for 10 min 10,000 x *g* at 4°C. The supernatant was discarded, and the pellet was resuspended in 1 mL of 10% TCA. Tubes were left to incubate for 5 min on ice and subsequently centrifuged for 10 min 10,000 x *g* at 4°C. The supernatant was discarded, and the pellet was manually resuspended and washed in 1 mL 1:1 ethanol:ethyl acetate mixture three times. After the final wash, pellets were resuspended in 500 µL 2.5 M guanidine hydrochloride. After centrifugation to remove any leftover debris, 220 µL of supernatant from each tube was placed in a 96-well plate and the absorbance was measured at 360 nm using a microplate reader. Protein carbonyl content was determined by subtracting the control value from the sample value and dividing by the extinction coefficient for DNPH. Protein content in the control samples was measured via a Bradford assay (Bradford, 1976), and carbonyl content was normalized to protein content.

4.2.4 Branchial and hepatic glutathione peroxidase activity

Branchial and hepatic GPx activity were measured using a commercial kit according to the manufacturer protocol (Glutathione Peroxidase Assay Kit; Cayman Chemicals). Briefly, 11 ± 2 mg (gill) and 10 ± 1 mg (liver) of tissue was homogenized with a handheld tissue homogenizer (2 x

30 s, 10 s rest in between) in 100 µL of cold homogenization buffer (50 mM Tris- HCl, pH 7.5, 5 mM EDTA, 1 mM DTT) and centrifuged for 15 minutes at 10,000 x *g* at 4°C. Sample activity was assessed via the oxidation of NADPH, where 20 µL of sample was added to 50 µL assay buffer (50 mM Tris-HCl, 5 mM EDTA, pH 7.6), 50 µL co-substrate mixture (lyophilized glutathione and glutathione reductase) and 50 µL NADPH. Reactions were initiated by adding 20 µL cumene hydroperoxide in a 96- well plate and the absorbance was measured every minute for 9 minutes at 340 nm using a microplate reader. The rate of reaction was used to determine final GPx activity in the samples and standardized to non-enzymatic activity.

4.2.5 Branchial Na^+, K^+-ATPase and H^+-ATPase activity

Branchial NKA and H^+-ATPase activity was measured according to the protocol outlined by Klaczek et al. (2022) with slight modifications. This protocol is based on the method described by McCormick (1993). Briefly, 31 ± 9 mg of thawed gill tissue was homogenized with a handheld tissue homogenizer for 35 s in SEID buffer (125 mM sucrose, 5 mM EGTA, 50 mM imidazole, 0.05 g 50 mL^{-1} sodium deoxycholate; pH 7.3) and centrifuged for 3 min at 5000 x *g* at 4°C. Supernatant was collected and assessed for NKA and H^+-ATPase activity. Specifically, NKA activity was measured via the ouabain sensitive component of ATP hydrolysis-coupled oxidation of NADH, and H^+-ATPase activity was measured via the N-ethylmaleimide inhibition sensitive component of NADH oxidation (Lin & Randall, 1993). Inhibitors were added to a standard reaction mixture (2.8 mM PEP, 3.5 mM ATP, 0.22 mM NADH, 4 U mL^{-1} LDH, 5 U mL^{-1} PK, 189 mM NaCl, 10.5 mM $MgCl_2 \cdot 6H_2O$, 42 mM KCl, 50 mM imidazole) and final activity was determined as the difference between uninhibited and inhibited reactions. Reactions were measured in a 96-well plate at 340 nm every 10 s for 30 min using a microplate reader and normalized to protein content via a Bradford assay (Bradford, 1976).

4.2.6 Branchial lactate dehydrogenase activity

Gill LDH activity was measured via slight modifications to the method of Vassault (1983). Briefly, 15 ± 2 mg of gill tissue was thawed and homogenized in a buffer (20 mM HEPES, 1 mM EDTA, 0.1% Triton X-100, pH 7.0) for 1 minute (2 x 30 s with 30 s rest in between) using a handheld tissue homogenizer. Samples were centrifuged (16, 000 x *g*, 5 minutes, 4°C) and 5 µL of supernatant was added to 200 µL of assay buffer (50 mM imidazole, 0.2 mM sodium pyruvate, 0.15 mM NADH; pH 7) in a 96-well plate. The decrease in NADH over a 15-minute period was determined every 30 seconds at 340 nm using a microplate reader and normalized to protein content via a Bradford assay (Bradford, 1976).

4.2.7 Statistics

All data were assessed for normality and homogeneity of variance using Shapiro-Wilk and Brown-Forsythe tests, respectively. Passing data were analyzed by one-way ANOVA and where an overall significant effect was identified, a Tukey's post hoc test was used to identify specific pairwise comparisons that differed. Liver PC and gill NKA data were log transformed to meet assumptions and were subsequently analyzed using a one-way ANOVA and Tukey's post hoc test. Data transformation failed to render gill LDH data appropriate for parametric analysis, thus a non-parametric Kruskal-Wallis test and Dunn's post hoc test were used. All analyses were performed, and graphs were created, in PRISM GraphPad 10. Significance for all data was assessed at an alpha level of 0.05. Unless otherwise stated, data are expressed as the means ± standard deviation.

4.3 Results

4.3.1 Waterborne selenite (Se(IV)) exposures

Water quality parameters were maintained within acceptable standard levels within and between all treatments (Table A2). Average conditions of 9.5°C ± 0.7°C, pH 8.05 ± 0.1 and DO 10.32 ± 0.36 mg L^{-1} were maintained. Measured Se(IV) exposure concentrations are reported in Table 4.1. As there were very minor fluctuations in exposure concentration over the 96-h period, the initial and final water concentration values were averaged to provide the mean exposure concentration.

4.3.2 LC_{50} determination

Fish survival in the 0, 1, 2, 4 and 8 mg L^{-1} was 100%, however in the 16 and 32 mg L^{-1} an average of 75% and 100% mortality was observed, respectively. The calculated 96-h LC_{50} value for Se(IV) was 15.55 (95% CI of 14.95-16.15) mg L^{-1} (Figure 4.1).

4.3.3 Branchial and hepatic protein carbonyl content

Exposing *O. clarkii lewisi* to elevated waterborne Se(IV) had a significant effect on gill and liver PC (Figure 4.2A, B; $p = 0.0251$ and $p = 0.0221$ respectively). Compared to the control, 4 mg L^{-1} significantly reduced gill PC by 47% (Figure 4.2A; $p = 0.0201$). Interestingly, 8 mg L^{-1} had no significant effect on gill PC (Figure 4.2A; $p = 0.1995$). In contrast to the gill data, 4 mg L^{-1} had no effect on liver PC (Figure 4.2B; $p = 0.0912$), but 8 mg L^{-1} reduced PC by 78% (Figure 4.2B; $p = 0.0225$). No significant difference in the level of PC was observed between the 4 mg L^{-1} and 8 mg L^{-1} treatment groups in either gill or liver.

4.3.4 Branchial and hepatic glutathione peroxidase activity

Selenite exposure at 8 mg L^{-1} resulted in a non-significant trend towards a decrease in GPx activity in gill tissue (Figure 4.3A; $p = 0.0676$). However, no significant effect was observed in hepatic GPx activity in response to elevated Se(IV) (Figure 4.3B; $p = 0.4618$).

4.3.5 Branchial Na^+, K^+-ATPase and H^+-ATPase activity

Increasing waterborne Se(IV) had a significant effect on gill NKA and H^+-ATPase activity (Figure 4.4A, B; $p = 0.0003$ for both). No effect was observed at 4 mg L^{-1} for NKA or H^+-ATPase (Figure 4.4A, B; $p = 0.4752$ and $p = 0.8360$ respectively). However, 8 mg L^{-1} increased NKA activity by 5.2-fold (Figure 4.4A; $p = 0.0004$) and H^+-ATPase activity by 3.1-fold compared to the control (Figure 4.4B; $p = 0.0005$). A significant increase in NKA and H^+-ATPase activity was also observed between 4 mg L^{-1} and 8 mg L^{-1} (Figure 4.4A, B; $p = 0.0038$ and $p = 0.0018$ respectively).

4.3.6 Branchial lactate dehydrogenase activity

The effect of waterborne Se(IV) on branchial LDH activity is seen in Figure 4.5. No significant effect was observed ($p = 0.563$).

Table 4.1 Measured selenite (Se(IV)) exposure concentrations as determined by ICP-OES. Reported values are means ± S.D. (n = 2-4 per treatment).

Nominal concentration (mg L^{-1})	Initial exposure concentration (mg L^{-1})	Final exposure concentration (mg L^{-1})	Mean exposure concentration (mg L^{-1})
0	0.04 ± 0.00	0.14 ± 0.08	0.09 ± 0.08
1	1.00 ± 0.25	0.83 ± 0.19	0.91 ± 0.21
2	1.92 ± 0.23	2.04 ± 0.14	1.98 ± 0.19
4	4.15 ± 0.21	3.93 ± 0.07	4.04 ± 0.18
8	8.05 ± 0.48	7.81 ± 0.52	7.93 ± 0.48
16	16.02 ± 0.34	16.34 ± 0.40	16.18 ± 0.38
32	32.37 ± 0.03	31.58 ± 1.38	31.97 ± 0.92

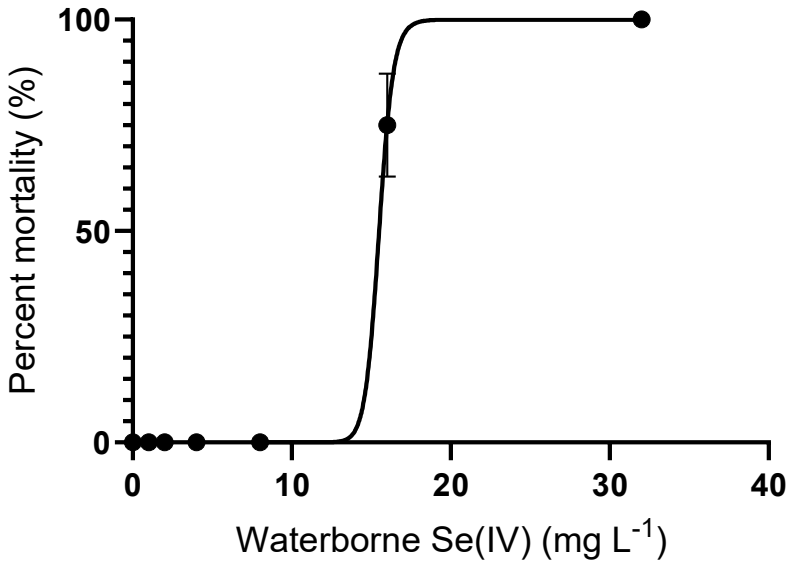

Figure 4.1 Calculated 96-h median lethal toxicity of waterborne Se(IV) (mg L^{-1}) to Westslope cutthroat trout, *Oncorhynchus clarkii lewisi,* in City of Edmonton dechlorinated water. Plotted points represent the means (± standard error) of 4 replicates. (N = 4).

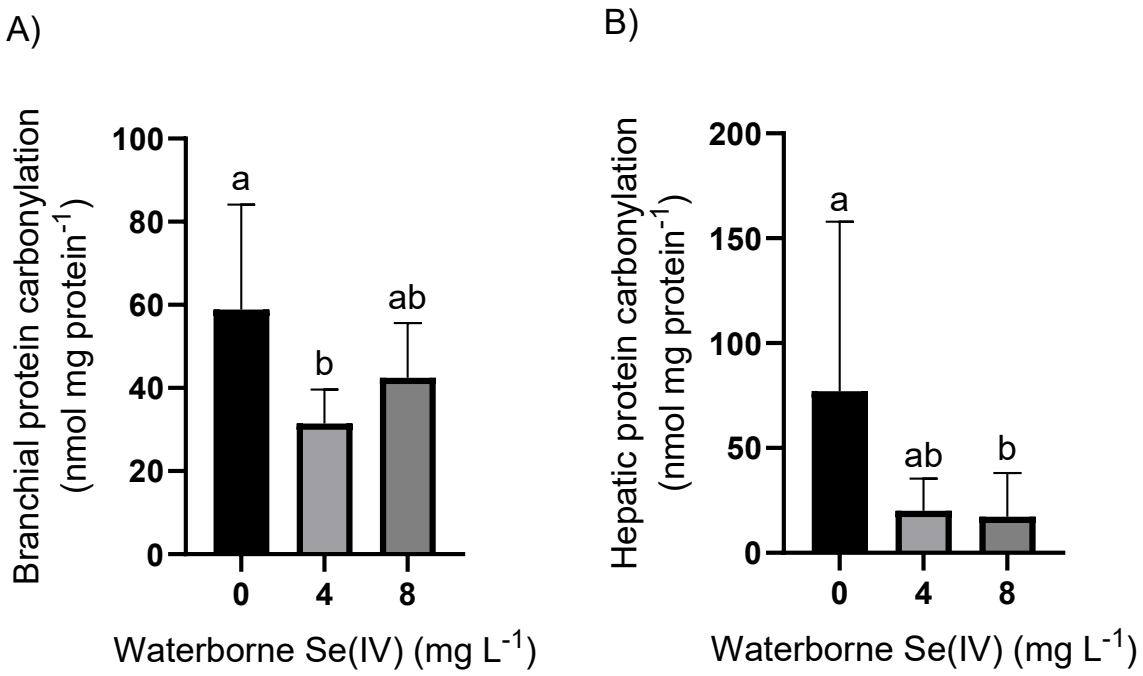

Figure 4.2 Westslope cutthroat trout (*Oncorhynchus clarkii lewisi*) A) branchial and B) hepatic protein carbonylation (nmol mg protein^{-1}) in response to a 6-h exposure to three waterborne Se(IV) concentrations: 0 mg L^{-1} (black bars), 4 mg L^{-1} (light grey bars) and 8 mg L^{-1} (medium grey bars). Bars represent the means (± standard deviation) of 8 replicates. Bars sharing letters are not statistically significantly different, as determined by one-way ANOVA followed by a post hoc Tukey's test.

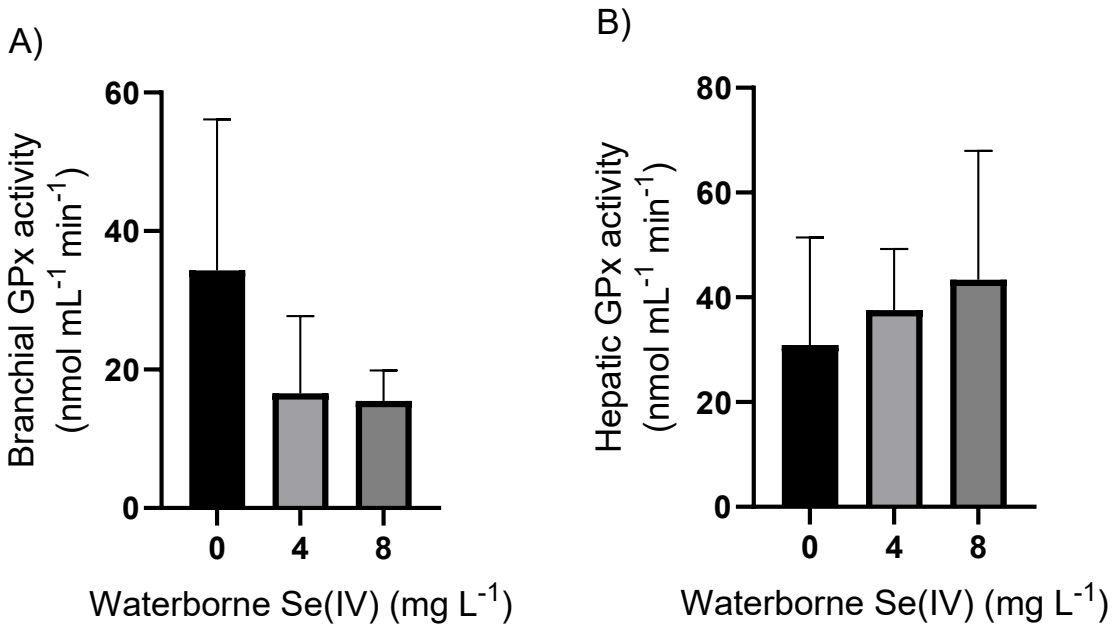

Figure 4.3 Westslope cutthroat trout (*Oncorhynchus clarkii lewisi*) A) branchial and B) hepatic glutathione peroxidase activity (nmol mL^{-1} min^{-1}) in response to a 6-h exposure to three waterborne Se(IV) concentrations: 0 mg L^{-1} (black bars), 4 mg L^{-1} (light grey bars) and 8 mg L^{-1} (medium grey bars). Bars represent the means (± standard deviation) of 5-8 replicates.

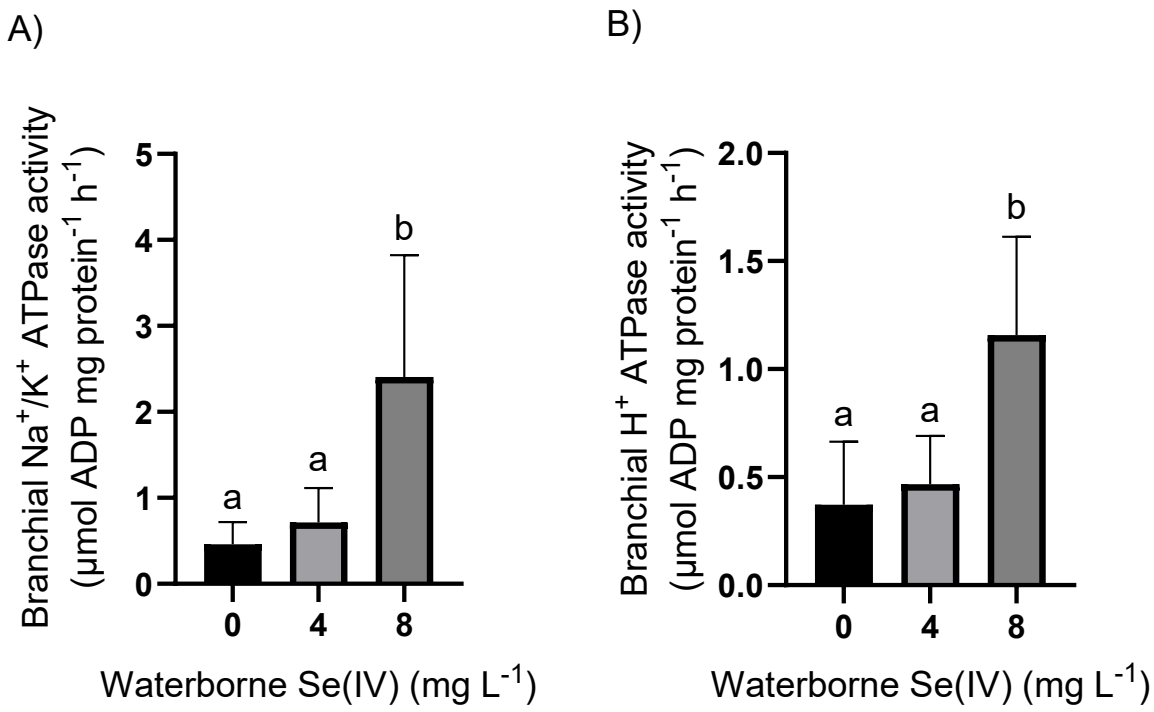

Figure 4.4 Westslope cutthroat trout (*Oncorhynchus clarkii lewisi*) branchial A) Na^+/K^+- ATPase and B) H^+-ATPase activity (µmol ADP mg $protein^{-1}$ h^{-1}) in response to a 6-h exposure to three waterborne Se(IV) concentrations: 0 mg L^{-1} (black bars), 4 mg L^{-1} (light grey bars) and 8 mg L^{-1} (medium grey bars). Bars represent the means (± standard deviation) of 7-8 replicates. Bars sharing letters are not statistically significantly different, as determined by one-way ANOVA followed by a post hoc Tukey's test.

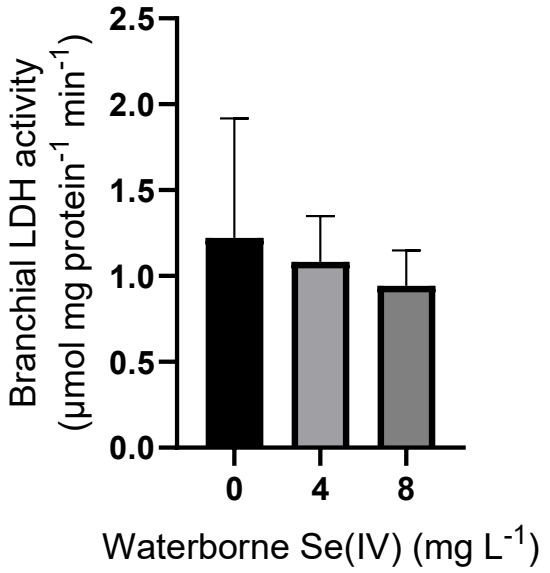

Figure 4.5 Westslope cutthroat trout (*Oncorhynchus clarkii lewisi*) branchial lactate dehydrogenase activity (µmol mg protein^{-1} min^{-1}) in response to a 6-h exposure to three waterborne Se(IV) concentrations: 0 mg L^{-1} (black bars), 4 mg L^{-1} (light grey bars) and 8 mg L^{-1} (medium grey bars). Bars represent the means (± standard deviation) of 8 replicates.

4.4 Discussion

This study supports previous observations that waterborne Se can contribute to toxicological impacts in fish species (see Table 1.4). The median lethal concentration for Se(IV) was calculated to be 15.55 mg L^{-1}, while at lower exposure concentrations reduced PC, and increased NKA and H^+-ATPase activity was observed. Other sub-lethal endpoints, such as GPx and LDH activity, remained unchanged. The Se(IV) concentrations at which these effects were observed suggests that *O. clarkii lewisi* is unlikely to experience significant toxic effects related to waterborne Se(IV), at least following an acute exposure.

The LC_{50} calculated in the present study suggests that waterborne Se(IV) related mortality of *O. clarkii lewisi* would only occur where Se(IV) concentrations are in the mg L^{-1} range. It is known that total selenium concentrations in wastewaters entering natural systems can be in this range (e.g. Santos et al., 2015); but there are limited measurements of environmental selenium that separates the element by its oxidation states. One study did measure Se(IV) concentrations of 57 µg L^{-1} downstream of mining activities in Quebec, Canada (Etteieb et al., 2021). Although this concentration would render this waterway as being highly polluted (Environment and Climate Change Canada (2022); water quality guideline value ranges from 1-3.1 µg L^{-1} for total selenium), it is still orders of magnitude lower than that required to result in *O. clarkii lewisi* mortality.

The sensitivity of *O. clarkii lewisi* to waterborne selenium is in the range of previous studies on salmonid fish. A study by Buhl & Hamilton (1991) examined the 96-h LC_{50}'s of aqueous Se(IV) exposure in juvenile Arctic grayling, and the closely related coho salmon and RBT. They found LC_{50} values of 34.30 mg L^{-1}, 7.83 mg L^{-1} and 9.00 mg L^{-1}, respectively (Buhl & Hamilton, 1991). However, it should be noted that Buhl & Hamilton used notably smaller fish (< 2.5 g) than

those in the current study (~8 g), and given that smaller fish tend to be more sensitive to environmental pollutants, then it is likely that LC_{50} values are relatively well-aligned among salmonid fish (e.g., Kanak et al., 2014).

Despite exposure to Se(IV) concentrations that were higher than half of the LC_{50} no effect of waterborne Se(IV) was observed on branchial or hepatic GPx activity. In contrast, Miller et al. (2007) found that after a 96-h exposure to 1.80 and 3.60 mg L^{-1} waterborne Se(IV), increased GPx activity was observed in RBT liver. An increase in hepatic GPx activity was also observed after exposing *O. mossambicus* to waterborne Se(IV) concentrations of 5-100 µg L^{-1} for 96-h (Gobi et al., 2018). Furthermore, dietary exposures also induced GPx activity in the liver of Chu's croaker (*Nibea coibor*), black sea bream (*Acanthopagrus schlegelii*) and red-tailed Brycon (*Brycon cephalus*) (Lin et al., 2021; Monteiro et al., 2009; Wang et al., 2019a). Increased GPx activity is also observed in the gills of fish exposed to Se(IV). For example, exposure of *O. mossambicus* to 5-25 µg L^{-1} waterborne Se(IV) for 96-h induced branchial GPx (Gobi et al., 2018). Furthermore, 1.5 mg L^{-1} of dietary selenium increased GPx activity in *Brycon cephalus* gills after 96-h (Monteiro et al., 2009).

Although previous studies have noted induction of GPx following Se(IV) exposure, other antioxidant enzyme pathways may be considered to be more important in defense against Se(IV) ROS induction. For example, Chen et al. (2020) also noted a lack of change in GPx activity in the tissues of *Oreochromis niloticus* following Se(IV) exposure, and suggested that catalase and glutathione S-transferase may be playing the more important roles in ROS scavenging. Consequently, it is possible the lack of effect on GPx activity in this study is due to other selenium sensitive antioxidant enzymes being upregulated, in lieu of GPx induction.

Although there was no change in GPx activity, it was clear that Se(IV) exposure did induce changes in tissue oxidative stress status. Acute exposure to elevated waterborne Se(IV) significantly reduced PC in both gill and liver. This finding contrasts previous studies of selenium impacts in fish. For example, upon a 96-h exposure to waterborne Se(IV) (5- 100 μg L^{-1}), PC levels were significantly increased in a dose-dependent manner in the gill and liver of *Oreochromis mossambicus* (Gobi et al., 2018). Furthermore, a study by Li et al. (2020) demonstrated an increase in PC in the liver and spleen of *Channa argus* after an 8 week exposure to 100 and 200 μg L^{-1} waterborne selenium (authors did not clarify the oxidative state of selenium, however). Other oxidative damage endpoints such as lipid peroxidation (measured via malondialdehyde (MDA) or thiobarbituric acid-reactive substances (TBARS)) have also been shown to increase under elevated selenium exposure (Berntssen et al., 2017; Gobi et al., 2018; Li et al., 2019; Misra & Niyogi, 2009). This induction of oxidative damage is thought to occur as selenium has the ability to oxidize thiols, which both limits the capacity of a cell to scavenge ROS, and leads to a chain reaction that generates ROS directly (Chen et al., 2007; Kim & Kang, 2015; Mézes & Balogh, 2009; Stewart et al., 1999). However, positive effects on cellular oxidative status, similar to the decline in PC in this study, have also been reported (Lin et al., 2021; Miller et al., 2007; Wang et al., 2019a). For example, lipid peroxidation was significantly reduced after 96-h exposure to 2.52 mg L^{-1} waterborne Se(IV) in RBT (Miller et al., 2007). Additionally, MDA levels decreased in serum and liver of *Nibea coibor* and *Acanthopagrus schlegelii* after 8 weeks of dietary selenium exposure (Lin et al., 2021; Wang et al., 2019a). It is hypothesized that this could be occurring due to the essential nature of selenium ultimately playing a protective role via activation of antioxidant defense mechanisms within the organism (Khan et al., 2017; Rotruck et al., 1973; Sarada et al., 2002). Indeed, once selenium is taken up it can be incorporated as selenocysteine into proteins to

form selenoproteins which have important roles in redox balance (Li et al., 2023). Furthermore, selenium can activate a signal transduction pathway related to increases in the gene expression of antioxidant enzymes (Li et al., 2023). However, further research would be required to support this hypothesis and explain how GPx and PC are related to Se(IV) exposure in *O. clarkii lewisi*.

A significant increase in branchial NKA and H^+-ATPase activity were observed at 8 mg L^{-1} waterborne Se(IV). The effect of Se(IV) on NKA in other aquatic animals is inconsistent. For example, studies in *Oncorhynchus mykiss* and *Carassius auratus* show a trend of increased gill NKA after 96-h, and a significant increase in gill NKA after 12-h to 120-h, respectively (Choi et al., 2015; Miller et al., 2007). On the other hand, decreased activity has been observed in *O. mossambicus* gills after 96-h at 100 µg L^{-1} waterborne Se(IV) (Gopi et al., 2021). Xie et al. (2016a) also observed a decrease in NKA activity in whole killifish (*Heterandria formosa*) supplemented with 2 µg g^{-1} dietary Se(IV). It has been hypothesized that trace-metal induced decreases in NKA activity could be a result of covalent binding to cysteine groups on the enzyme ultimately affecting NKA function (Gopi et al., 2021). Conversely, the increase in activity could be attributed to the organism's attempt to compensate for ion loss. Indeed, an increase in H^+-ATPase was also observed in this study. As H^+-ATPase also plays a role in ion balance this could further support this idea. Although research into the response of H^+-ATPase in aquatic organisms under elevated metal exposures, particularly selenium, is lacking, a study on rice demonstrated a similar finding where selenium increased H^+-ATPase activity (Lin et al., 2012). Thus, it is possible that the increase in NKA and H^+-ATPase activity in the present study could be compensation for effects of Se(IV) on ion regulation. Studies examining plasma ion concentrations over Se(IV) exposure time would help to test this hypothesis.

No effect of elevated waterborne Se(IV) was observed on branchial LDH activity. This is opposite of what was predicted given that previous studies have observed increased LDH activity under similar exposure concentrations (Kumar et al., 2018; Ramesh et al., 2014). Indeed, significant increases in gill LDH of *Pangasius hypophthalmus* were induced by 4.5-6 mg L^{-1} selenium over 96-h (Kumar et al., 2018). Furthermore, *Labeo rohita* exhibited increasing liver LDH over 7-35 days from 2.38 mg L^{-1} waterborne Se(IV) (Ramesh et al., 2014). Since LDH is a key metabolic enzyme, it has been hypothesized that Se(IV) may induce increased LDH activity via metabolic changes in the liver and induction of anaerobic metabolism (Farhana & Lappin, 2023; Ramesh et al., 2014). That oxidative damage status (PC) actually improved with waterborne Se(IV) exposure in this study, it seems unlikely that there was sufficient cellular stress to induce LDH. It is therefore possible that *O. clarkii lewisi* are more tolerant to waterborne Se(IV) than the previously-studied fish (e.g., selenium LC_{50} of 5.82 mg L^{-1} for *P. hypophthalmus*; Kumar et al, 2018), thus negligible tissue damage occurred at these concentrations over the exposure period resulting in an unchanged LDH activity. As LDH is associated with anaerobic metabolism, it is notable that Se(IV) exposure does induce hypoxia in carp species, including *L. rohita* (Dhara et al., 2022), and so that could be the explanation as to why LDH also changes in this species. Future studies would be useful to investigate whether Se(IV) exposure in *O. clarkii lewisi* also alters oxygen consumption rates and whether any changes in that endpoint are consistent with our observations of unaltered branchial LDH activity.

4.5 Conclusion

The current study indicated that *O. clarkii lewisi* has a similar tolerance to waterborne Se(IV) as other salmonid fish species. A 96-h LC_{50} of 15.55 (95% CI of 14.95-16.15) mg L^{-1} and significant effects on PC, NKA and H^+-ATPase were observed at 4 and 8 mg L^{-1}. Alternatively, no

effects on GPx or LDH were seen. While these concentrations of Se(IV) are unlikely to occur in natural aquatic systems, even in heavily polluted areas, where only up to 2.7 mg L^{-1} of other selenium species and/or species mixtures have been observed globally (Table 1.1). Thus, further research into longer term effects at lower concentrations for this species would provide valuable information. Indeed, the results of this study may point towards a potential beneficial effect of selenium; however, over a more environmentally realistic concentration and chronic exposure an alternate harmful effect may persist. Thus, these biochemical endpoints will be useful for further investigation of Se(IV) toxicity in this and other fish species and allow us to gain insight into potential protective measures for selenium-contaminated sites.

Chapter 5
General discussion

5.1 Summary

This thesis demonstrates how different water chemistries can affect the uptake and accumulation of Se(IV) in aquatic organisms and the relative toxicity of waterborne Se(IV) to species of direct relevance to selenium-impacted waters. For example, this thesis documented one of the first studies to examine how waterborne Se(IV) is being taken up across epithelia in an invertebrate and how water chemistry can affect Se(IV) accumulation in fish. Furthermore, I determined lethal thresholds and biochemical effects a of waterborne Se(IV) in a poorly-studied and threatened trout species.

In Chapter 2 the mechanistic uptake of waterborne Se(IV) was evaluated in *Daphnia magna*. I originally hypothesized that as Se(IV) is present as an anion in water, uptake would occur through an anionic transporter. The results of this study supported my hypothesis as uptake of waterborne Se(IV) was significantly affected by anionic water composition in *D. magna.* Specifically, waterborne Se(IV) uptake was significantly affected by the presence of bicarbonate and phosphate in the exposure water. At high Se(IV) concentrations bicarbonate significantly reduced Se(IV) uptake, while at low Se(IV) concentrations, the presence of phosphate significantly reduced Se(IV) uptake. These findings were supported by the effects of transport inhibitors, with blockers of phosphate uptake (PFA) and bicarbonate uptake (DIDS), also affecting Se(IV) uptake. These data suggests that waterborne Se(IV) uptake may have a low, bicarbonate transporter-mediated, and high, phosphate transporter-mediated, affinity pathway in *D. magna*.

In Chapter 3, I evaluated how water chemistry can affect waterborne Se(IV) accumulation in two trout species (*Oncorhynchus clarkii lewisi, Oncorhynchus mykiss*). I originally hypothesized that changes in anionic water composition would affect Se(IV) accumulation in both trout species tissues. The results of this study could only partially support this hypothesis with

respect to *O. clarkii lewisi* but did not support this hypothesis with respect to *O. mykiss*. Westslope cutthroat trout liver Se(IV) accumulation was significantly affected by water phosphate concentration, while PFA, the phosphate transporter inhibitor, significantly reduced gill Se(IV) burden. Conversely, there was no significant effect of any water chemistry manipulation on the accumulation of Se(IV) in any RBT tissue.

In Chapter 4, I examined lethal and sub-lethal effects of waterborne Se(IV) on the local species, *O. clarkii lewisi*. As they require clean water and have specific habitat needs (Costello & Rubidge, 2006), I originally hypothesized that they would have a high sensitivity to Se(IV) and that biochemical endpoints known to be sensitive to Se(IV) in other species would be impacted by acute Se(IV) exposure. The results of this study did not sufficiently support this hypothesis, as *O. clarkii lewisi* demonstrated a relatively high LC_{50} value (in the range measured previously for other salmonid fish), and biochemical changes that were not necessarily reflective of a toxic impact. These data suggest that WCT may be relatively tolerant to acute waterborne Se(IV) exposure. Indeed, branchial and hepatic PC was decreased in response to high Se(IV) concentrations, while branchial NKA and H^+-ATPase were significantly increased in response to elevated Se(IV). No effect of waterborne Se(IV) on branchial or hepatic GPx or branchial LDH were observed. These data further support the idea that the sensitivity of waterborne Se(IV) varies between species (see Table 1.3 and 1.4. for other species overview).

Overall, the results of this thesis demonstrate that the mechanisms of uptake and patterns of accumulation of waterborne Se(IV) may be species-dependent and that model organism sensitivities may not be representative of species of greatest relevance to the natural environments impacted by selenium wastewaters. For example, factors affecting waterborne Se(IV) accumulation in *O. clarkii lewisi* did not elicit an effect in *O. mykiss* tissue accumulation. However,

there were some similarities between the different studies described in this thesis. For example, *Daphnia* and *O. clarkii lewisi* appear to have Se(IV) handling components that are mediated by phosphate transporters, which suggests that the presence of phosphate in Se(IV) contaminated waters may provide protection against Se(IV) accumulation and toxicity. Furthermore, the biochemical effects of elevated selenium on *O. clarkii lewisi* align with those seen in other freshwater fish (see Table 1.4 for other species overview).

5.2 Future directions

Research into selenium toxicity in aquatic biota is of significant real-world value. However, more studies should be conducted to further investigate its uptake and develop a comprehensive understanding of the sensitivity of non-model organisms to selenium exposure. Specifically, future research should focus on the mechanistic uptake of selenium, the changes selenium induces on the antioxidant defense pathway, characterization of the sensitivities of non-model organisms and how different water chemistries can impact tissue burdens.

Gaining a better understanding of how selenium species are transported across epithelia and accumulate in tissues of different trophic levels is important for gaining insight into potential modes of toxicity and protective measures. Indeed, given that the diet is considered the main uptake route for selenium at higher trophic levels (e.g. Besser et al., 1993), understanding how selenium is accumulating in lower trophic levels is important not only for the species themselves but for their consumers. Given there was an effect of phosphate at lower Se(IV) concentrations, conducting further experiments at lower concentrations and over chronic timelines will prove beneficial to determine if these effects persist in natural settings. Furthermore, there was an effect of bicarbonate and DIDS, but no effect of chloride so future studies should examine how bicarbonate is interfering with Se(IV) and if this is a pathway of relevance in the environment.

Additionally, studies of selenium uptake and toxicity in invertebrates and lower trophic levels is lacking, thus investigating if these effects persist in other species could provide valuable insight for trophic transfer.

Since the selenium accumulation patterns of WCT and RBT differed with respect to the effect of water chemistry, examining pathways involved in selenium uptake across a wider range of species would be beneficial. Because RBT are a widely used model organism (Thorgaard et al., 2002; USEPA, 2002), it is especially important to understand whether the lack of effect of water chemistry manipulation in this species is conserved or unique. This will better allow extrapolation of studies conducted in this species to other aquatic biota and could facilitate better water quality management in contaminated areas. Indeed, basing water quality guidelines on RBT may be over-protective or under-protective of selenium toxicity in local species. However, knowledge on how selenium is being taken up and accumulating in most fish species is lacking, with only one study (to my knowledge) investigating this in RBT (Misra et al., 2012). Furthermore, since there was no effect of modifying water anions in the current study, further studies investigating other putative transport pathways, including those related to cations, may be warranted. Additionally, as mentioned previously, the diet is the main route of uptake for selenium in fish. Thus, examining if factors affecting waterborne Se(IV) tissue burden also affect dietary Se(IV) tissue burden could be examined. Indeed, it is known that dietary uptake of phosphate is vital for fish development, where SLC34 transporters have been documented in their intestines (Verri & Werner, 2019). Thus, determining if the mechanistic uptake and accumulation of Se(IV) is similar across exposure routes could prove beneficial in determining water quality criteria and providing protective measures against toxicity.

Lastly, it has been suggested that selenium may play a role in other antioxidant defense enzymes, not just GPx, as they can share close relationships (Chen et al., 2020). Indeed, GPx and GST can utilize the same substrate (Deponte, 2013), thus further examination of oxidative stress under selenium exposures is an area of future required research. Specifically, as studies on WCT are lacking, further examination into the reason(s) for reduced PC by identifying upregulated antioxidant enzymes could allow for a more comprehensive understanding of effects of Se(IV) in this species. As knowledge of selenium interactions with other antioxidant enzymes remains unclear, further exploration into if selenium plays an essential role in these enzymes could provide a better physiological understanding of the essentiality of selenium in fish. Examining the sub-lethal effects of waterborne Se(IV) on WCT at more environmentally realistic concentrations under chronic or sub-chronic conditions and seeing if similar effects are induced by dietary intake also warrants further investigation.

Overall, the research presented in this thesis lacks relevance to most natural systems due to the relatively high concentrations of Se(IV) used. Indeed, only heavily contaminated areas have Se(IV) concentrations in the high $\mu g\ L^{-1}$ or $mg\ L^{-1}$ range (see Table 1.1). However, the data presented provides clear insight into the mechanisms of waterborne Se(IV) uptake in a freshwater invertebrate and two trout species. Given that it has been suggested that sulphate and bicarbonate dependent processes play a role in isolated cells (Misra et al., 2012), investigating more anion and cation water compositions should be considered. Lastly, further examination into the effects of selenium on antioxidant defense and protection against oxidative stress in WCT is warranted. In general, this research contributes to an enhanced overall understanding of selenium toxicity and the potential protective role of water chemistry in modifying Se(IV) handling and toxicity, providing valuable insight into potential protective measures in selenium contaminated areas.

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Appendix

Table A1: Statistical approach and outcomes for selenite uptake data as a function of experimental water chemistry.

Treatment	Selenite concentration (µM)	Data transformation	Bonferroni-adjusted alpha level	P value
Gluconate	1	Log	0.0083	0.046
	2		0.0083	**0.005**
	4		0.0083	0.059
	8		0.0083	**0.004**
	16		0.0083	**<0.0001**
	32		0.0083	**<0.0001**
Bicarbonate	1	Log	0.0083	0.088
	2		0.0083	0.012
	4		0.0083	0.013
	8	Log	0.0083	**<0.0001**
	16	Log	0.0083	**<0.0001**
	32	Log	0.0083	**<0.0001**
DIDS	1		0.017	**0.003**
	8		0.017	**0.001**
	32	KW ANOVA	0.017	**<0.001**
Chloride	1	Log	0.0083	0.773
	2		0.0083	0.223
	4		0.0083	0.044
	8		0.0083	0.117
	16		0.0083	0.187
	32		0.0083	0.032
Sulphate	1	Log	0.0083	0.414
	2	Square Root	0.0083	0.464
	4		0.0083	0.664
	8		0.0083	0.122
	16		0.0083	0.063
	32		0.0083	0.932
Phosphate	1	Log	0.0083	**<0.0001**
	2		0.0083	**<0.0001**
	4		0.0083	**<0.0001**
	8	Log	0.0083	**<0.0001**
	16		0.0083	0.075
	32	Log	0.0083	**<0.0001**
Selenate	0.75 nM		0.05	0.078
NAD^+	2		0.05	0.978
PFA	2	Log	0.05	**<0.001**

Bolded values in p-value column represent statistically significant data.

Table A2: Water quality parameters for acute 96 h selenite (Se(IV)) exposure. Temperature, dissolved oxygen (DO) and pH were verified at 0, 24, 48, 72 and 96 h. The mean over time was calculated for all replicates per treatment. Means ± S.D. (n = 2-4 per treatment).

Treatment (mg L^{-1})	Temperature (°C)	DO (mg L^{-1})	pH
0	9.79 ± 0.72	10.23 ± 0.24	7.99 ± 0.06
2	9.15 ± 0.31	10.39 ± 0.25	7.99 ± 0.03
4	9.37 ± 0.65	10.34 ± 0.42	8.00 ± 0.05
8	9.32 ± 0.69	10.31 ± 0.46	8.01 ± 0.05
16	9.38 ± 0.69	10.26 ± 0.38	8.02 ± 0.04
32	9.54 ± 0.65	10.33 ± 0.34	8.02 ± 0.05
64	9.95 ± 0.52	10.20 ± 0.33	8.03 ± 0.02

Table A3: Mean values for Westslope cutthroat trout (*Oncorhynchus clarkii lewisi*) weight, standard length (SL) and total length (TL) used for acute 96 h selenite (Se(IV)) exposure. Means ± S.D. (n = 14-28 per treatment).

Treatment (mg L^{-1})	Weight (g)	SL (cm)	TL (cm)
0	7.60 ± 2.55	8.28 ± 0.95	9.18 ± 1.02
2	7.63 ± 1.79	8.39 ± 0.62	9.37 ± 0.62
4	8.36 ± 1.45	8.60 ± 0.54	9.55 ± 0.56
8	8.72 ± 2.33	8.69 ± 0.74	9.64 ± 0.80
16	7.58 ± 2.21	8.23 ± 0.78	9.15 ± 0.86
32	8.24 ± 2.11	8.24 ± 0.69	9.25 ± 0.70
64	9.26 ± 2.96	8.41 ± 1.07	9.39 ± 1.08