INTERACTIVE EFFECTS OF SELENIUM ON ARSENIC AND CADMIUM INDUCED TOXICITY IN RAINBOW TROUT (*Oncorhynchus mykiss*)

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ABSTRACT

The existing scientific literature suggests that selenium can modify arsenite or cadmium toxicity in fish; however, our current understanding of the biochemical pathways involved in such interactions is obtained predominantly from mammalian experimental systems, and the literature on aquatic organisms is sparse. To address this knowledge gap, the modulatory effects of selenium on cadmium or arsenite toxicity was investigated using a model freshwater fish, rainbow trout (*Oncorhynchus mykiss*). Interactions at the cellular level were investigated using trout hepatocytes in primary culture. Selenite and selenomethionine were used as the inorganic and organic chemical forms of selenium to understand the chemical species-dependent effects. Interactions at the organismal level were studied by feeding the fish with *Artemia* based diets supplemented with selenomethionine in combination with cadmium or arsenite for 30 days. At the cellular level, cadmium and arsenite disrupted redox homeostasis which was alleviated by low-moderate doses of selenite and selenomethionine (< 25 μM). Further analysis revealed that selenite antagonized arsenite-induced oxidative stress by augmenting enzymatic antioxidants, whereas selenomethionine upregulated the GSH-dependent non-enzymatic antioxidative pathway. At the organismal level, supplementing the diet with only cadmium (40 μg/g diet) or arsenite (80 μg/g diet) increased the tissue level deposition of the respective elements and caused oxidative stress in the liver. However, medium dose (10 μg/g diet) of selenomethionine reduced cadmium accumulation in the liver and alleviated oxidative stress. In contrast, supplementation of diet with selenomethionine (both at low and high levels) in combination with arsenite resulted in higher degree of oxidative stress relative to the fish treated with arsenite alone. Furthermore, fish co-treated with arsenite and selenomethionine accumulated significantly higher levels of arsenic in liver, kidney and muscle relative to fish treated with arsenite alone. Similarly, the synchrotron-based X-ray fluorescence imaging also revealed a dose-dependent increase in the co-localization

of arsenic and selenium in the brain of fish co-treated with dietary arsenite and selenomethionine, whereas no arsenic deposition in the brain was recorded in fish treated with dietary arsenite alone. Overall, the results indicated that selenium at moderate doses could antagonize cadmium-induced oxidative stress; however, selenomethionine can interact metabolically with arsenite at systemic level and increase its toxicity in fish.

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Dedication

To my parents and my motherland

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%	Percent
As_2O_3	Arsenite
~	Approximately
<	Less than
>	Greater than
±	Denotes error of a statistic
°C	Degree Celsius
µM	Micromole
•OH	Hydroxyl ion
$^1O_2$	Singlet oxygen
ANOVA	Analysis of Variance
As	Arsenic
As III	Trivalent arsenite
As IV	Pentavalent arsenate
ATA	3-amino-1,2,4-triazole
ATP	Adenosine triphosphate
BSO	Buthionine sulfoximine
Ca^{+2}	Calcium cation
CAT	Catalase enzyme
Cd	Cadmium
Cd^{+2}	Cadmium cation
$CdCl_2$	Cadmium chloride
cm	Centimeter
CM-H_2DCFDA	50,6-chloromethyl-20,70-dichlorodihydro-fluorescein diacetate
CTCF	Corrected total cell fluorescence

Cu	Copper
DETC	Diethyldithiocarbamic acid sodium salt trihydrate
DFO	Dimethylformamide
DMA	Dimethylarsenic acid
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
ETC	Electron transport chain
Fe	Iron
g	Gram
GPx	Glutathione peroxidase enzyme
GR	Glutathione reductase enzyme
GSH	Reduced glutathione
GSSeH	Glutathioneselenol
GSSeSG	Selenodiglutathione
GSSG	Oxidized glutathione
GTP	Guanosine-5'-triphosphate
h	Hour
H^+	Hydrogen ion
H_2O	Molecular water
H_2O_2	Hydrogen peroxide
H_2Se	Hydrogen selenide
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)
HSP	Heat shock protein
KCl	Potassium chloride
keV	Kilo electronvolt
Kg	Kilogram
KH_2PO_4	Potassium dihydrogen phosphate
L	Liter

L-15	Leibovitz cell culture media
LC50	Median lethal concentration
mg	Milligram
$MgSO_4.7H_2O$	Magnesium sulfate heptahydrate
min	Minute
ml	Milliliter
mM	Millimole
MMA	Monomethylarsonic acid
MS	Mercaptosuccinic acid
n	Sample size
N	Denotes normality of a solution
Na	Symbol for sodium metal
$Na_2HPO_4.7H_2O$	Disodium hydrogen phosphate heptahydrate
NAC	N-acetyl-L-cysteine
NaCl	Sodium chloride
NAD(P)H	Reduced form of Nicotinamide adenine dinucleotide phosphate
$NADP^+$	Nicotinamide adenine dinucleotide phosphate
NaOH	Sodium hydroxide
nm	Nanometer
nmol	Nanomole
NO	Nitric oxide molecular formula
O_2	Molecular oxygen
$O_2^{\bullet-}$	Superoxide anion
OPT	o-Phthalaldehyde
pH	Potential of hydrogen
POP	Persistent organic pollutant
RDA	Recommended dietary allowance

RNS	Reactive nitrogen species
ROS	Reactive oxygen species
S.E.M.	Standard error of mean
Se	Symbol for selenium
Se^0	Elemental selenium
SECIS	Selenocysteine insertion sequence
SeCys	Selenocysteine
SeMet	Selenomethionine
SeO_3^{-2}	Ionic form of selenite
SeO_4^{-2}	Ionic form of selenate
Se-Se	Diselenide
SOD	Superoxide dismutase enzyme
S-Se	Selenenylsulfide
S-Se-S	Selenotrisulfide
TCA	Tricarboxylic acid
TCA	Trichloroacetic acid
TEMPO	(2,2,6,6-tetramethylpiperidin-1-yl)oxidanyl
Trx	Thioredoxin
TrxR	Thioredoxin reductase
v/v	Volume/volume
vs.	Versus
XFI	X-ray fluorescence imaging
Zn	Zinc
µg	Microgram

CHAPTER 1: General Introduction

1.1. Introduction

Contamination of aquatic habitats from trace elements is a major issue in many parts of the world. Trace elements exist naturally in the geosphere, and due to various geological and anthropogenic activities may find their way into aquatic habitats. Contamination from metals like aluminum, copper, silver, and lead occurs mostly due to their targeted mining since they are the elements of human utility. However, the effluents that contaminate water bodies also consist of highly toxic metals and metalloids (henceforth referred to as metal), such as cadmium (Cd), arsenic (As), and selenium (Se), that are the by-products of mining and smelting processes of the 'targeted' elements.

In a natural environment, with multiple biotic and abiotic components, organisms are exposed to more than one stressor which may cause additive, synergistic or antagonistic effects through crosstalk of cellular pathways (Schulte, 2007). This is also true for metal toxicity, as organisms are often exposed to multiple metals in the natural environment. Interactions among stressors make it more challenging to predict the ecotoxicological implications of biotic and abiotic stressors. Therefore, investigation of such interactions is essential and more ecologically relevant.

Among various physiological mechanisms underpinning metal-induced toxicity, oxidative stress is of particular significance to aquatic toxicologists (Di Giulio et al., 1989), and an increasing body of literature emphasizes this concern (Wood, 2012). The term 'oxidative stress' is defined as a "*disturbance in prooxidant-antioxidant balance in favor of the former, leading to damage*" (Sies,

1991). A more practical definition of oxidative stress was given by Sies (2000) as "*an increase in formation of pro-oxidants such as hydrogen peroxide, which is accompanied by a loss of glutathione and the formation of glutathione disulfide...*". Metals, in general, can disrupt oxidative homeostasis in two ways: (i) through production of reactive oxygen species (ROS) through redox recycling of metals or Fenton's like reaction (Ercal et al., 2001; Jomova et al., 2012), and (ii) *via* depletion or impairment of cellular antioxidant defense system (e.g. glutathione) leading to increase in intracellular peroxide ions (Stohs and Bagchi, 1995).

1.2. Selenium as an environmental pollutant

Selenium is present globally in geological formations and is mobilized through a variety of natural and anthropogenic activities (Maher et al., 2010). Although natural weathering of seleniferous soil may add selenium to water, anthropogenic activities such as mining aggravate this process. Mining of coal, phosphate, and uranium are of particular concern (Lussier et al., 2003; Presser et al., 2004; Ramirez-Solis et al., 2004). Targeted refining of selenium for the industrial purpose also adds a significant amount of selenium to water bodies through the discharge electrolytic water. Other significant sources of aquatic selenium contamination are burning of coal in thermal plants, burning of fossil fuels, pharmaceutical industry waste, and application of selenium in agriculture. Selenium is a critical component in some *quantum dots* used in electronic products based on nanotechnology, and this source could be a major contributor to aquatic selenium contamination in coming future (Janz et al., 2010).

The toxicity of selenium is influenced by its chemical speciation, which is quite complex and determined by various abiotic and biotic factors (Janz, 2012). Selenium in freshwater exists in four oxidation states (+6, +4, 0 and – 2). Due to its proclivity to form oxyanions in water, selenium mainly exists as inorganic salts of selenite (SeO_3^{-2}) and selenate (SeO_4^{-2}). Acute toxicity of

selenium to fish is usually caused by waterborne exposure to selenite or selenite, with the former being more toxic than the latter. Selenite was found to be 3-7 folds more toxic to fish in comparison to selenate (Hamilton and Buhl, 1990). The 96 h LC_{50} values for sodium selenite and sodium selenate in juvenile rainbow trout (*Oncorhynchus mykiss*) range from 4.2 to 9.0 mg Se L^{-1} and 32 to 47 mg Se L^{-1}, respectively (Buhl and Hamilton, 1991; Hodson et al., 1980). The organic forms of selenium (organoselenide) also exist and are formed as a result of biotransformation. The organoselenides form an important component of the fish diet (Maher et al., 2010). Selenomethionine (SeMet) is the most predominant form of organoselenide present in the freshwater food chain (Janz, 2012; Maher et al., 2010) and is responsible for chronic toxicity to fish through dietary exposure.

Traditionally, the focus of selenium research was mainly on its nutritional and toxicological effects on mammals and other animals. However, more recently, selenium has been identified as one of the most hazardous aquatic pollutants after mercury (Janz, 2012; Luoma and Presser, 2009), and as a result, there has been a rise in the research concerning its toxic effects on the overall sustainability of the environment. Although selenium is a metalloid, because of highly persistent nature, and high bioaccumulation and biomagnification factor it behaves like a persistent organic pollutant (POP). The primary producers can accumulate up to 1,000,000-fold higher selenium, relative to the water column (Stewart et al., 2010). The accumulated inorganic selenium is readily biotransformed to organoselenides and biomagnified with the increase in trophic level (Janz, 2012). Today, contamination of aquatic habitats with selenium is a global environmental safety issue which needs proper assessment and remediation (Lemly, 2004).

1.3. Physiological effects of selenium exposure in fish

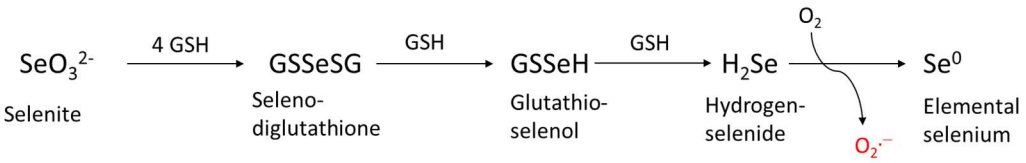
1.3.1. Toxicity of selenium to fish

Exposure to elevated concentrations of both organic and inorganic selenium in fish is known to cause deleterious effects in all the major organs such as kidney, liver, brain, heart, gonads (Berntssen et al., 2017; Lemly, 2002; Naderi et al., 2017). A survey of literature on dietary and waterborne selenium requirements of fish supports a whole-body threshold of 4 μg g^{-1} in fish and 3 μg g^{-1} in diet beyond which selenosis occurs (Hamilton, 2004). Selenium and sulfur share many physical and chemical properties, because of which it was proposed that selenium can replace sulfur in methionine and cysteine to form selenomethionine and selenocysteine, respectively. Since the sulfurous residues form disulfide bonds and are responsible for maintenance of the tertiary structure of the proteins, their replacement could disrupt the structural and functional integrity of various proteins. Substitution of methionine with selenomethionine in yolk proteins was believed to cause teratogenesis (Lemly, 2002).

More recently it has been argued that cysteine, and not methionine, is a major amino acid that contributes towards disulfide linkages (Hatahet et al., 2014; Reczek and Chandel, 2015). Moreover, the formation of selenocysteine and its incorporation into the protein chain is a highly regulated process and does not occur merely by substitution of the sulfur atom. Therefore, substitution of sulfur in the protein chain may not be the main reason behind selenosis. Instead, it is now proposed that the pro-oxidative nature of selenium at elevated concentrations could be the cause of its cytotoxicity and teratogenesis (Kupsco and Schlenk, 2014; Misra et al., 2010; Misra and Niyogi, 2009; Palace et al., 2004).

As mentioned previously, the toxicity of selenium is governed by its chemical speciation. In fish, selenite and selenomethionine are metabolized differently, and thus generate oxidative

stress through different biochemical pathways (Misra et al., 2010; Stewart et al., 1999). Painter, in 1941, proposed for the first time that selenite produces its toxic effects by interacting with cellular thiols like GSH (Painter, 1941). Later, the metabolic relationship between GSH and selenite was elucidated, and it was proposed that the metabolic reduction of selenite not only reduces the cellular pool of GSH, but also produces superoxide radical ($O_2^{\cdot -}$) as a by-product (see Equation 1.1) (Seko et al., 1989).



……. (1.1)

On the other hand, selenomethionine, through the action of methionase enzyme, generates methylselenol which undergoes spontaneous redox recycling in the presence of GSH. It is during this redox recycling reaction that superoxide ion is formed (Palace et al., 2004) (Fig. 1.1). In addition, the pro-oxidative nature of inorganic selenium also leads to increased mitochondrial membrane permeability which activates a cascade of intrinsic apoptotic pathway assisted by mitochondrial cytochrome C. In summary, selenium in excess creates an oxidizing atmosphere within the cell which drives a cell towards apoptotic pathways (Kim et al., 2006).

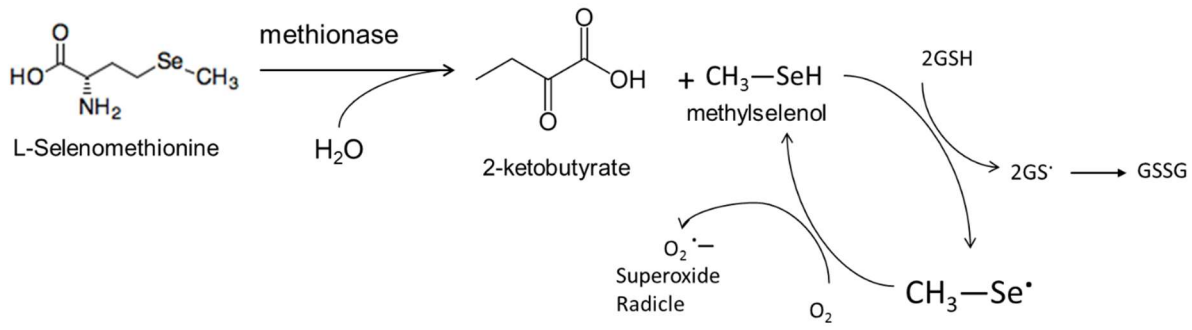


Figure 1.1: Selenomethionine under the influence of enzyme methionase generates methylselenol which undergoes a spontaneous redox recycling in the presence of GSH to generate superoxide radical (Figure adapted from Palace et al., 2004).

1.3.2. Essentiality of selenium

The biochemical role of selenium in redox homeostasis was first demonstrated experimentally by Rotruck et al., (1972). A year later, in 1973, two independent research groups established that glutathione peroxidase (GPx), which is an antioxidant, was a selenoprotein and has selenium as its essential structural constituent (Flohe et al., 1973; Rotruck et al., 1973). Since then, a large number of scientific reports have subsequently shown that selenium is an integral part of many more proteins that help in the maintenance of cellular redox balance (Burk, 2002; Tapiero et al., 2003). The redox reactive properties of selenium are mediated by the selenolate moiety (SeCys-Se-) of the selenoproteins which acts as a nucleophile and reduces the toxic free radicals (Reich and Hondal, 2016).

Although main role of selenium is to maintain cellular redox homeostasis (Reich and Hondal, 2016; Zwolak and Zaporowska, 2012), there are many selenoproteins with unknown biological roles. Approximately 30 seleno-compounds have been identified in mammals with known functions of about 12 (Arteel and Sies, 2001). After the discovery of specialized selenocysteinyl-

tRNA, which incorporates selenium into selenocysteine through recognition of UGA codon in selenocysteine insertion sequence (SECIS) of mRNA (Gonzalez-Flores et al., 2013), selenocysteine is now regarded as 21st amino acid (Böck et al., 1991). This discovery has further stimulated the research in selenium, and the SECIS has been discovered in many species of plants (Fajardo et al., 2014), mammals (Pietschmann et al., 2014), and fish (Kryukov and Gladyshev, 2000). It is also known that selenocysteine is more catalytically redox reactive than cysteine, and probably this is why the SECIS was developed and retained during evolution of aerobes (H.-Y. Kim et al., 2006; Lobanov et al., 2009). A highly specialized genetic mechanism to incorporate selenium into proteins is a clear indication of the importance of this element in biology.

1.3.2.1. Essentiality of selenium to fish

Dietary deficiency of selenium in fish is known to result in reduced growth rate (Hu et al., 2016), impaired immune function (Pacitti et al., 2016), and even mortality (Khan et al., 2017; Wang et al., 2013). The detrimental effects of selenium deficiency in fish have been associated with reduced antioxidant status (Bell et al., 1987; Hu et al., 2016; Khan et al., 2017; Wang et al., 2013). Unlike the recommended dietary allowance (RDA) of selenium for humans, the scientific literature do not have a consensus on dietary requirement of selenium for fish because the experimentally derived values can depend on three factors: (i) the chemical species of selenium, (ii) exposure period, and (iii) fish species. The optimum dietary requirement of selenium for fish can range from 0.3 µg g^{-1} to 20 µg g^{-1} of diet (Hodson and Hilton, 1983; Janz, 2012; Le and Fotedar, 2014). However, an analysis of literature also suggests that the optimal dietary requirement of selenium for fish is between 5 to 25 µg Se Kg^{-1} body weight (Janz, 2012), which is significantly higher than the RDA for mammals. A dietary dose of 55 µg selenium per day is recommended for an adult human (Institute of Medicine, 2000). Considering 60 Kg to be the average weight of an adult

human, the daily dietary requirement of selenium is 0.92 μg Kg^{-1} body weight, which is 5 to 25 folds less than the requirement for fish. Perhaps the higher dietary requirement of selenium for fish is to maintain their selenoproteome which is the largest amongst all the vertebrates (Lobanov et al., 2009, 2008).

The physiological roles of selenium in fish are similar to the roles observed in mammals; however, there are some selenoproteins that are specific to only fish. For example, fish specific selenoproteins have been identified in zebrafish and pufferfish that have a structural role in eye lens (Castellano et al., 2005, 2004). Oxidoreductases, especially the GPx, are the best-characterized selenoproteins in fish (Janz, 2012; Khan et al., 2017). The function of GPx is primarily to reduce hydroperoxides to corresponding alcohols at the expense of glutathione (GSH). There are five known GPx in vertebrates, and at least four have been identified in fish (Kryukov and Gladyshev, 2000; Thisse et al., 2003). Since GPx is involved in the metabolism of hydroperoxides and GSH, its activity in combination with measures of membrane lipid peroxidation, and thiol ratio is a sensitive biochemical marker of oxidative stress in ecotoxicological studies of fish (Branco et al., 2012; Sattin et al., 2015; Wendel, 1980). Thioredoxin (Trx) and thioredoxin reductase (TrxR), which are the essential components of the intracellular redox system and important regulators of ROS accumulation, have also been characterized in fish (Pacitti et al., 2014). Vertebrate Trx is a selenoprotein with selenocysteine in its active site. New selenoproteins isoforms and their role in redox homeostasis, are being identified in fish every year. The overwhelming amount of evidence from scientific literature supports the antioxidative role of selenium in fish and cannot be overstated.

1.4. Cadmium

1.4.1. Cadmium as an environmental pollutant

Cadmium (Cd) is a ubiquitous toxic element with no known biological utility except as a zinc replacement at the catalytic site of a type of carbonic anhydrase in some marine diatoms (Maret and Moulis, 2013). Mean concentration of cadmium in Earth's crust is very low (~0.2 mg Cd Kg^{-1}) and thus natural weathering of rocks or water run-off usually does not contribute much to aquatic contamination except in rare cases where soil may have unusually high cadmium (e.g., Jamaica and Devon Island in Canadian Arctic) (McGeer et al., 2012). Instead, cadmium contamination of the water bodies originates primarily from anthropogenic activities like mining and smelting, and its use in utility products like nickel-cadmium batteries, paints, phosphate fertilizers, stabilizers, and nanoparticles.

Toxicity of cadmium to aquatic organisms depends on its bioavailability which, in turn, depends on its chemical speciation. The free cation (Cd^{+2}) is the most bioavailable and most toxic form of cadmium (McGeer et al., 2012). In water, a wide variety of organic and inorganic ligands can reduce cadmium toxicity by forming complexes with it, and thereby reducing the availability of free Cd^{+2} (Soumya Niyogi and Wood, 2004; Playle et al., 1993). However, under most common conditions and at environmentally relevant pH, cadmium occurs primarily in its free cationic form (Cd^{+2}) (McGeer et al., 2012).

Cadmium through waterborne and dietary exposures is highly bioaccumulative, and liver and kidney are the main sites of accumulation in fish (Ciardullo et al., 2008; de Conto Cinier et al., 1997; McGeer et al., 2012; Szebedinszky et al., 2001). Moreover, cadmium has very long depuration time which further contributes to its high body burden (Kondera et al., 2014).

1.4.2. Toxicity of cadmium to fish

Cadmium is toxic to fish even at extremely low concentrations. Exposure to toxic concentrations of cadmium can result in reduced growth, teratogenesis, impaired immune and behavioral functions, reproductive failure, and mortality (Driessnack et al., 2017, 2016; Eissa et al., 2010; Groh et al., 2015; Ismail and Yusof, 2011; Jin et al., 2015). Acute toxicity of cadmium arises primarily from competitive inhibition of Ca^{+2} uptake at gills (Niyogi and Wood, 2004). Physiological mechanisms of chronic toxicity due to cadmium exposure are not well understood, but the deleterious effects of exposure may arise due to disruption of oxidative homeostasis (Fig. 1.2) (Bertin and Averbeck, 2006; Nunes et al., 2014; Zhang et al., 2017; Zheng et al., 2016). Chronic toxic effects of cadmium exposure have been found to correlate with depletion of glutathione (Jin et al., 2015), binding of cadmium to the thiol groups of cellular proteins and enzymes (Bertin and Averbeck, 2006), or substitution of essential metals such as zinc in metalloenzymes (Hartwig, 2001).

1.4.2.1. Cadmium-induced oxidative stress

One of the major reasons for cadmium-induced toxicity is disruption of oxidative homeostasis and induction of oxidative stress (Fig. 1.2) (Bertin and Averbeck, 2006; Nunes et al., 2014; Zhang et al., 2017; Zheng et al., 2016). Mitochondria are the main site of cellular oxidative respiration and ROS generation. Accumulation of ROS in mitochondria is kept under check by an array of antioxidative enzymes such as catalase, glutathione reductase, superoxide dismutase, as well as by a variety of reducing agents like GSH, ascorbate, β-carotene, and α-tocopherol that sequester ROS (Orrenius et al., 2007; Sies, 1997). Cadmium can inhibit electron transport chain by binding with complex III located in the inner mitochondrial membrane (Wang et al., 2004). The electron transport chain, under normal circumstances, can efficiently handle the train of electrons

from one complex to the other. However, inhibition of complex III inhibits the transfer electrons to the next respiratory complex, and the electrons are released prematurely out of the system which reacts with molecular oxygen in an uncontrolled fashion to form various ROS (Orrenius et al., 2007). Also, binding of cadmium to complex III may result in accumulation of semi-ubiquinones (Cannino et al., 2009; Wang et al., 2004), which is a radical anion generated within complex III due to an incomplete transfer of electrons. Generation of semi-ubiquinones may also generate ROS (Wang et al., 2004). Inhibition of complex III and uncoupling of mitochondrial electron transport chain was reported in rainbow trout (*Oncorhynchus mykiss*) hepatocytes after chronic water-borne exposure to a sublethal concentration of cadmium (Adiele et al., 2012, 2011). Generation of ROS under chronic cadmium exposure may also overwhelm the cellular machinery responsible for maintenance of redox homeostasis and various ROS scavengers, such as GSH, may be depleted (Rani et al., 2014; Wang et al., 2015). In addition, proteins involved in DNA repair are sensitive targets of cadmium toxicity (Ling et al., 2017). Over the past few decades, there have been extensive studies on cadmium bioaccumulation and toxicity in aquatic organisms, and cadmium ecotoxicology continues to receive further attention (Wang and Rainbow, 2006).

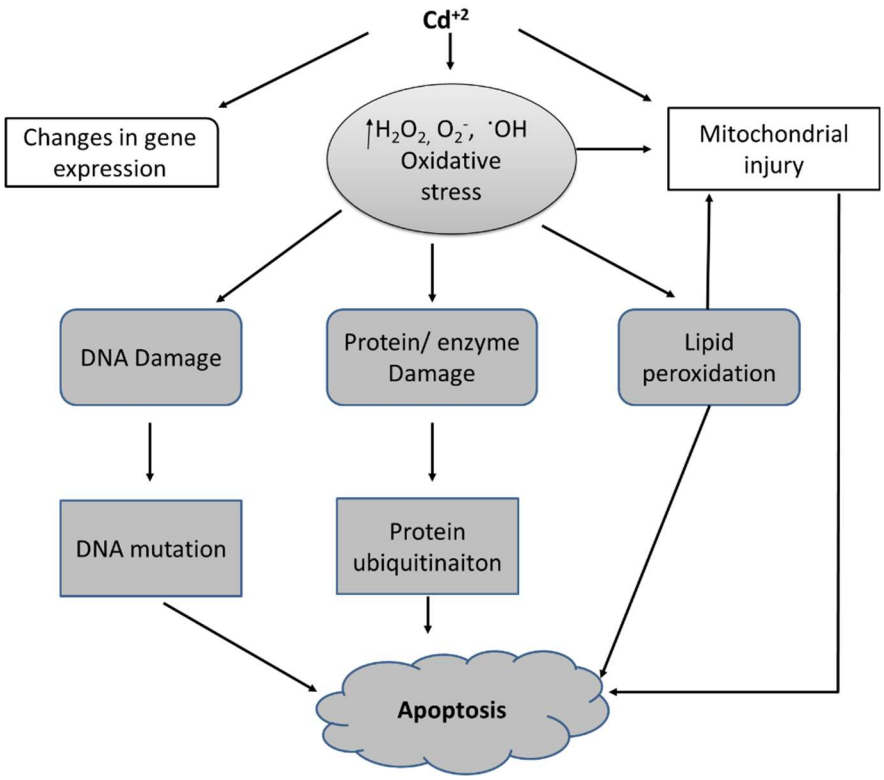


Figure 1.2: Effects of Cd on various cellular processes leading to cytotoxicity. Cadmium exposure causes oxidative stress by generating ROS and overwhelming the cellular anti-oxidative machinery. The ROS generated in response to Cd exposure damage biomolecules such as proteins, DNA, lipids in membranes of cell and mitochondria. Cd exposure also affects DNA repair system causing mutations and carcinogenicity. If the adaptive cellular response is not able to contain the damage induced by Cd exposure, then the cell proceeds towards apoptosis. Modified from Bertin and Averbeck (2006).

1.5. Arsenic

1.5.1. Arsenic as an environmental pollutant

Arsenic (As) is a metalloid that occurs ubiquitously in Earth's crust in various geological formations, usually in conjunction with sulfur and metals (Kumagai and Sumi, 2007). From such

depositions, arsenic may find its way into the atmosphere and then into the aquatic habitats that serve as the ultimate sink for pollutants. Natural events like volcanic activities and low-temperature volatilization from natural deposits contribute towards atmospheric arsenic, but anthropogenic activities constitute a significant source and contribute about 2/3rd of the total atmospheric arsenic flux (Chilvers and Peterson, 1987). Primary anthropogenic sources of arsenic are electronic industries (admixture in semiconductor production), ore production and processing, metal treatment, metal galvanizing, chemical industries (dyes and colors, wood preservatives, pesticides, pyrotechnics), and pharmaceutical products (Matschullat, 2000). These are the sources that also contribute towards environmental selenium contamination. Thus, arsenic and selenium may coexist as aquatic contaminants and may influence each other's toxicity. In fresh water, arsenic primarily exists in inorganic forms as oxyanion of trivalent arsenite (As III) or pentavalent arsenate (As IV), and organically as monomethylarsonic acid (MMA) and dimethylarsenic acid (DMA) (Anawar, 2012).

1.5.2. Toxicity of arsenic

The toxicity of arsenic is dependent on its chemical form and oxidation state with inorganic forms being more toxic than their organic counterparts. Also, among the inorganic forms of arsenic, the trivalent arsenites are more toxic than the pentavalent arsenates (Hughes, 2002). In comparison to many other toxic metals and metalloids , inorganic arsenic is less toxic, however its widespread distribution and persistent nature make it a priority pollutant (McIntyre and Linton, 2012; Smedley and Kinniburgh, 2002). Arsenic can bioaccumulate in fish, but can be influenced by its route of exposure, bioavailability, and chemical species in the water column (Ciardullo et al., 2010; Russell J. Erickson et al., 2011; Jankong et al., 2007). Exposure to toxic concentrations of inorganic arsenic to fish results in depression of growth, impaired immune system, poor cognitive and reproductive

function, detrimental effects on all organs, and mortality (Kumari et al., 2017; Olivares et al., 2016; Ray et al., 2017; Szymkowicz et al., 2017). Toxic to all multicellular organisms, arsenic has no known biological roles in higher organisms although some *arsenate reductase* possessing bacteria metabolize arsenic to derive energy (Green, 1918). Arsenic and phosphorus are both group 15 elements and have chemical resemblance due to similar electron cloud and a same number of valence electrons, and because of this chemical resemblance, arsenite may uncouple the synthesis of ATP synthesis (Luz et al., 2016). Inorganic arsenic may also replace phosphorus on active sites of enzymes and impair metabolic reactions (McIntyre and Linton, 2012). Apart from these biological interferences, the most important deleterious effect of inorganic arsenic exposure is disturbance of cellular redox homeostasis which affects all macro biomolecules such as enzymes, membrane lipids and nucleic acids (Chitta et al., 2013).

1.5.2.1. Arsenic-induced oxidative stress

One of the most widely accepted and studied mechanisms of arsenic toxicity is its effect on cellular redox homeostasis by inducing ROS (Jomova et al., 2011; Kumagai and Sumi, 2007). Exposure to inorganic arsenicals is also known to induce ROS in fish (McIntyre and Linton, 2012; Sarkar et al., 2017; Selvaraj et al., 2013a); however, our understanding of the mechanisms of arsenic-mediated oxidative stress is derived primarily from work done on mammalian systems. Major species of ROS induced by exposure to inorganic arsenic are superoxide anion ($O_2^{\bullet-}$), hydroxyl ion (•OH), singlet oxygen ($^1O_2$), hydrogen peroxide (H_2O_2) and peroxyl radicals (Flora, 2011).

Arsenite can generate ROS through NAD(P)H oxidase assisted process (Kumagai and Sumi, 2007) (Fig. 1.3). The NAD(P)H oxidase is a membrane-bound enzyme that transfers the electrons from intracellular NAD(P)H across the cell membrane to reduce molecular oxygen to

superoxide anion (see Eq. 1.2). It was demonstrated in mammalian endothelial cell culture that arsenite acts as an extracellular signal for the Ras proteins (cdc42) which activates NAD(P)H oxidase to generate ROS (Qian et al., 2005).

$$NAD(P)H + 2O_2 \rightarrow NADP^+ + 2O_2^- + H^+ \quad \text{(1.2)}$$

Arsenite also generates ROS by affecting nitric oxide (NO) synthase enzyme system (Fig. 1.3). Nitric oxide synthase iso-enzymes are coupled to produce NO from L-arginine and molecular oxygen without generating superoxides. However, exposure to arsenic disrupts this coupling and ROS is generated (Kumagai and Pi, 2004; Kumagai and Sumi, 2007). The physiological effects of arsenic-induced oxidative stress in fish include increased tissue lipid peroxidation and Caspase-3 induced apoptosis (Datta et al., 2009; Greani et al., 2017a; Sarkar et al., 2017; Selvaraj et al., 2013a).

The monomethylated (MMA III) and the demethylated (DMA III) metabolites of inorganic arsenic are known to be the potent inhibitors of antioxidative enzymes such as glutathione peroxidase, glutathione reductase, and thioredoxin reductase (Chouchane and Snow, 2001; Lin et al., 2001; Petrick et al., 2001; Rodriguez et al., 2005). Therefore, the metabolic methylation of inorganic arsenic, which was previously considered as a detoxification process, is now known to potentiate the toxicity of inorganic arsenicals by compromising the cellular antioxidative machinery.

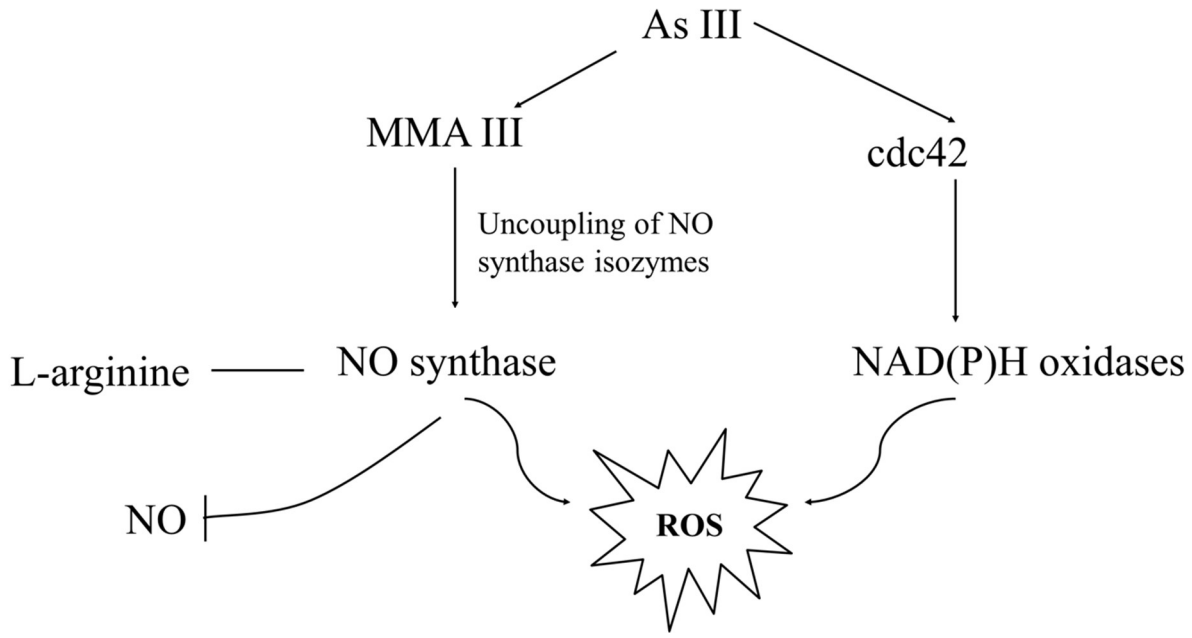


Figure 1.3: Inorganic arsenite acts as an extracellular signal for small GTPase Rac family of proteins of which cdc42 is a part. Activation of Rac proteins triggers the downstream cascade of ROS mediated signaling via activation of NAD(P)H oxidases (Figure modified from Kumagai and Sumi, 2007).

1.6. Interactions of selenium with arsenic and cadmium

1.6.1. Interactions of selenium with arsenic

Selenium is known to have both antagonistic and synergistic interactions with a large number of trace elements in fish; however, most of our understanding on the mechanistic basis of their interactions is derived mainly from mammalian studies (Alp et al., 2011; Janz, 2012; Ponomarenko, O., 2017; Selvaraj et al., 2012; Zwolak and Zaporowska, 2012). Moxon in 1938, first discovered that arsenite could antagonize the toxicity of inorganic selenium in rats. Later, work by Moxon et al., (1945), and Levander and Baumann (1966) suggested that arsenic and selenium could interact metabolically and facilitated their excretion through biliary route. Currently, arsenic-

selenium antagonism in mammals is an active field of research, and is even being used to prevent chronic arsenicosis through selenium fortified diets in human populations (Krohn et al., 2016; Sah et al., 2013). It has been proposed that the antioxidative properties of selenium (Banni et al., 2011; Ponton et al., 2016), and its modulatory effect on the tissue-level distribution of arsenic (Jamba et al., 1997) could be the reasons behind their antagonistic interactions in animals. The antioxidative properties of organic and inorganic forms of selenium against arsenic-induced oxidative stress are well characterized in mammals. In mammalian experiment models, selenium has been demonstrated to restore the redox-status of the tissues by upregulating the thiol balance and activities of anti-oxidative enzymes (Rossman and Uddin, 2004; Xu et al., 2013; Xue et al., 2010). Although the available literature mostly presents evidence and arguments in favor of arsenic-selenium antagonism, some mammalian studies have also suggested that methylated selenium or the methylated metabolites of selenium can have synergistic toxic effects with arsenite (Kraus and Ganther, 1989; Levander, 1977). Inorganic selenium and its methylated forms may also potentiate arsenite toxicity by competitively inhibiting the complete methylation of arsenite, and thus increase the retention of more toxic monomethylated arsenicals in the tissues (Styblo and Thomas, 2001; Walton et al., 2003).

In addition to the anti-oxidative properties of selenoproteins against arsenite, formation of a seleno-bis(*S*-glutathionyl) arsinium compound in liver has also been proposed as a potential mechanism by which selenium may antagonize arsenite toxicity (Gailer, 2007, 2009; Gailer et al., 2000). It has been demonstrated in mammalian experimental models that arsenite and inorganic selenium are reduced in the presence of GSH to form bis(*S*-glutathionyl) arsinium and highly nucleophilic hydrogen selenide ions in the liver. Because of the reactive nature of hydrogen

selenide, it reacts with the bis(*S*-glutathionyl) arsinium ion to form a water soluble seleno-bis(*S*-glutathionyl) arsinium complex, which is then methylated to replace glutathionyl and excreted as [$(CH_3)_2AsSe^-$] *via* biliary route (Fig. 1.4) (Gailer, 2007, 2009; Gailer et al., 2000; Ponomarenko, O., 2017; Sun et al., 2014). However, it has also been proposed that under chronic exposure to arsenite, formation of a As-Se complex can deplete the flux of selenium to the liver and compromise the synthesis of anntioxidative selenoproteins such as GPx (Birringer et al., 2002).

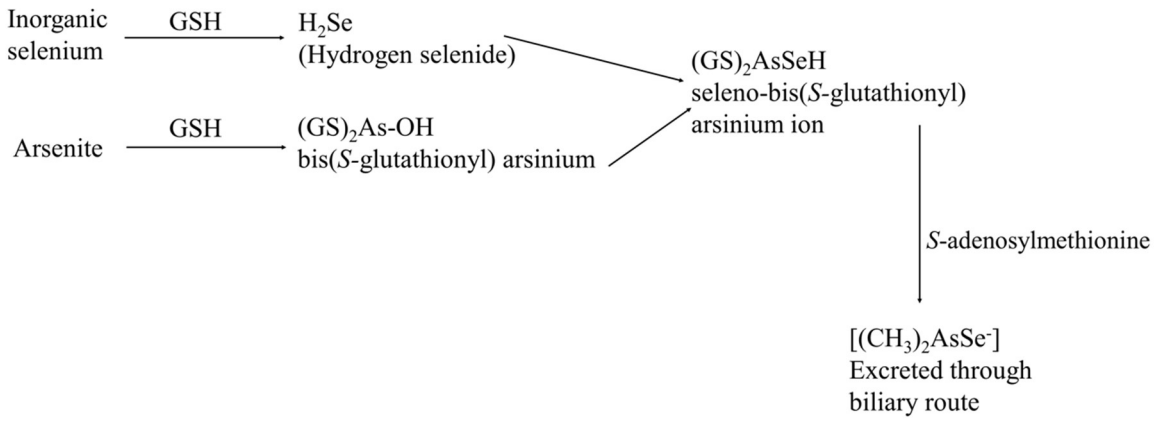


Figure 1.4: Structural basis of antagonism between arsenite and inorganic selenium. Figure modified from Sun et al., 2014.

The interactions between selenium and arsenic have been studied sporadically in fish. To date, only a couple of *in vitro* studies have examined the arsenic-selenium interactions in piscine cells, and both of these studies reported the antagonistic effects of selenium against arsenic toxicity (Alp et al., 2011; Selvaraj et al., 2012). For example, both selenate and selenite were able to reduce acute arsenite and arsenate induced cell mortalities in fin and gill cell culture derived from Bluegill (*Lepomis macrochirus*) (Babich et al., 1989). Selenite was also demonstrated to have a protective role against arsenite (As_2O_3) induced toxicity in fish hepatoma cell line through a reduction in ROS

generation, reduced DNA damage, increased GPx activity, reduction in apoptosis and prevention of mitochondrial membrane damage (Selvaraj et al., 2012).

1.6.2. Interactions of selenium with cadmium

Experiments conducted on mammalian and avian experimental models suggest that selenium can prevent cadmium-induced toxicity through the restoration of oxidative homeostasis, or escalation of the cellular stress response. For example, Liu et al., (2014) demonstrated through an *in vitro* experiment on chicken splenic lymphocytes that selenite augments the activity of anti-oxidative enzymes and reduces ROS mediated apoptosis. Similarly, Chen et al. (2012) also demonstrated that selenite could augment cellular stress response against cadmium-induced toxicity by up-regulation of various proteins of HSP family in chicken splenic lymphocytes. Concurrent exposure to selenite with cadmium is known to restore cellular energetics in rat renal cells (El-Sharaky et al., 2007). Organic and inorganic selenium has also been found to alleviate cadmium-induced oxidative stress in fish (Banni et al., 2011; Orun et al., 2008; Talas et al., 2008; Xie et al., 2016), however all of these studies were carried out mainly by using non-toxic selenium exposure doses, as well as extremely high cadmium concentrations during waterborne exposure. Clearly, further studies are required on the interactive effects of selenium and cadmium in fish, specifically to enhance our understanding of how this interaction is modulated by the different exposure doses of selenium (non-toxic and toxic) as well as its chemical speciation (inorganic *vs*. organic) at both cellular and systemic levels.

1.7. Research objectives

Rationale: My research was designed to study the mechanistic underpinnings of interactive effects of selenium with arsenite or inorganic cadmium toxicity in a model freshwater fish, rainbow trout (*Oncorhynchus mykiss*). Interactions of selenium with arsenite or cadmium have been studied

sporadically, and most evidence on these interactions are derived from non-aquatic experimental systems, particularly from mammals and birds. Currently, there is complete lack of information on the biochemical pathways through which selenium modulates arsenite-induced oxidative stress in aquatic animals. Also, the *in vivo* investigations into selenium-arsenite interactions in aquatic animals and the effects of such interactions on the tissue-level distribution of metals is lacking. As mentioned previously, the scientific reports on selenium-cadmium interaction already exist for piscine experimental models. However, these studies have limited environmental relevance because these studies were carried out only with physiologically optimum doses of selenium. Also, the fish were exposed mainly to waterborne cadmium concentrations (0.4 – 2.0 mg L^{-1}) that were well above the range of environmentally relevant cadmium exposure levels. It is essential to understand how selenium can influence cadmium toxicity at levels that exceed the physiological threshold and may cause toxicity to fish by itself. Since it is now known that diet is a significant route of exposure to cadmium (Harrison and Klaverkamp, 1989; Liu et al., 2015; Xu and Wang, 2002), the studies investigating interactions of selenium with dietary cadmium are also warranted.

Hypothesis: This research project is based on the principal hypothesis that "Selenium will modulate arsenite and cadmium-induced toxicity in freshwater fish but in a dose-specific manner, with antagonistic interactions at low to intermediate exposure levels and synergistic effects at higher doses".

Research objectives: My research has four main objectives which are as follows:

i) To provide insights into the cellular pathways by which different chemical species [inorganic (selenite) *vs.* organic (selenomethionine)] and exposure concentration of selenium influences cadmium-induced oxidative stress at the cellular level *(Chapter 2)*

The interactions between selenium and cadmium were characterized in isolated hepatocytes of rainbow trout in primary culture. Modulatory effects of selenite and selenomethionine as inorganic and organic forms of selenium, respectively, on cadmium-induced oxidative stress were examined. The interactive effects on major anti-oxidative enzymes and cellular redox potential were investigated. Confocal microscopy was also used to analyze the generation of ROS within the hepatocytes under different experimental treatments. Pharmacological antioxidants, with biochemical activities similar to cellular enzymatic and non-enzymatic anti-oxidants, were also used to distinguish the anti-oxidative pathways involved in amelioration of cadmium-induced oxidative stress.

ii) To characterize the hepatic pathways by which different chemical species [inorganic (selenite) *vs.* organic (selenomethionine)] and exposure concentration of selenium modulate arsenite-induced oxidative stress at the cellular level *(Chapter 3)*

The biochemical pathways by which selenium modulates arsenite hepatotoxicity were characterized using isolated hepatocytes of rainbow trout in primary culture. Selenite and selenomethionine were used to investigate the modulatory effects of inorganic and organic forms of selenium, respectively. The influence of various exposure treatment on the activities of major anti-oxidative enzymes and cellular redox potential were studied. Effects of selenium on the arsenite-induced generation of ROS was visualized by confocal microscopy. Pharmacological antioxidants, with biochemical activities similar to cellular enzymatic and non-enzymatic anti-oxidants, were also used to distinguish the anti-oxidative pathways involved in amelioration of arsenic-induced oxidative stress.

iii) To understand the interactive effects of chronic exposure to dietary cadmium and selenomethionine on tissue-specific accumulation and toxicity of cadmium in rainbow trout (*Oncorhynchus mykiss*) *(Chapter 4)*

The fish were exposed to diets supplemented with cadmium in combination with three different doses (low, medium, and high) of selenomethionine for 30 days. Hepatosomatic index and condition factor were used as the general biomarkers of stress and energetic status of fish to dietary treatments. Lipid peroxidation, activities of hepatic anti-oxidative enzymes, and thiol ratio (GSH:GSSG) were used as biomarkers of oxidative stress. Redistribution of cadmium from critically important tissues such as liver, kidney, and muscle as one of the strategies for amelioration of cadmium-toxicity under the influence of selenomethionine was also investigated.

iv) Characterization of the modulatory effects of selenomethionine on arsenite-induced hepato-toxicity and tissue-specific accumulation of arsenic during chronic dietary exposure *(Chapter 5)*

Juvenile rainbow trout were exposed to an environmentally relevant dietary dose of arsenite in combination with different levels of dietary selenium [control (low), moderate and high; as selenomethionine] for 30-days to examine the modulatory effects of selenium on the tissue-specific accumulation of arsenic and arsenite-induced hepatotoxicity. Hepatic lipid peroxidation, activities of anti-oxidative enzymes, and thiol ratio (GSH:GSSG) were used to understand the effects of selenomethionine on arsenite-toxicity. Modulation of arsenic accumulation in the critically important liver, kidney, and muscle were investigated as a strategy by which selenium may ameliorate arsenite toxicity. In addition, synchrotron-based X-ray absorption spectroscopy (see appendix for more information; Fig. C1 S1) was also used to understand how the interactions between selenium and arsenite may influence their distribution and localization pattern in fish brain.

CHAPTER 2: An *in vitro* examination of selenium-cadmium antagonism using primary cultures of rainbow trout (*Oncorhynchus mykiss*) hepatocytes

Preface

The aim of this chapter is to address the 1st objective of my doctoral research work which is to provide insights into the cellular pathways by which different chemical species [inorganic (selenite) *vs.* organic (selenomethionine)] and exposure dose of selenium influences cadmium-induced oxidative stress at the cellular level. The hepatocytes of rainbow trout in primary culture were used to determine the antagonistic effects of selenite and selenomethionine against cadmium hepatotoxicity without systemic interference.

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Author contributions

Ankur Jamwal (University of Saskatchewan) conceived, designed, and conducted the experiment, generated and analyzed the data, prepared all figures, drafted and revised the manuscript.

Mohammad Naderi (University of Saskatchewan) provided technical assistance with biochemical assays and data analysis.

Som Niyogi (University of Saskatchewan) provided inspiration, scientific input and guidance, commented on and edited the manuscript, and provided funding for the research.

2.1. Introduction

Cadmium (Cd) has been categorized as a global priority pollutant because of its ubiquitous presence, bio-accumulative nature and potential to induce toxic effects at relatively low concentrations (Campbell, 2006). Cadmium is a non-essential metal, and toxic to all life forms, including fish. Cadmium is a calcium antagonist, and known to cause toxicity in fish by disrupting branchial calcium uptake and homeostasis, especially during acute exposure (Niyogi and Wood, 2004). The toxicity of Cd has also been attributed to the disruption of oxidative homeostasis (Faverney et al., 2004; Wang et al., 2004). Cellular oxidative homeostasis is primarily maintained by various enzymatic antioxidants such as catalase (CAT), glutathione peroxidase (GPx), superoxide dismutase (SOD), as well as by multiple non-enzymatic antioxidants such as glutathione, ascorbate and metallothionein (Lushchak, 2011). However, exposure to cadmium often leads to the disruption of antioxidative enzymes and/or depletion of the non-enzymatic antioxidant pool, resulting in a concomitant cellular accumulation of reactive oxygen species (ROS) and oxidative damage (Bertin and Averbeck, 2006; Waisberg et al., 2003).

The intracellular handling of cadmium can be influenced by the crosstalk of cellular pathways involved in the metabolism and regulation of essential/nutrient elements, which may elicit additive, synergistic or antagonistic effects. Selenium (Se) is one such essential element that has been suggested to influence the toxicity of cadmium in different organisms, including mammals and fish (see Zwolak and Zaporowska, 2012 for review). Selenium is essential for the synthesis of selenoproteins, which have various critical adaptive and housekeeping functions in organisms, including the maintenance of cellular oxidative homeostasis (Zwolak and Zaporowska, 2012). Among all the living organisms, fish are known to have the highest requirement of selenium, as they possess the largest selenoproteome (Lobanov et al., 2009). For example, fish are known to have 30-37 selenoproteins, whereas mammals contain only 23-25 selenoproteins (Lobanov et al.,

2009). Selenium exists in the environment in both organic and inorganic forms (Hodson et al., 1983). Selenomethionine (SeMet) is the most common form of organic selenium found in fish diet, whereas selenite (SeO_3^{2-}) is usually the most abundant soluble form of inorganic selenium found in natural waters under normal conditions (Maher et al., 2010). Since selenium, in the form of selenoproteins, is known to have antioxidative properties, it can be hypothesized that selenium may ameliorate cadmium-induced cellular oxidative stress, and thus provide protection against the toxicity of cadmium. However, it is also interesting to note here that selenium is also an important aquatic pollutant (Janz et al., 2010), and when present above the threshold level in biological systems, can rapidly turn into a pro-oxidant (Hodson et al., 1983). It has been demonstrated that exposure to high levels of both selenite and selenomethionine causes cellular oxidative stress in fish, essentially by disrupting thiol redox and inducing ROS generation (Misra et al., 2012a; Misra and Niyogi, 2009). Thus, the protective effects of selenium against cadmium-induced cytotoxicity might be influenced by selenium exposure dose, with antagonistic effect at low exposure levels and additive or synergistic effects at high exposure levels.

Previous mammalian studies have demonstrated the protective effects of both selenite and selenomethionine against cadmium cytotoxicity (Messaoudi et al., 2010; Zhou et al., 2009; Zwolak and Zaporowska, 2012); however, the precise mechanistic underpinnings of this antagonism are not fully understood. The protective effects of selenium against the organismal toxicity of cadmium have also been reported in fish exposed to sub-lethal cadmium in the presence of selenium (Banni et al., 2011; Talas et al., 2008). However, all of these previous studies have investigated the ameliorative effects of relatively low exposure levels of selenium against cadmium toxicity, and how this effect is modulated by high selenium exposure level is yet to be characterized. Moreover, it is important to note that the chemical speciation of selenium may also influence cellular effects of cadmium exposure, since inorganic and organic selenium are known to be metabolized through

different cellular pathways (Misra et al., 2010; Palace et al., 2004; Seko et al., 1989). This is particularly important in fish since they acquire selenium primarily through their diet as selenomethionine (Maher et al., 2010). Although the antagonistic behavior of selenite and cadmium has been suggested to be mediated by the amelioration of oxidative stress (Newairy et al., 2007; Zhou et al., 2009), it is not known whether the cytoprotective effect of selenomethionine against cadmium cytotoxicity occurs through a similar mechanism.

The main objectives of this study were twofold: (i) to examine how the chemical speciation [inorganic (selenite) *vs.* organic (selenomethionine)] and exposure dose of selenium influences cadmium induced cytotoxicity at the cellular level, and (ii) to provide a deeper insight into the cellular pathways underlying the antagonism of selenium and cadmium in fish. Rainbow trout (*Oncorhynchus mykiss*) hepatocytes in primary culture were used as the model *in vitro* experimental system in the present study, since hepatocytes are the functional units of liver and one of the main sites of selenium and cadmium metabolism (Okuno et al., 2001; Suzuki, 2005; Waalkes, 2000).

2.2. Methods

2.2.1. Chemicals

High purity, cell culture tested sodium selenite (Na_2SeO_3, purity ~ 98%), seleno-L-methionine (purity > 98 %), cadmium chloride ($CdCl_2$, purity~99.99 %), cell dissociation solution non-enzymatic (catalogue# C1419), CelLytic MT™ solution, Dulbecco's phosphate buffered saline and trichloroacetic acid (TCA) were purchased from Sigma Aldrich, USA. Antibiotic and antimicotic solution, and L-15 media were purchased from Invitrogen, Canada. Aquacalm™ (Metomidate hydrochloride) was purchased from Syndel Laboratories Ltd, Canada. All other chemicals were purchased from VWR International, Canada.

2.2.2. Experimental animals

Rainbow trout weighing 600-700 g were used for the experiments. Fertilised eggs from reference rainbow trout females were hatched in the department of biology, University of Saskatchewan. The spawn were reared until they reached 600-700 g in dechlorinated Saskatoon City water at a rate of 2 l min^{-1} under constant aeration. Fish were maintained at a photoperiod of 16 h light: 8 h dark and a water temperature of 12 ± 1 ºC. The fish were fed once daily with commercial diets at a ration of 2 % of body weight.

2.2.3. Hepatocyte isolation and culture

Trout hepatocytes were isolated using a two-step collagenase perfusion technique as described by Mommsen et al, (1994) with slight modifications (Misra and Niyogi, 2009; Moon et al., 1985). Briefly, fish were euthanized with an overdose of Aquacalm (0.5 g l^{-1}) in dechlorinated water. The hepatic portal vein was cannulated with PE-50 tubing and perfused with ice-cold modified Hank's Media (136.9 mM NaCl, 5.4 mM KCl, 0.8 mM $MgSO_4.7H_2O$, 0.33 mM $Na_2HPO_4.7H_2O$, 0.44 mM KH_2PO_4, 5.0 mM HEPES, 5.0 mM Na-HEPES, pH 7.63). Perfusion with Hank's media was continued until the liver was completely blanched, after that the perfusion line was switched to the medium containing 0.2 mg ml^{-1} collagenase in Hank's Media. Perfusion with collagenase was performed until liver was fully digested. Digested liver was chopped into small pieces with a razor blade and the dissociated cells were filtered, first through 260 µm and then through 73 µm mesh size strainers. The cells obtained in the filtrate were centrifuged at 100 x g for 5 minutes at 4 ºC and washed twice in Hank's media. This was followed by a single washing with the Hank's media containing BSA (2 %) and $CaCl_2$ (1.5 mM). The cells were then incubated for 30 min in L-15 media (pH 7.63) containing antimicrobial and antimicotic solution on an ice bath. The settled down cells were collected by aspirating out the media on top. The cells were then

re-suspended in 25 ml of L-15 media and their viability was determined by trypan blue exclusion test (Strober, 2001). The suspensions showing more than 85 % cell viability were used for the experiments. The cells were plated in 6-well Primaria plates (BD Falcon, USA) at a density of 0.3 x 10^6 cells cm^{-2} and incubated, in dark at 15 ^oC for 24 h using a low temperature incubator (Fisher Scientific, Canada), to form monolayer before their use in the experiment.

2.2.4. Exposure of hepatocytes to cadmium, alone or in combination with selenium

At first, the hepatocytes were exposed only to an increasing range of cadmium concentrations (0 – 500 μM) in order to determine the dose-dependent effect of cadmium on cell viability. After 24 h of culture, following isolation, the media from culture plates was aspirated out and the hepatocytes were exposed to the media containing different concentrations of $CdCl_2$. Cadmium exposures were conducted for 48 h with a change of exposure media at 24 h. A consistent 30 % reduction in cell viability was observed at 100 μM cadmium exposure dose (n=4; data not shown). This concentration was therefore chosen for all of the subsequent experiments described below.

To determine how selenium influences cadmium-induced cytotoxicity, hepatocytes were exposed to 100 μM cadmium, alone or in combination with different concentrations (25, 50, 100, 250 and 500 μM) of selenite (SeO_3^{-2}) or selenomethionine (SeMet) for 48 h. Our preliminary work revealed that Se did not influence cadmium-induced loss of cell viability at concentrations below < 25 μM (selenite or selenomethionine) (data not shown). The hepatocytes in the control group were treated similarly with the media without any added cadmium or selenium. The exposure media was spiked with appropriate amounts of freshly prepared solutions of $CdCl_2$, selenite or selenomethionine prior to each exposure. The exposure media was also changed after 24 h of exposure as described above. The osmolality of the exposure media was measured using a 5100C

vapour pressure osmometer (Wescor Inc., USA), and no change was recorded in any treatment due to the addition of $CdCl_2$, SeO_3^{-2} or SeMet. At the end of the exposure period, cells were collected from the culture plate using a non-enzymatic cell dissociation solution. Cell viability was measured immediately by the trypan blue exclusion test. The experiment was performed five times using hepatocytes isolated from an individual fish at each time.

For the measurement of enzymatic activities, the harvested cells were centrifuged at 500 x g for 5 min and washed with Dulbecco's Phosphate Buffer Saline three times and then lysed with 500 µl of CelLytic-M reagent. The lysate was centrifuged at 25,000 x g for 20 min at 4 ^{o}C to pellet the cellular debris. The supernatant was collected and stored at -80 ^{o}C for the enzymatic analysis. For the measurement of oxidized and reduced glutathione, cells were treated as before, except that an ice-cold 5% TCA solution was used along with CelLytic-M reagent during the cell lysis. The cell lysate was split into two fractions (250 µl each). One fraction was stored as such at -80 ^{o}C for measurement of reduced glutathione (GSH). A 20 µl aliquot of 0.04 M of N-ethylmaleimide was added immediately to the other fraction in order to prevent the oxidation of GSH, and stored at -80 ^{o}C for measurement of oxidised glutathione (GSSG). The Bradford method was used for estimation of protein content of the samples (Bradford, 1976).

2.2.5. Exposure of hepatocytes to pharmacological antioxidants in the presence of cadmium

Pharmacological antioxidants, (2,2,6,6-tetramethylpiperidin-1-yl)oxidanyl (TEMPO) and *N*-acetyl-*L*-cysteine (NAC), were used to compare with the antioxidative effects of selenium in trout hepatocytes. Hepatocytes were exposed to 100 µM cadmium, alone as well as with TEMPO or NAC (100 µM) for 48 h, as described previously for exposures with selenium. At the end of the exposure, cells were harvested and cell viability was assessed as mentioned above.

2.2.6. Measurement of cellular thiol redox balance (GSH:GSSG ratio)

Cellular thiol redox balance is assessed traditionally by measuring GSH to GSSG ratio (Hwang et al., 1992; Jones, 2010; Mallikarjun et al., 2012). The concentration of the reduced (GSH) and oxidized (GSSG) glutathione in the cell lysates was measured using a fluorometric method (Hissin and Hilf, 1976), modified to a 96-well microplate based assay (Misra and Niyogi, 2009). In order to confirm the linearity of the reaction rate in the adopted method, commercially purified GSH and GSSG were used to calibrate the standard curve. The measurement of GSH was performed in a final reaction mixture volume of 200 µl, which contained 180 µl of phosphate–EDTA buffer (0.1 M sodium phosphate–0.005 M EDTA, pH 8.0), 10 µl of o-Phthalaldehyde (OPT, 100 µg per 100 µl methanol) and 10 µl of sample. The reaction mixture was incubated for 15 min at room temperature, and the fluorescence was measured in a multimode microplate reader (Varioskan Flash, Thermo Fisher Scientific, Finland) at excitation and emission wavelengths of 350 nm and 450 nm, respectively. The GSH content was expressed as µg per mg of protein. GSSG was measured similarly, except the final reaction mixture volume (200 µl) contained 140 µl of 0.1 N NaOH, 20 µl of o-Phthalaldehyde (OPT, 100 µg per 100 µl methanol) and 40 µl of sample. The GSSG content was also expressed as µg per mg of protein. Finally, GSH content of each replicate was divided by its corresponding GSSG content and expressed as a ratio.

2.2.7. Measurement of antioxidant enzyme activities

We measured the activities of three antioxidant enzymes, SOD, CAT, and GPx using 96-well microplates and a multimode plate reader (Varioskan Flash, Thermo Fisher Scientific, Finland). Enzyme activities were measured in the hepatocytes exposed to 100 µM cadmium alone or in combination with 25 or 250 µM selenite or selenomethionine. The enzyme activities were measured using SOD (Catalogue #706002), CAT (Catalogue #707002), and GPx (Catalogue

#706002) activity kits as per the manufacturer's (Cayman chemical company, USA) instructions. SOD activity was expressed as % of control. Activities of CAT and GPx were expressed as nmol/min/mg protein. One unit of CAT was defined as the amount of enzyme that will cause the formation of 1.0 nmol formaldehyde at 25 ºC. One unit of GPx was defined as the amount of enzyme that will cause the oxidation of 1.0 nmol of NAD(P)H to $NADP^+$ per minute at 25 ºC.

2.2.8. Measurement of intracellular reactive oxygen species (ROS) generation using Confocal Microscopy

Confocal microscopy was used to investigate the effect of selenite and selenomethionine on ROS production in hepatocytes exposed to 100 µM cadmium. An ROS sensitive fluorescent dye, 50,6-chloromethyl-20,70-dichlorodihydro-fluorescein diacetate (CM-H_2DCFDA) was used for this purpose. CM-H_2DCFDA passively diffuses into cells, where its acetate groups are cleaved by intracellular esterases. Following the cleavage of acetate groups, the dye gets oxidized by intracellular ROS to yield a fluorescent adduct which can be evaluated under a confocal microscope with maximum excitation and emission spectra of 495 nm and 529 nm, respectively. Fluorescent intensity can be measured and quantified, thus, providing a measure of intracellular ROS generation.

A 2 mM stock of CM-H_2DCFDA was prepared in anhydrous dimethylformamide (DFO) which was diluted to 5 µM in L-15 media for use in the experiments. Final concentration of DFO in the exposure media was less than 1 %. For this experiment, hepatocytes were cultured on glass bottom dishes coated with poly-D-lysine (Mat Tek Corporation, USA) for 24 h. The 24 h culture was then exposed to 5 µM of CM-H_2DCFDA for 45 min at 15 ºC and washed three times with L-15 media without phenol red. Subsequently, the hepatocytes were exposed to media containing 100 µM cadmium, alone or in combination with a low concentration (25 µM) or a high concentration

(250 µM) of selenite or SeMet for 2 h. An exposure period of 2 h was employed instead of 24 h, primarily to capture the effects during the early exposure phase and also to prevent any leakage of the dye from the cells due to potential ROS induced membrane damage. At the end of the exposure period, intracellular ROS production in each treatment were measured at room temperature (21 ^{o}C) using the 488 nm excitation Argon laser beam and emission was collected using 505–530 nm band pass filter of the confocal microscope (Zeiss Axiovert LSM 510 Meta Confocal System, Carl Zeiss, Germany). Similar microscopic settings for the imaging were maintained throughout to allow conformity of the results.

ImageJ software (National Institutes of Health, Bethesda, Maryland, USA) was used to quantify the fluorescence intensity of the cells. Integrated density of all the cells under the view of microscope was measured from which mean background intensity was subtracted. The final fluorescent intensity was expressed as corrected total cell fluorescence (CTCF) using the following formula:

CTCF = Integrated Density - (Area of selected cell x Mean fluorescence of background readings)

2.2.9. Statistical analysis

All data are presented as mean ± standard error of mean (S.E.M.). Sample size 'n' indicates the number of true independent evaluations, each conducted with cells isolated from a different fish. The experiment involved manipulation of only Se as an independent variable therefore significant differences among the treatment groups were analyzed by one-way analysis of variance (1-WAY ANOVA) followed by Tukey's multiple comparison test (SigmaPlot, version 11, Systat Software, Inc., USA). The assumptions of ANOVA, normality of distribution and homogeneity of variances, were verified using the Shapiro-Wilk and Levene's tests, respectively. A p-value of $\leq$ 0.05 was considered to be significant while comparing different treatments.

2.3. Results

2.3.1. Effects of selenite and selenomethionine on cadmium induced cytotoxicity

Hepatocyte viability after 48 h of exposure to 100 μM cadmium, alone or in combination with selenite or selenomethionine (25, 50, 100, 250, and 500 μM) is illustrated in Fig. 2.1. There was a significant effect of cadmium exposure, independently or in combination with different concentrations of selenium, on cell viability ($F_{11,48} = 13.29$; $p < 0.001$) . Exposure to cadmium alone reduced hepatocyte survival by 30 % ($p < 0.001$). Co-exposure of hepatocytes to cadmium with selenite or selenomethionine reduced cadmium induced cytotoxicity. However, the protective effect of selenium against cadmium toxicity was observed only at 25 μM selenite or selenomethionine concentration, where the cell viability was restored and did not differ from the control group ($p = 0.65$). No such protective effect of selenium against the cytotoxicity of cadmium was observed at 50 – 250 μM of selenite or SeMet ($p > 0.05$). Co-exposure of 100 μM cadmium and 500 μM selenium (selenite or SeMet) was found to be more toxic than 100 μM cadmium alone, as the hepatocyte viability decreased significantly in the former treatment relative to the latter ($p = 0.025$).

2.3.2. Effects of pharmacological antioxidants on cadmium induced cytotoxicity

The effects of pharmacological antioxidants on cadmium-induced cytotoxicity are presented in Fig. 2.2. Exposure of hepatocytes to cadmium, alone or in combination with pharmacological antioxidants, had a significant effect on cell viability ($F_{3,16} = 13.92$; $p < 0.001$). Co-exposure of hepatocytes to 100 μM cadmium and 100 μM TEMPO or NAC completely restored the cell viability ($p < 0.001$). Exposure to only 100 μM cadmium reduced cell viability by about 30% ($p < 0.001$) in comparison to the control. However, there was no statistically significant

difference in cell viability between the control and the co-exposure of cadmium with TEMPO or NAC.

2.3.3. Effects of selenite and selenomethionine on cadmium induced changes in cellular thiol redox balance

Changes in GSH:GSSG ratio in hepatocytes exposed to 100 μM cadmium alone or in combination with selenite or selenomethionine are illustrated in Fig. 2.3. Cellular thiol redox balance was significantly influenced by cadmium exposure, alone or in combination with different concentrations of selenium ($F_{11,36} = 8.65$; $p < 0.001$). Exposure to cadmium alone resulted in a significant decrease in GSH:GSSG ratio ($p < 0.001$). Similar to the effect of selenium on cadmium induced loss in cell viability, 25 μM of selenium, either as selenite or selenomethionine, was able to fully restore the GSH:GSSG ratio as no difference relative to the control was observed. However, exposure of hepatocytes to ≥ 50 μM selenite or selenomethionine did not alter the cadmium induced decrease in cellular thiol redox ($p > 0.05$).

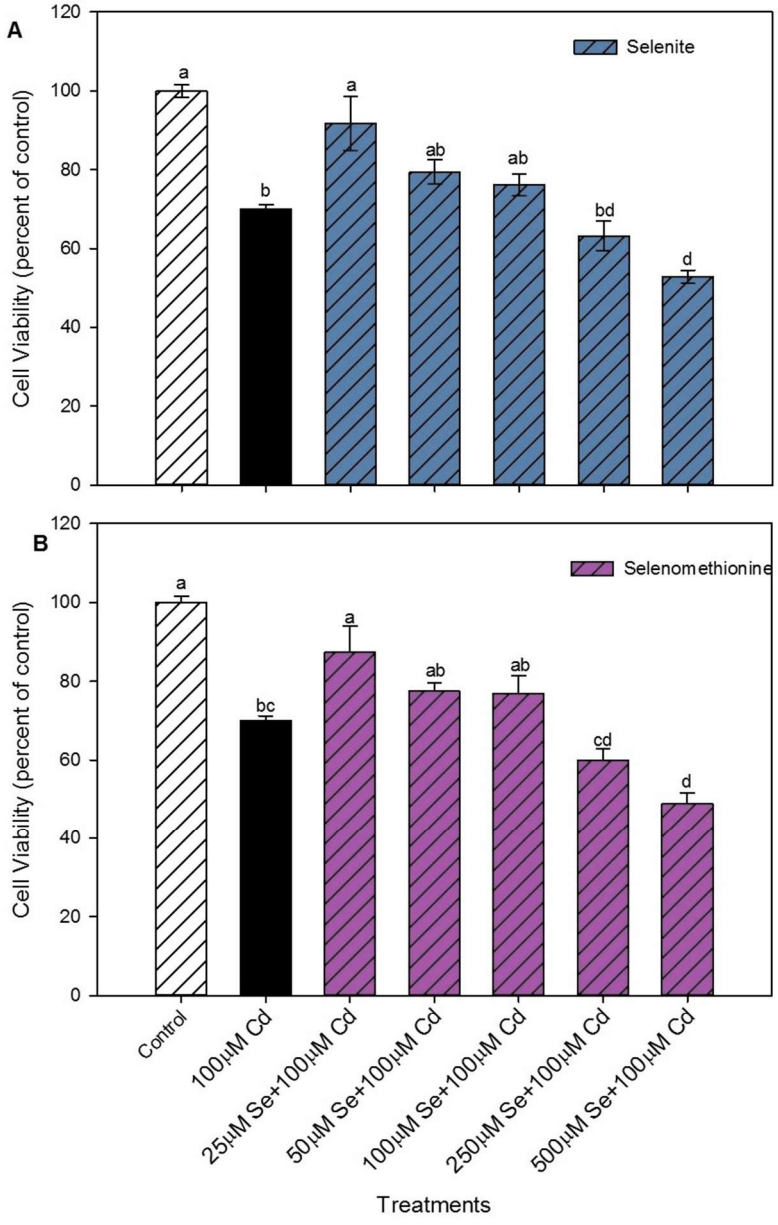


Figure 2.1: Changes in cell viability when rainbow trout hepatocytes were exposed to 100 μM cadmium (Cd), alone or in combination with different concentrations (25 – 500 μM) of selenite (A) or selenomethionine (B). Data are presented as mean ± S.E.M. (n = 5), where n represents the number of true independent measurements, each performed with cells isolated from a different fish. Mean values with different letters are statistically significant ($p < 0.05$).

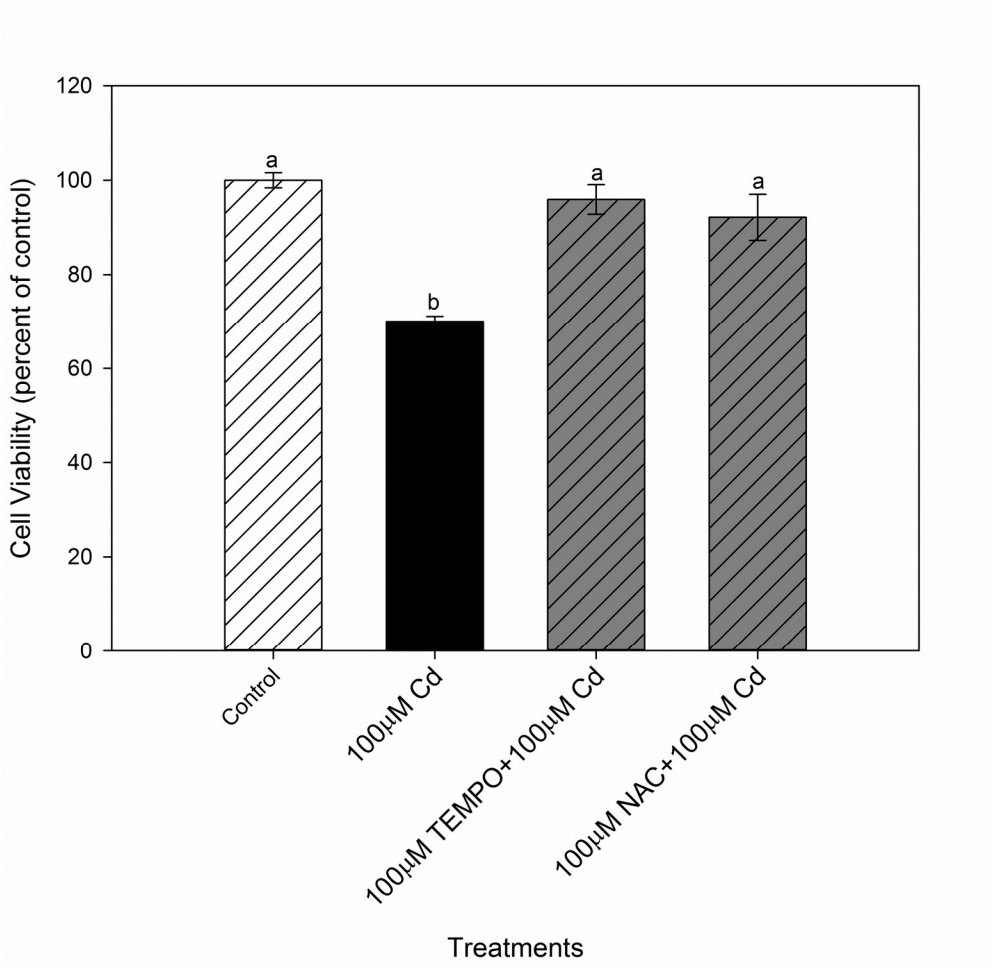


Figure 2.2: Changes in cell viability when rainbow trout hepatocytes were exposed to 100 μM cadmium (Cd), alone or in combination with pharmacological antioxidants, TEMPO (100 μM) or NAC (100 μM). Data are presented as mean ± S.E.M. (n = 5), where n represents the number of true independent measurements, each performed with cells isolated from a different fish. Mean values with different letters are statistically significant ($p < 0.05$).

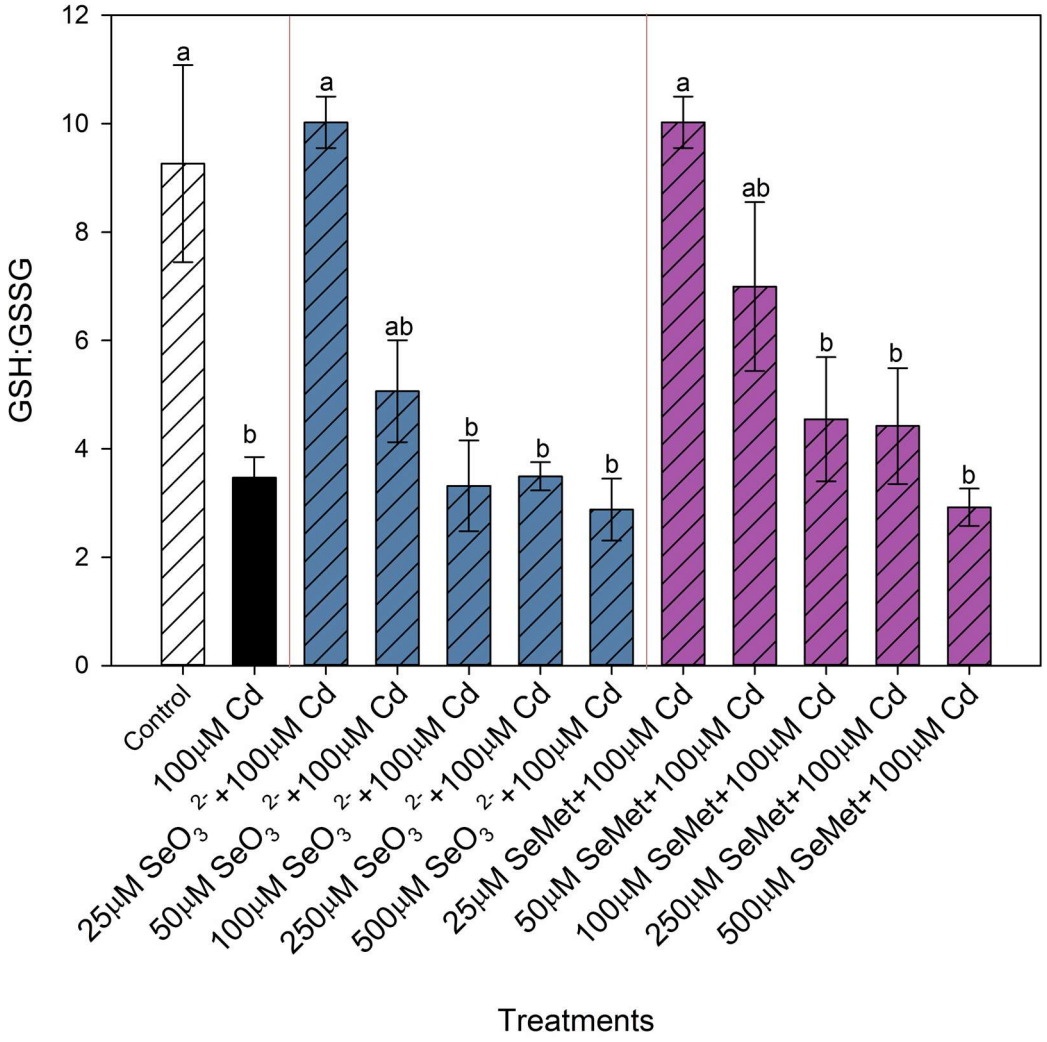


Figure 2.3: Changes in cellular thiol redox status, expressed as ratio of reduced (GSH) to oxidized (GSSG) glutathione in rainbow trout hepatocytes exposed to 100 µM cadmium (Cd), alone or in combination with different concentrations (25 – 500 µM) of selenite (SeO_3^{2-}) or selenomethionine (SeMet). Data are presented as mean ± S.E.M. (n = 5), where n represents the number of true independent measurements, each performed with cells isolated from a different fish. Mean values with different letters are statistically significant ($p < 0.05$).

2.3.4. Effects of selenite and selenomethionine on cadmium induced changes in antioxidative enzyme activities

Cadmium exposure, independently or in combination with selenium, had a significant effect on SOD activity ($F_{5,22} = 42.0$; $p < 0.001$). Exposure to 100 µM cadmium alone reduced SOD activity by 40 %, which was significantly different relative to the control ($p < 0.001$) (Fig. 2.4 A). A similar decrease in the activity was also observed when hepatocytes were co-exposed to cadmium and 250 µM selenium ($p < 0.001$). However, the SOD activity recovered back to the control level when hepatocytes were exposed to cadmium in the presence of 25 µM SeMet, whereas a treatment with 25 µM selenite resulted in a partial recovery of cadmium induced decrease in SOD activity.

Cadmium exposure, independently or in combination with selenium, had a significant effect on CAT activity ($F_{5,24} = 9.24$; $p < 0.001$) Exposure of hepatocytes to 100 µM cadmium alone resulted in a > 50% decrease in the activity of CAT, which was significantly lower relative to the control (Fig. 2.4 B). The enzyme activity, however, was similar to the control in hepatocytes exposed to cadmium in combination with 25 µM selenite or selenomethionine. In contrast, co-exposure to 100 µM cadmium and 250 µM of selenite or selenomethionine resulted in a similar decrease in CAT activity as caused by 100 µM cadmium alone.

GPx activity exhibited a similar pattern as observed with CAT, when treated with 100 µM cadmium alone or together with 25 µM or 250 µM selenite or selenomethionine ($F_{5,25} = 6.77$; $p < 0.001$) (Fig. 2.4 C). The GPx activity decreased significantly (~35%), in hepatocytes treated with cadmium alone or in combination with 250 µM selenite or selenomethionine, relative to the control. However, a partial recovery of GPx activity was recorded in hepatocytes exposed to 100 µM cadmium in combination with 25 µM selenite or selenomethionine.

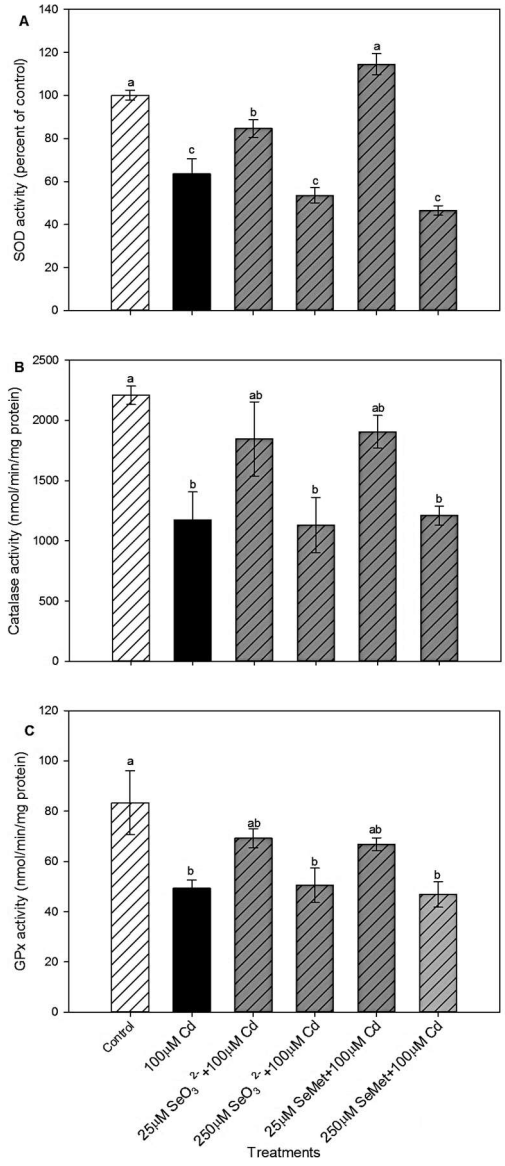


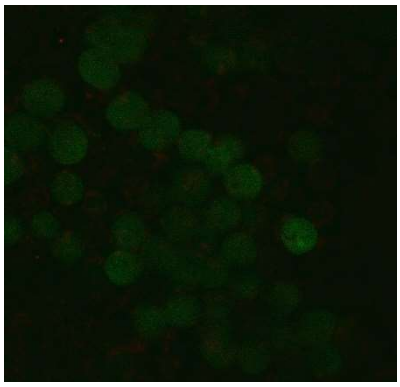
Figure 2.4: Changes in the activities of antioxidant enzymes (A) superoxide dismutase (SOD), (B) catalase (CAT), and (C) glutathione peroxidase (GPx) in in rainbow trout hepatocytes exposed to 100 μM cadmium (Cd), alone or in combination with a low (25 μM) or high (250 μM) concentration of selenite (SeO_3^{2-}) or selenomethionine (SeMet). Data are presented as mean ± S.E.M. (n = 4 – 5), where n represents the number of true independent measurements, each performed with cells isolated from a different fish. Mean values with different letters are statistically significant ($p < 0.05$).

2.3.5. Effects of selenite and selenomethionine on cadmium-induced intracellular ROS generation

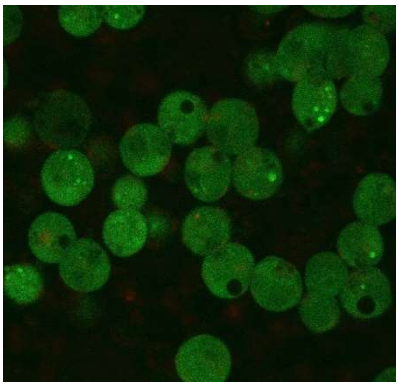
The fluorescence intensity from ROS generation was measured as corrected total cell fluorescence (CTCF) and is illustrated in Fig. 2.5 A and B. There was a significant increase in intracellular ROS generation in hepatocytes exposed to 100 µM cadmium alone relative to the control ($F_{5,18} = 13.72$; $p < 0.001$) (Fig. 2.5 A i and ii, and B). However, co-exposure of hepatocytes to cadmium with 25 µM of selenite or selenomethionine resulted in a significant decrease in intracellular ROS level, and no difference in ROS generation was recorded in comparison to the control (Fig 2.5 A iii and iv, and B). In contrast, no difference in intracellular ROS generation was observed between the treatments of 100 µM Cd alone, and in combination with 250 µM selenite or selenomethionine (Fig. 2.5 A iv and vi, and B). Intracellular ROS level remained significantly high in hepatocytes exposed to cadmium, with or without 250 µM selenium, relative to the control ($p = 0.001$).

A.

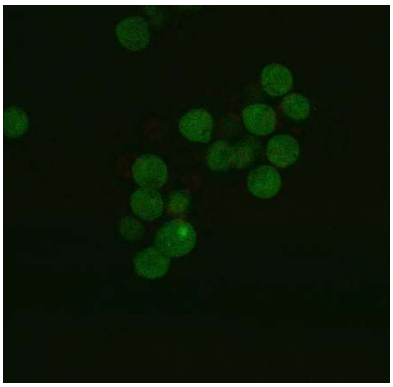
i) Control



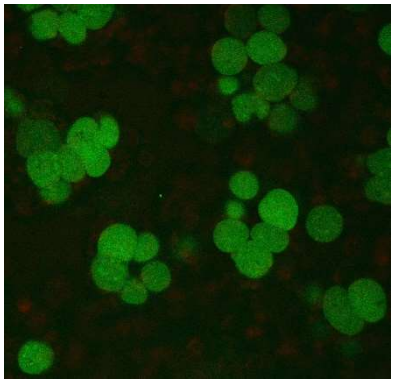
ii) 100µM Cd



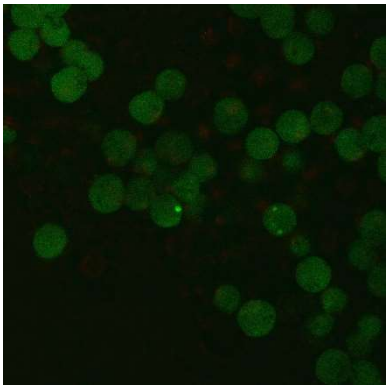
iii) 25 µM SeO_3^{2-}+100 µM Cd



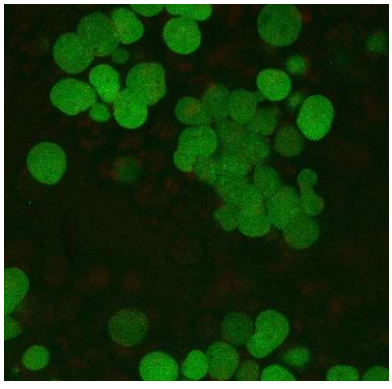
iV) 250 µM SeO_3^{2-}+100 µM Cd



v) 25 µM SeMet+100 µM Cd



vi) 250µM SeMet+100 µM Cd



B.

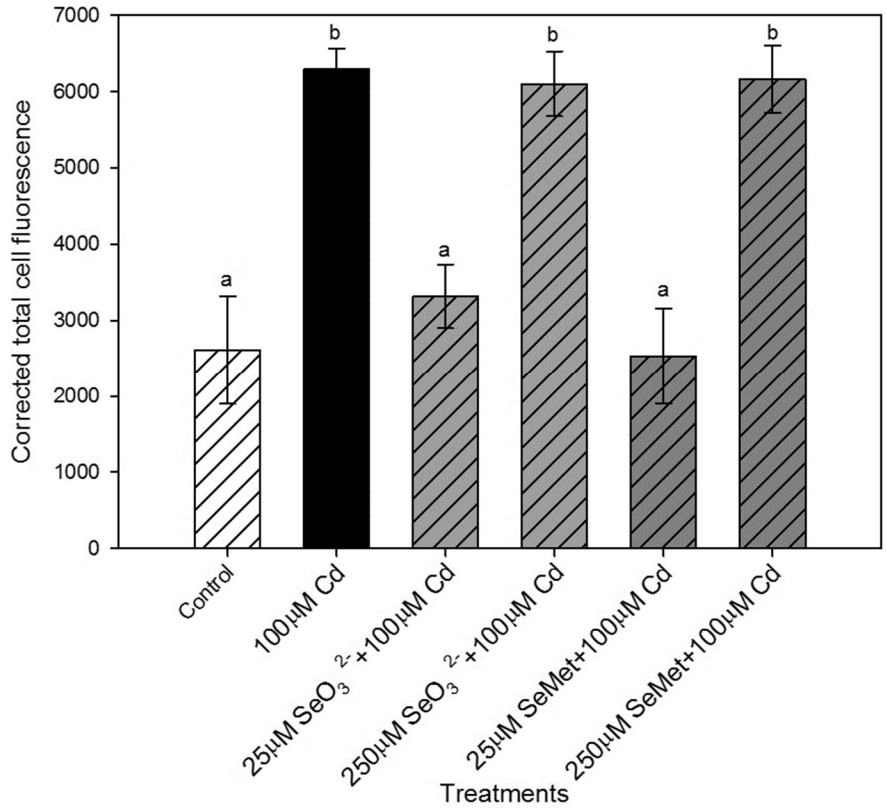


Figure 2.5: Representative confocal fluorescent images (A) and corrected total fluorescent intensity (B) of isolated rainbow trout hepatocytes exposed to 100µM cadmium (Cd), alone or in combination with low (25µM) or high (250µM) concentration of selenite (SeO_3^{2-}) or selenomethionine (SeMet) for a period of 2 h. The cells were loaded with CM-H_2DCFDA for 45 min followed by exposure to various treatments. The intensity of fluorescent signals was measured using ImageJ software (National Institutes of Health, Bethesda, Maryland, USA). Data are presented as mean ± S.E.M. of average fluorescence intensity of 15–20 cells from each replicate and the experiment was repeated four times using four different fish. Mean values with different letters are statistically significant ($p < 0.05$).

2.4. Discussion

In the present study, we have examined the mechanistic underpinnings of selenium-cadmium antagonism at the cellular level in rainbow trout hepatocytes in primary culture. Two different seleno-compounds, selenite (inorganic) and selenomethionine (organic), over a broad range of exposure dose (25 – 500 μM), were used to understand the chemical species specific and dose dependent effects of selenium on cadmium cytotoxicity. In general, our findings supported our original hypothesis that selenium, irrespective of its chemical form, ameliorated cadmium induced oxidative stress by augmenting cellular antioxidative machinery, albeit the protective effect was evident only at the lowest selenium exposure dose (25 μM) used in this study.

Our results demonstrated that a low dose of selenium (25 μM) restored the cadmium induced loss in cell viability, however selenium at concentrations > 50 μM did not elicit any protective effect. Treatment with both selenite and selenomethionine produced similar protective effect against the cytotoxicity of cadmium. Similar protective effects of selenite and/or selenomethionine (10 – 50 μM) against cadmium-induced loss in cell viability were reported previously in mammalian *in vitro* studies with human erythroleukemia K-562 cells (Frisk et al., 2002), and porcine LLC-PK1 renal epithelial cells (Liu et al., 2007). The findings of the present study are also consistent with previous *in vivo* observations which suggested that supplementation of fish diet with low to moderate levels of selenium could reduce waterborne cadmium toxicity in fish (Abdel-Tawwab and Wafeek, 2010; Banni et al., 2011). Interestingly however, our study also revealed that an exposure to 100 μM cadmium in conjunction with the highest dose of selenite or selenomethionine used (500 μM) was more toxic to the trout hepatocytes than 100 μM cadmium alone. Although selenium is an essential element and has antioxidative functions (Hamilton, 2004; Reich and Hondal, 2016; Schwarz et al., 1957), it can rapidly turn into a pro-oxidant when its

concentration exceeds a certain threshold (Hodson et al., 1983; Janz et al., 2010; Maher et al., 2010). Previous studies have demonstrated that both selenite and selenomethionine induce oxidative stress and markedly decrease viability of trout hepatocytes when their exposure concentrations exceed 200 μM (Misra et al., 2012a; Misra and Niyogi, 2009). Thus, the co-exposure of cadmium with selenite or selenomethionine at high dose levels elicited an additive adverse effect on cell viability, likely because of the oxidative stress induced by both elements.

Previous experimental evidences suggest that one of the primary mechanisms of cadmium cytotoxicity is the induction of oxidative stress, mediated mainly by the depletion of glutathione (Macías-Mayorga et al., 2015; Mohamed et al., 2008), and increased accumulation of intracellular of ROS (Faverney et al., 2004; Wang et al., 2004). In our study, a complete recovery in cell viability was observed when the hepatocytes were exposed to cadmium in the presence of pharmacological antioxidants, TEMPO or NAC (Fig. 2.2). This strongly suggests that the decrease in the viability of trout hepatocytes exposed only to cadmium occurred due to the induction of oxidative stress. TEMPO and NAC are both pharmacological antioxidants, but they act through different mechanisms. TEMPO is known to be a ROS scavenger (Sandhir et al., 2015), whereas NAC restores cellular thiol redox balance by facilitating GSH synthesis (Ramen, 2015). Since both TEMPO and NAC were able to ameliorate cadmium induced cytotoxicity, it is reasonable to suggest that selenium at a low dose level (25 μM) was able restore the cadmium induced loss in cell viability *via* mechanisms similar to both of these two pharmacological antioxidants.

Analysis of the GSH:GSSG ratio in the present study revealed that exposure to 100 μM cadmium alone caused a significant drop in cellular thiol redox potential (Fig. 2.3). Again, a complete recovery of GSH:GSSG ratio was observed when trout hepatocytes were exposed to 100 μM cadmium in conjunction with 25 μM of selenite or selenomethionine. In contrast, no significant

improvement in thiol redox was noticed when cells were exposed to cadmium along with higher concentrations of selenium, especially >100 μM (Fig. 2.3). It has been reported previously that the exposure to toxic concentrations of cadmium causes a decline in cellular pool of GSH and thiol containing proteins in mollusk (Ivanina et al., 2008; Macías-Mayorga et al., 2015), and freshwater fish (Hermenean et al., 2015). A dose dependent decline in GSH:GSSG ratio in response to cadmium exposure was also observed in a mammalian renal cell line (Nair et al., 2015). Reduced glutathione can scavenge free ionic species of cadmium (Cd^{2+}), which is the main driver for cadmium cytotoxicity (Waisberg et al., 2003). In addition to this, the thiol group of GSH in its reduced state is able to donate an electron to ROS and thereby neutralize it (Wu et al., 2004). During this process, GSH itself becomes oxidized and readily reacts with another oxidized glutathione to form GSSG. Cellular pool of GSH is replenished by two cellular processes: (i) *de novo* synthesis of GSH, catalysed by γ-glutamylcysteine ligase (Quintana-Cabrera et al., 2012), and (ii) reduction of GSSG to GSH by glutathione reductase enzyme (Wu et al., 2004). During continuous exposure to cadmium, *de novo* synthesis of GSH by γ-glutamylcysteine ligase and GSH regeneration from GSSG recycling could get overwhelmed, leading to the depletion of cellular pool of GSH and build-up of GSSG, resulting in reduced cellular thiol redox potential (Liu et al., 1990). This might have occurred in the present study which resulted in the reduction of GSH:GSSG ratio in trout hepatocytes exposed to cadmium alone. A cell with reduced redox potential is more susceptible to cadmium toxicity because of its negative implications on several biochemical pathways that depend on reduced intracellular environment (Maracine and Segner, 1998; Waisberg et al., 2003).

Selenium, at optimal concentrations is known to upregulate *de novo* synthesis of GSH (Chung and Maines, 1981; Richie et al., 2011). An increase in GSH:GSSG ratio was reported by Fontagné-Dicharry *et al.,* when rainbow trout were fed with selenomethionine/selenite supplemented diet (Fontagné-Dicharry et al., 2015). Moreover, it has been demonstrated by Chung

and Maines (1981), that selenite could upregulate the activity of γ-glutamylcysteine ligase in rat liver, which can lead to increased GSH synthesis. This could be a likely mechanism by which selenium, at a low exposure dose (25 μM), was able to augment cellular GSH:GSSG ratio observed in the present study. The improved cellular thiol ratio, in turn, probably played an important role in ameliorating cadmium induced oxidative stress and loss of cell viability. On the other hand, exposure to high doses of selenium (both selenite and selenomethionine) can lead to a reduced thiol ratio (Misra et al., 2012a; Misra and Niyogi, 2009). It has been suggested that the cellular metabolism of selenite, when present in excess, occurs *via* its reaction with GSH, which leads to the generation of superoxide anion ($O_2^{\bullet-}$) (Lin and Spallholz, 1993; Seko et al., 1989). This causes a depletion of cellular GSH pool, and thereby induce cytotoxicity. In contrast, selenomethionine, when present in abundance, is metabolised into methylselenol by the enzyme, L-methionine-γ-lyase. Subsequently, methylselenol undergoes redox cycling, which requires GSH, and produce $O_2^{\bullet-}$ in the process (Misra et al., 2010). Therefore, the decrease in GSH:GSSG ratio, observed in the present study when trout hepatocytes were exposed to high doses of selenite or SeMet, was likely mediated by the cellular metabolism of selenite and SeMet, which also resulted in increased intracellular ROS generation and oxidative stress.

In the present study, we analysed the activities of three major enzymatic antioxidants, SOD, CAT, and GPx. These enzymes represent the first line of defence against ROS. SOD is responsible for dismutation of $O_2^{\bullet-}$ into H_2O and H_2O_2 (Fukai and Ushio-Fukai, 2011), whereas CAT or GPx reduce H_2O_2 to non-toxic H_2O and O_2 (Chelikani et al., 2004; Mills, 1957). These enzymes are also used as oxidative biomarkers because their activities are usually induced in response to mild oxidative stress as a compensatory mechanism. However, a rapid increase in intracellular ROS generation can overwhelm the antioxidative mechanisms, resulting in the suppression of

antioxidant enzymes (Petersen et al., 2014). In our study, a short-term (2 h) exposure to cadmium was found to cause a marked increase in intracellular ROS level, which could have overwhelmed the cellular antioxidative response capacities, leading to an apparent decline in the activity of these enzymes. The reduced activities of these key antioxidative enzymes, in turn, would reduce the capacity to neutralize ROS, leading to various cytotoxic effects. Therefore, the suppressed activity of enzymatic antioxidants could be one of the major cellular implications of cadmium toxicity. Furthermore, metalloenzymes such as SOD, CAT and GPx require an essential metal as cofactor to function, and cadmium is known to inhibit metalloenzymes by substituting metal cofactors (Casalino et al., 1997; Martelli et al., 2006). It is possible that cadmium at the dose used in our study might have been able to impair the functionality of these enzymes by replacing essential metals from their active sites. Reduced activity of enzymatic antioxidants following exposure to cadmium has been reported in several *in vitro* mammalian studies, using hamster ovarian cell line (Yang et al., 1996), and in cultures of rat pneumocytes (Tátrai et al., 2001), male gonadal cells (Yang et al., 2003), and hepatocytes (Müller, 1986). Similarly, *in vivo* studies conducted with different mammalian and piscine species also reported decreased activities of these antioxidant enzymes during exposure to cadmium (Banni et al., 2011; Cao et al., 2010; Jia et al., 2011; Manigandan et al., 2015; Qu et al., 2014; Waisberg et al., 2003).

We have also demonstrated in this study that a low exposure dose of selenium (25 μM of selenite or selenomethionine) was able to alleviate the activities of these antioxidant enzymes. Our results are in agreement with previous *in vivo* studies that reported upregulation of antioxidative enzymes, when fish were treated with low doses of selenite (Nazıroğlu et al., 2004; Zafar et al., 2003). This effect is probably attributable to the role of selenium in the maintenance of enzymes involved in redox reactions. Selenium causes this effect indirectly through GSH, which maintains

the redox status of the enzymes (Arteel and Sies, 2001). As discussed previously, low concentration of selenium was found to increase the cellular GSH:GSSG ratio in our study. It is perhaps this elevated cellular thiol status that facilitated the increase of antioxidative enzyme activities. We have also demonstrated in this study that when hepatocytes were exposed to cadmium along with high selenium (250 μM), no induction of antioxidant enzymes was observed. This occurred likely because selenium, when present in excess, can interact with the thiol moieties of antioxidative enzymes and impair their functions. For example, selenium, when present in high concentrations, has been reported to inhibit GPx activity in mammals, by the formation of selenotrisulfide (S-Se-S), selenenylsulfide (S-Se), and diselenide bonds (Se-Se), and also by the catalysis of disulfide bond (S-S) (Ganther, 1999).

As discussed above, cadmium exposure can increase intracellular ROS generation indirectly by depleting thiol levels as well as suppressing enzymatic antioxidants. In addition, cadimum is known to inhibit and uncouple complex III of the mitochondrial electron transport chain (ETC) and cause proton leak, which ultimately leads to the generation of ROS (Faverney et al., 2004; Wang et al., 2004). Inorganic cadmium can also replace essential metals like Fe, Zn, or Cu from various intracellular sites that binds them and keep their cytosolic concentrations low (Banni et al., 2011; Martelli et al., 2006). Increased concentration of these pro-oxidative metals in the cytosol promote generation of ROS through Fenton's reaction (Stohs and Bagchi, 1995). It has been demonstrated previously that increased ROS production is linked to reduced cell viability (Kelts et al., 2015; Laville et al., 2004). In the present study we also demonstrated that exposure to cadmium significantly increased intracellular ROS production, which corresponded with a reduction in cell viability. However, co-exposure of 100 μM cadmium with 25 μM selenite or selenomethionine was found to reduce ROS production back to the level observed in the control,

along with a full recovery of cell viability. It is apparent that the reduction in intracellular ROS generation during treatment with the lowest dose of selenium occurred due to increased ROS scavenging capacity, as a result of elevated cellular thiol redox and antioxidative enzyme activities. In contrast, we found that co-exposures of cadmium with 250 μM selenite or selenomethionine did not alter the intracellular ROS generation as well as cell viability, relative to that in the cells exposed to cadmium alone. This was to be expected since high selenium treatment did not produce any improvement in cellular thiol redox or antioxidative enzyme activities.

In addition to the restoration of oxidative homeostasis, selenium may also alleviate cadmium induced toxicity by formation of metabolic complex. For example, Gasiewicz & Smith (1978) demonstrated formation of Cd-Se complex by gel filtration chromatography in rat plasma and erythrocytes through *in vivo* and *in vitro* experiments. Formation of chemically and biologically inert Cd-Se complex was also reported in yeast (*Saccharomyces cerevisiae*) (Dauplais et al., 2013). However, no interaction of selenium with cadmium was noted in the absence of erythrocytes *in vitro* (Gasiewicz & Smith, 1978). Similar interactions of selenium with cadmium may also influence their toxicity in fish and need further exploration.

2.5. Conclusion

The present study demonstrated that both selenite and selenomethionine could protect the hepatocytes of rainbow trout against toxicity of cadmium, but this cytoprotective effect of selenium occurs only at a low/non-toxic exposure dose (25 μM). Cadmium was found to decrease cell viability, which corresponded with increased intracellular ROS generation, and decreased cellular thiol redox and antioxidative enzyme activities. Selenium at a low exposure concentration was found to alleviate cadmium-induced intracellular ROS generation by restoring the cellular thiol redox potential and capacity of enzymatic antioxidants. This indicates that selenium at low

exposure levels could act as an antidote for cadmium poisoning in fish and potentially in other organisms including humans. In contrast, selenium at a high exposure dose was not found to be protective against cadmium toxicity, as it did not induce any change in cellular thiol redox status or capacity of enzymatic antioxidants to overcome the oxidative stress caused by cadmium exposure. Overall, our study demonstrated that the antagonistic effects of selenium on cadmium-induced cytotoxicity occur *via* both enzymatic and non-enzymatic antioxidative mechanisms.

CHAPTER 3: Dose and chemical species-specific effects of selenium against arsenite toxicity in cultured hepatocytes of rainbow trout (*Oncorhynchus mykiss*)

Preface

The aim of this chapter is to address the 2st objective of my doctoral research work which is to characterize the hepatic pathways by which different chemical species [inorganic (selenite) *vs.* organic (selenomethionine)] and exposure dose of selenium modulate arsenite induced oxidative stress at the cellular level. The hepatocytes of rainbow trout in primary culture were used to determine the mechanistic basis of interactions between selenium and arsenite without systemic interference.

The content of Chapter 3 was reprinted (adapted) from *Metallomics*, (DOI: 10.1039/C7MT00006E) A. Jamwal, and S. Niyogi, "Dose and chemical species-specific effects of selenium against arsenite toxicity in cultured hepatocytes of rainbow trout (*Oncorhynchus mykiss*)". Copyright 2017, with permission from Royal Society of Chemistry.

Author contributions

Ankur Jamwal (University of Saskatchewan) conceived, designed, and conducted the experiment, generated and analyzed the data, prepared all figures, drafted and revised the manuscript.

Som Niyogi (University of Saskatchewan) provided inspiration, scientific input and guidance, commented on and edited the manuscript, and provided funding for the research.

3.1. Introduction

Arsenic (As) is a highly toxic element that occurs ubiquitously in Earth's crust, usually in conjunction with sulphur and metals (Kumagai and Sumi, 2007). It ranks first on the Superfund list of hazardous substances (ATSDR, 2016) and is also listed as a group 1 carcinogen by the International Agency for Research on Cancer (IARC, 2016). Arsenicosis or arsenite poisoning usually occurs through contaminated water which is a serious environmental concern because of large number of contaminated sites present in many parts of the world (Smedley and Kinniburgh, 2002). Toxicity of arsenic in humans and animals is usually exhibited through a variety of health effects including dysfunction of critical enzymes and cellular damage.

It is generally believed that arsenic, especially in its trivalent form (arsenite; As-III), causes toxicity primarily by inducing reactive oxygen (ROS) reactive nitrogen species (RNS) generation, which eventually leads to the disruption cellular redox homeostasis (Flora, 2011; Shi et al., 2004). Although the reasons underlying the arsenic-mediated ROS and RNS production are not fully understood, it has been suggested that this occurs as a result of arsenic-induced changes in the structure of mitochondrial membrane and subsequent disruption of membrane redox potential (Valko et al., 2015). The cellular pathways regulated by flavin enzymes (e.g., NAD(P)H oxidase, and NO synthase isozymes) have also been suggested to generate ROS and RNS during arsenic exposure (Kumagai and Sumi, 2007; Shi et al., 2004). In addition to increased ROS and RNS generation, arsenic exposure results in impaired activities of redox active peptides and enzymes such as glutathione (GSH), superoxide dismutase (SOD), glutathione peroxidase (GPx), glutathione reductase (GR), glutathione–*S*-transferase (GST), and catalase (CAT), which can further add to the severity of oxidative stress (Flora, 2011). Arsenite is also known to inhibit the activity of redox active biomolecules by binding to the functional sulfhydryl groups (Shen et al., 2013).

Similar to many other trace elements, toxicity of arsenite may also be influenced by the crosstalk of intra-cellular pathways involved in the metabolism and regulation of other essential and non-essential elements. These intracellular interactions, depending on the dose of interacting elements in the exposure, may either antagonize or accentuate the toxic effects of each other. Previous studies in mammalian systems suggest that selenium (Se) is one such important essential element, which can influence the metabolism and toxicity of arsenite (Davis et al., 2000; Levander, 1977; McIntyre and Linton, 2012), although the mechanistic underpinnings of such interactions are yet to be fully understood. Moreover, the cellular and physiological implications of selenium-arsenic interactions have been examined sporadically in non-mammalian organisms, particularly in aquatic organisms such as fish (Babich et al., 1989; Selvaraj et al., 2012). The significance of examining the cellular interactions of selenium and arsenite in fish can be emphasized by the fact that they possess the largest and most diverse selenoproteome (Lobanov et al., 2008). Lobanov et al. (2009) conducted a phylogenetic analysis of selenoproteins and suggested that in the terrestrial animals some of the organoselenium moieties were substituted by cysteine-containing homologues in order to reduce the reliance on the trace elements (Lobanov et al., 2009). Fish is more dependent on selenoproteins than mammals to maintain redox balance in the body, and thus have a much greater physiological requirements of selenium (Lobanov et al., 2009; Mariotti et al., 2012). The physiological roles of many selenoproteins in fish are still unknown, nonetheless it has been established that selenium lends the peroxidase activity to the enzymes, which is why selenoproteins play a critical role in the maintenance of cellular redox balance (Reich and Hondal, 2016).

Selenium exists in the environment in both organic and inorganic forms (Maher et al., 2010). Selenite (SeO_3^{2-}) is usually the most abundant water soluble inorganic form under normoxic conditions, whereas selenomethoinine (SeMet) comprises the most common organic form of selenium that is predominantly available to fish through diet (Maher et al., 2010). Although the

antioxidative properties of both inorganic and organic selenium have been demonstrated previously (Selvaraj et al., 2013b; Xu et al., 2013), the potential mechanisms by which selenium modulates the activities of key enzymatic and non-enzymatic antioxidants remain unclear. Furthermore, the anti-oxidative role of selenium is strictly dose dependent, and when present (both as selenite and SeMet) above the physiological threshold level, can itself turn into a pro-oxidant and induce oxidative stress (Jamwal et al., 2016; Misra et al., 2012a; Misra and Niyogi, 2009; Wallenberg et al., 2014). Moreover, the metabolism of organic and inorganic selenium, particularly when present in excess, occurs *via* different cellular pathways (Misra et al., 2010; Seko et al., 1989). Thus, it can be assumed that the interaction of selenium and arsenite can be influenced by both the dose and chemical speciation of selenium in the exposure. Although the dose and chemical species-specific ameliorative effects of selenium against arsenite cytotoxicity have been reported sporadically in mammalian systems (Alp et al., 2011; Rossman and Uddin, 2004), such investigations have not been performed in any non-mammalian models including fish. Given the greater physiological essentiality of selenium and its important role in maintaining redox homeostasis in fish, selenium and arsenite interactions in fish may elicit different cellular responses compared to the mammals, and thus requires an in-depth investigation.

The present study was designed to carry out an in-depth examination of how the dose and chemical speciation of selenium affect the cytotoxicity of arsenite in a model freshwater fish, rainbow trout (*Oncorhynchus mykiss*). In particular, we focused on identifying the specific antioxidative pathways by which inorganic or organic selenium (selenite or SeMet, respectively) moderates arsenite cytotoxicity. Rainbow trout hepatocytes in primary culture were employed as the *in vitro* experimental system in the present study, because hepatocytes are the main functional units of the liver - the primary site of selenium and arsenite metabolism in animals (Sun et al.,

2014). We hypothesized that selenium will influence arsenite cytotoxicity, but in a dose-specific manner with antagonistic interactions occurring only at low to intermediate exposure levels.

3.2. Methods

3.2.1. Chemicals

High purity, cell culture tested sodium selenite (Na_2SeO_3, purity ~ 98%), seleno-L-methionine (purity>98%), non-enzymatic cell dissociation solution (catalogue# C1419), CelLytic MT™ solution, Dulbecco's phosphate buffered saline, L-15 medium (Leibovitz), 3-amino-1,2,4-triazole (ATA), buthionine sulfoximine (BSO), diethyldithiocarbamic acid sodium salt trihydrate (DETC), mercaptosuccinic acid (MS), and trichloroacetic acid (TCA) were purchased from Sigma Aldrich, USA. Antibiotic and antimycotic solution, were purchased from Invitrogen, Canada. Aquacalm™ (Metomidate hydrochloride) was purchased from Syndel Laboratories Ltd, Canada. All other chemicals, including sodium arsenite ($NaAsO_2$), were purchased from VWR International, Canada.

3.2.2. Experimental animals

Rainbow trout (*Oncorhynchus mykiss*), weighing 600 – 700 g were used for the experiments. Fertilised eggs from reference rainbow trout females were hatched in the Department of Biology, University of Saskatchewan. The juveniles were reared on a ration of 2 % body weight until they attained 600 – 700 g. Dechlorinated Saskatoon City water with a flow rate of 2 l min^{-1} under constant aeration was used for husbandry. A photoperiod of 16 h light: 8 h dark and a water temperature of 12 ± 1 °C was maintained during the rearing period.

3.2.3. Hepatocyte culture and experimental treatments

Trout hepatocytes were isolated using a two-step collagenase perfusion technique as described elsewhere (Jamwal et al., 2016). Following isolation, the cells were plated in a 6-well Primaria plates (BD Falcon, USA) at a density of 0.3 x 10^6 cells per cm^2 and incubated in the dark at 15 ºC for 24 h using a low temperature incubator (Fisher Scientific, Canada), to form a monolayer before their use in the experiment.

Initially, to determine the dose-dependent effect of arsenite on cell viability, the hepatocytes were exposed only to an increasing concentration range of arsenite (0 – 500µM). Following 24 h of culture, the media from the culture plates was aspirated out and the hepatocytes were exposed to fresh media spiked with different concentrations of arsenite (as sodium arsenite) for 24 h. A consistent 40 % reduction in cell viability (61 % ± 6.08) was observed at the concentration of 100 µM arsenite (n = 5; supplementary data, Fig. C3.S1). This concentration was therefore chosen for all the subsequent experiments described below.

To determine the influence of selenium on arsenite-induced cytotoxicity, hepatocytes were exposed to freshly prepared solutions of 100 µM arsenite, independently or in combination with different concentrations (5, 10, 20, and 40 µM) of selenite (SeO_3^{2-}) or selenomethionine (SeMet) for 24 h. The hepatocytes were also exposed to the exposure media spiked solely with 5 – 40 µM of selenite or selenomethinine for 24 h to evaluate the independent effects of selenium on the hepatocyte viability. The hepatocytes in the control group were treated with a similar change in media without any added arsenic or selenium. The exposure dose levels of selenium (5 – 40 µM) was selected on the basis of our preliminary experiments which demonstrated that the combination of 100 µM arsenite and >40 µM selenite or selenomethionine either did not affect arsenite cytotoxicity or was more toxic to the hepatocytes than 100 µM arsenite alone (see supplementary

data, Fig. C3.S2 A & B). The osmolality of the exposure media was measured using a 5100C vapour pressure osmometer (Wescor Inc., USA), and no significant change was recorded in any treatment due to the addition of $NaAsO_2$, selenite or selenomethionine. At the end of the exposure period, cells were gently harvested from the culture plate using a non-enzymatic cell dissociation solution. Cell viability was measured immediately by the trypan blue exclusion test using the Countess Automated Cell Counter (Invitrogen, Canada). The experiment was performed five times using hepatocytes isolated from an individual fish at each time.

3.2.4. Processing of treated hepatocytes for bio-chemical measurements

For the measurement of biochemical end-points, the harvested cells were centrifuged at 500 x g for 5 min and washed three times with Dulbecco's Phosphate Buffer Saline and then lysed with 1000 µl of CelLytic-M reagent. The lysate was centrifuged at 25,000 x g for 20 min at 4 ºC to pellet the cellular debris. The supernatant was collected and stored at -80 ºC for the enzymatic analysis. For the measurements of oxidized (GSSG) and reduced glutathione (GSH), the cells were also lysed as mentioned before. From the cell lysate 20 µl was taken for estimation of protein content by the Bradford method (Bradford, 1976). The remaining cell lysate was immediately deproteinised with 100 µl of 5% TCA. The deproteinised solution was then centrifuged at 12,000 x g at 4 ºC for 25 min to pellet cellular debris. The supernatant was collected and split into two fractions (500 µl each). One fraction was stored as such at -80 ºC for the measurement of reduced glutathione (GSH). A 20 µL aliquot of 0.04 M of N-ethylmaleimide was added immediately to the other fraction to prevent the oxidation of GSH, and stored at -80 ºC for the measurement of oxidised glutathione (GSSG).

3.2.5. Measurement of cellular thiol redox balance (GSH:GSSG ratio)

The concentration of the reduced (GSH) and oxidized (GSSG) glutathione, as an estimate of cellular redox balance, was measured using a fluorometric method (Hissin and Hilf, 1976), modified to a 96-well microplate based assay. A calibration standard curve was prepared from commercially purified GSH and GSSG and used to confirm the linearity of the reaction rate in the adopted method. The final reaction mixture (200 µl) for the measurement of GSH contained 180 µl of phosphate–EDTA buffer (0.1 M sodium phosphate–0.005 M EDTA, pH 8.0), 10 µl of o-Phthalaldehyde (OPT, 100 µg per 100 µl methanol) and 10 µl of sample. The reaction mixture was incubated at room temperature for 15 min before measuring the fluorescence at excitation and emission wavelengths of 350 nm and 450 nm, respectively in a multimode microplate reader (Varioskan Flash, Thermo Fisher Scientific, Finland). The GSH content was expressed as µg per mg of protein. GSSG was measured in the same way except that the final reaction mixture volume contained 140 µl of 0.1 N NaOH, 20 µl of o-Phthalaldehyde (OPT, 100 µg per 100 µl methanol) and 40 µl of sample. The GSSG content was also expressed as µg per mg of protein. Finally, the GSH:GSSG ratio was obtained by dividing GSH concentration with its corresponding GSSG content.

3.2.6. Measurement of antioxidant enzyme activities

We measured the activities of three antioxidant enzymes, superoxide dismutase (SOD, catalase (CAT), and glutathione peroxidase (GPx), in 96-well microplates using a multi-well plate reader (Varioskan Flash, Thermo Electron Corporation,Finland). The enzyme activities were measured using SOD (Catalogue #706002), CAT (Catalogue #707002), and GPx (Catalogue #706002) activity kits as per the manufacturer's (Cayman chemical company, USA) instructions. SOD activity was expressed as U/mg protein. One unit (U) of SOD is defined as the amount of

enzyme needed to exhibit 50% dismutation of the superoxide radical. Activities of CAT and GPx were expressed as nmol min^{-1} mg $protein^{-1}$.

3.2.7. Measurement of intracellular reactive oxygen species (ROS) generation using Confocal Microscopy

The effect of selenite and selenomethionine on ROS production in hepatocytes exposed to 100 μM arsenite was examined as described in Jamwal *et al.,* (2016) using 50,6-chloromethyl-20,70-dichlorodihydro-fluorescein diacetate (CM-H_2DCFDA) as an ROS sensitive probe (Jamwal et al., 2016). A 2mM stock solution of the ROS sensitive dye was prepared by dissolving CM-H_2DCFDA in anhydrous dimethylformamide (DFO). The dye was protected from direct light during all the stages of preparation and exposure. From the stock, 5 μM working solution was prepared in L-15 media for use in the experiments. Final concentration of DFO in the exposure media was less than 1% (v/v). For this experiment, hepatocytes were cultured on glass bottom dishes coated with poly-D-lysine (Mat Tek Corporation, USA) for 24 h to form a monolayer. The 24 h culture was then pre-incubated with 5 μM of CM-H_2DCFDA for 45 min at 15 ^oC and washed three times with L-15 media that did not contain phenol red. This step was performed to remove the background fluorescence from phenol red and unabsorbed dye. Subsequently, the hepatocytes were exposed to media containing 100 μM arsenite, alone or in combination with a low concentration (20 μM selenite or 10 μM selenomethionine) or a high concentration (40 μM) of selenite or selenomethionine for 2 h. An exposure period of 2 h was employed instead of 24 h, to capture the early effects of treatments when most hepatocytes were under oxidative stress but still viable. At the end of the exposure period, the intensity of fluorescence due to intracellular ROS production was measured at room temperature (21 ^oC) using the 488 nm excitation Argon laser beam, and the emission was collected using 505 – 530 nm band pass filter of the confocal

microscope (Zeiss Axiovert LSM 510 Meta Confocal System, Carl Zeiss, Germany). Similar microscopic settings for the imaging were maintained throughout the experiment to allow for conformity of the results.

ImageJ software (National Institutes of Health, Bethesda, Maryland, USA) was used to quantify the fluorescence intensity of the cells. Integrated density of all the cells under the view of microscope was measured from which mean background intensity was subtracted. The final fluorescent intensity was expressed as corrected total cell fluorescence (CTCF) using the following formula:

CTCF = Integrated Density - (Area of selected cell X Mean fluorescence of background readings)

3.2.8. Pharmacological treatments

Pharmacological antioxidants, (2,2,6,6-tetramethylpiperidin-1-yl) oxidanyl (TEMPO) and *N*-acetyl-*L*-cysteine (NAC), were used to compare with the antioxidative effects of Se in trout hepatocytes. Hepatocytes were exposed to 100 µM arsenite, alone or in combination with isomolar concentrations of TEMPO or NAC for 24 h, as described previously for exposures with selenium. At the end of the exposure, cells were harvested and cell viability was assessed as described before.

We also examined the effects of specific enzyme inhibitors to understand the role of GSH and antioxidative enzymes (CAT, SOD, and GPx) in mediating the antagonistic effects of selenium against arsenite-induced oxidative stress. Since we observed that 20 µM selenite or 10 µM selenomethionine were most protective against the cytotoxicity of 100 µM arsenite (see Fig. 1. B, C.), only these two concentrations were used in these assays. Trout hepatocytes were pre-exposed to 1000 µl of media containing 1 mM concentration of one of the following inhibitors: BSO (GSH inhibitor), ATA (Catalase inhibitor), DETC (SOD inhibitor), or MS (GPx inhibitor). Pre-incubation ensured the loading of cells with inhibitors before the main treatment, and thus prevented the

interference from housekeeping concentrations of the target enzymes. After 2 h of incubation in dark, 1000 µl of media was added that contained 200 µM arsenite and 40 µM selenite or 20 µM selenomethionine. Therefore, the composition of final incubation media was 0.5 mM inhibitor, 100 µM arsenite, and 20 µM selenite or 10 µM SeMet. The hepatocytes were then incubated for 24 h in the final incubation media. At the end of the exposure period, the hepatocyte viability was assessed as described previously. A positive control experiment was also conducted to examine the efficacy of the anti-oxidative enzymes inhibitors. The hepatocytes were incubated with 0.5 mM ATA, DETC or MS for 24 h, after which the hepatocytes were processed and the activities of the enzymes were measured using the procedures mentioned previously.

3.2.9. Statistical analysis

All statistical analyses were performed using the IBM SPSS statistical software package (Version 20.0.0) and graphs were plotted with SigmaPlot (version 12, Systat Software, Inc., USA). The data are presented as mean ± standard error of mean (S.E.M.). Sample size 'n' indicates the number of true independent evaluations, each conducted with cells isolated from a different fish. Interactive effects of selenium and arsenite on hepatocyte viability were analysed by two-factor analysis of variance (2-WAY ANOVA). Interactive effects of selenium, arsenite and enzyme inhibitors were analysed by three-way analysis of variance (3-WAY ANOVA). Estimation of the effects of treatments on enzyme activities, GSH:GSSG ratio, and CTCF involved manipulation of only selenium as an independent variable, therefore significant differences among the treatment groups were analyzed by one-way analysis of variance (1-WAY ANOVA) followed by Tukey's multiple comparison test (SigmaPlot, version 11, Systat Software, Inc., USA). The positive controls (inhibitors of anti-oxidative enzymes) were compared to the true controls (without the inhibitors) by t-test. The assumptions of ANOVA, normality of distribution and homogeneity of

variances, were verified using the Shapiro-Wilk and Levene's tests, respectively. A p-value of ≤ 0.05 was considered to be significant while comparing different treatments.

3.3. Results

3.3.1. Effects of selenite and selenomethionine on arsenite-induced cytotoxicity

There was no significant effect of independent Se treatments (5-40 µM selenite or SeMet) on cell viability ($p > 0.05$; Fig. 3.1 A). However, there was a statistically significant interaction between arsenite and selenite (but not with selenomethionine) on hepatocyte viability (2-way ANOVA; $F_{4,40} = 3.43$; $p = 0.02$). Exposure to only 100 µM arsenite significantly reduced (36%) cell viability in comparison to the control ($p<0.001$). Co-treatment with arsenite and selenium (selenite or selenomethionine) ameliorated arsenite cytotoxicity, but only at moderate selenium concentrations used in this study, with no effect at low or high Se exposure levels (Fig. 3.1 B and C). For example, an exposure to 10 and 20 µM selenite in conjunction with 100 µM arsenite significantly improved cell viability [14% ($p = 0.02$) and 23% ($p < 0.001$), respectively] relative to that in the 100 µM arsenite only treatment, however no such effect was observed during co-treatment with 5 or 40 µM selenite (Fig. 3.1 B). Similarly, a combined treatment of 10 µM selenomethionine and 100 µM arsenite significantly improved cell viability (14%) in comparison to that in the 100 µM arsenite only treatment ($p = 0.03$), but no protective effect against arsenite cytotoxicity occurred during co-treatment with 5, 20 or 40 µM selenomethionine (Fig. 3.1 C).

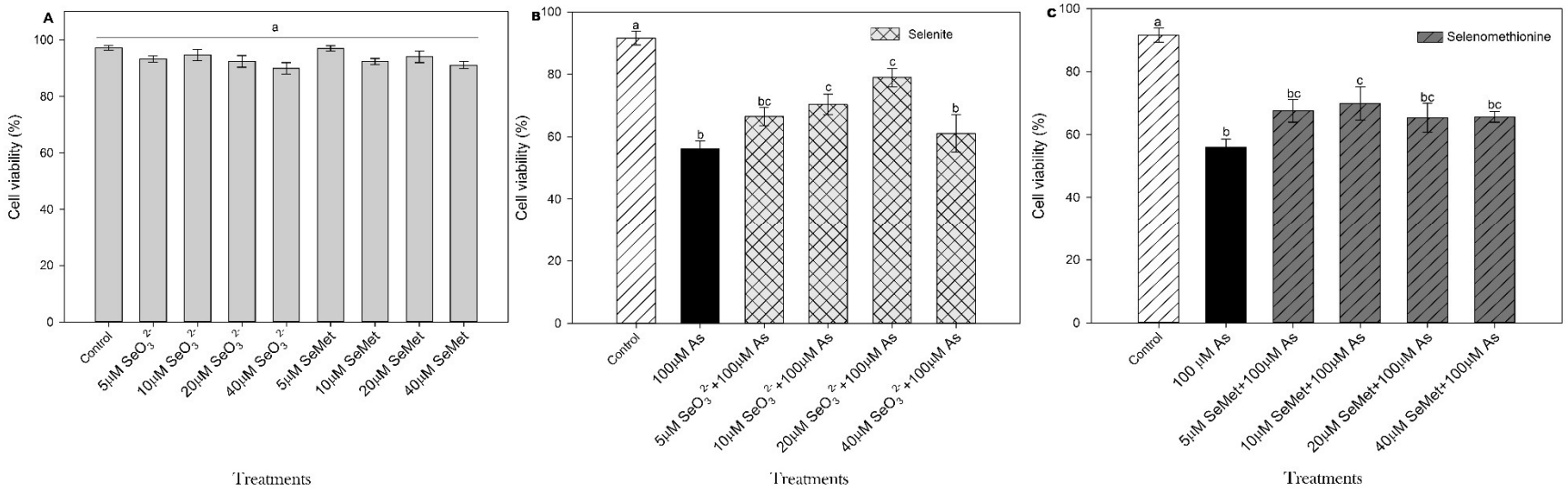


Figure 3.1: Changes in the viability of rainbow trout hepatocytes when exposed to (A) only 5µM – 40µM selenite or selenomethionine (SeMet); (B) 100µM arsenite (As), alone or in combination with different concentrations (5µM – 40µM) of selenite, or (C) 100µM arsenite, alone or in combination with different concentrations (5µM – 40µM) of selenomethionine. Data are presented as mean ± S.E.M. (n = 5), where n represents the number of true independent measurements, each performed with cells isolated from a different fish. Different alphabetical notations on the bars indicate statistical differences between groups of means ($p < 0.05$) as determined by ANOVA and post-hoc test.

3.3.2. Effects of selenite and selenomethionine on arsenite-induced intracellular ROS generation

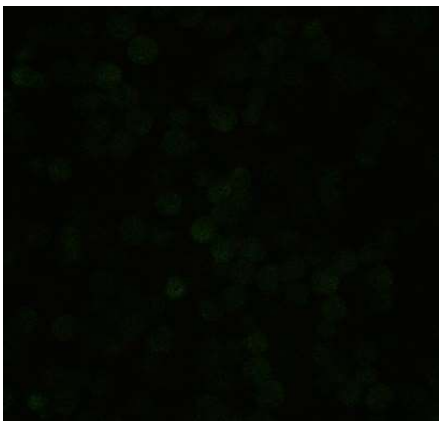
Intensity of green fluorescence from CM-H_2DCFDA, which is proportional to the extent of intracellular ROS generation, was measured as the corrected total cell fluorescence (CTCF), and is presented in Fig. 3.2 A and 3.2 B. There was an overall significant effect of treatments on intracellular ROS generation in the hepatocytes ($F_{5,24} = 13.7$; $p < 0.001$). An exposure to 100 µM arsenite resulted in a significant increase in CTCF relative to the control ($p < 0.001$). However, the co-exposure of hepatocytes to 100 µM arsenite and 20 µM selenite or 10 µM selenomethionine resulted in a significant decrease in the intracellular ROS production in comparison to that in the 100 µM arsenite only treatment (Fig. 3.2 A iii and v, and 3.2 B). In contrast, no significant decrease in intracellular ROS production was recorded in trout hepatocytes during co-exposure with 40 µM selenite or SeMet (Fig. 3.2A iv and vi, and 3.2 B).

3.3.3. Effects of pharmacological antioxidants on arsenite-induced cytotoxicity

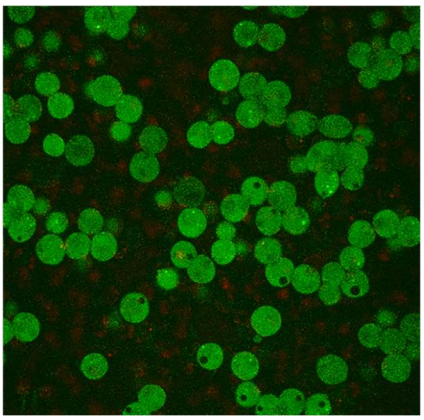
The effect of pharmacological antioxidants on arsenite-induced cytotoxicity are illustrated in Fig. 3.3. Exposure of hepatocytes to 100 µM arsenite, independently or with equimolar concentration of pharmacological antioxidants had a significant influence on their viability ($F_{3,16} = 46.4$; $p < 0.001$). An independent exposure to 100 µM arsenite reduced cell viability by 40 % ($p < 0.001$) relative to the control. Pharmacological anti-oxidants, however, significantly decreased the toxicity of arsenite in trout hepatocytes. Co-treatment of hepatocytes to 100 µM TEMPO with an equimolar concentration of arsenite significantly improved the cell viability relative to the hepatocytes treated exclusively with 100 µM arsenite ($p < 0.001$). NAC was also found to significantly increase the viability of arsenite-exposed cells ($p = 0.01$), although the effect was not as strong as that of TEMPO.

A.

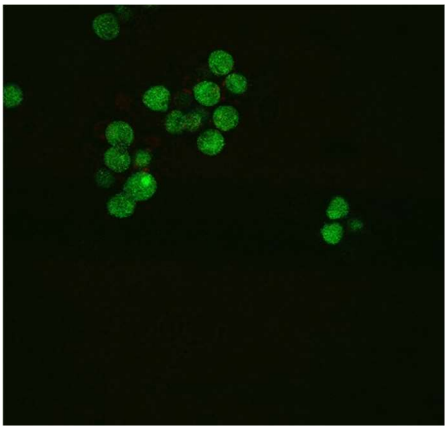
i) Control



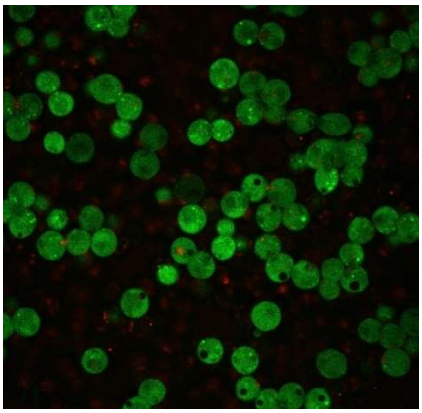
ii) 100µM As



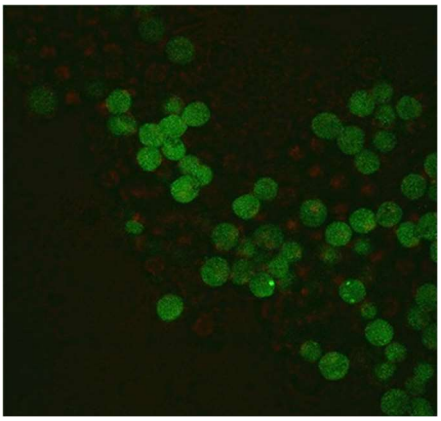
iii) 20 µM SeO_3^{2-}+100 µM As



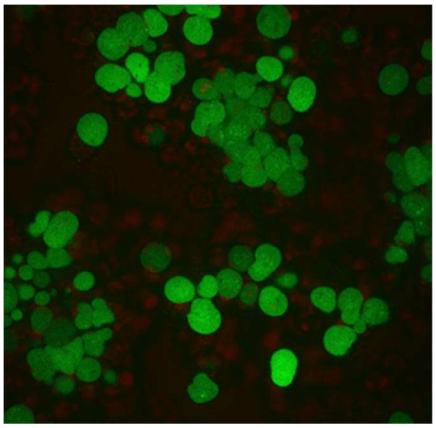
iv) 40 µM SeO_3^{2-}+100 µM As



v) 10 µM SeMet+100 µM As



vi) 40µM SeMet+100 µM As



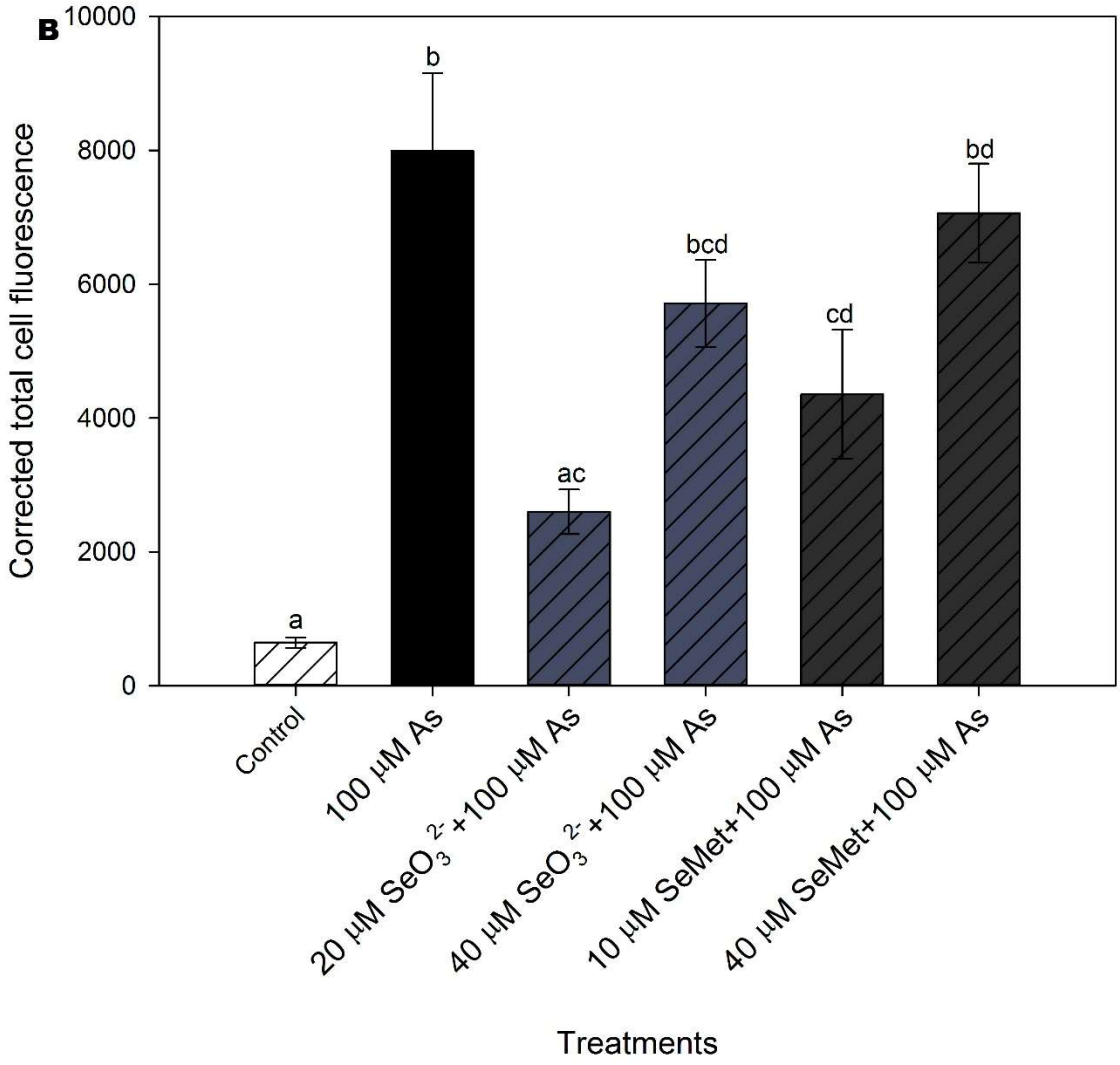


Figure 3.2: Representative confocal fluorescent images (A) and corrected total fluorescent intensity (B) of isolated rainbow trout hepatocytes exposed to 100µM arsenite (As), alone or in combination with low (10-20µM) or high (40µM) concentration of selenite (SeO32-) or low selenomethionine (SeMet) for a period of 2 h. The cells were loaded with CM-H2DCFDA for 45 min followed by exposure to various treatments. The intensity of fluorescent signals (green colour) was measured using ImageJ software (National Institutes of Health, Bethesda, Maryland, USA). Data are presented as mean ± S.E.M. of average fluorescence intensity of 15–20 cells from each replicate and the experiment was repeated four times using four different fish. Different alphabetical notations on the bars indicate statistical differences between groups of means ($p < 0.05$) as determined by ANOVA and post-hoc test.

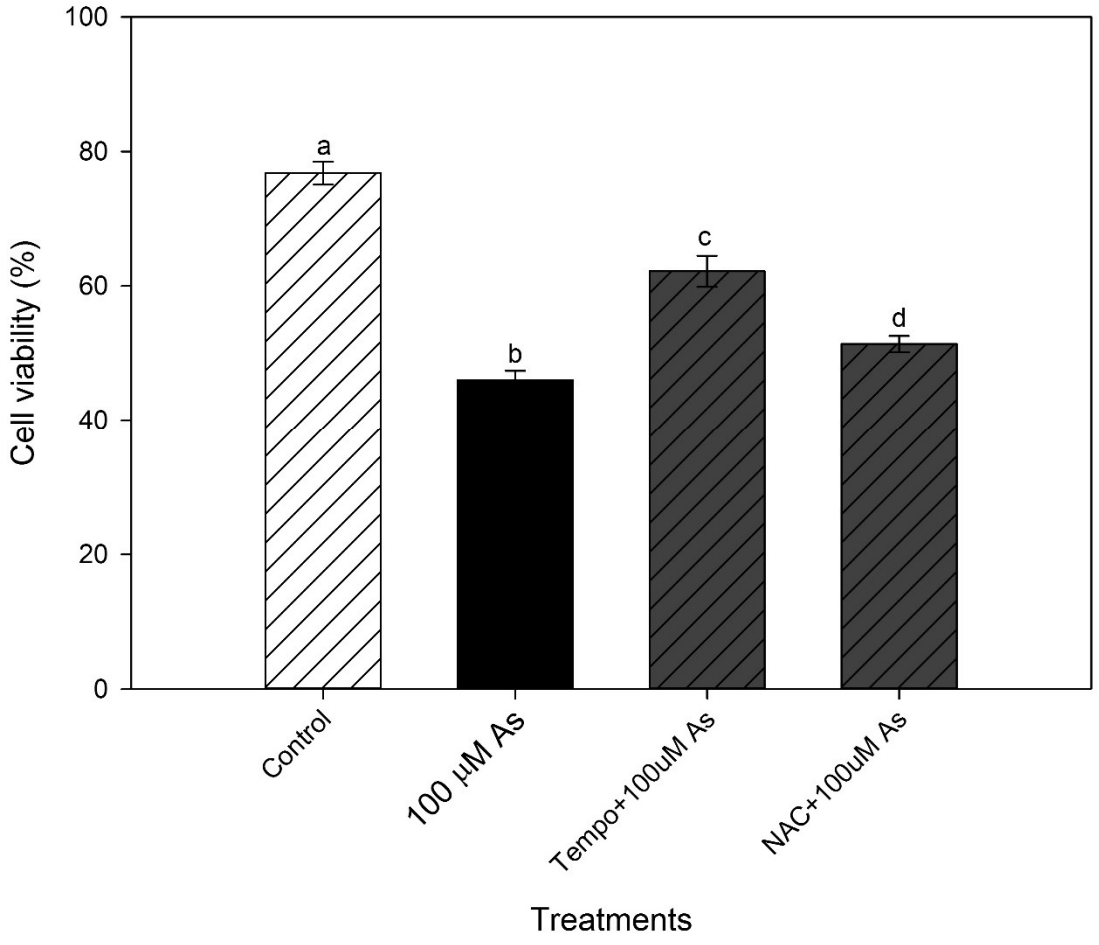


Figure 3.3: Changes in cell viability when rainbow trout hepatocytes were exposed to 100µM As, alone or in combination with pharmacological antioxidants, TEMPO (100µM) or NAC (100µM). Data are presented as mean ± S.E.M. (n = 5), where n represents the number of true independent measurements, each performed with cells isolated from a different fish. Different alphabetical notations on the bars indicate statistical differences between groups of means ($p < 0.05$) as determined by ANOVA and post-hoc test.

3.3.4. Effects of selenite and selenomethionine on arsenite-induced changes in cellular thiol redox balance

Exposure to arsenite, alone or in combination with selenium, significantly influenced intracellular thiol content and ratio in the trout hepatocytes ($p<0.001$; Fig. 3.4 A, and 3.4 B). Exposure to arsenite only caused 83% reduction in GSH content, in comparison to the control (Fig. 3.4 A). However, co-exposure to 10 and 40 µM selenite with 100 µM arsenite resulted in a significant increase in the intracellular GSH concentration in comparison to 100 µM arsenite only treatment ($p<0.001$; Fig. 3.4 A). A consistent increase in intracellular GSH content was also observed when hepatocytes were co-treated with 5 – 40 µM selenomethionine and 100 µM arsenite ($p < 0.001$. Fig. 3.4 A). The hepatic GSSG content was very low in comparison to the GSH and ranged from 1.8 to 8.1 µg mg protein^{-1}. The intracellular GSSG concentration was almost 50 % lower in the hepatocytes treated with only 100 µM arsenite, in comparison to the control (Fig. 3.4 A). Nevertheless, due to high variation in the GSSG content of the control hepatocytes, this change was not statistically significant ($p = 0.93$; Fig. 3.4 A). The GSSG content in the hepatocytes treated with 20 and 40 µM selenite, in combination with 100 µM arsenite, increased by up to 10 folds than the cells treated with 100 µM arsenite alone ($p < 0.005$). Similarly, in comparison to the 100 µM arsenite only treatment, the GSSG content was significantly elevated in the hepatocytes co-treated with 10 – 40 µM selenomethionine and 100 µM arsenite ($p < 0.02$). With respect to the GSH to GSSG ratio, exposure to arsenite alone caused a 70 % reduction in the ratio, in comparison to the control ($p < 0.001$; Fig. 3.4 B). Co-exposure to 5 and 10 µM selenite with 100 µM arsenite elevated thiol ratio by 30 % and 40 % respectively, in comparison to 100 µM arsenite only treatment ($p < 0.03$). Similarly, co-exposure to 5 µM selenomethionine and 100 µM arsenite increased thiol ratio by 40 % in comparison to 100 µM arsenite alone treatment ($p < 0.03$). However, selenite at

concentrations ≥ 20 μM as well as selenomethionine at concentrations ≥ 10 μM did not alter the arsenite-induced decrease in thiol ratio ($p > 0.05$).

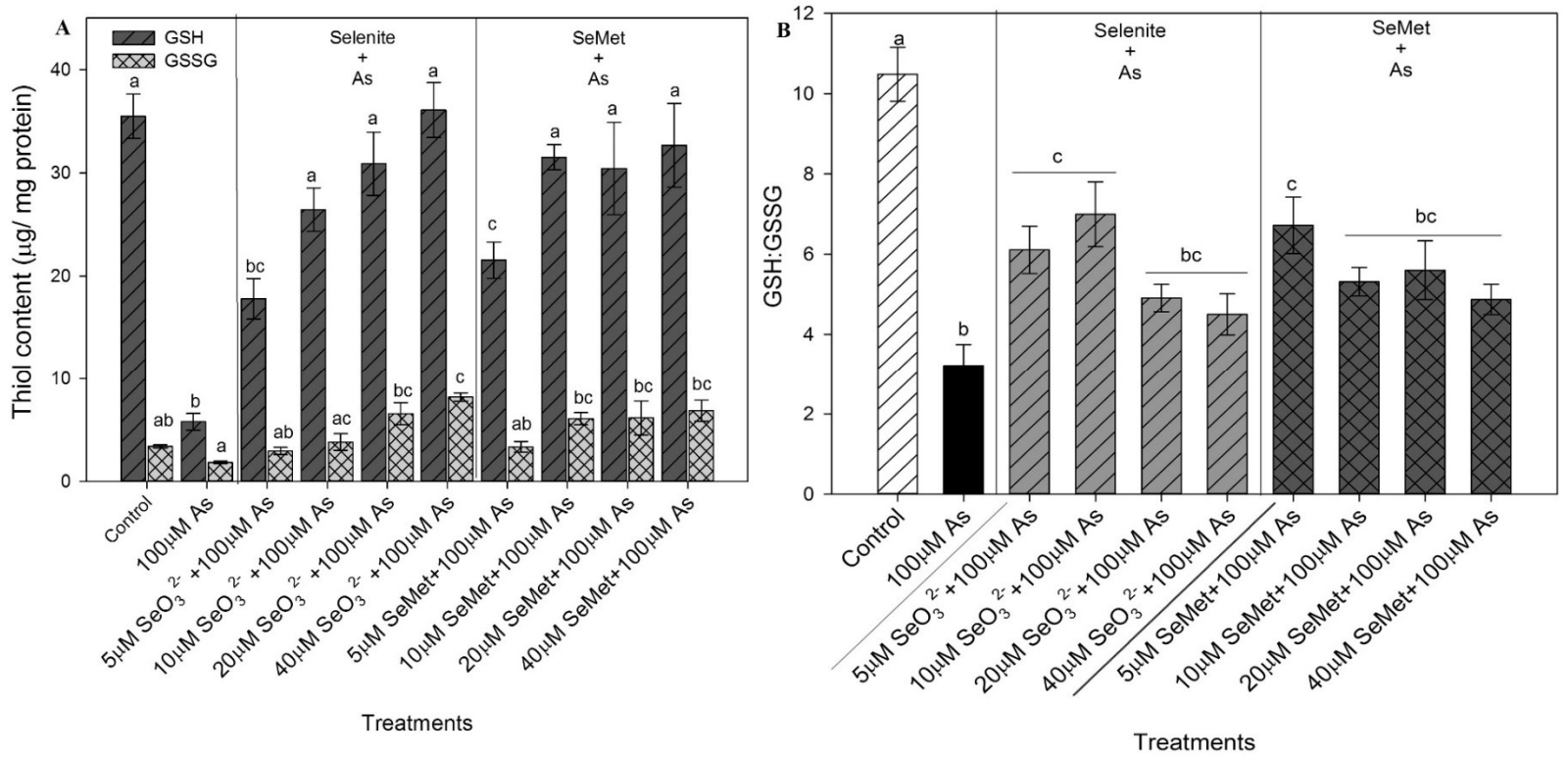


Figure 3.4: Changes in cellular thiol content (A), and thiol redox status, expressed as a ratio of reduced (GSH) to oxidized (GSSG) glutathione (B), in rainbow trout hepatocytes exposed to 100µM arsenite (As), alone or in combination with different concentrations (5µM – 40µM) of selenite (SeO_3^{2-}) or selenomethionine (SeMet). Data are presented as mean ± S.E.M. (n = 5), where n represents the number of true independent measurements, each performed with cells isolated from a different fish. Different alphabetical notations on the bars indicate statistical differences between groups of means ($p < 0.05$) as determined by ANOVA and post-hoc test.

3.3.5. Effects of selenite and selenomethionine on arsenite-induced changes in antioxidative enzyme activities

Arsenite exposure, independently or in combination with selenite, significantly influenced the activity of SOD enzyme ($F_{5,24} = 6.6$; $p = 0.001$; Fig. 3.5 A). Exposure to arsenite alone inhibited the activity of SOD by 39 % in comparison to the control ($p < 0.001$). A complete recovery in the activity of SOD was recorded when the hepatocytes were exposed to 100 µM arsenite in combination with 10 µM selenite, however this effect of selenite against arsenite was not observed at concentrations above or below 10 µM (Fig. 3.5 A). We did not observe any significant effect of selenomethionine, at any concentration tested, on arsenite-induced inhibition of SOD activity ($p>0.05$; Fig. 3.5 B).

Exposure of hepatocytes to 100 µM arsenite alone resulted in a significant reduction (25 %) in the activity of GPx enzyme in comparison to the control ($F_{5,24} = 5.6$; $p = 0.001$; Fig. 3.6). Co-exposure of 100 µM arsenite and 5-10 µM selenite did not have any effect on GPx activity relative to that in 100 µM arsenite alone ($p < 0.38$; Fig. 3.6 A). However, the GPx activity was restored back to the control level when the cells were co-exposed to arsenite with 20-40 µM selenite (Fig. 3.6 A). SeMet also ameliorated the reduction in GPx activity caused by arsenite exposure ($F_{5,24} = 5.9$; $p = 0.001$; Fig. 3.6 B). A complete restoration of GPx activity was observed when the cells were exposed to 5 µM selenomethionine in combination with 100 µM arsenite (Fig. 3.6 B), however this protective effect was not particularly evident at concentrations of ≥10 µM selenomethionine (Fig. 3.6 B).

Both selenite and selenomethionine had similar effects on the arsenite-induced changes in CAT activity. Arsenite exposure, independently or in combination with selenium, had a significant effect on the activity of CAT enzyme ($F_{5,24} = 26.3$; $p < 0.001$; Fig. 3.7). A significant upregulation

in the activity of CAT, in comparison to the control, was observed when the hepatocytes were exposed exclusively to 100 μM arsenite ($p < 0.001$). However, exposure to selenite or selenomethionine, irrespective of their exposure concentrations (5 – 40 μM), resulted in a consistently significant suppression of arsenite-induced increase in CAT activity, although it was still relatively higher than the control (Fig. 3.7 A and 3.7 B).

3.3.6. Effects of the pharmacological inhibitors of the antioxidative enzymes (CAT, SOD, and GPX) and GSH on the viability of hepatocytes and the activities of the enzymes

Effects of the anti-oxidative enzymes and GSH inhibitors on the viability of trout hepatocytes exposed to arsenite in combination with the most protective levels of selenite (20 μM) and selenomethionine (10 μM) are presented in Fig. 3.8. Exposure to the pharmacological inhibitors of the anti-oxidative enzymes alone led to a marginal (10-20%) decrease in the viability of hepatocytes, in comparison to the control ($p<0.001$; supplementary data, Fig C3.S3). Although all the pharmacological inhibitors used in this study eliminated the protective effect of 20 μM selenite against the toxicity of 100 μM arsenite ($p < 0.05$), the effect of DETC (SOD inhibitor) was most pronounced since the cell viability decreased further than that in the arsenite only exposure (Fig. 3.8). In contrast, the protective effect of selenomethionine against the arsenite-induced loss of cell viability was eliminated by BSO, DETC and MS ($p < 0.05$), but was not influenced by ATA at all ($p > 0.05$; Fig. 3.8). The GPx inhibitor MS elicited the maximum response as the loss in cell viability was even greater than that observed with exposure to 100 μM arsenite alone. The pharmacological inhibitors also reduced the activities of their respective enzymes ($p<0.001$; supplementary data, Table C3.A1). The activity of CAT was reduced by 37 %, in comparison to the control, by 0.5 mM ATA ($p < 0.05$). Similarly, DETC, and MS also reduced the activities of

SOD and GPx by 47.3 % and 42.7 %, respectively, relative to the control ($p<0.001$; supplementary data, Table C3.A1).

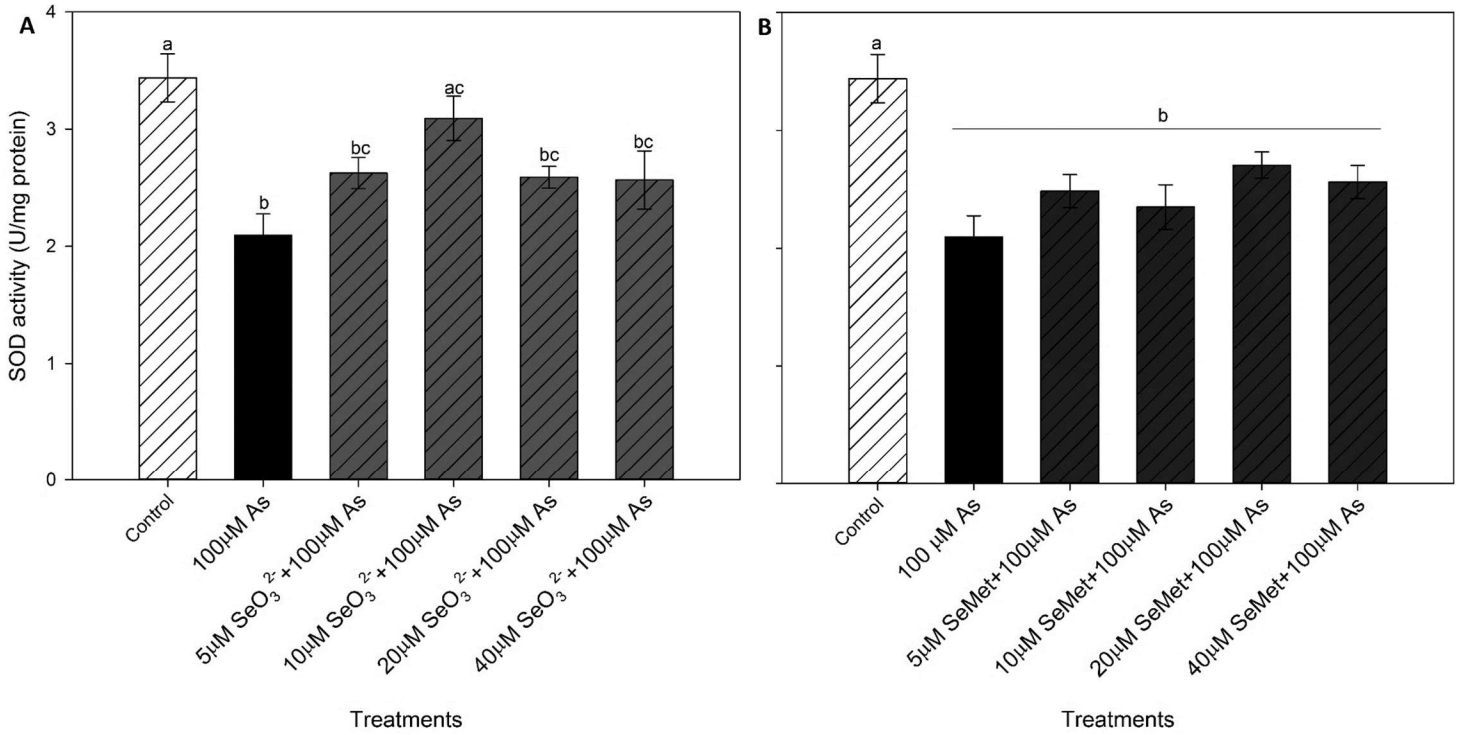


Figure 3.5: Changes in activities of superoxide dismutase (SOD) in rainbow trout hepatocytes upon exposure to 100µM arsenite (As), alone or in combination with different concentrations (5µM – 40µM) of **A.** Selenite (SeO_3^{2-}), or **B.** Selenomethionine (SeMet). Data are presented as mean ± S.E.M. ($n = 5$), where n represents the number of true independent measurements, each performed with cells isolated from a different fish. Different alphabetical notations on the bars indicate statistical differences between groups of means ($p < 0.05$) as determined by ANOVA and post-hoc test.

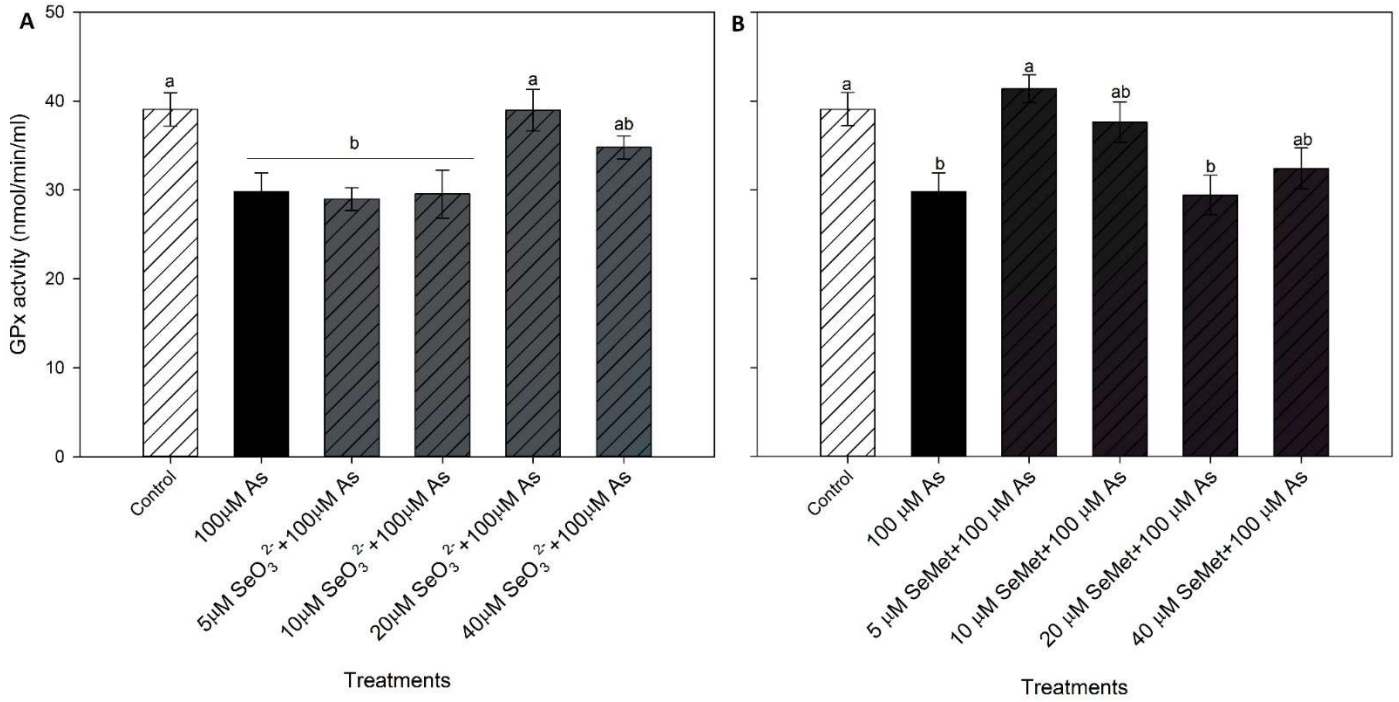


Figure 3.6: Changes in activities of glutathione peroxidase (GPx) in rainbow trout hepatocytes upon exposure to 100µM arsenite (As), alone or in combination with different concentrations (5µM – 40µM) of **A.** Selenite (SeO_3^{2-}), or **B.** Selenomethionine (SeMet). Data are presented as mean ± S.E.M. ($n = 5$), where n represents the number of true independent measurements, each performed with cells isolated from a different fish. Different alphabetical notations on the bars indicate statistical differences between groups of means ($p < 0.05$) as determined by ANOVA and post-hoc test.

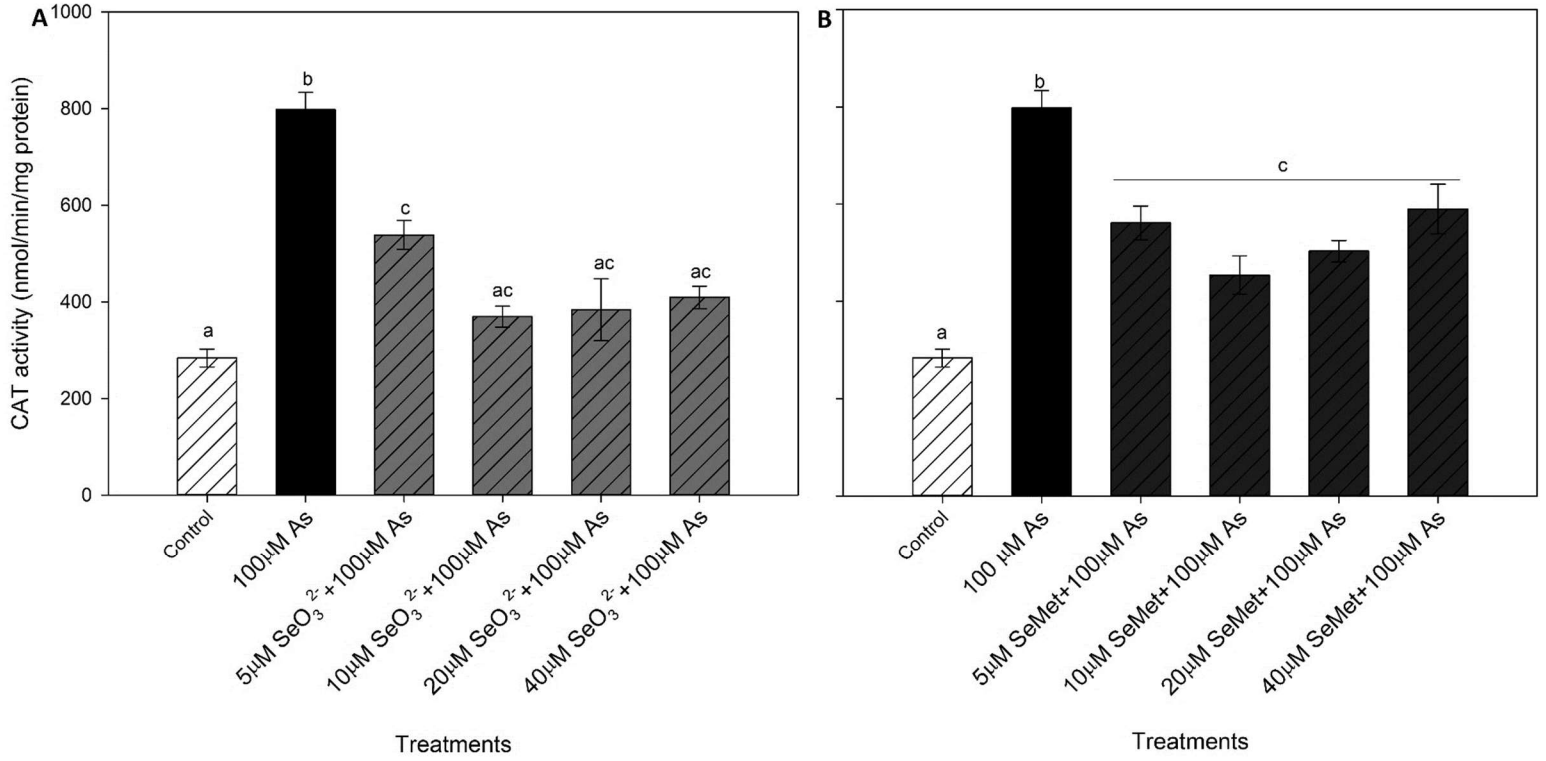


Figure 3.7: Changes in activities of catalase (CAT) in rainbow trout hepatocytes upon exposure to 100µM arsenite (As), alone or in combination with different concentrations (5µM – 40µM) of **A.** Selenite (SeO_3^{2-}), or **B.** Selenomethionine (SeMet). Data are presented as mean ± S.E.M. (n = 5), where n represents the number of true independent measurements, each performed with cells isolated from a different fish. Different alphabetical notations on the bars indicate statistical differences between groups of means ($p < 0.05$) as determined by ANOVA and post-hoc test.

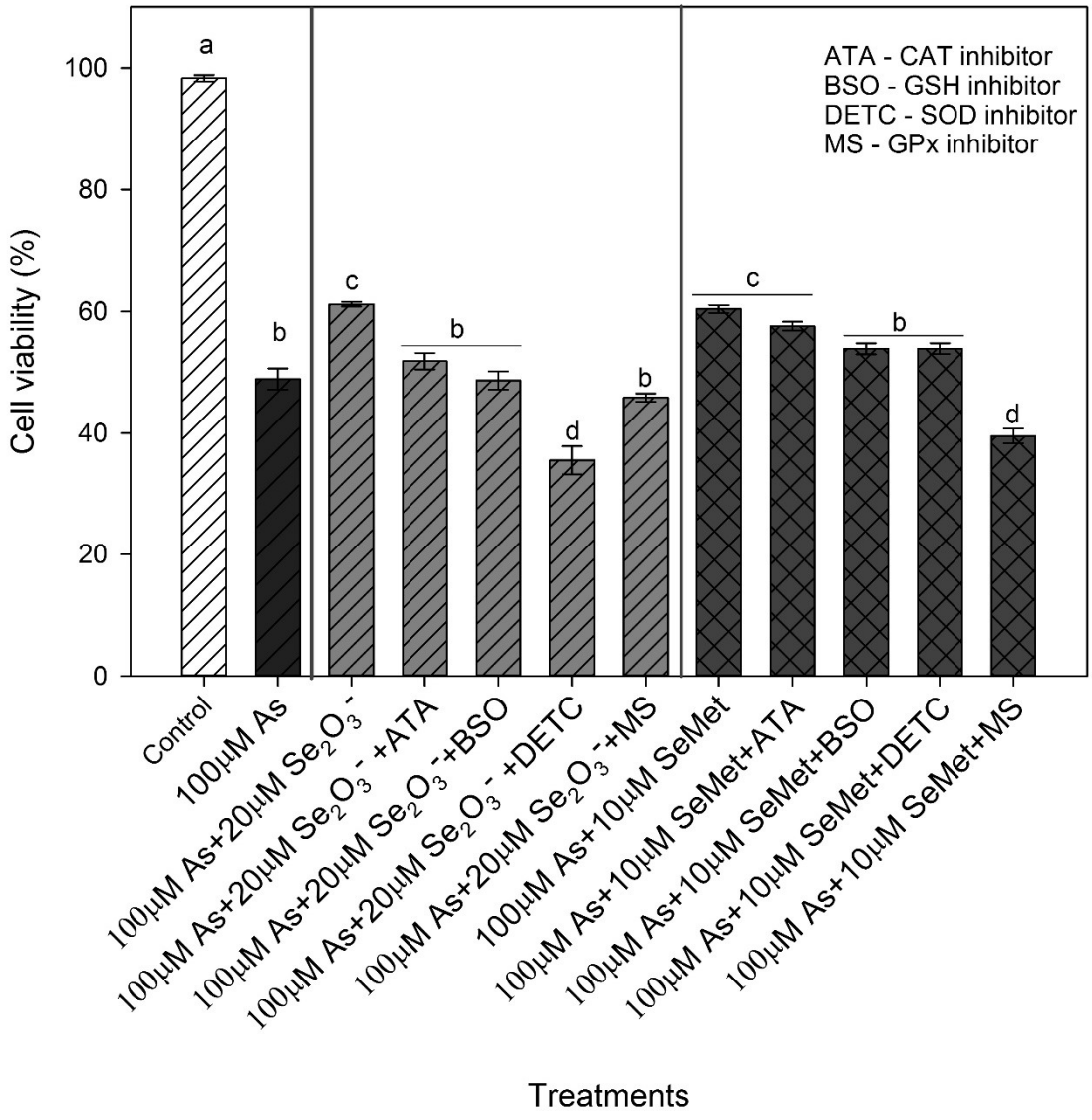


Figure 3.8: Changes in cell viability when rainbow trout hepatocytes were exposed to 100μM arsenite (As), alone or in combination with 20 μM selenite (SeO_3^{2-}) or 10 μM selenomethionine (SeMet), and inhibitors of anti-oxidative enzymes. Data are presented as mean ± S.E.M. (n = 5), where n represents the number of true independent measurements, each performed with cells isolated from a different fish. Different alphabetical notations on the bars indicate statistical differences between groups of means ($p < 0.05$) as determined by ANOVA and post-hoc test.

3.4. Discussion

To the best of our knowledge, the current study is first to examine how arsenite cytotoxicity is modulated not just by the dose but by the chemical speciation of selenium as well in a non-mammalian model experimental system. The main objective of our study was to unravel the cellular pathways involved in the ameliorative effects of selenium against arsenite-induced cytotoxicity using the primary cultures of rainbow trout hepatocytes as the experimental model. Both inorganic (selenite) and organic (selenomethionine) selenium were used at 5 – 40 μM concentrations to evaluate the dose and chemical species dependent effects of selenium. Our study revealed that both selenite and selenomethionine were able to ameliorate the toxicity of arsenite (100 μM) in trout hepatocytes. Interestingly, though, the protective effects of selenite and selenomethionine were observed only at specific exposure concentrations. For example, selenite at concentrations <10 μM and > 20 μM did not provide any protective effects against arsenite cytotoxicity, whereas selenomethionine did not elicit any ameliorative effects at concentrations over 10 μM. Similar to the observations in the current study, both selenite and selenomethionine were also found to ameliorate cadmium toxicity in rainbow trout hepatocytes in a dose-specific manner, with no protective effects at >25 μM Se concentrations (Jamwal et al., 2016). It is to be noted though that treatment with selenite and selenomethionine resulted in a partial recovery of arsenite-induced cell viability in the current study, whereas it produced a complete recovery of cell viability in trout hepatocytes exposed to 100 μM cadmium for 48 h. This suggests that arsenite may cause cytotoxicity not just by inducing oxidative stress, but other pathway(s) likely contributes to its toxicity as well.

Protective effects of selenium against arsenite cytotoxicity have also been reported previously in different *in vitro* mammalian experimental models. Antagonistic effects of selenium

on arsenite toxicity were demonstrated in human malignant skin and renal cells (Chitta et al., 2013; Wang and Guo, 2011). Similar observations were also reported in rat kidney cells where selenium ameliorated the toxic effects of arsenic (Berry and Galle, 1994). A couple of previous studies have also provided evidence of antagonism between selenium (only as selenite) and arsenite in piscine experimental models. Babich et al. (1989) examined selenium-arsenite interactions in fibroblastic cell line of a Bluegill (*Lepomis macrochirus*), where partial protective effects of selenite against arsenite cytotoxicity were observed over an exposure period of 24 h. Similar *in vitro* protective effects of selenite (1, and 5 μM) against 100 μM arsenite cytotoxcity were also reported in teleost hepatocellular carcinoma line 1 during an exposure period of 20 h (PLHC-1) (Selvaraj et al., 2012). However, the present study provides the first evidence of similar ameliorative effect of selenomethionine, which is the predominant form in which fish acquire selenium (Janz, 2012; Maher et al., 2010), against arsenite cytotoxicity in fish. In the present study, we demonstrated that selenite was more protective than selenomethionine against arsenite cytotoxicity in trout hepatocytes (Fig. 3.1 B and 3.1 C). Rossman and Udin (2004) also examined the protective effects of selenite and selenomethionine, at comparable exposure dose levels as used in the present study over an expoure period of 6 weeks, and found that selenite was more protective than selenomethionine against arsenite cytotoxicity in human osteosarcoma cells (Rossman and Uddin, 2004). Thus, our findings on the chemical species-specific effects of selenium against arsenite cytotoxicity in trout hepatocytes are in agreement with previous observations in mammalian cells. It is to be noted though that contradictory evidence also exists in the mammalian literature on the ameliorative effects of inorganic and organic selenium against arsenite toxicity. For example, Alp et al. (2011) reported that only selenomethionine, not selenite, reduced arsenite cytotoxicity in human kidney cells over an exposure period of 24 h, although the exposure doses of selenite and

selenomethionine (30 – 100 μM) were relatively higher than that used in the present study (5 – 40 μM).

Multiple studies, both *in vitro* and *in vivo*, indicate that arsenite can generate ROS by involving flavin enzymes such as NAD(P)H oxidase, and NO synthase isozymes (Flora, 2011; Kumagai and Sumi, 2007; Selvaraj et al., 2012). Metabolic intermediates of inorganic arsenic such as dimethylarsinic acid (DMA) can also react with molecular oxygen to generate superoxide ($O_2^{\bullet-}$) and peroxyl radicals that cause oxidative damage, including breaks in DNA strands, and ultimately lead to apoptosis (Yamanaka et al., 1990, 1989). In the present study, we have also demonstrated that exposure to 100 μM arsenite alone significantly increased intracellular ROS production (Fig. 3.2). However, co-exposure of trout hepatocytes to 100 μM arsenite and 20 μM selenite or 10 μM selenomethionine significantly reduced the intracellular ROS level (Fig. 3.2 A iii and v, and B), which also coincided with a significant recovery in the cell viability relative to that in the 100 μM arsenite only treatment (Fig 3.1 B and C). This observation indicated that selenium at low exposure levels ameliorates arsenite cytotoxicity likely by augmenting cellular ROS scavenging activity. To counter the effects of deleterious ROS, cells have evolved both enzymatic and non-enzymatic anti-oxidative machineries (Sies, 1997). One of the major components of the non-enzymatic machinery is the ROS scavenger, glutathione (GSH) (Sies, 1999, 1997). On the other hand, the enzymatic machinery involves three major anti-oxidative enzymes - superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) (Sies, 1997). Pharmacological or exogenous anti-oxidants, such as the ones used in this study, either mimic the activity of natural anti-oxidative agents in the cells or stimulate their activity. In our study, we observed that the treatment of hepatocytes with 100 μM TEMPO and NAC in conjunction with isomolar concentration of arsenite increased the cell viability relative to that in arsenite treatment alone (Fig. 3.3). Both, TEMPO and

NAC, are pharmacological anti-oxidants, but differ in their mechanisms of action. TEMPO mimics the activity of SOD enzyme and scavenges ROS (Samuni et al., 1990; Sandhir et al., 2015), whereas NAC facilitates GSH synthesis and therefore acts *via* non-enzymatic pathway (Ramen, 2015). Our results suggest that selenite and selenomethionine may ameliorate arsenite-induced oxidative stress by facilitating ROS scavenging, which could involve both enzymatic and non-enzymatic pathways. To further investigate the precise pathways involved in selenite and selenomethionine mediated amelioration of arsenite-toxicity, we analyzed the cellular GSH:GSSG ratio and the activity of anti-oxidative enzymes.

Reduced glutathione (GSH) is a redox buffer that can readily donate its electron to reduce ROS. During this process, GSH itself becomes oxidized and reacts with another oxidized glutathione to form glutathione disulphide (GSSG) (Sies, 1999). Since the formation of intracellular GSSG is coupled with the oxidation of GSH, a decrease in their ratio (GSH:GSSG) indicates the loss of cellular redox potential, and the onset of cellular oxidative stress (Jamwal et al., 2016; Zitka et al., 2012). Analysis of GSH:GSSG ratio in the present study revealed that the exposure of trout hepatocytes to 100 μM arsenite independently caused a sharp decline in the cellular redox potential (Fig. 3.4), further indicating arsenite-induced oxidative stress. These results are consistent with previous studies that demonstrated a reduction in GSH:GSSG ratio associated with the exposure to arsenite (Bhattacharya and Bhattacharya, 2007; Flora et al., 2009). Arsenic has a strong affinity to bind with the sulfhydryl groups (Shi et al., 2004). The functional capacity of GSH depends on the presence of sulfhydryl groups at its active sites, which can be inhibited due to the binding of arsenic to sulfhydryl groups (Miller et al., 2002). In addition, it has now been established that the metabolic reduction of inorganic arsenic occurs only in the presence of thiol compounds such as GSH (Watanabe and Hirano, 2013). Consequently, an exposure to sodium arsenite is expected to rapidly deplete the cellular pools of GSH, as it will be required for both the

mitigation of oxidative stress and metabolism of arsenic. It is therefore reasonable to argue that these factors likely contributed to the arsenite-induced decrease in cellular GSH:GSSG ratio observed in the present study.

Interestingly, we also observed that GSH:GSSG ratio recovered, at least partially, when arsenite-exposed hepatocytes were co-treated with low concentrations of selenite (5 and 10 μM) and selenomethionine (5 μM) (Fig. 3.4). Previous studies suggest that selenium helps in the maintenance of cellular thiol redox balance through a combination of 3 processes: (i) *de novo* synthesis of GSH, catalyzed by γ-glutamyl-cysteine ligase (Chung and Maines, 1981; Richie et al., 2011), (ii) reduction of GSSG to GSH by glutathione reductase (GR) enzyme (Chung and Maines, 1981), and/or (iii) alleviation of oxidative stress by other biochemical mechanisms so that GSH is spared. The alleviation of arsenite-induced reduction in cellular thiol redox potential at low concentrations of Se (selenite and selenomethionine) observed in the present study was likely mediated by a combination of these processes (Fig. 3.4 B). Although we did not estimate the activity of γ-glutamyl-cysteine ligase, an elevated GSH content in the hepatocytes, when treated only with 10 – 20 μM selenite or 5 – 40 μM SeMet, suggests an upregulation of *de novo* GSH synthesis (Fig. 3.4 A). No significant improvement in the cellular thiol redox potential was recorded when the cells were co-treated with 100 μM arsenite and >10 μM selenite, or >5 μM selenomethionine (Fig. 3.4 B). The metabolism of selenite, when present in concentrations excess of physiological requirements, occurs *via* cellular pathways that require GSH and generate $O_2^{\bullet-}$ as a metabolic by-product (Lin and Spallholz, 1993; Seko et al., 1989). Similarly, selenomethionine, when present in excess, is metabolized into methylselenol (a redox active compound), which subsequently utilizes GSH to undergo redox cycling and produce $O_2^{\bullet-}$ in the process (Misra et al., 2010; Okuno et al., 2001). Therefore, the lack of recovery of GSH:GSSG ratio in arsenite-exposed

cells co-treated with higher concentration of selenium observed in the present study (Fig. 3.4 B) occurred likely because of the cellular metabolism of excess selenite and selenomethionine, resulting in conversion of cellular GSH pool to GSSG.

In addition to the non-enzymatic antioxidant pathway (thiol ratio), we also evaluated the response of key enzymatic anti-oxidants in trout hepatocytes treated with arsenite and/or selenium (Fig. 3.5 – 3.7). An exposure to 100 μM arsenite alone inhibited the activities of SOD (Fig. 3.5) and GPx (Fig. 3.6) enzymes, whereas it upregulated the activity of CAT (Fig. 3.7). Arsenic-induced inhibition of SOD and GPx has been reported previously in mammalian systems (Ramanathan et al., 2002; Shila et al., 2005). The most common forms of SOD in eukaryotic cells require copper, zinc and manganese as co-factors to carry out their functions (Fukai and Ushio-Fukai, 2011). Arsenite has been known to induce alterations in redox-active metals associated with proteins and cause intoxication (Valko et al., 2015). It has also been demonstrated in human fibroblast cells that generation of excessive superoxide/lipid peroxide ions in response to high concentration of arsenite can inhibit SOD activity (Yang et al., 2007). Thus, it is possible that arsenite-induced inhibition of SOD activity observed in our study might have occurred *via* similar mechanisms. Furthermore, arsenite is known to be an inhibitor of selenoprotein synthesis (Ganyc et al., 2007; Rodríguez-sosa and García-montalvo, 2013), which could be the probable mechanism of arsenite-induced reduction in activity of GPx, which is a selenoprotein. Upregulation of the activity of anti-oxidative enzymes in response to oxidative stress is a compensatory cellular mechanism for maintaining redox homeostasis. The elevated activity of CAT observed in our study might have occurred as a compensatory response to arsenite-induced increase in cellular ROS production (Shi et al., 2004), especially when the activities of other two important enzymatic anti-oxidants (SOD and GPx) were downregulated.

We have also observed in this study that treatment with both selenite and SeMet ameliorated the arsenite-induced alterations in the activities of antioxidative enzymes, except for the SOD activity in cells treated with selenomethionine (Fig. 3.5 – 3.7). It has been demonstrated previously that selenomethionine can ameliorate arsenite toxicity by influencing the activities of enzymatic antioxidants (Zwolak and Zaporowska, 2012), however we have demonstrated for the first time that this effect is dependent on the chemical speciation of selenium. We demonstrated that only selenite was able to upregulate SOD activity, which was inhibited by arsenite exposure (Fig. 3.5). In addition, we also found that the inhibition of SOD activity with a pharmacological blocker, DETC, resulted in a complete elimination of the protective effect of selenite against arsenite cytotoxicity (Fig. 3.8). Inhibition of other enzymatic (CAT and GPx) as well as non-enzymatic (GSH) antioxidants also reduced the protective effect of selenite, although the magnitude of reduction was modest and not as pronounced as observed with the inhibition of SOD activity (Fig. 3.8). This indicated that selenite protects against the adverse effects of arsenite-induced production of ROS mainly by augmenting SOD activity. Interestingly, the cellular anti-oxidant response to selenite cytotoxicity also involves selective upregulation of SOD, whereas other anti-oxidants, such as CAT and GPx, are not upregulated at the same magnitude (Weekley et al., 2014). On the other hand, treatment with selenomethionine alleviated arsenite-induced decrease in the GPx activity, but not the SOD (Fig. 3.6). This suggested that unlike selenite, selenomethionine provides its antagonistic effects against arsenite cytotoxicity primarily by augmenting the activity of GPx. This was further substantiated by the observation that pharmacological blocking of GPx activity completely eliminated the protective effect of selenomethionine against arsenite cytotoxicity (Fig. 3.8). Furthermore, the pharmacological blocking of other enzymatic (SOD and CAT) as well as non-enzymatic (GSH) anti-oxidants either did not influence at all or marginally reduced the

protective effects of selenomethionine against arsenite cytotoxicity (Fig. 3.8). The anti-oxidant enzyme GPx ameliorates oxidative stress by catalyzing the reactions by which GSH neutralizes ROS (Arteel and Sies, 2001), and thus the upregulation of GPx activity is merely a facilitation of the non-enzymatic pathway for maintaining redox homeostasis.

It is evident from our findings that both selenite and selenomethionine elicited antagonistic effects against arsenite-induced oxidative stress by both enzymatic and non-enzymatic pathways, which ultimately lead to a decrease in the intracellular ROS accumulation (Fig. 3.2 A iii and v, and 3.2 B). Mammalian studies have previously reported that selenite at low exposure concentrations can augment the activity of SOD (Messarah et al., 2012; Zwolak and Zaporowska, 2012), which can be attributed to the upregulation of redox active selenoproteins which maintain the cellular redox status and may therefore prevent redox injury to anti-oxidative enzymes (Reich and Hondal, 2016). On the other hand, GPx activity is highly dependent on cellular thiol status, since GPx is extremely specific for GSH as its substrate (Messarah et al., 2012; Shila et al., 2005; Wendel, 1980). It is also to be noted here that selenomethionine has been suggested to have GPx-like activity (Arteel and Sies, 2001; Walter and Roy, 1971). In the present study, it was observed that co-treatment of 100 μM arsenite and 5 μM selenomethionine led to the recovery of arsenite-induced decrease in cellular thiol status, which also coincided with a concurrent increase in the GPx activity. This indicates that selenomethionine ameliorated arsenite-induced oxidative stress by improving cellular thiol status, which also subsequently contributed to the recovery of GPx activity.

3.5. Conclusion

The present study demonstrated that both, selenite and selenomethionine, at low exposure levels can ameliorate arsenite toxicity in rainbow trout hepatocytes. Exposure to arsenite (100 μM) was found to decrease the cell viability, which corresponded with an increased accumulation of

intracellular ROS, decreased thiol status, and reduced activity of enzymatic antioxidants (SOD and GPx). Co-exposure of hepatocytes to low-intermediate levels of selenomethionine (10 – 20 μM selenite and 10 μM selenomethionine) and 100 μM arsenite resulted in a significant (although partial) recovery of cell viability. Improvement in cell viability corresponded with decreased accumulation of intracellular ROS, indicating the antioxidative properties of selnomethionine. Interestingly however, our investigation into the cellular mechanisms underlying the antagonistic interactions of selenium and arsenite revealed that inorganic (selenite) and organic (selenomethionine) forms of selenium elicit their protective response through different anti-oxidative pathways. Selenite alleviates arsenite-induced oxidative stress essentially by upregulating the SOD activity, whereas selenomethionine provides similar protective response predominantly *via* non-enzymatic anti-oxidative pathway that involves GSH. The characterization of chemical species-specific responses of selenomethionine against arsenite toxicity is novel, and has important toxicological implications since selenium and arsenic often co-occur in contaminated aquatic ecosystems. Our study indicates that selenium can be an effective antidote to arsenic poisoning in fish and potentially in other organisms as well, but only at a specific exposure levels (low-moderate) as the protective effects were not evident at high exposure levels of selenium.

CHAPTER 4: Interactive effects of chronic exposure to dietary cadmium and selenomethionine on tissue-specific accumulation and toxicity of cadmium in rainbow trout (*Oncorhynchus mykiss*)

Preface

The aim of this chapter is to address the 3rd objective of my doctoral research work which is to understand the interactive effects of chronic exposure to dietary cadmium and selenomethionine on tissue-specific accumulation and toxicity of cadmium in rainbow trout (*Oncorhynchus mykiss*). The findings of this chapter complement the findings of Chapter 2 and further our understanding on the mechanistic basis of how selenomethionine influences the toxic effects of cadmium at the organismal level (under the influence of systemic interference).

4.1. Introduction

Cadmium (Cd) is a non-essential element and known to induce toxic effects in all biological forms at relatively low exposure levels (Benavides et al., 2005; Trevors et al., 1986). Although it is a rare and a naturally occurring element, the anthropogenic activities have dominated the cadmium biogeochemical cycle, which has elevated its concentrations in many aquatic ecosystems (Cullen and Maldonado, 2013). In natural waters, cadmium is highly persistent and can biomagnify along the food chain (Croteau et al.,. 2005). Thus, diet is an important source of Cd in aquatic organisms in addition to the water, and several studies have suggested that dietary Cd is more

bioaccumulative than waterborne Cd in fish (Guo et al., 2017; Harrison and Klaverkamp, 1989; Maunder et al., 2011; Ranaldi and Gagnon, 2009). Therefore, the carnivorous fish, such as rainbow trout (*Oncorhynchus mykiss*), that occupy higher trophic levels in the food chain can be more susceptible than the herbivorous fish to Cd toxicity through diet in metal contaminated aquatic habitats (Croteau et al., 2005).

The toxicity of chronic cadmium exposure in fish are often associated with the disruption of cellular redox balance due to the increased production and accumulation of reactive oxygen species (ROS) (Baldisserotto et al., 2005; Cuypers et al., 2010; Verbost et al., 1989; Wood et al., 2006). Cadmium has very low standard reduction potential and cannot generate ROS *via* direct redox reactions under normal physiological conditions (Cuypers et al., 2010). However, cadmium can increase the cytoplasmic concentration of free redox-reactive metals, such as iron, by replacing them in various metalloproteins. The free redox-reactive metals (e.g., iron) in cytoplasm can generate ROS *via* Fenton reaction (Cuypers et al., 2010). Cadmium can also cause leaks in mitochondrial membrane, and induce NADPH-oxidases that generate superoxide anions ($O_2^{\bullet -}$) (Cuypers et al., 2010; Onukwufor et al., 2017). In addition, cadmium can also induce oxidative stress by disrupting the activities of enzymatic antioxidants (e.g., catalase, superoxide dismutase) and/or depleting the non-enzymatic antioxidant pool (e.g., glutathione), which can ultimately lead to the accumulation of intracellular ROS (Bertin and Averbeck, 2006; Cuypers et al., 2010; Waisberg et al., 2003).

In natural waters that receive effluents from mining and smelting industries, cadmium often co-exists with other metals and metalloids (Barwick and Maher, 2003; Ponton et al., 2016; Yuan, 2017). These elements, depending on their concentration and chemical speciation, can interact with each other, and antagonize or exacerbate their toxicity in exposed organisms. Selenium (Se) is one

such element that has been reported to have an antagonistic relationship with cadmium (Messaoudi et al., 2010; Zwolak and Zaporowska, 2012; Jamwal et al., 2016). Selenium is essential for the synthesis of various selenoproteins that have vital adaptive and housekeeping functions, including the maintenance of cellular redox homeostasis (Reich and Hondal, 2016). On the other hand, selenium can also be extremely toxic to fish, when its concentration in the body exceeds the physiological threshold (Janz, 2012). Recent studies have indicated that exposure to elevated organic and inorganic forms of selenium causes toxicity in fish by inducing ROS production and oxidative stress (J.-H. Kim and Kang, 2015; Kupsco and Schlenk, 2014; Thomas and Janz, 2016). Although fish can accumulate selenium *via* both water and diet, the predominant source of selenium exposure to fish is the diet, especially in its organic form (selenomethionine) (Janz, 2012).

The ameliorative effects of both inorganic and organic forms of selenium against chronic cadmium toxicity in fish have been reported in a few previous studies (Banni et al., 2011; Talas et al., 2008; Xie et al., 2016). Talas et al. (2008) and Banni et al. (2011) suggested that selenite, *via* water and diet, respectively, alleviates oxidative stress in fish during chronic exposure to waterborne cadmium. More recently, Xie et al. (2016) also demonstrated that pre-exposure to selenium-supplemented diet (both in the form of selenite and selenomethionine) led to lower oxidative stress and whole body cadmium burden in fish during short-term exposure to waterborne cadmium. Although these studies provided useful insights into the interaction of cadmium and selenium in fish, their environmental implications are limited. This is mainly because all of these studies were carried out using physiologically optimum or non-toxic exposure levels of selenium, and also at waterborne cadmium concentrations (0.4 – 2.0 mg L^{-1}) that were well above the range of environmentally relevant cadmium exposure levels. To date, it is not yet understood how the interactions of selenium and cadmium are influenced by different selenium exposure levels,

particularly at levels that exceed the physiological threshold and may cause toxicity to fish by itself. Moreover, no previous studies have investigated how the interaction of cadmium and selenium modulates the accumulation pattern of both elements in critical body organs (e.g., liver, kidney, muscle) in fish. Interestingly, it has been reported in a recent field study that higher selenium levels in the liver were correlated with lower hepatic oxidative stress in fish naturally exposed to metals including cadmium (Ponton et al., 2016). This indicates that the physiological response in fish during chronic exposure to cadmium and selenium may be directly linked with the tissue-specific burden of each element, and thus worthy of further investigation.

The present study was designed to conduct an in-depth investigation of the interactive effects of dietary cadmium and selenium exposure in fish, and also to elucidate how these effects are influenced by selenium exposure levels. Juvenile rainbow trout were chronically exposed to an environmentally relevant dietary cadmium concentration, concurrently with different levels of dietary selenium (control (low), moderate and high; as selenomethionine). The specific objectives of this study were to examine: (i) how selenomethionine (SeMet) affects cadmium-induced alteration in morphophysiological indices (condition factor, hepato-somatic index) of fish, (ii) how cadmium-induced changes in hepatic anti-oxidative responses and oxidative stress markers are modulated by dietary SeMet exposure, and also (iii) how the interaction of cadmium and SeMet alters the tissue-specific burden of each element in fish. We hypothesized that SeMet would ameliorate cadmium toxicity by augmenting the cellular redox potential as well as by reducing cadmium accumulation in critically important tissues such as liver and kidney, but these protective effects would be evident only at the moderate selenium exposure level, with no protection or potential exacerbation of toxicity at the high selenium exposure level.

4.2. Materials and Methods

4.2.1. Chemicals

L(+)-Selenomethionine (>99% purity) was procured from Fisher Scientific, Canada. High purity Cadmium chloride (>99.99 % trace metal basis) was purchased from Sigma Aldrich, USA. Aquacalm^{TM} (Metomidate hydrochloride) was purchased from Syndel Laboratories Ltd., Canada. The reference standards and reagents for graphite-furnace atomic absorption spectroscopy were purchased from PerkinElmer, Canada. All other chemicals and reagents were procured from VWR, Canada, unless stated otherwise.

4.2.2. Experimental Fish

Rainbow trout juveniles weighing 30 – 35 g were used in the study. Fertilized eggs from adult rainbow trout females were hatched in the Aquatic Toxicology Research Facility (ATRF) at the University of Saskatchewan, Canada. Fish were raised on commercial dried pellet feed, fed at 2 % body weight (wet weight) ratio throughout the husbandry period. Fish were reared in dechlorinated, aerated water [hardness 159 mg L^{-1}, alkalinity 105 mg L^{-1} (both as $CaCO_3$), dissolved organic carbon (DOC) 2.1 mg L^{-1}, pH 7.5 – 7.8, Se 5.2 µg L^{-1}, Cd < 0.1 µg L^{-1}] in a flow-through system (1 L min^{-1}). The photoperiod was set at 14:10 h light to dark cycle and water temperature was maintained at 12 ºC.

4.2.3. Diet preparation

Brine shrimp (*Artemia franciscana*) containing 5.8 % crude protein, 0.6 % crude fat, 0.25 % crude fiber (all on a wet wt. basis) was used to prepare the experimental diets. First, the shrimps were rinsed several times with deionized water and then ground in a commercial blender with a known amount of selenomethionine and/or cadmium (as $CdCl_2$) solution, which was prepared with deionized water. The mixture was blended for about 10 min to ensure homogenous mixing.

Carboxymethylcellulose (2 %, in dry wt.) was also included as a feed binder during the blending process. The mix was frozen at −20 ºC and subsequently freeze-dried (Labconco Freezone, USA). The feed was cut into small pellets (~0.3 cm) and stored at -20 ºC until its use in the experiment. The control diet was prepared similarly without the addition of selenomethionine or $CdCl_2$. The concentrations of selenium and cadmium in each of the experimental diets were verified in an atomic absorption spectrometer, prior to their use in the experimental exposure (see below for details).

4.2.4. Experimental treatments and sampling

Using a fully factorial experimental design, fish were treated with six different dietary treatments (see Table 4.1). Fish in each treatment were fed once daily at 2 % of their body weight for 30 days. A total of 60 fish were divided equally into twelve 180 L tanks (5 fish in each tank, 2 replicates per treatment). The six different diets used in the present study were identified as: Control (no added cadmium or selenium), cadmium only (40 μg g^{-1} dry wt.), medium SeMet only (10 μg g^{-1} dry wt.), high SeMet only (40 μg g^{-1} dry wt.), cadmium + medium SeMet (40 and 10 μg g^{-1} dry wt., respectively), and cadmium + high SeMet (40 μg g^{-1} dry wt. for each). The concentrations of cadmium and selenium in the diets mentioned here are nominal concentrations, and the measured concentration of cadmium and selenium are presented in Table 4.1. Typically, fish in each treatment consumed the diet within 3 minutes of its presentation. Each tank received continuous aeration, and the water flow-through rate was maintained at 8 L min^{-1} to ensure rapid flushing of selenomethionine or cadmium, that could have potentially leached out of the diet. Nonetheless, water samples were collected from each treatment tank once a week 1 h post feeding. Fish were starved for 24 h before sampling on day 30^{th}, and 4 fish from each replicate treatment were randomly sampled to obtain a sample size (*n*) of 8. Fish were euthanized with an overdose of

Aquacalm^{TM}. Length and weight of each fish were recorded. Subsequently, the muscle, liver, and kidney were dissected out, weighed to the nearest 0.01 mg, and then frozen immediately in liquid nitrogen and stored at -80 ºC until their analysis.

4.2.5. Measurement of hepatic thiol content

The hepatic concentration of reduced (GSH) and oxidized (GSSG) glutathione were measured as an estimate of cellular redox balance using a fluorometric method (Hissin and Hilf, 1976), modified to a 96-microwell plate based assay as described in Jamwal and Niyogi (2017). The liver samples were homogenized in HEPES-EGTA sample buffer, with 1:5 sample to buffer ratio (w/v). A 20 µl homogenate was taken for the estimation of protein. The remaining homogenate was immediately deproteinized with 100 µl of 5 % TCA and centrifuged at 12,000 x g at 4 ºC for 25 min. The supernatant was collected and split into two fractions of 500 µl each. One fraction was immediately stored in -80 ºC for the estimation of GSH. The second fraction was used for the estimation of GSSG, and mixed immediately with a 20 µl aliquot of 0.04 M of *N*-ethylmaleimide to prevent the spontaneous oxidation of GSH to GSSG. For the estimation of GSH, a 200 µl final reaction mixture was prepared, which contained 180 µl of phosphate-EDTA buffer (0.1 M sodium phosphate, 0.005 M EDTA, pH 8.0), 10 µl of *o*-phthalaldehyde (OPT, 100 µg per 100 µl methanol), and 10 µl of sample. The reaction mixture was incubated for 15 min and read fluorometrically at excitation and emission wavelengths of 350 nm and 450 nm, respectively, in a multimode microplate reader (Varioskan Flash, thermos Fisher Scientific, Finland). The concentration of GSSG was measured in the same way except that the final reaction mixture volume (200 µl) contained 140 µl of 0.1 N NaOH, 20 µl of *o*-phthalaldehyde (OPT, 100 µg per 100 µl methanol) and 40 µl of sample. The GSH and GSSG content was expressed as µg per mg of protein. Finally, the GSH : GSSG ratio was obtained by dividing GSH concentration with its

corresponding GSSG content. Bradford method, modified for 96-well microplate based assay, was used to determine the protein content in the samples (Bradford, 1976).

4.2.6. Measurement of hepatic antioxidant enzyme activities

Frozen liver samples were thawed and then homogenized in 5 volumes (w/v) of chilled HEPES-EGTA sample buffer (1 mM EGTA, 210 mM mannitol, 70 mM sucrose, and 20 mM HEPES, pH 7.2) using a hand-held homogenizer. The homogenate was centrifuged at 25,000 x g for 20 min at 4 ^{o}C and the supernatant was collected. A 50 µl of the supernatant was used to estimate the protein content, as described previously. The activities of three antioxidant enzymes, catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GPx), were measured using commercially available SOD (Catalogue #706002), CAT (Catalogue #707002), and GPx (Catalogue #703102) activity kits, following the manufacturer's instructions (Cayman chemical company, USA). The activities of CAT, SOD, and GPx were expressed as nmol/ mg protein, U mg protein^{-1}, and nmol min^{-1} mg protein^{-1}, respectively. One unit of SOD was defined as the amount of enzyme needed to achieve the dismutation of 50 % superoxide anions.

4.2.7. Estimation of hepatic lipid peroxidation (LPO)

A 96 micro-well plate based thiobarbituric acid reactive substance (TBARS) assay kit (Catalogue # 10009055; Cayman chemical company, USA) was used for the estimation of lipid peroxidation (LPO) in the livers. For the estimation of LPO, liver samples were homogenized in 1:10 volume (w/v) of chilled TBARS-kit buffer. The homogenate was centrifuged at 1,600 x g for 10 min at 4 ^{o}C to collect the supernatant. A 20 µl of the supernatant was taken to estimate the protein content, as mentioned before. The hepatic LPO was measured as per the instruction manual of the kit at excitation and emission wavelengths of 530 nm and 550 nm, respectively, in a multimode microplate reader. The LPO was expressed as nmol MDA mg tissue protein^{-1}.

4.2.8. Measurement of selenium and cadmium in water, diet and fish tissue

Experimental water samples were acidified (1% HNO_3) and analyzed for selenium and cadmium concentrations using a graphite-furnace atomic absorption spectrometer (Analyst 800, PerkinElmer, USA). The experimental diets and fish tissues were digested in 5 volumes of 2 N HNO_3 at 60 ºC for 48 h and then centrifuged at 1,000 x g for 20 min. The supernatant was collected and diluted appropriately in 0.1 % HNO_3 prior to their analysis. Appropriate method blanks and certified standards for both cadmium and selenium were used to maintain the quality control and quality assurance of total selenium and cadmium analysis (PerkinElmer, USA). A certified reference material (DOLT-3; National Research Council, Canada) was also analyzed similarly to validate the efficiency of the applied method, and the recovery of Cd and Se was found to be 105 % and 93 %, respectively.

4.2.9. Calculations and statistical analysis

Hepato-somatic index [HSI = weight of liver (g) weight of the fish (g) $^{-1}$] and condition factor [K = 100 x weight of the fish (g) length (cm)$^{-3}$] were calculated for individual fish. All statistical analysis and the graphs were plotted using SigmaPlot (version 12, Systat Software, Inc., USA). The data are presented as mean ± standard error of mean (S.E.M.). Interactive effects of selenomethionine and cadmium were analyzed by a two-way analysis of variance (2-WAY ANOVA). Pairwise multiple comparisons (*post hoc*) with Holm-Sidak correction was performed to determine the effect of different treatments. The assumptions of ANOVA (normality of distribution and heteroscedasticity) were verified using Shapiro-Wilk and Brown-Forsythe test, respectively. Data were subjected to logarithmic transformation when the assumption of normality was not met. A p-value of ≤ 0.05 was considered to be significant while comparing different treatments.

Table 4.1. Measured selenium and cadmium concentrations in different diets (µg g^{-1} feed dry weight).

Treatments	Se (µg g^{-1})	Cd (µg/g^{-1})
Control	1.2 ± 0.7^{a}	0.8 ± 0.2^{A}
Cadmium only	1.3 ± 0.9^{a}	42.4 ± 1.7^{B}
Medium SeMet	12.2 ± 1.9^{b}	1.2 ± 0.7^{A}
High SeMet	46.1 ± 3.1^{c}	1.6 ± 0.5^{A}
Cadmium + Med SeMet	13.7 ± 1.2^{b}	47.5 ± 1.7^{B}
Cadmium + High SeMet	47.8 ± 2.1^{c}	51.2 ± 1.3^{B}

Values are mean ± SEM, $n = 3$ for the selenium and cadmium in the diets. Values with different alphabetic superscripts represent statistical difference in the concentration of a particular element between different treatments ($p < 0.001$). Similar superscripts represent no statistical difference ($p > 0.05$), as determined by 1-WAY ANOVA and *post hoc* analysis.

4.3. Results

4.3.1. Fish morphometrics [*Hepato-somatic index (HSI) and condition factor (K-factor)*]

No mortality was recorded in any treatment over 30 days of experimental exposure. The interaction between dietary cadmium and selenomethionine significantly influenced the HSI ($F_{2,37}$ = 4.2; p = 0.023; Table 4.2). There was a significant reduction (~30 %) of HSI in fish treated with cadmium only diet, relative to the control group ($p < 0.001$), whereas HSI in fish treated with medium SeMet only diet was ~36 % higher than the control group ($p < 0.001$). Fish treated with high SeMet diet, alone or in combination with cadmium, also exhibited a significantly lower HSI values, which were similar to that observed in fish treated with cadmium only diet ($p > 0.05$; Table 4.2). Interestingly, however, fish fed with cadmium + medium SeMet diet did not show any significant difference in HSI value relative to the control fish (p = 0.80; Table 4.2). No statistically significant interaction between cadmium and selenomethionine was recorded for *K-factor* ($F_{2,37}$ = 0.67; p = 0.52; Table 4.2), and a significant decrease, relative to the control, was observed only in fish treated cadmium only diet (Table 4.2).

Table 4.2. Physiological indicators of health of juvenile rainbow trout exposed to different treatments.

Treatments	HSI	Condition factor (*K*)
Control	0.022 ± 0.0008^{a}	1.29 ± 0.041^{A}
Cadmium only	0.014 ± 0.0002^{b}	1.10 ± 0.025^{B}
Medium SeMet	0.029 ± 0.0005^{c}	1.35 ± 0.030^{C}
High SeMet	0.016 ± 0.0002^{b}	1.29 ± 0.028^{A}
Cadmium + Med SeMet	0.022 ± 0.0004^{a}	1.33 ± 0.031^{A}
Cadmium + High SeMet	0.015 ± 0.0004^{b}	1.24 ± 0.03^{A}

Values are mean ± SEM, n = 5 - 8. HSI = hepatosomatic index. Values with different alphabetic superscripts represent statistical difference among the treatments for a particular parameter ($p < 0.001$). Similar superscripts represent no statistical difference ($p > 0.05$), as determined by 2-WAY ANOVA and *post hoc* analysis.

4.3.2. Hepatic thiol redox balance

Two-Way ANOVA did not reveal any significant interactive effect of cadmium and selenomethionine on GSH, GSSG, or the thiol ratio (GSH:GSSG). However, there was a significant simple-main effect of the treatments on the thiol redox balance (Fig. 4.1). Exposure to cadmium only diet led to a significant decrease and increase (both ~50%) in the hepatic GSH and GSSG contents, respectively, which ultimately resulted in a markedly significant reduction in the hepatic GSH:GSSG ratio relative to the control ($p < 0.001$; Fig. 4.1). The concentrations of GSH, GSSG, and the thiol ratio were not significantly different among fish treated with SeMet only diets (medium and high) as well as cadmium + medium SeMet diet, relative to the control (Fig. 4.1). However, the hepatic GSH level and GSH:GSSG ratio were significantly reduced (26 % and 60 %, respectively) in fish treated with cadmium + high SeMet diet in comparison to the control ($p < 0.001$; Fig. 4.1). The magnitude of reduction in hepatic GSH content and thiol ratio in fish treated with cadmium + high SeMet diet was similar to that observed in fish treated with cadmium only diet (Fig. 4.1).

4.3.3. Hepatic antioxidative enzyme activities

The effects of different dietary treatments on the activities of hepatic antioxidative enzymes (CAT, SOD, and GPx) are illustrated in Fig. 4.2. Dietary exposure to selenomethionine and cadmium had a significant interactive effect on CAT activity ($F_{2,33} = 9.2$; $p < 0.001$). Dietary exposure to cadmium, alone and in combination with high SeMet, resulted in a significant increase (~40% in both treatments) in CAT activity relative to the control ($p < 0.001$; Fig. 4.2 A). In contrast, no significant change in CAT activity was recorded following treatments with medium and high SeMet only diets as well as cadmium + medium SeMet diet ($p > 0.05$; Fig. 4.2 A).

Similar to CAT, the hepatic SOD activity was also significantly affected by the interaction of cadmium and selenomethionine ($F_{2,39} = 8.1$; $p < 0.001$). Exposure to dietary cadmium alone resulted in a significant increase (2.5 fold) in SOD activity relative to the control ($p < 0.001$), however no change in SOD activity was recorded following treatment with cadmium + medium SeMet diet (Fig 4.2 B). The SOD activity in SeMet only (medium and high) and cadmium + high SeMet dietary treatments was also somewhat elevated, and not significantly different relative to that observed in the cadmium only dietary treatment.

No significant interaction between dietary cadmium and selenomethionine was found for hepatic GPx activity ($F_{2,24} = 2.6$; $p = 0.1$). Exposure to dietary cadmium, alone and in combination with medium or high SeMet, resulted in significant increases (35 – 70%) in GPx activity relative to the control ($p < 0.001$), however, the magnitude of increase was more modest (~35%) and significantly lower in the cadmium + medium SeMet treatment relative to the other two cadmium treatments (Fig. 4.2 C). The GPx activity in both medium and high SeMet alone dietary treatments was similar to that in control ($p = 0.008$; Fig. 4.2 C).

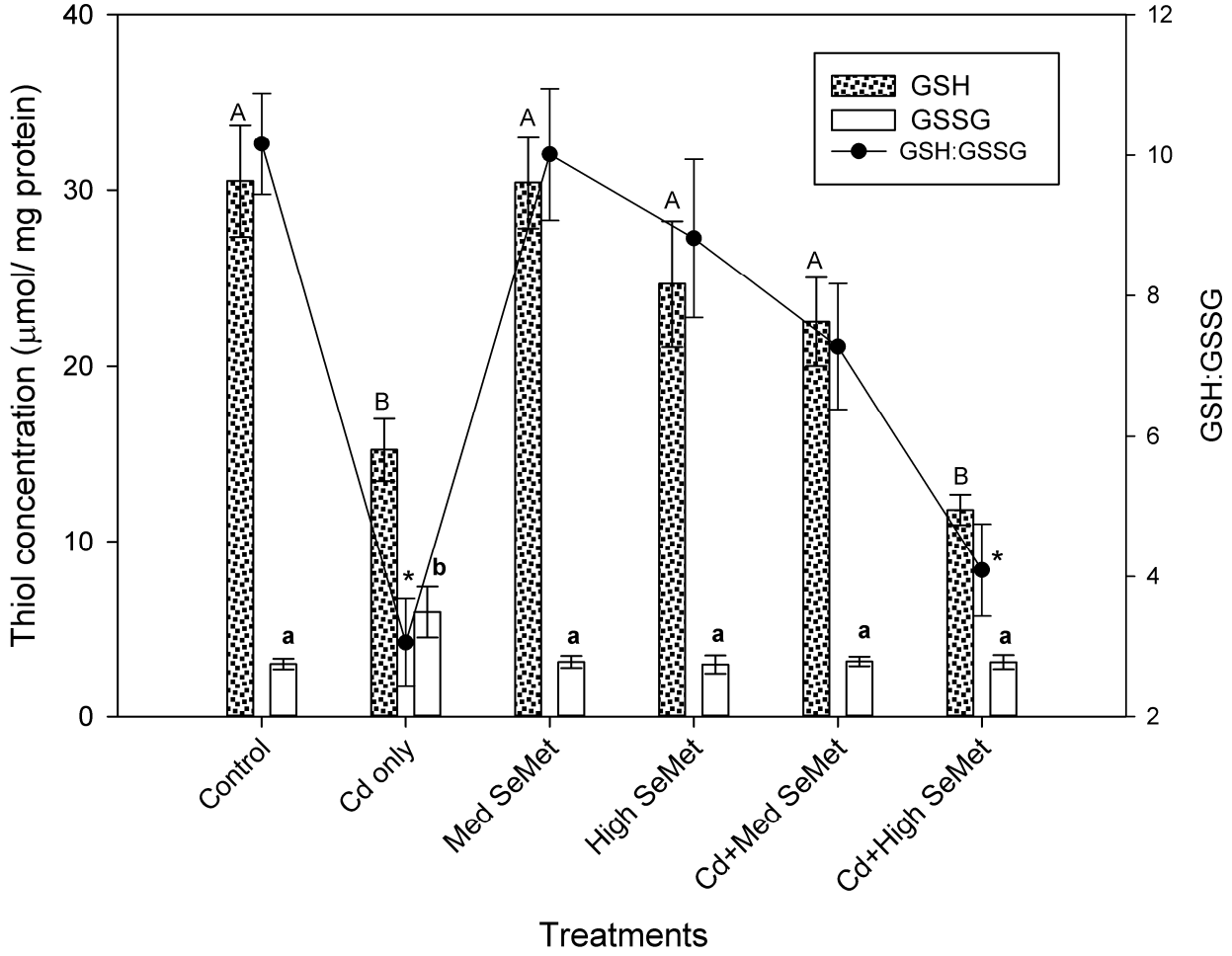


Figure 4.1: Concentration of reduced (GSH) and oxidized (GSSG) glutathione, and their ratio (GSH:GSSG) in the livers of rainbow trout exposed to various treatments. Data are presented as mean ± SEM (n = 6 - 8). For GSH and GSSG the statistical significance is denoted with capital letters, and small letters respectively. The bars with different letters are statistically different ($p < 0.05$). For GSH:GSSG, an asterisk (*) represents statistically significant difference in comparison to the control group and the data-points without an asterisk are statistically similar to each other ($p < 0.05$).

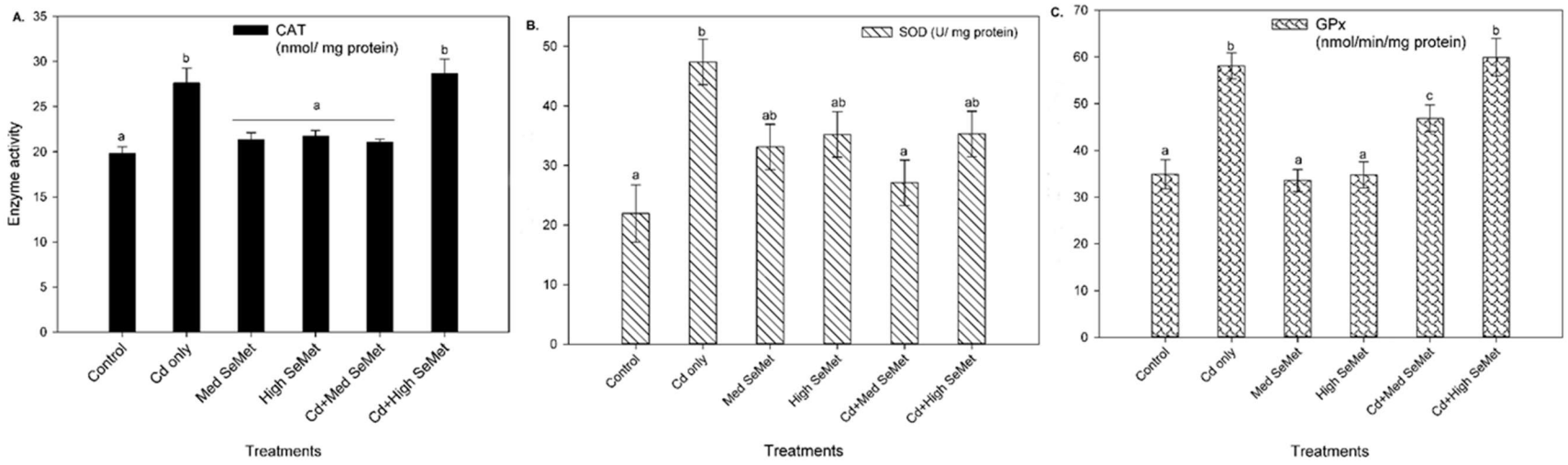


Figure 4.2: Changes in the activities of (A.) catalase (CAT), (B.) superoxide dismutase (SOD), and (C.) glutathione peroxidase (GPx) upon exposure to various treatments. Data are presented as mean ± SEM (n = 5 – 8). In each figure panel (A, B, or C), experimental treatments with different alphabetical notations are significantly different ($p < 0.005$) from each other, while the treatments with common alphabetical notations have no significant difference among them, as determined by 2-WAY ANOVA and *post hoc* analysis.

4.3.4. Hepatic lipid peroxidation (LPO)

There was a significant interaction between dietary cadmium and selenomethionine on the hepatic lipid peroxidation ($F_{2,29} = 4.3$; $p = 0.02$). Hepatic MDA level increased by more than 2-folds following treatment with dietary cadmium, alone and in combination with high SeMet, relative to the control ($p < 0.001$ and $p < 0.001$, respectively; Fig. 4.3). However, no change in hepatic MDA level was observed between the control and cadmium + medium SeMet treatments. Similarly, the hepatic MDA level in both medium and high SeMet only treatments was not significantly different relative to the control (Fig. 4.3).

4.3.5. Tissue specific selenium and cadmium accumulation

A significant interaction between cadmium and selenomethionine was recorded for the accumulation of cadmium in liver ($F_{2,40} = 3.44$; $p = 0.042$), kidney ($F_{2,37} = 13.4$; $p < 0.001$), and muscle ($F_{2,35} = 8.2$; $p = 0.001$). The cadmium burden in all of these tissues in fish fed with SeMet only diets (medium and high) was similar to that in control ($p > 0.005$; Fig. 4.4). Treatment of fish with cadmium only diet resulted in significantly increased cadmium accumulation in all of the tissues. The hepatic cadmium concentration increased by ~50-fold in fish exposed to dietary cadmium alone, relative to the control ($p < 0.001$; Fig. 4.4 A). A marked increase in cadmium burden in the kidney (~115-fold) and muscle (~160-fold) was also observed following treatment with cadmium only diet ($p <0.001$; Fig 4.4 B and C). Interestingly, however, the supplementation of dietary cadmium with medium and high levels of SeMet significantly reduced cadmium accumulation in the liver and kidney ($p < 0.05$), although cadmium levels in both tissues remained significantly higher than the control (Fig 4.4 A and B). The ameliorative effect of selenomethionine on cadmium accumulation was more pronounced in the kidney than the liver, as the magnitude of decrease in cadmium accumulation was much greater (1.4 – 2.7 fold) in the kidney relative to that

in the liver (1.2 – 1.6 fold). In contrast, supplementing dietary cadmium with either medium or high level of SeMet did not reduce the cadmium burden in the muscle. No significant change in muscle cadmium accumulation was observed between cadmium only and cadmium + medium SeMet dietary treatments ($p = 0.09$), whereas a significant increase in muscle cadmium accumulation was recorded following treatment with cadmium + high SeMet diet, in comparison to the cadmium only dietary treatment ($p < 0.001$; Fig 4.4 C).

Accumulation of selenium in the liver, kidney and muscle was also significantly affected by the interaction between cadmium and SeMet ($F_{2,38} = 11.6$; $p < 0.001$, $F_{2,38} = 8.9$; $p < 0.001$; $F_{2,39} = 33.2$; $p < 0.001$, respectively). Selenium accumulation in both liver and kidney increased significantly following treatment with cadmium only diet (not supplemented with selenomethionine) relative to the control, whereas a significant decrease of selenium accumulation was observed in the muscle ($p < 0.001$; Fig 4.5). There was a significant dose dependent increase in Se accumulation in liver, kidney and muscle following treatment with medium and high SeMet only diets, in comparison to the control ($p < 0.001$; Fig. 4.5). The presence of cadmium in the diet supplemented with medium level SeMet did not significantly alter the selenium concentration in any of tissues examined relative to that in the medium SeMet only treatment. However, a significant decrease in selenium accumulation was observed in all of the tissues following treatment with cadmium + high SeMet diet in comparison to high SeMet only diet. The magnitude of this effect was more pronounced in the muscle (a 2-fold reduction; $p < 0.001$; Fig. 4.5 C), than in the liver ($p = 0.002$; Fig. 4.5 A) and kidney ($p < 0.001$; Fig. 4.5 B), where the decrease in selenium accumulation was modest (1.3 – 1.5 fold).

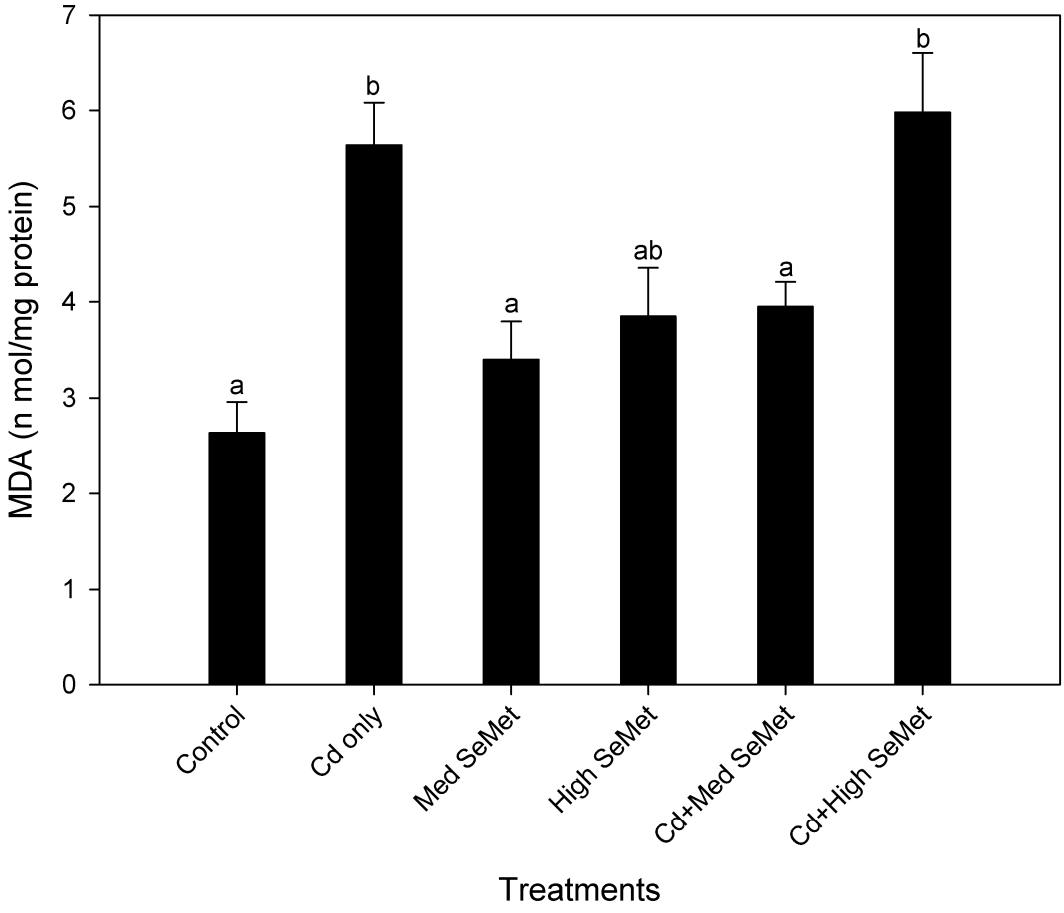


Figure 4.3: Concentration of hepatic malondialdehyde (MDA) measured as a biomarker of lipid peroxidation (LPO) due to different treatments to which the rainbow trout were exposed. Data are presented as mean ± SEM (n = 6 – 8). The experimental treatments with different alphabetical notations are significantly different ($p < 0.005$) from each other, while the treatments with common alphabetical notations have no significant difference among them, as determined by 2-WAY ANOVA and *post hoc* analysis.

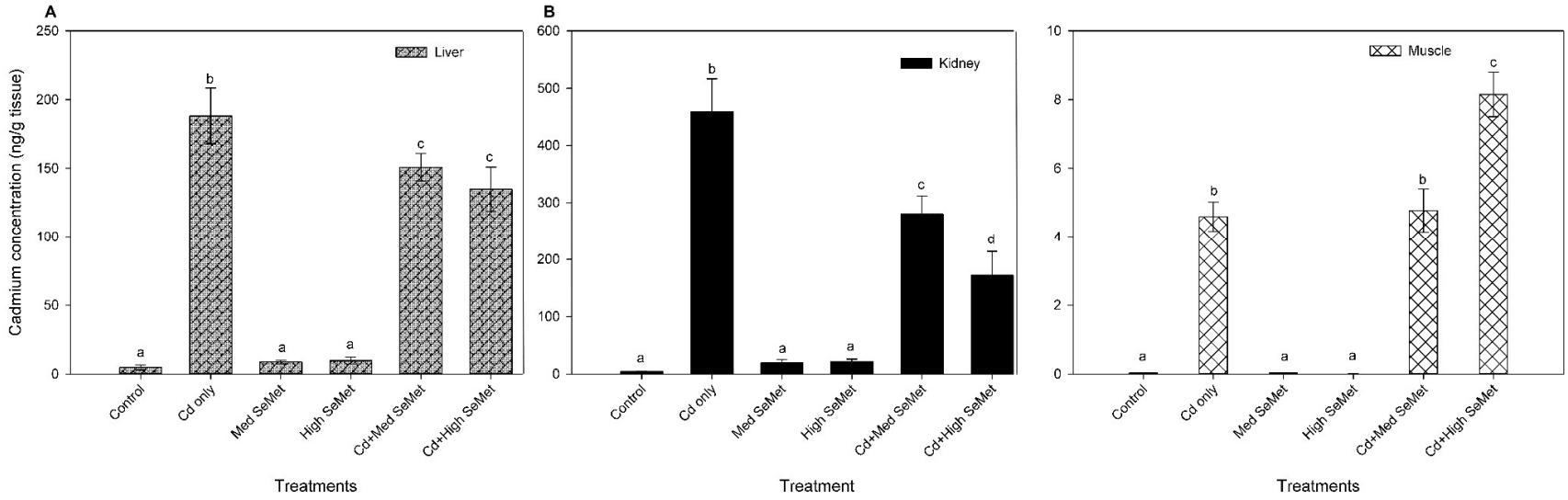


Figure 4.4: Concentration of Cd in liver (A.) kidney (B.), and muscle (C) in rainbow trout exposed to various treatments. Values are mean ± SEM (n = 6-8). In each figure panel (A, B, or C), experimental treatments with different alphabetical notations are significantly different ($p < 0.005$) from each other, while the treatments with common alphabetical notations have no significant difference among them, as determined by 2-WAY ANOVA and *post hoc* analysis.

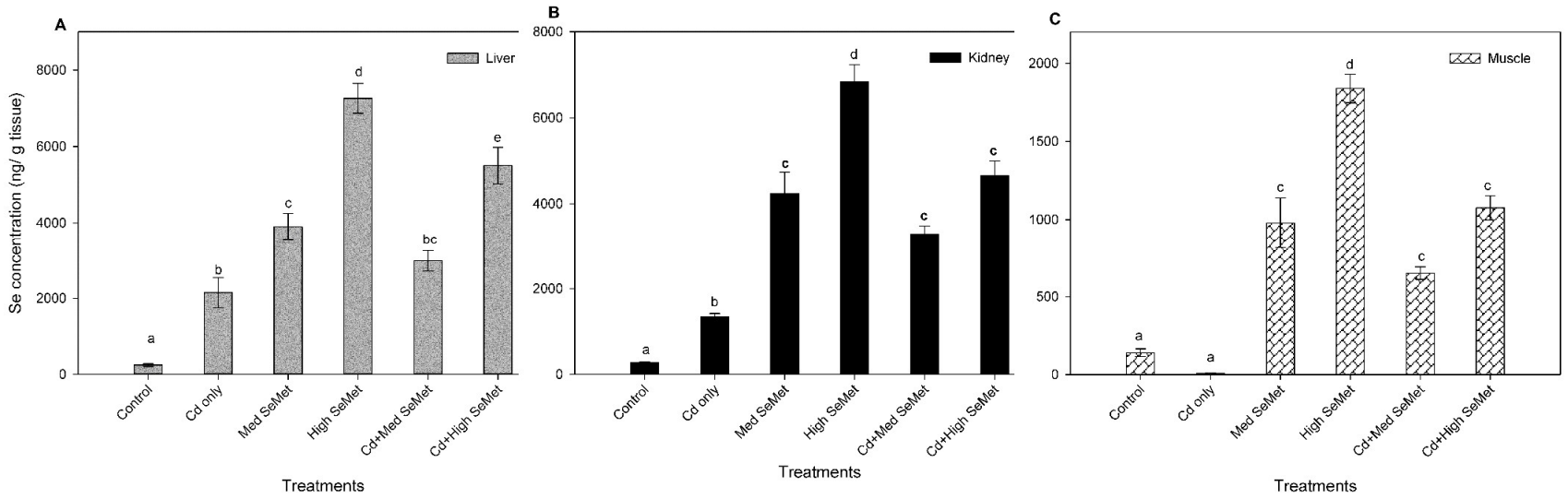


Figure 4.5: Concentration of Se in liver (A.) kidney (B.), and muscle (C) in rainbow trout exposed to various treatments. Values are mean ± SEM (n = 6-8). In each figure panel (A, B, or C), experimental treatments with different alphabetical notations are significantly different ($p < 0.005$) from each other, while the treatments with common alphabetical notations have no significant difference among them, as determined by 2-WAY ANOVA and *post hoc* analysis.

4.4. Discussion

To the best of our knowledge, the current study is the first to examine the interactive effects of chronic exposure to cadmium and SeMet *via* diet in fish. We used exposure concentrations of cadmium and SeMet that fish might experience in the metal-contaminated natural environments, and thus are environmentally relevant. The measured concentration of cadmium in cadmium-supplemented diets used in the present study varied between 42 – 48 μg g^{-1} dry wt. (Table 4.1), which was comparable to the cadmium levels reported in fish prey species collected from the metal-contaminated natural waters (Farag et al., 1999). On the other hand, the selenium concentration (1.2 ± 0.7 μg g^{-1}) recorded in the control diet was within the range of physiologically optimum dietary selenium requirement (0.1 – 4.7 μg g^{-1} dry wt.) in rainbow trout (Hodson and Hilton, 1983). The measured selenium concentrations in diets supplemented with medium and high levels of SeMet used in our study were in the range of 12 – 14 and 46 – 48 μg g^{-1} dry wt., respectively (Table 4.1). Similar range of selenium level has also been reported in the aquatic food chain in several Se-impacted North American sites (Hamilton, 2004).

The HSI and *K*-factor are the most common indirect measures for estimating the energetic status in fish (Chellappa et al., 1995). Fish, like other vertebrates, under normal physiological condition convert the excess dietary energy into glycogen and fat, which are then stored mainly in the liver and adipose tissues (Enes et al., 2009). However, under chronic stress such as exposure to elevated levels of metals, the energy requirement for maintaining the homeostatic processes (e.g., synthesis of detoxifying proteins, and antioxidative enzymes) may exceed the energy assimilated from the diet. As a consequence, the hepatic glycogen reserves are used to compensate the energy deficiency, which reduces the overall mass of the liver. The reduced liver mass, in proportion to the body mass of the fish, is reflected by a decrease in the HSI. In addition, the increased energetic

cost of homeostasis may also reduce the fat reserves, muscular development and growth of fish, which can result in a decreased *K*-factor. Our observations in the present study suggest that chronic exposure to dietary cadmium alone was energetically costly to the fish, as indicated by the reduced HSI and *K*-factor (Table 4.2). Interestingly, however, both HSI and *K*-factor values recovered back to the control levels when fish were treated with dietary cadmium supplemented with medium, but not high, level of SeMet. It is important to note here that we recorded a decrease in HSI when fish were treated with the diet supplemented exclusively with high, not medium, SeMet level, indicating that the high SeMet only diet used in our study increased the metabolic cost as well, and thus was somewhat toxic to the fish.

Cadmium is known to elicit its toxic effects by disrupting the cellular redox homeostasis, mainly *via* the depletion of glutathione (GSH) (Rani et al., 2014; Sandbichler and Höckner, 2016; Wang et al., 2008). In the present study, we examined the biochemical effects of interaction between cadmium and SeMet in the liver, which is the primary site of their metabolism (Nordberg, 1984). In addition, the deleterious effects of dietary cadmium exposure are usually observed first in the hepatic tissue (Godt et al., 2006). Analysis of the reduced and oxidized thiol content in the liver revealed that exposure to elevated dietary cadmium alone markedly reduced the GSH:GSSG ratio (Fig. 4.1). This is consistent with our previous findings in the primary cultures of rainbow trout hepatocytes, where the acute exposure to a toxic cadmium concentration was found to sharply decrease the thiol ratio (Jamwal et al., 2016). Cadmium-induced depletion of GSH and an associated increase in GSSG content has also been reported previously in several *in vivo* studies in fish (Jin et al., 2015; Karaytug et al., 2014; Pereira et al., 2016). The tripeptide GSH acts as a non-enzymatic redox buffer and the first line of defense against cadmium intoxication (Sandbichler and Höckner, 2016; Singhal et al., 1987). It prevents cadmium toxicity by scavenging the free cationic

Cd^{+2}, which is the most toxic form of cadmium (Waisberg et al., 2003). In addition, GSH also mitigates cadmium-induced oxidative stress by transferring its electron to ROS, thereby neutralizing it (Wu et al., 2004). During this process, the reduced form of glutathione (GSH) is oxidized to GSSG, and the overall thiol redox potential (GSH:GSSG) decreases, compromising the cellular redox homeostasis.

The cellular pool of GSH can be maintained by a combination of 3 different processes (Chung and Maines, 1981; Jamwal and Niyogi, 2017; Richie et al., 2011): (i) *de novo* synthesis of GSH, catalyzed by γ-glutamyl-cysteine ligase, (ii) recycling of GSSG to GSH by glutathione reductase, and/or (iii) alleviation of oxidative stress by other biochemical mechanisms so that GSH is spared. Under chronic cadmium-exposure, the GSH:GSSG ratio can decrease if the rate of GSH oxidation exceeds GSSG recycling or when the *de novo* GSH synthesis is compromised. In the present study, the decrease in hepatic GSH:GSSG ratio following treatment with elevated dietary cadmium alone suggested the occurrence of oxidative stress. Reduced thiol ratio makes tissues more susceptible to various other stressors besides oxidative damage because many other homeostatic and housekeeping cellular processes depend on a reduced intracellular environment (Waisberg et al., 2003). Interestingly, we observed that the decrease in thiol ratio recorded during exposure to elevated dietary cadmium was completely alleviated, when the diet was supplemented with medium level of SeMet, but not with high SeMet level (Fig. 4.1). Selenium, when present at an optimum dose, is known to upregulate the activity of γ-glutamylcysteine ligase and increase the *de novo* GSH synthesis (Chung and Maines, 1981). In our previous *in vitro* study with trout hepatocytes, a low dose of SeMet (25 μM) was also found to completely restore cadmium-induced (100 μM) reduction in thiol ratio (Jamwal et al., 2016). The recovery of thiol ratio in fish treated with cadmium + medium SeMet diet was probably contributed, in part, by the upregulation of *de*

novo GSH synthesis, which is evident from a significant increase in the hepatic GSH content relative to that in fish treated with cadmium only diet. The restoration of thiol redox balance might have ameliorated cadmium-induced oxidative stress, which also corresponded with the improved morphometrics (HSI and *K*-factor) of fish treated with cadmium + medium SeMet diet (Table 4.2).

In addition to GSH, the enzymatic antioxidants such as SOD, CAT and GPx are also responsible for the maintenance of cellular redox homeostasis. These enzymes play a crucial role in neutralizing the toxic ROS, and therefore their activities are commonly used as markers of oxidative stress. SOD converts toxic $O_2^{\bullet-}$ into H_2O and H_2O_2, whereas CAT and GPx reduce H_2O_2 into non-toxic H_2O and O_2 (Chelikani et al., 2004; Fukai and Ushio-Fukai, 2011; Mills, 1957). On the other hand, LPO is an index of oxidative damage of cellular lipid structures, since ROS (e.g., such as $O_2^{\bullet-}$) can react with fatty acid molecules and produce MDA as one of the end products (Yagi, 1998). In the current study, we observed that exposure to elevated dietary cadmium alone upregulated the activities of all 3 antioxidative enzymes examined (SOD, CAT, and GPx; Fig. 4.2), which might have occurred as a defensive response in order to cope with cadmium-induced oxidative stress, as suggested in several previous studies (Almeida et al., 2002; Basha and Rani, 2003; Cuypers et al., 2010; Nair et al., 2015). This is also consistent with our concurrent observation of decreased hepatic thiol ratio as well as increased hepatic lipid peroxidation in fish treated with cadmium only diet (Fig. 4.1 and 4.3), as both of these responses indicate increased accumulation of intracellular ROS. Similar to Cd, exposure to elevated levels of SeMet is also known to cause oxidative stress in fish (Hao et al., 2014; J.-H. Kim and Kang, 2015; Kupsco and Schlenk, 2014; Thomas and Janz, 2016). However, we did not observe any significant change in any of the hepatic oxidative stress parameters in fish treated with the diet supplemented exclusively with high SeMet. Our findings are in agreement with Decker (2015), who also reported no change

in hepatic thiol balance and lipid peroxidation in rainbow trout chronically exposed to the same high SeMet level in the diet (Decker, 2015). It should, however, be noted that we recorded a significant reduction in the HSI of fish treated exclusively with high SeMet diet, as discussed previously. This suggests that despite the increased the metabolic cost, high SeMet in the diet did not impair the anti-oxidative balance in rainbow trout.

Although the cellular compensatory responses may enable an organism to survive a chronic stress, it is energetically expensive and unsustainable over long term. In the present study, a high energetic burden of such compensatory response might have contributed to reduced morphometrics in fish exposed to elevated dietary cadmium alone. Interestingly again, no significant increase in the activity of any of the antioxidative enzymes or hepatic lipid peroxidation were recorded in fish treated with cadmium + medium SeMet diet (Fig. 4.2 and 4.3). Consistent with our observations, Talas et al. (2008) and Banni et al. (2011) also reported protective effects, albeit partial, of low levels of selenium (as selenite, *via* water or diet) against the alterations in hepatic antioxidative enzymes and/or increased lipid peroxidation in rainbow trout and zebrafish (*Danio rerio*), respectively, during chronic exposure to waterborne cadmium. Recently, Xie et al. (2016) also demonstrated that pre-exposure to low levels of selenite and SeMet *via* diet ameliorates the adverse effects of short-term (5 days) waterborne cadmium exposure on whole-body CAT activity and lipid peroxidation in killifish (*Heterandria formosa*). It is important to note here that although high SeMet on its own did not elicit an oxidative stress response, its combination with cadmium resulted in increased lipid peroxidation and antioxidative enzyme activities in the liver. This indicates that high SeMet in combination with cadmium overwhelmed the capacity of maintaining redox homeostasis in rainbow trout. Collectively, our observations suggest that SeMet protects against the cadmium-induced oxidative stress in fish essentially by restoring cellular redox balance, but only at a moderate dose level.

In the present study, we also observed that chronic exposure to elevated dietary cadmium increased the tissue-specific cadmium accumulation in rainbow trout, primarily in the kidney followed by liver and muscle. Similar tissue-specific pattern of cadmium accumulation was also reported in previous studies where trout were chronically exposed to dietary cadmium (Kwong et al., 2011; Ng et al., 2009; Szebedinszky et al., 2001)**.** However, co-exposure to cadmium and SeMet (medium and high levels) *via* diet was found to significantly reduce cadmium burden in the liver and kidney, but not in the muscle (Fig. 4.4). In contrast to our findings, Banni et al. (2011) did not record any significant decrease in cadmium accumulation in the liver and ovary in zebrafish exposed to waterborne cadmium (0.4 mg L^{-1}) and fed with a selenium-supplemented diet (2 μg g^{-1} dry wt., as selenite) for 21 days. This might have occurred because of the low dietary selenium exposure level used by Banni et al. (2011) relative to the present study (10 – 50 μg g^{-1} dry wt.), and/or differences in the chemical speciation of selenium (selenite *vs*. SeMet) and route of cadmium exposure (waterborne *vs*. dietary). Nevertheless, our findings are consistent with previous mammalian studies, which also demonstrated that selenium reduces the cadmium accumulation in the liver and kidney (Chen et al., 1975; Whanger et al., 1980). These mammalian studies suggested that the selenium-induced reduction in hepatic and renal cadmium was correlated with increased affinity of cadmium for high molecular weight proteins instead of low molecular weight proteins (e.g., metallothioneins) (Chen et al., 1975; Whanger et al., 1980). Since cadmium in the body is known to be primarily complexed by metallothioneins in hepatic and renal tissues for long-term storage (Nordberg, 1984; Rani et al., 2014), any reduction in their affinity for binding to each other may reduce cadmium accumulation in these tissues. However, unlike in the liver and kidney, an increase in cadmium accumulation in the muscle was observed in the present study during co-exposure to dietary cadmium in combination with high SeMet level. Selenium has been found to

increase cadmium concentration in the blood (Chen et al., 1975; Magos and Webb, 1980), which might have led to increased cadmium uptake in the muscle. In addition, redistribution of cadmium from low-molecular weight proteins to high molecular weight proteins could have also contributed to increased cadmium burden in the muscle, because the muscular tissue is enriched with high molecular weight proteins containing actin and myosin monomers which contain cadmium-binding thiol domains (Bailey and Perry, 1947; Cuypers et al., 2010; Jiang et al., 2017; Otterbein et al., 2001) It is important to note here that although both medium and high level of dietary SeMet decreased hepatic and renal cadmium burden, cadmium toxicity (increased hepatic oxidative stress and reduced morphometrics) was ameliorated only by the medium SeMet level in the diet. This suggests that SeMet-induced reduction in tissue cadmium burden was likely not the predominant factor behind the reduction of cadmium toxicity in fish.

In the present study, dietary exposure to SeMet alone increased tissue-specific selenium accumulation in rainbow trout in a dose-dependent manner except in the muscle. A similar pattern of tissue-specific selenium accumulation was previously reported in rainbow trout chronically exposed to dietary SeMet (40 μg selenium g^{-1} dry wt.)(Misra et al., 2012b). More importantly though, we also observed that the presence of admium in the diet supplemented with high, not medium, level of SeMet resulted in a significant decrease in selenium accumulation in all three tissues examined relative to that in the treatment with only high SeMet diet (Fig. 4.5). This indicates that the antagonistic effect of dietary cadmium on tissue-specific selenium accumulation occurs when cadmium and selenium exist in 1:1 molar ratio in the diet. The influence of cadmium on tissue-specific selenium accumulation has not been investigated previously in any species, and the precise mechanisms by which cadmium reduces selenium accumulation, and *vice versa*, are presently unknown.

Selenium has also been reported to form chemical complex with cadmium in mammals and may therefore influence the distribution of both elements in tissues. For example, Gasiewicz & Smith (1978) demonstrated formation of Cd-Se complex by gel filtration chromatography in rat plasma and erythrocytes through *in vivo* and *in vitro* experiments. Formation of chemically and biologically inert Cd-Se complex was also reported in yeast (*Saccharomyces cerevisiae*) (Dauplais et al., 2013). It is possible that formation of similar Cd-Se complex could have also influenced the tissue-level distribution of both cadmium and selenium in the present study. However, such metabolic interactions between cadmium and SeMet have not been studied in any aquatic animal so far and only further investigations into this can reveal their inter-relationship in fish.

4.5. Conclusion

We demonstrated in the current study that dietary supplementation of SeMet ameliorated the toxicity of chronic dietary cadmium exposure in rainbow trout. However, this protective effect was evident only at the moderate SeMet exposure level (~ 10 μg Se g^{-1} dry wt.), with no reduction in cadmium toxicity at the high dietary SeMet exposure level (~ 45 μg Se g^{-1} dry wt.). Chronic exposure to dietary cadmium caused increased hepatic lipid peroxidation and loss of thiol redox balance, and reduced morphometrics in fish, which were completely alleviated during co-exposure to the moderate level of dietary selenomethionine. Dietary supplementation of s SeMet, both at the moderate and high level, was also found to reduce cadmium accumulation in the liver and kidney during chronic dietary exposure to cadmium. Overall, our study suggests that SeMet essentially protects fish against the chronic toxicity of dietary cadmium exposure *via* its anti-oxidative functions.

CHAPTER 5: Characterization of the modulatory effects of selenomethionine on arsenite induced hepato-toxicity and tissue-specific accumulation of arsenic during chronic dietary exposure in rainbow trout (*Oncorhynchus mykiss*)

Preface

The aim of this chapter is to address the 4th objective of my doctoral research work which is to characterize the modulatory effects of selenomethionine on arsenite-induced hepatotoxicity and tissue-specific accumulation of arsenic during chronic dietary exposure. The findings of this chapter complement the findings of Chapter 3 and further our understanding on the mechanistic basis of how selenomethionine influence the toxic effects of arsenite at the organismal level (under the influence of systemic regulation).

5.1. Introduction

Arsenic is a well-known environmental toxin and a priority water pollutant that is mobilized from its geological deposits by both natural and anthropogenic activities (Shen et al., 2013; Winter, 2017). In the water bodies, arsenic is highly persistent and can easily accumulate to toxic concentrations. Currently, millions of people worldwide are at the risk of chronic exposure to toxic forms of arsenic through contaminated water with consequences that affects immune system, and all major organs systems to cause carcinogenic and noncarcinogenic pathologies (Shakoor et al., 2017). Consistent with the studies on humans and other mammals, exposure to inorganic arsenic also affects multiple vital organs of fish, and is also associated with reduced growth, poor reproductive performance, weak immune response, and impaired behavioral and cognitive functions (de Castro et al., 2009; Kumari et al., 2017; Szymkowicz et al., 2017).

The toxic effects of arsenic have been attributed to the generation of reactive oxygen species (ROS) and reactive nitrogen species (RNS), which eventually cause oxidative stress (Flora, 2011; Watanabe and Hirano, 2013). Although the precise mechanisms underlying arsenic mediated oxidative stress are not fully understood, it has been suggested that arsenite can disrupt redox potential by inducing changes in the mitochondrial membrane (Flora, 2011; Selvaraj et al., 2013a). In addition to the role of mitochondria, arsenite induced upregulation of flavin enzymes, such as NAD(P)H oxidase, is also known to generate ROS (Kumagai and Sumi, 2007). Inorganic arsenic is also known to disrupt cellular redox homeostasis indirectly by inhibiting antioxidative enzymes such as catalase (CAT), superoxide dismutase (SOD), and glutathione peroxidase (GPx) (Flora, 2011; Jamwal and Niyogi, 2017). Arsenic-induced oxidative stress can alter the integrity of cellular biomolecules and signaling pathways, which is linked to carcinogenic as well as non-carcinogenic disorders (Shi et al., 2004).

In aquatic ecosystem, arsenic can occur in four different oxidation states; however, the pentavalent arsenate [As(V)], and trivalent arsenite [As(III)] are the most environmentally and toxicologically relevant (Carlin et al., 2016; Zhu et al., 2014). Under normoxic conditions, As(V) is usually the dominant oxidation state, whereas As(III) is more abundant in hypoxic and acidic waters (Zhu et al., 2014). However, following the uptake by the living organisms, most of the absorbed As(V) is reduced to more toxic As(III) (Russell J. Erickson et al., 2011; McIntyre and Linton, 2012; Zhu et al., 2014). Most toxicological assessments of inorganic forms of arsenic in aquatic animals are conducted through waterborne exposures, and dietary exposure has received very little attention to date. It has been demonstrated that aquatic invertebrates can bioaccumulate arsenic to toxic concentrations and intoxicate the fish that feed on them even when the arsenic in water is below lowest-observed-effect-concentration (LOEC) (CCME, 2001; R. J. Erickson et al.,

2011; US-EPA, 2015). Therefore, dietary exposure of arsenic is a more sensitive marker of environmental arsenic contamination to aquatic animals, including fish.

The toxicity of arsenite is also confounded by its interactions with other metals, non-metals, and metalloids. Selenium (Se) is one such element that is known to interact with As (Zeng et al., 2005; Zwolak and Zaporowska, 2012). Unlike arsenic, selenium is essential for the synthesis of selenoproteins that have various adaptive and housekeeping functions, especially in the maintenance of cellular redox homeostasis (Reich and Hondal, 2016). However, selenium is also a freshwater priority aquatic pollutant, and can induce ROS production and oxidative stress when its concentration in the body exceeds the physiological threshold (J.-H. Kim and Kang, 2015; Kupsco and Schlenk, 2014; Thomas and Janz, 2016; USEPA, 2014). Although a fish can be exposed to selenium *via* both water and diet, the latter is a predominant route of exposure, especially in its organic form (selenomethionine) (Janz, 2012). Therefore, it is important to investigate how dietary selenomethionine modulates arsenite toxicity in a dose dependent manner.

The interaction between selenium and inorganic arsenic was first reported by Moxon and DuBois, (1939) in rats. Later, the ameliorative effect of selenium against arsenic (as arsenate and arsenite) toxicity was also demonstrated in the fish epithelial cell lines (Babich et al., 1989). More recently, selenium was shown to partially antagonize arsenite toxicity in fish hepatocytes by restoring cellular redox homeostasis (Jamwal and Niyogi, 2017; Selvaraj et al., 2012); although, the ameliorative effects of selenium were seen only at relatively low concentration (10 – 20 μM) (Jamwal and Niyogi, 2017). These *in vitro* studies provide a *prima facie* evidence that selenium can antagonize arsenite-induced oxidative stress in fish. However, there is a complete lack of information on how these two metalloids interact in any aquatic animal *in vivo*, and our understanding on this subject is derived solely from mammalian literature. In addition to the

antioxidative effects, mammalian literature also suggests that selenium can form complexes with arsenite, which is either biologically inert or less toxic (Burns et al., 2008; Gailer, 2007; Korbas et al., 2008). It has also been suggested that selenium can reduce tissue accumulation of arsenic by increasing its depuration rate (Hilmy et al., 1991; Krohn et al., 2016; Sah et al., 2013). However, some reports have also indicated that inorganic arsenic can have synergistic toxic effects with methylated selenium or methylated metabolites of selenium (Kraus and Ganther, 1989; Levander, 1977). More recently, it was suggested that selenium can prevent complete methylation of inorganic arsenic, and thus increase retention of monomethylated arsenicals in the tissues, which are more toxic than their inorganic counterparts (Styblo and Thomas, 2001; Walton et al., 2003). These studies indicate that there is no general consensus on how arsenic and selenium may interact even within the mammalian system. Since both arsenic and selenium are important aquatic pollutants, a cohesive scientific enquiry on aquatic animals is also warranted linking their interaction with oxidative stress and tissue-level distribution.

The present study is the first in-depth investigation on how the interactions between arsenite and selenium in the fish diet are influenced by different selenium exposure concentrations. The juvenile rainbow trout were exposed to an environmentally relevant dietary dose of arsenite in combination with diferent doses of dietary selenium [control (low), moderate and high; as selenomethionine]. The specific objectives of this study were to examine: (i) how the interaction between dietary selenomethionine and arsenite affect the morphophysiological indices (condition factor, hepatosomatic index) of fish, (ii) the modulatory effects of selenomethionine on the arsenite-induced hepatic oxidative stress, and (iii) how the interactions between arsenite and selenomethionine influence the tissue-level deposition of each element. Furthermore, arsenic can cross blood-brain barrier and have deleterious effects on neurological development and congnitive

functions, in both mammals and fish (Au and Kwong, 2008; de Castro et al., 2009; Palaniappan and Vijayasundaram, 2009; Prakash et al., 2016), we used synchrotron based X-ray fluorescence imaging to map the depostion of arsenic and selenium in the telencephalic region of the fish brain. Telencephalon is a highly specialized region of the fish cerebellum that is involved in critical behavioural and learning functions such as breeding, parental care, learning, and foraging (Gómez et al., 2011; Rodriguez et al., 2005). We hypothesized that selenium would influence arsenite toxicity in a dose-specific manner by modulating hepatic redox potential, and accumulation of arsenic in critically important tissues such as kidney, liver, muscle and brain.

5.2. Materials and methods

5.2.1. Experimental Fish

Rainbow trout eggs were hatched in the Aquatic Toxicology Research Facility (ATRF), at the University of Saskatchewan. The fish were raised in dechlorinated, aerated water [alkalinity 105 mg L^{-1} (both as $CaCO_3$), hardness 159 mg L^{-1}, dissolved organic carbon (DOC) 2.1 mg L^{-1}, pH 7.5 – 7.8, Se 5.2 µg L^{-1}, Cd < 0.1 µg L^{-1}] in a flow-through system (1 L min^{-1}). The photoperiod was set at 14:10 h light to dark cycle and water temperature was maintained at 12 ºC. The fish were fed at 2 % body weight ratio (wet wt.) with commercial feed during the husbandry period. The juveniles, weighing 30 – 35 g, were used for the experiment.

5.2.2. Diet preparation

The diets were prepared using frozen brine shrimp (*Artemia franciscana*). Nominal concentrations of Se (10 and 40 µg g^{-1} dw) and As (80 µg g^{-1} dw), in the form of selenomethionine and sodium arsenite ($NaAsO_2$), respectively were dissolved in de-ionized distilled water, added to frozen *Artemia*, and blended in commercial blender for 10 min. Carboxymethyl cellulose (2 % w/w. dw basis) was used as a feed binder. The mix was frozen at −20 ºC and freeze-dried (Labconco

Freezone, USA). The freeze-dried feed was cut into small pieces (~0.5 cm), and stored in air-tight containers in -20 ºC until used. The control diet (normal selenium and normal arsenite levels) was prepared similarly, without the addition of selenomethionine or $NaAsO_2$. The final concentration of selenium and arsenic in the diets was analyzed by atomic absorption spectroscopy (AAS), as described later.

5.2.3. Experimental treatments and sampling

The fish were divided into six different treatment groups which are identified as: control (normal selenium and arsenic), arsenite only (normal selenium and 80 µg arsenic g^{-1} diet), medium SeMet only (10 µg selenium g^{-1} diet), High SeMet only (40 µg selenium g^{-1} diet), arsenite + medium SeMet (10 µg selenium + 80 µg arsenite g^{-1} diet), arsenite + high SeMet (40 µg selenium + 80 µg arsenite g^{-1} diet). Each treatment was run in duplicate, with five fish in each replicate. Five fish from each treatment group were weighed randomly every week to maintain the feeding ration at 2 % body weight. The fish consumed the diet within 3 minutes of feeding. Each tank received continuous aeration and the water flow-through rate was maintained at 8 L min^{-1} to ensure rapid flushing of metabolites, excreta, selenomethionine or arsenic that could have potentially leached out of the diet. Fish were starved for 24 h before the final take down of the experiment. On the 30th day, fish from each treatment were euthanized with an overdose of Aquacalm^{TM} (metomidate hydrochloride; Syndel, USA). Four fish from each replicate treatment were randomly sampled to obtain a sample size (*n*) of 8. Length and weight of each fish was measured. Liver, kidney, brain and muscle were dissected from each fish. Liver from each was also weighed for the estimation of hepatosomatic index (HSI). Liver, kidney, and muscle were immediately frozen in liquid nitrogen and stored in -80 ºC until used. Fish brains were embedded in optimal cutting temperature (OCT)

compound and frozen in liquid nitrogen. The frozen brain samples were sectioned to 12 μm thick sections using a cryotome and mounted on Thermanox plastic coverslips (Gibco BRL).

5.2.4. Estimation of hepatic lipid peroxidation (LPO)

The extent of lipid peroxidation (LPO) in fish livers was estimated by thiobarbituric acid reactive substance (TBARS) assay. A 96 micro-well plate based commercial TBARS kit (Catalogue # 10009055; Cayman chemical company, USA) was used for the estimation of LPO. The hepatic LPO was expressed as nmol MDA mg^{-1} tissue protein.

5.2.5. Measurement of hepatic thiol content

The hepatic concentration of reduced (GSH) and oxidized (GSSG) glutathione were measured using fluorometric method (Hissin and Hilf, 1976), modified to a 96-well microplate based assay as described in Jamwal and Niyogi (2007). The GSH and GSSG contents were expressed as μg mg $protein^{-1}$. The hepatic GSH content was divided by GSSG concentration to obtain thiol ratio (GSH:GSSG).

5.2.6. Measurement of antioxidant enzyme activities

The thawed livers were homogenized in 5 times its volume (w/v) of chilled HEPES-EGTA sample buffer (1mM EGTA, 210 mM mannitol, 70 mM sucrose, and 20mM HEPES, pH 7.2) using a hand-held homogenizer. The homogenate was centrifuged at 25,000 x g for 20 min at 4 ºC to obtain supernatant with enzyme fraction. The activities of three antioxidant enzymes, catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GPx) were estimated using SOD (Catalogue #706002), CAT (Catalogue #707002), and GPx (Catalogue #703102) kits as per the manufacturer's instructions (Cayman chemical company, USA). The activities of CAT, SOD, and GPx were expressed as nmol mg $protein^{-1}$, U mg $protein^{-1}$ and nmol min^{-1} mg $protein^{-1}$,

respectively. One unit of SOD is defined as the amount of enzyme needed to exhibit dismutation of the superoxide radical by 50%.

5.2.7. Measurement of selenium and cadmium in water, diet and tissue

The concentration of selenium and arsenic in water, diet and tissues was measured using a graphite-furnace atomic absorption spectroscopy (Analyst 800, PerkinElmer, USA). The water samples were acidified with 1% HNO_3, and filtered through 0.45 µm filter before analysis. The experimental diets and tissues were digested in 5 volumes of 2 N HNO_3 at 60^{o}C for 48 h, and then was centrifuged at 10,000 RCF for 20 min. The supernatant was extracted and diluted with 0.2 % HNO_3, to analyze for total selenium and arsenic concentrations using a graphite-furnace atomic absorption spectrometer (AAnalyst 800, Perkin Elmer, USA). Appropriate method blanks and certified standards for both metals were used to maintain the quality control and assurance of selenium and arsenic analysis (PerkinElmer, USA). Certified reference materials (DOLT-3; National Research Council, Canada) were also used to validate the results. The recovery of selenium and arsenic were 97 % and 93 %, respectively.

5.2.8. X-ray fluorescence imaging (XFI)

The pallium region of the telencephalon was scanned for the imaging. Due to limited beamtime, only two to three representative brain sections from each treatment were scanned. The XFI data was collected at the 20-ID-B,C beamline of the Advanced Photon Source in Argonne National Laboratory, Illinois following the protocol and experimental set-up as described by McDonald et al., (2015). The Rh-coated Kirkpatrick-Baez mirrors were used to obtain X-ray beams of size 2.5 µm vertical and 2.7 µm horizontal, and incident at 45^{o} on the samples. The X-ray beam was monochromated by diffraction from silica crystals. The incident X-ray energy for the imaging was selected at 13.45 keV which was above both selenium K-edge and the arsenic K-edge, but

below bromine K-edge to avoid bromine fluorescence from plastic components of the setup. Initially, fluorescence signals from the larger area of the telencephalon were collected in "fly scan" mode with 15 µm beam size and 0.2 s dwell time to detect the regions of arsenic and selenium localization. Later, smaller regions of interest were scanned for higher resolution with 5 µm step size and 0.6 s dwell time. The integrated fluorescence spectra of smaller regions of interest were extracted to determine the average elemental concentration in µg cm^{-2} and quantification was performed by comparison to the spectra from CdSe and GaAs elemental standards with known concentration (15.6 µg cm^{-2} and 16.3 µg cm^{-2}, respectively). The concentration of arsenic and selenium was quantified as areal density *a* and expressed in units of µg cm^{-2}. Analysis of the image was performed using SMAK MicroAnalysis toolkit (https://www.sams-xrays.com/smak).

5.2.9. Calculations and statistical analysis

Hepato-somatic index and condition factor (*K*-factor) were used as the physiological indicators of energy status of fish and calculated as follows: HSI = weight of liver (g)/ weight of the fish (g)] and condition factor (*K*) = 100 X weight of the fish (g)/ length $(cm)^3$. SigmaPlot (version 12.5, Systat Software, Inc. USA) was used for statistical analysis and preparation of graphs, except for the analysis and graphing of the X-ray fluorescence imaging (XFI) data. The data are presented as mean ± standard error of mean (S.E.M.). Interactive effects of SeMet and As were analyzed by two-way analysis of variance (2-WAY ANOVA). Pairwise multiple comparison (*post hoc*) with Holm-Sidak correction was performed to determine the effect of different treatments. The assumptions of ANOVA, such as normality of distribution and heteroscedasticity, were verified using Shapiro-Wilk and Brown-Forsythe test, respectively. Data was subjected to logarithmic transformation when the assumption of normality was violated. A *p*-value of ≤ 0.05 was considered to be significant while comparing different treatments. A Pearson correlation

analysis was conducted to assess the relationship between hepatic arsenic accumulation, lipid peroxidation and the activities of enzymatic antioxidants. For the correlation plots of XFI data, the error in both abscissa and ordinate axis were accounted to minimize the sum of square of the perpendicular distance from each point to the line. A custom designed program was used for the computation and graphing of the XFI data (MacDonald et al., 2015).

5.3. Results

5.3.1. Fish morphometrics [*Hepato-somatic index (HSI) and condition factor (K-factor)*]

No fish mortality was recorded in any of the treatments during the experimental exposure period. The interaction between arsenite and selenomethionine had a statistically significant effect on the fish HSI ($F_{2,31} = 6.82$; $p = 0.003$; Table 5.1). Supplementing the diet with arsenite, alone or in combination with high selenomethionine reduced the HSI by 50 %, ($p < 0.001$), while the reduction in HSI in fish treated with arsenite and medium SeMet was about 30 % relative to the control ($p < 0.001$). The diet supplemented with only high selenomethionine also had a deleterious effect and reduced the HSI by 20 %, when compared to the control ($p = 0.003$); however, the fish fed with medium SeMet diet demonstrated an increase in the HSI by the same proportion (20 %) relative to the control ($p = 0.008$). No significant interaction between arsenite and selenomethionine was recorded for *K-factor* ($F_{2,37} = 1.12$; $p = 0.339$; Table 5.1); however, a significant decrease, relative to the control, was observed only in the fish treated with arsenite only diet ($p = 0.031$; Table 5.1).

Table 5.1. Physiological indicators of health of juvenile rainbow trout exposed to different dietary treatments of arsenite and/or selenomethionine (SeMet).

Treatments	HSI	Condition factor (*K*)
Control	0.025 ± 0.0011^{a}	1.38 ± 0.088^{A}
Arsenite only	0.013 ± 0.0006^{b}	1.17 ± 0.023^{B}
Medium SeMet	0.030 ± 0.0014^{c}	1.35 ± 0.049^{A}
High SeMet	0.020 ± 0.0010^{d}	1.29 ± 0.019^{A}
Arsenite + Med SeMet	0.017 ± 0.0009^{bd}	1.22 ± 0.050^{A}
Arsenite + High SeMet	0.013 ± 0.0004^{b}	1.21 ± 0.057^{A}

Values are mean ± SEM, n = 5 - 8. HSI = hepatosomatic index. Values with different alphabetic superscripts represent statistical difference among the treatments for a particular parameter ($p < 0.001$). Similar superscripts represent no statistical difference ($p > 0.05$), as determined by 2-WAY ANOVA and *post hoc* analysis.

5.3.2. Hepatic thiol redox balance

A statistically significant interaction between arsenite and selenomethionine was recorded for hepatic GSSG and thiol ratio (GSH:GSSG), but not for GSH. However, there was a significant simple-main effect of the treatments on the GSH (Fig. 5.1). Exposure to arsenite only diet significantly reduced GSH content by 2.3 folds, whereas it increased GSSG level by 1.5-fold. As a result, there was a significant reduction in the GSH:GSSG ratio, relative to the control ($p < 0.001$; Fig. 5.1). The hepatic concentrations of thiols and their relative ratio did not differ significantly from the control when fish were fed with the diet supplemented with medium or high selenomethionine only ($p > 0.05$; Fig. 5.1). However, the GSH and GSH:GSSG ratio were significantly reduced, in comparison to the control, when the diets were supplemented with arsenite and selenomethionine ($p < 0.001$; Fig. 5.1). Supplementing the arsenite diet with medium selenomethionine reduced GSH and GSH:GSSG ratio by 1.7 and 2.2-folds, respectively relative to control ($p < 0.001$), whereas the reduction in GSH and GSH:GSSG in fish fed with arsenite + high selenomethionine diet was 3 and 4-folds, respectively ($p < 0.001$; Fig. 5.1).

5.3.3. Hepatic lipid peroxidation (LPO)

There was a significant effect of the interaction between arsenite and selenomethionine on the hepatic lipid peroxidation ($F_{2,30} = 7.423$; $p = 0.002$). The hepatic MDA level in fish fed with dietary arsenite, alone or in combination with medium selenomethionine, was 2.5 to 3-folds higher than the control ($p < 0.001$; Fig. 5.2). The hepatic lipid peroxidation following the treatment with arsenite + high selenomethionine was even higher than in fish treated with arsenite only diet. The hepatic MDA levels following treatment with arsenite + high selenomethionine were 5-folds and 2-folds greater than in control and arsenite only treatment group, respectively ($p < 0.001$; Fig. 5.2). A Pearson correlation analysis also revealed that the degree of lipid peroxidation was higher with increased accumulation of arsenic in the hepatic tissue ($r = 0.306$; $p = 0.039$; Table 5.2). However, no change in the hepatic MDA levels, relative to control, was observed in fish treated exclusively with medium selenomethionine and high selenomethionine dietary treatments ($p > 0.90$; Fig. 5.2).

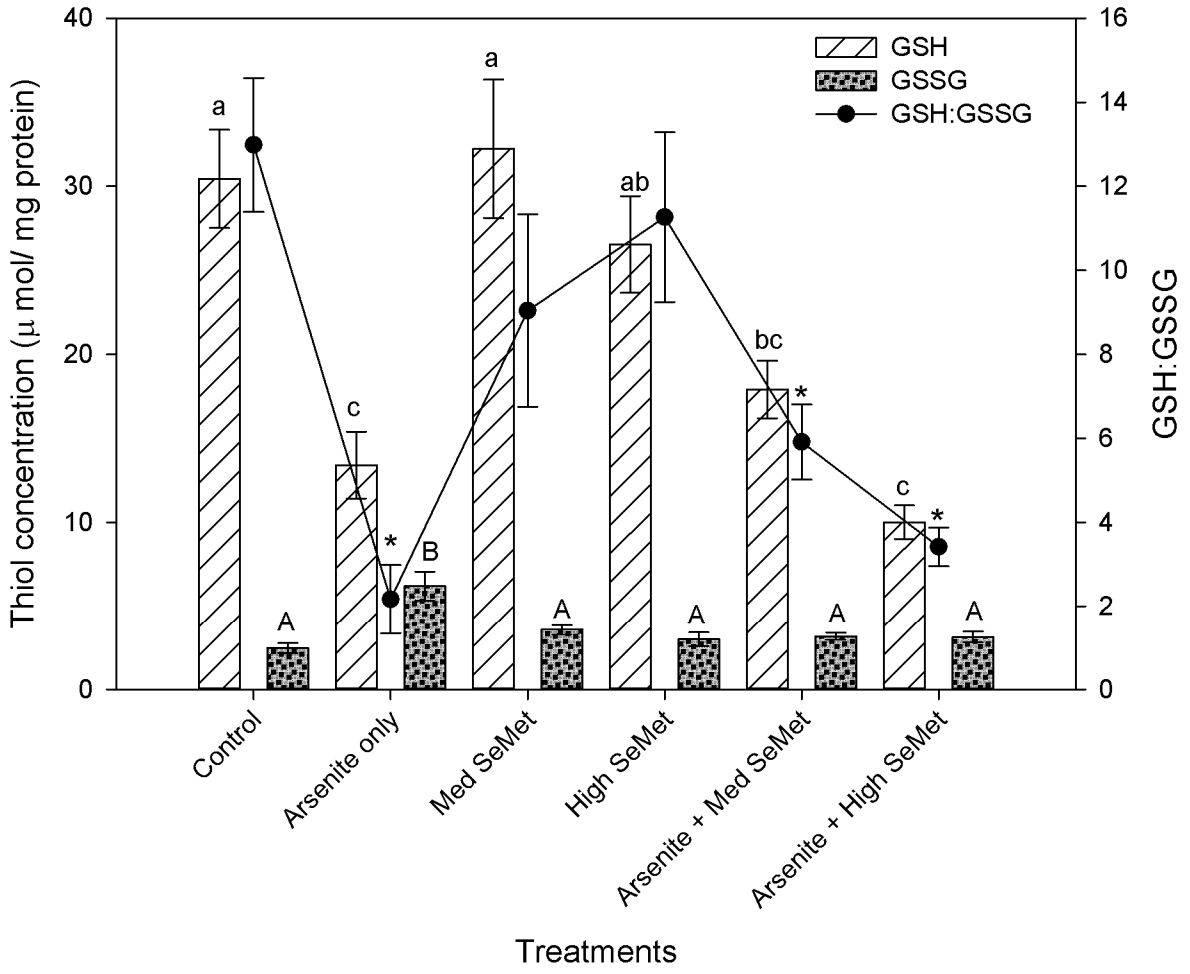


Figure 5.1: Concentration of reduced (GSH) and oxidized (GSSG) glutathione, and their ratio (GSH:GSSG) in the liver of rainbow trout exposed to various treatments. Data are presented as mean ± SEM (n = 6). For GSH and GSSG the statistical significance is denoted with small letters, and capital letters respectively. The bars with different letters are statistically different ($p < 0.05$). For GSH:GSSG, an asterisk (*) represents statistically significant difference in comparison to the control group and the data-points without an asterisk are statistically similar to each other ($p < 0.05$).

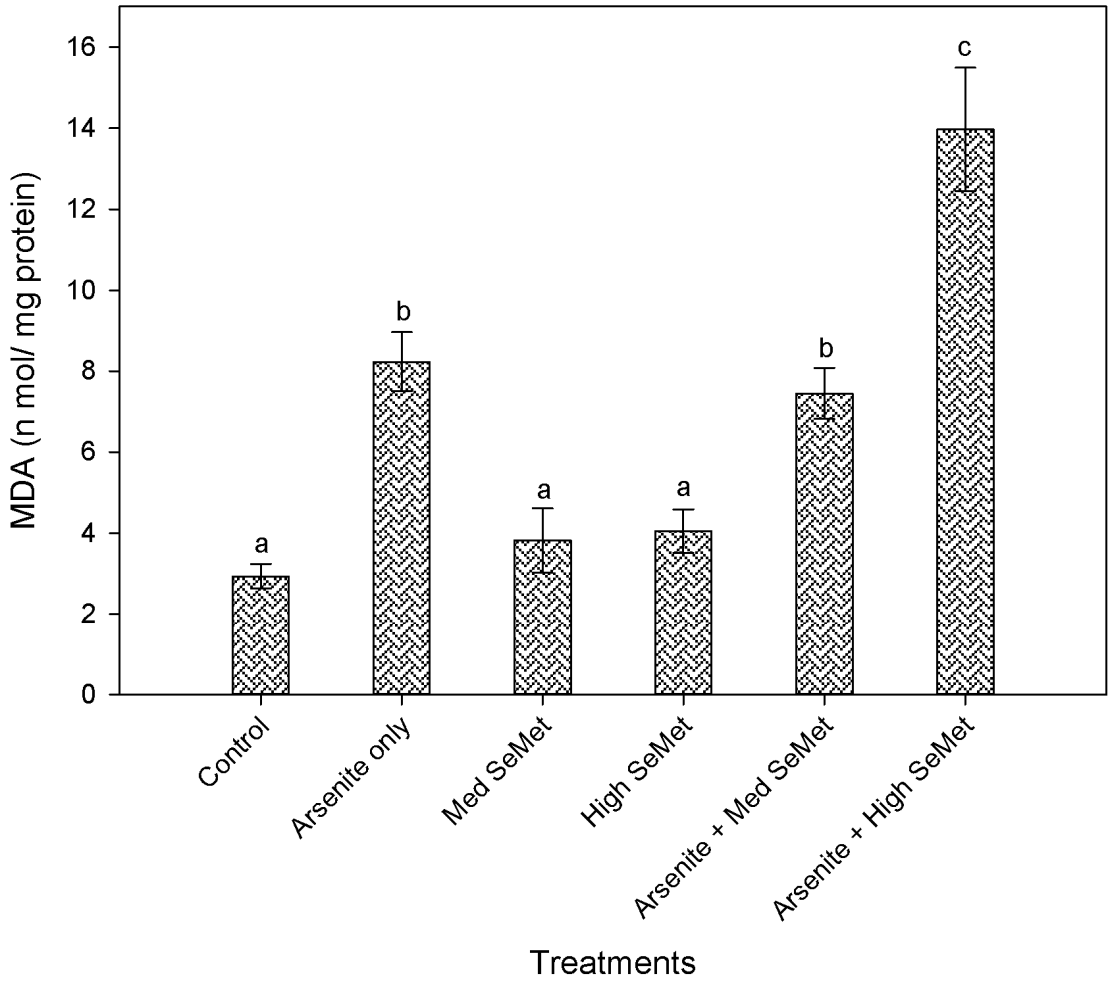


Figure 5.2: Concentration of hepatic malondialdehyde (MDA) measured as a biomarker of lipid peroxidation (LPO) exposed to different dietary treatments of arsenite and/or selenomethionine (SeMet). Data are presented as mean ± SEM ($n = 6 – 8$). The experimental treatments with different alphabetical notations are significantly different ($p < 0.005$) from each other, while the treatments with common alphabetical notations have no significant difference among them, as determined by 2-WAY ANOVA and *post hoc* analysis.

5.3.4. Hepatic antioxidative enzymes

The effects of dietary treatments on the activities of the hepatic antioxidative enzymes (CAT, SOD, and GPx) are illustrated in the Fig. 5.3. There was no interactive effect of dietary arsenite and selenomethionine on the CAT activity ($F_{2,31} = 0.05$; $p = 0.95$). However, the simple-main effects revealed that exposure to arsenite, alone or in combination with medium or high selenomethionine resulted in a significant increase (~50%) in the CAT activity, in comparison to the control ($p < 0.001$; Fig. 5.3 A). A two-tailed Pearson correlation analysis revealed a strong correlation between CAT activity and hepatic arsenic accumulation ($r = 0.532$; $p = 0.001$; Table 5.2). In contrast, no significant change in CAT activity was recorded in fish treated exclusively with medium and high selenomethionine only diets ($p > 0.1$; Fig. 5.3 A).

Similar to the CAT activity, there was no interactive effect of arsenite and selenomethionine on the activity of the hepatic SOD enzyme ($F_{2,32} = 0.469$; $p = 0.63$), although exposure to arsenite only diet led to a 1.5-fold increase in the activity of SOD in contrast to the control ($p < 0.001$; Fig. 5.3 B). Dietary exposure to both, arsenite + medium selenomethionine, and arsenite + high selenomethionine diets resulted in more than 2-folds increase in the SOD activity when compared to the control ($p < 0.001$, Fig. 5.3 B). A significant correlation was also revealed between SOD activity and hepatic concentration of arsenic ($r = 0.733$; $p = 0.002$; Table 3). However, no change in the activity of hepatic SOD, in contrast to the control, was observed when fish were fed with medium and high selenomethionine only diets ($p > 0.5$; Fig. 5.3 A).

An interactive effect of dietary arsenite and selenomethionine on hepatic GPx was revealed by the 2-Way ANOVA test ($F_{2,26} = 3.95$; $p = 0.032$). No effect on the GPx activity was recorded due to medium and high selenomethionine only diets in comparison to the control ($p > 0.1$; Fig. 5.3 C). Dietary exposure to arsenite alone led to a significant increase (36 %) in the hepatic GPx

activity, in contrast to the control ($p = 0.035$; Fig. 5.3 C). Interestingly, dietary exposure to arsenite + high selenomethionine resulted in an increase in the hepatic GPx activity, which was 112 % and 56 % higher than the control and arsenite only treatment groups, respectively ($p < 0.001$; Fig. 5.3 C). Similarly, dietary exposure to arsenite + medium selenomethionine resulted in the GPx activity that was 77 % and 30 % higher than the fish fed with control and arsenite only diets, respectively ($p < 0.001$; Fig. 5.3 C). Moreover, the GPx activity following treatment with arsenite + high selenomethionine was also statistically higher than the fish fed with arsenite + medium selenomethionine diet ($p = 0.04$; Fig. 5.3 C). Similar to CAT and SOD, the activity of GPx enzyme was also strongly correlated with the accumulation of arsenic in liver tissue ($r = 0.397$; $p = 0.27$; Table 5.2).

Table 5.2. Pearson correlation coefficient of analyzed hepatic anti-oxidative enzymes or lipid peroxidation and hepatic arsenic (As) concentration.

Enzymatic anti-oxidant	**Pearson correlation coefficient (r)**	**p - value**
Catalase (CAT)	0.532	0.001
Superoxide dismutase (SOD)	0.490	0.002
Glutathione peroxidase (GPx)	0.397	0.027
Lipid peroxidation (LPO)	0.306	0.039

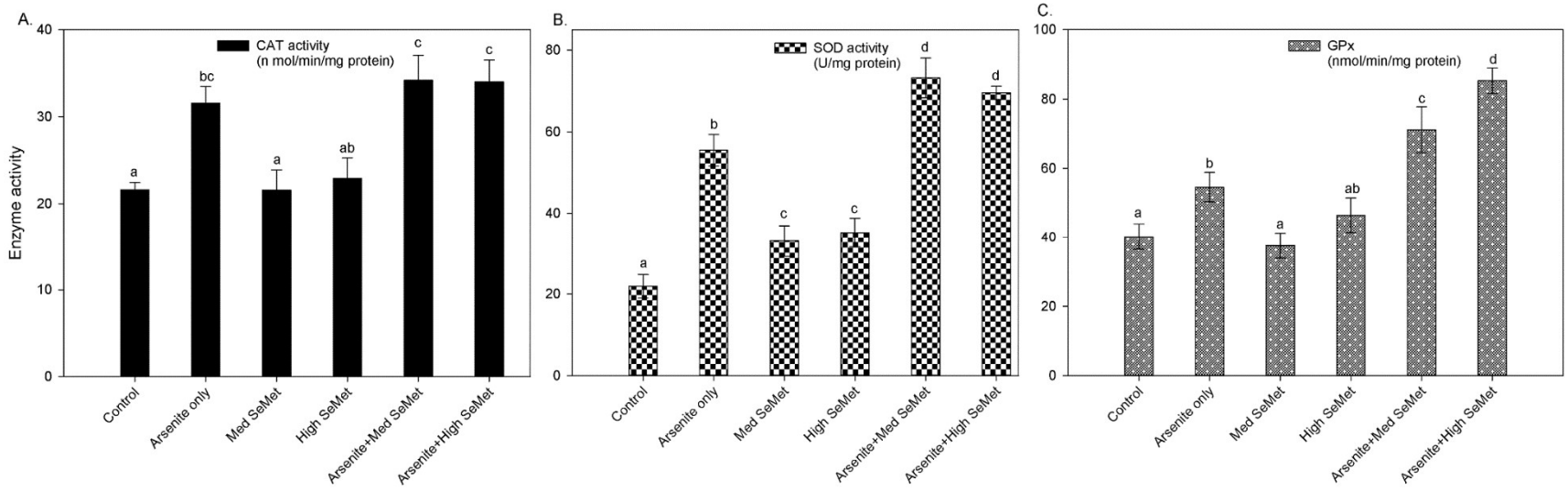


Figure 5.3: Changes in the hepatic activities of (A.) catalase (CAT), (B.) superoxide dismutase (SOD), and (C.) glutathione peroxidase (GPx) exposed to different dietary treatments of arsenite and/or selenomethionine (SeMet). Data are presented as mean ± SEM (n = 5 – 8). In each figure panel (A, B, or C), experimental treatments with different alphabetical notations are significantly different ($p <$ 0.005) from each other, while the treatments with common alphabetical notations have no significant difference among them, as determined by 2-WAY ANOVA and *post hoc* analysis.

5.3.5. Accumulation of arsenic and selenium in hepatic, renal, and muscle tissue

A significant interaction between dietary arsenite and selenomethionine existed for the accumulation of arsenic in liver ($F_{2,33} = 7.99$; $p = 0.001$), but not in kidney ($F_{2,31} = 2.90$; $p = 0.07$) and muscle ($F_{2,33} = 2.353$; $p = 0.11$). Following the treatment with arsenite only diet, a significant accumulation of arsenic was observed in all the tissues ($p < 0.001$) with a maximum (38 folds) increase in the liver, followed by a modest increase in the kidney and muscle (3 and 2-folds, respectively), relative to the control (Fig. 5.4 A-C). The range of arsenic concentrations observed in all of the tissues from selenomethionine only dietary treatments (medium or arsenite) was similar (0.3 – 1.5 μg g^{-1} tissue), and significantly higher than their respective controls ($p < 0.007$; Fig. 5.4). The concentration of arsenic in kidney and muscle from selenomethionine only dietary treatments (medium or high) was also statistically similar to the arsenite only treatment group ($p > 0.08$; Fig. 5.4 B, C). Although the hepatic concentration of arsenic in medium or high selenomethionine dietary groups was statistically higher than the control ($p > 0.007$), it was significantly lower than the hepatic arsenic levels observed in the arsenite only treatment ($p > 0.001$; Fig. 5.4A). The dietary treatment of arsenite and medium or high selenomethionine also resulted in significantly higher arsenic levels in all the tissues, in comparison to the control ($p < 0.001$; Fig 5.4 A-C). Interestingly, the arsenic accumulation in muscle following treatment with arsenite + high selenomethionine diet was twice as high as the arsenic concentration in muscle from arsenite only dietary treatment ($p = 0.039$; Fig. 5.4 C).

An interactive effect of dietary arsenite and selenomethionine on selenium accumulation was revealed by 2-Way ANOVA in the liver ($F_{2,33} = 9.69$; $p < 0.001$) and muscle ($F_{2,31} = 9.123$; $p < 0.001$), but not in the kidney ($F_{2,31} = 0.894$; $p = 0.419$). The selenium accumulation in all of the tissues following treatment with arsenite only was significantly lower than the control ($p < 0.001$;

Fig. 5.5). There was a 4-fold and 6-fold increase in the hepatic Se concentration, relative to the control, following treatment with medium and high selenomethionine only diets, respectively ($p < 0.001$; Fig. 5.5 A). Although the concentration of selenium in renal and muscle tissue also increased after treatment with selenomethionine only diets (both medium and high), the selenium accumulation between the two treatment groups was not statistically different ($p > 0.367$; Fig. 5.5 B, C). Presence of arsenite in the diet supplemented with medium selenomethionine did not change the selenium accumulation pattern relative to that in the medium selenomethionine only treatment. However, a similar 2-fold decrease in the Se accumulation was recorded in the liver and kidney following treatment with arsenite + high selenomethionine in comparison to the high selenomethionine only diet ($p < 0.001$; Fig. 5.5 A and B).

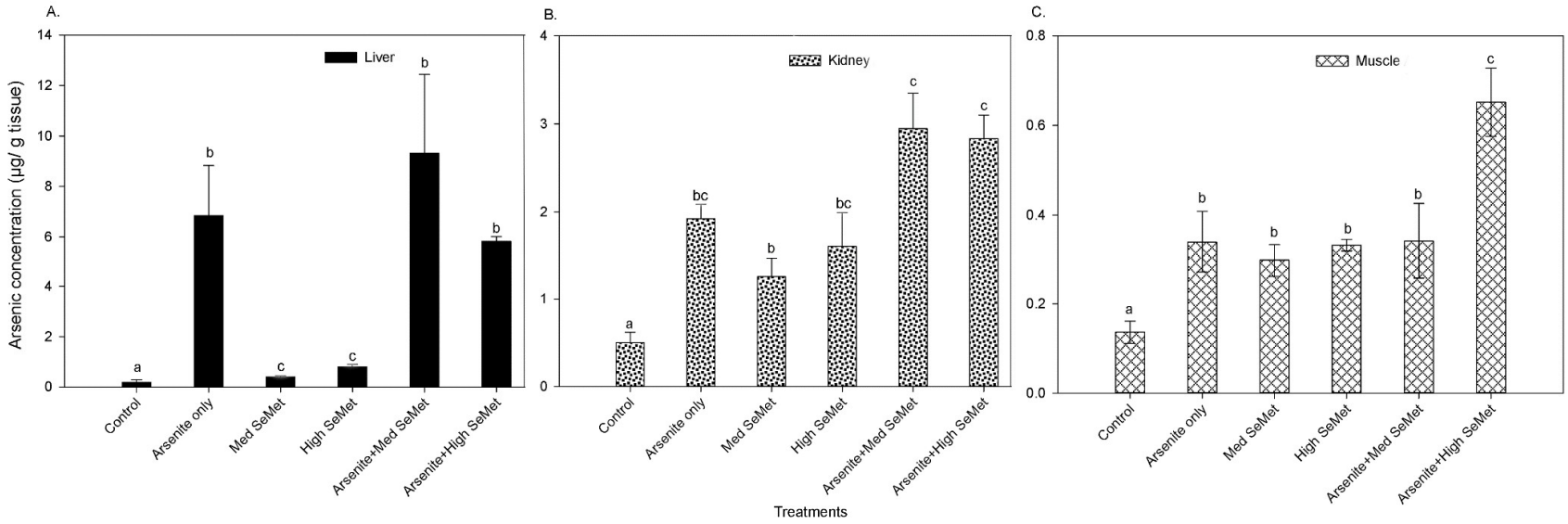


Figure 5.4: Concentration of arsenic in the liver (A) kidney (B), and muscle (C) in rainbow trout exposed to dietary treatments with arsenite and/or selenomethionine. Values are mean ± SEM (n = 6-8). In each figure panel (A, B, or C), experimental treatments with different alphabetical notations are significantly different ($p < 0.005$) from each other, while the treatments with common alphabetical notations have no significant difference among them, as determined by 2-WAY ANOVA and *post hoc* analysis.

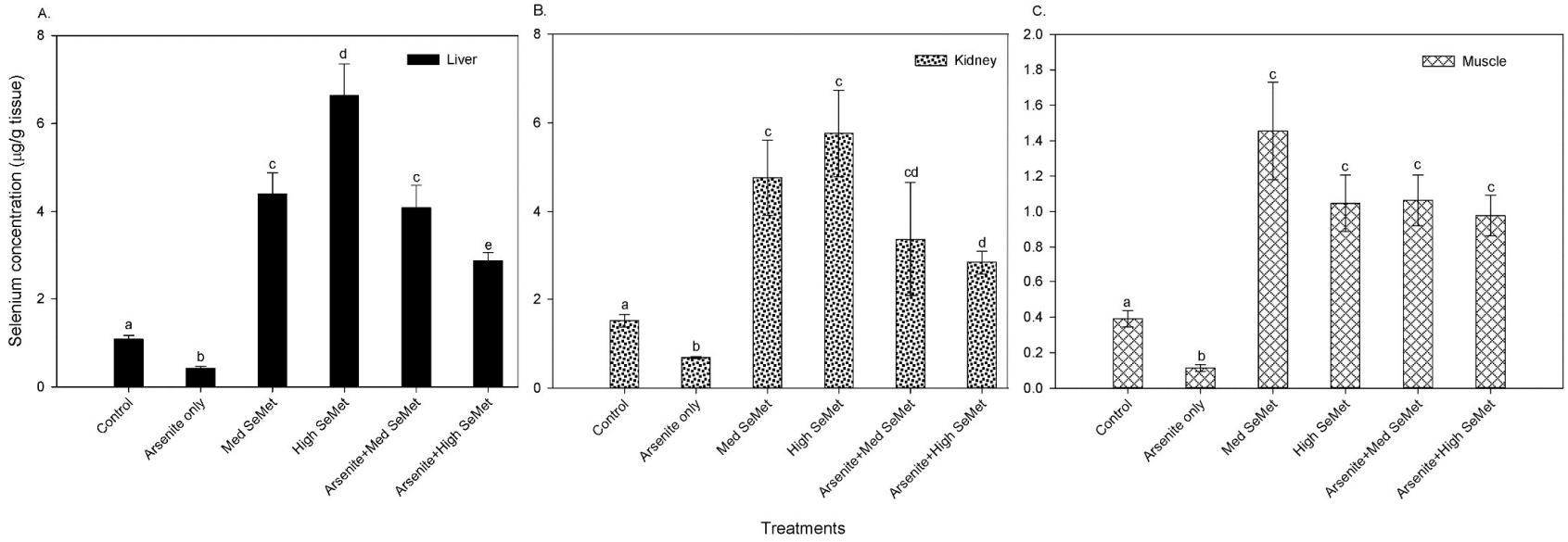


Figure 5.5: Concentration of selenium in liver (A.) kidney (B.), and muscle (C) in rainbow trout exposed to dietary treatments with arsenite and/or selenomethionine (SeMet). Values are mean ± SEM (n = 6-8). In each figure panel (A, B, or C), experimental treatments with different alphabetical notations are significantly different ($p < 0.005$) from each other, while the treatments with common alphabetical notations have no significant difference among them, as determined by 2-WAY ANOVA and *post hoc* analysis.

5.3.6. X-ray fluorescence imaging (XFI) of the fish telencephalon

The images from the representative brain sections showing the spatial distribution of arsenic and selenium in the telencephalic pallium are presented in the Fig. 5.6 – 5.8. The control showed no detectable amount of arsenic or selenium in the brain (Fig. 5.6 B). Following treatment with arsenite only diet, arsenic was detected in few randomly distributed hotspots within the pallium of the rainbow trout brain; however, selenium was below the limit of detection (Fig. 5.6 C). Supplementation of the diet with selenomethionine, alone or in combination with arsenite, resulted in uptake of both arsenic and selenium which were highly co-localized along the telencephalic ventricle (Fig. 5.7 and 5.8). In addition to the spatial co-localization, the concentration of arsenic and selenium also exhibited a high degree of correlation ($R^2 > 0.90$; Fig. 5.9). Following treatment with medium selenomethionine only diet, arsenic and selenium existed in equimolar ratio in their regions of maximum accumulation (hotspots), and their spatial densities ranged between 0.01 and 0.02 μg cm^{-2}. However, in fish fed with high selenomethionine only diet, the concentration of arsenic exceeded selenium by 3 – 5 folds.

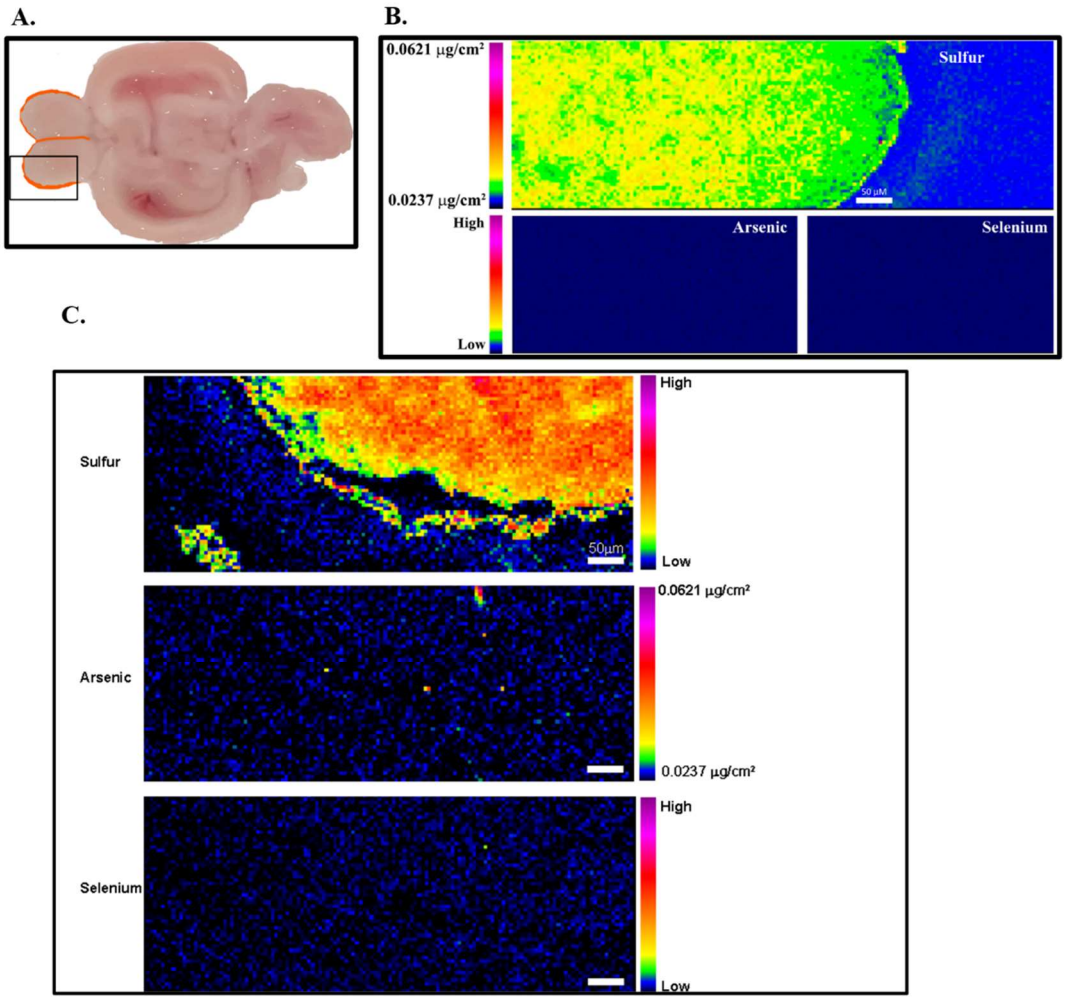


Figure 5.6: (A.) Transverse section of a fish brain embedded in OCT solution highlighting the telencephalic ventricle (TV) region in orange colour. The black rectangle indicates the region of the telencephalon scanned for the purpose of XFI. (B.) X-ray fluorescence images of the telencephalic region of the fish brain demonstrating the spatial localization of sulfur (top), arsenic (bottom-left) and selenium (bottom-right) in fish exposed to control diet (C.) X-ray fluorescence images of the telencephalic region of the fish brain demonstrating the spatial localization of sulfur (top), arsenic (middle) and selenium (bottom) in fish exposed to only arsenite supplemented diet. Each section was imaged with a step size of 5 µm and dwell time of 0.6 s. The concentration of arsenic and selenium are illustrated as areal density (µg cm^{-2}). The distribution of sulfur is presented to illustrate the area of telencephalon that was scanned.

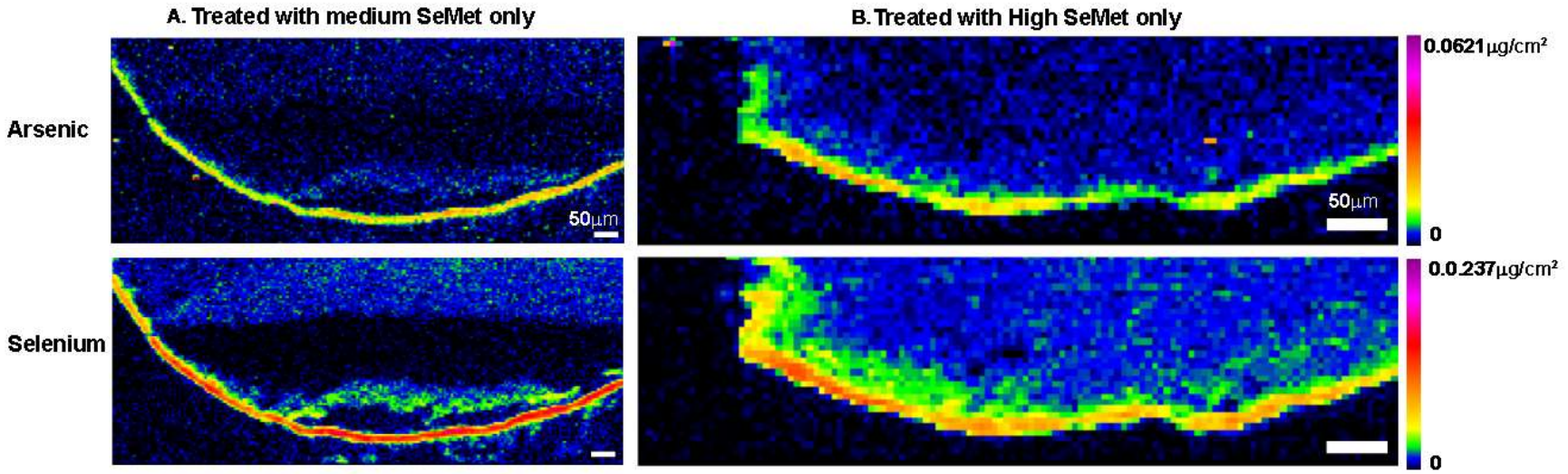


Figure 5.7: X-ray fluorescence images of the telencephalic region of the fish brain demonstrating the spatial localization of arsenic (top) and selenium (bottom) in fish exposed to diets supplemented with medium selenomethionine (left) or high selenomethionine (right) only. Each section was imaged with a step size of 5 µm and dwell time of 0.6 s. The concentration of arsenic and selenium are illustrated as areal density ranges (µg cm^{-2}).

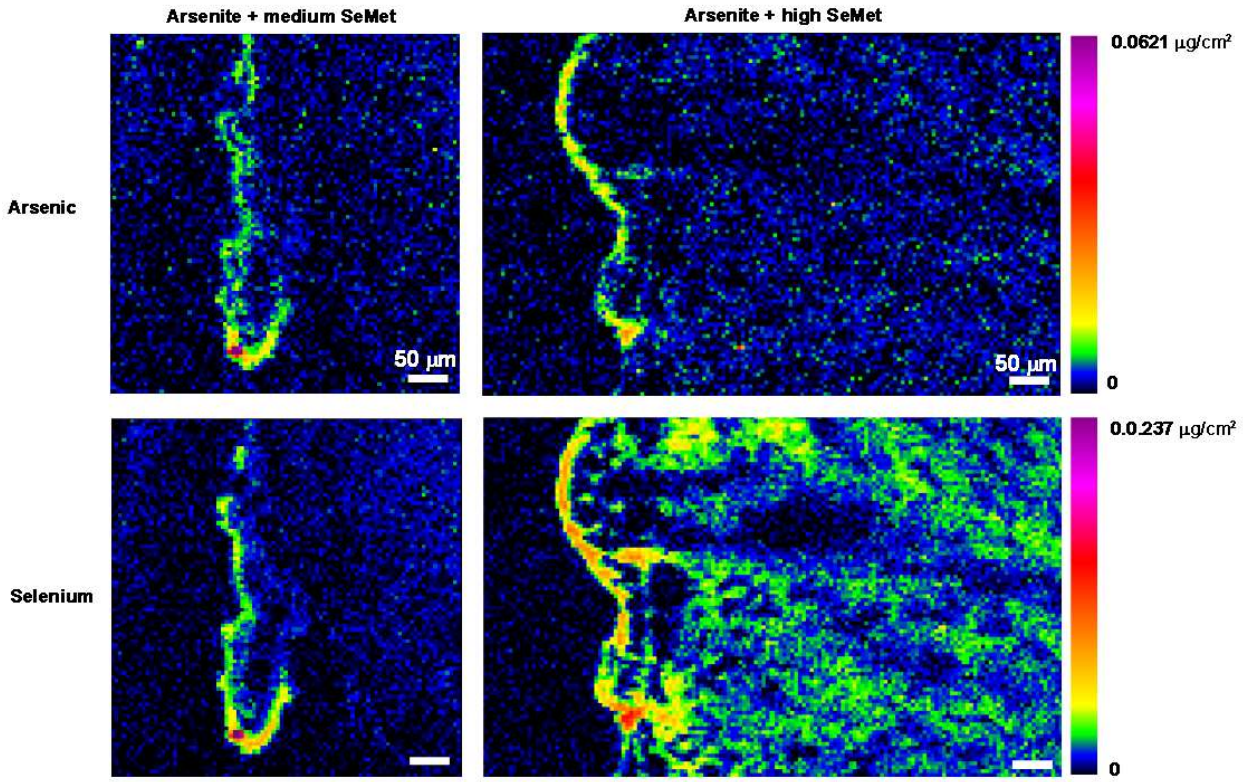


Figure 5.8: X-ray fluorescence images of the telencephalic region of the brain demonstrating the spatial localization of arsenic (top) and selenium (bottom) in fish exposed to dietary arsenite supplemented with medium selenomethionine (left) or high selenomethionine (right). Each section was imaged with a step size of 5 µm and dwell time of 0.6 s. The concentration of arsenic and selenium are illustrated as areal density ranges (µg cm^{-2}).

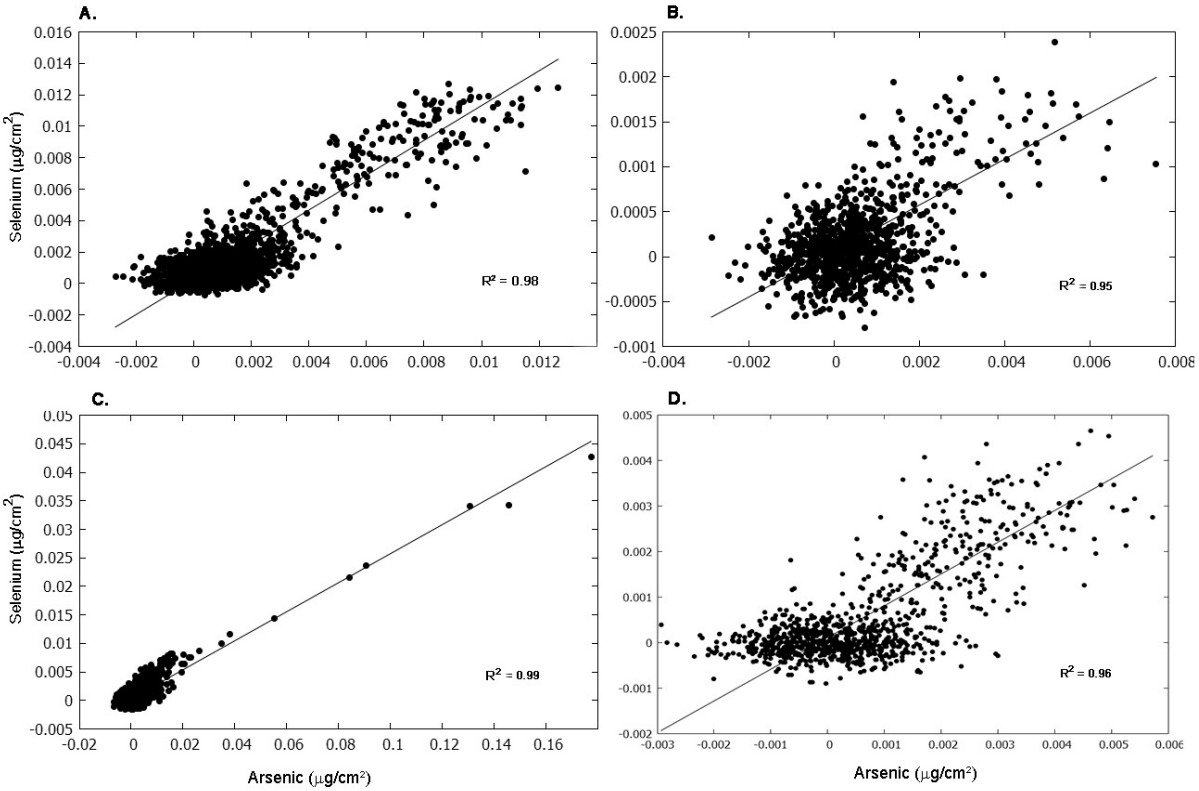


Figure 5.9: Correlation plots of arsenic *vs.* selenium for the representative images from the treatments, (A.) medium selenomethionine only, (B.) high selenomethionine only, (C.) arsenite + medium selenomethionine, and (D.) arsenite + high selenomethionine. The straight lines represent the lines of best fit and were obtained from the linear regression analysis of the XFI data. The correlation coefficient (R^2) of the data are mentioned in the respective graphical illustration.

5.4. Discussion

To the best of our knowledge, this is the first study to investigate the interaction between dietary arsenite and selenomethionine in any aquatic animal. Our main objective was to examine the modulatory effects of environmentally relevant doses of dietary selenomethionine on arsenite induced oxidative stress and its tissue-level accumulation in freshwater fish. The measured dietary selenium concentration (1.2 ± 0.7 μg g^{-1}) in the control diet was within the physiologically optimum range for dietary selenium requirement (0.1 – 4.7 μg g^{-1} dry wt.) in rainbow trout (Hodson and Hilton, 1983). The measured selenium concentration in the diets supplemented with medium and high levels of selenomethionine was in the range of 12 – 14 and 46 – 48 μg g^{-1} dry wt., respectively (Table. 5.3). Similar concentrations of selenium have also been reported in the aquatic food chain of the selenium contaminated sites in North American (Hamilton, 2004). Similarly, the macroinvertebrates living in the arsenic contaminated freshwater habitats have been reported to accumulate arsenic similar to the dietary concentrations used in this experiment (78 – 88 μg g^{-1} dry wt) (Phillips, 1990).

Table 5.3. Measured selenium and arsenic concentrations in different diets (μg g^{-1} feed dry weight).

Treatments	Se (μg g^{-1})	As (μg g^{-1})
Control	1.2 ± 0.7^{a}	3.4 ± 0.8^{A}
Arsenite only	1.3 ± 0.9^{a}	88.4 ± 2.7^{B}
Medium SeMet	12.2 ± 1.9^{b}	2.8 ± 0.6^{A}
High SeMet	46.1 ± 3.1^{c}	3.3 ± 0.9^{A}
Arsenite + Med SeMet	13.7 ± 1.2^{b}	78.4 ± 3.0^{B}
Arsenite + High SeMet	47.8 ± 2.1^{c}	81.6 ± 3.3^{B}

Values are mean ± SEM, $n = 3$ for the selenium and arsenic in the diets. Values with different alphabetic superscripts represent statistical difference in the concentration of a particular element between different treatments ($p < 0.001$). Similar superscripts represent no statistical difference ($p > 0.05$), as determined by 1-WAY ANOVA and *post hoc* analysis.

The HSI and *K*-factor are one of the most commonly used general biomarkers of stress and environmental pollution at the tissue-level (Al-Ghais, 2013; Chellappa et al., 1995). Under normal physiological conditions, fish converts the excess dietary energy in liver and adipose tissue in the form of glycogen and fat reserves (Enes et al., 2009). However, when under chronic stress, such as exposure to aquatic pollutants, the energy needed to maintain homeostasis may exceed the assimilated dietary energy. As a result, a fish may use its hepatic glycogen reserves to compensate for the dietary energy deficit, which in the long run reduces the mass of the liver. The reduced liver weight, in proportion to the total weight of the body, is reflected by a decreased HSI. In addition, a fish may also utilize its muscular fat deposits to meet the energetic cost of homeostasis, and result in a decreased *K*-factor. In the present study, we observed that chronic exposure to dietary arsenite, alone or in combination with medium or high selenomethionine, reduced the HSI (Table 5.1), and was therefore energetically stressful to the fish. It should also be noted that the there was a slight but modest decrease in the HSI due to chronic exposure to high dose of selenomethionine alone, which alludes to the mild toxic nature of the high dose of selenomethionine used in this study. The *K*-factor, in contrast, seemed to be a less sensitive indicator and reflected chronic stress only when fish were fed with diet supplemented with arsenite alone.

Cells have evolved an efficient anti-oxidative strategy to mitigate the deleterious effects of ROS that are generated during aerobic respiration; however, exposure to a toxic dose of trace elements, such as arsenic and selenium, can increase the rate of ROS production and overwhelm the anti-oxidative machinery. Reduced form of glutathione (GSH) is a part of cellular anti-oxidative machinery that readily neutralizes ROS by donating its electron, and during this process itself becomes oxidized to glutathione disulfide (GSSG). Since the cellular concentrations of GSH and GSSG are coupled with each other, a decrease in the concentration of GSH relative to GSSG

(reduced GSH:GSSG ratio) is often used as an indicator of oxidative stress. Previous literature indicates that toxicity due to chronic arsenite exposure is highly correlated with the reduced GSH levels in the tissues (Adeyemi et al., 2015; Sarkar et al., 2014; Xu et al., 2017). Therefore, maintenance of cellular GSH pool is of critical importance for defending against arsenite-induced toxicity. The cellular pool of GSH can be maintained by a combination of 3 processes (Chung and Maines, 1981; Jamwal and Niyogi, 2017; Richie et al., 2011): (i) the *de novo* synthesis of GSH, catalyzed by γ-glutamyl-cysteine ligase (ii) recycling of GSSG to GSH by glutathione reductase enzyme, and (iii) alleviation of oxidative stress by other anti-oxidants so that GSH is spared. Under chronic arsenite exposure, the hepatic thiol ratio can be reduced if the rate of oxidation of GSH overwhelms the GSSG recycling, or if the *de novo* synthesis of GSH is compromised. In the present study, a sharp decline in the hepatic GSH:GSSG ratio (Fig. 1) and a concomitant increase in the lipid peroxidation (Fig. 2) following the treatment with arsenite, alone or in combination with selenomethionine (medium or high), suggests increased cellular ROS accumulation. Although ours is the first study to provide a direct evidence of hepatic oxidative stress in response to dietary arsenite in a fish, previous experiments with waterborne arsenite have also reported hepatotoxicity in fish due to impaired redox homeostasis (Adeyemi et al., 2015; Greani et al., 2017a; Sarkar et al., 2014). In addition to generation of ROS, the arsenites, or the trivalent from of arsenic, also have strong affinity for the sulfhydryl groups (Shen et al., 2013). It is important to note here that the presence of a sulfhydryl group lends GSH its biological activity; therefore, binding of arsenite to this active group can inhibit the functional capacity of GSH and impair cellular redox balance. Moreover, the metabolic reduction of inorganic arsenic occurs only in the presence of the thiol compounds which can further reduce the cellular GSH concentration (Aposhian et al., 2004; Watanabe and Hirano, 2013). Therefore, exposure to the toxic doses of arsenic is expected to

contribute to the reduced GSH:GSSG ratio through a combination of processes involving its metabolism, generation of ROS, and direct binding with GSH.

We also observed a partial recovery of hepatic GSH:GSSG ratio with arsenite + medium selenomethionine dietary treatment (Fig. 5.1). It is known from previous studies that selenium, at optimal doses, can upregulate *de novo* synthesis of GSH (Chung and Maines, 1981; Jamwal and Niyogi, 2017; Richie et al., 2011). A modest increase in the hepatic GSH concentration with a dietary combination of arsenite and medium selenomethionine could be a result of selenium-dependent upregulation of GSH synthesis (Fig. 5.1). A similar increase in the thiol ratio and GSH concentration during short-term (24 h) exposure to low doses of selenomethionine has also been observed previously with rainbow trout hepatocytes (Jamwal et al., 2016; Jamwal and Niyogi, 2017). It should, however, be noted that the increase in the thiol ratio, in the present study, was not sufficient to prevent hepatic lipid peroxidation which was still elevated, as reflected by the MDA levels in the arsenite + medium selenomethionine treatment group (Fig. 5.2). Moreover, the dietary treatment of arsenite and high selenomethionine resulted in hepatic lipid peroxidation which was even higher than the arsenite only treatment group. This increase in lipid peroxidation was also accompanied by low GSH concentration and reduced thiol ratio in the trout liver. Based on an earlier estimate, long-term exposure to selenium exceeding 13 $\mu g\ g^{-1}$ diet could be toxic to rainbow trout (Hodson and Hilton, 1983). Previous studies also demonstrated that both organic and inorganic forms of selenium, beyond a certain threshold concentration, can induce oxidative stress in fish (J.-H. Kim and Kang, 2015; Kupsco and Schlenk, 2014; Thomas and Janz, 2016). Even though we did not observe any detrimental changes in oxidative stress parameters exclusively due to high selenomethionine exposure, it is possible that dietary exposure to arsenite and selenomethionine was more toxic than their individual effects. Previously, Decker (2015) has also

reported no change in the hepatic thiol contents and lipid preoxidation when rainbow trout were chronically exposed to similar high dose (~ 37 μg g^{-1} diet) of dietary selenomethionine as used in the present study. However, a significant reduction in the HSI due to high dietary selenomethionine (Table 5.1) in the present study suggests that the high dose of selenomethionine, in isolation, involved high metabolic costs but did not impair cellular redox homeostatic machinery. It is possible that combination of high selenomethionine with arsenite overwhelmed the liver redox homeostatic machinery and resulted in higher lipid peroxidation. Although there are no precedents demonstrating the additive effects of arsenite and selenomethionine on lipid peroxidation, mammalian literature suggests that they both can potentiate each other's toxicity through metabolic interactions (Kraus and Ganther, 1989; Levander, 1977; Styblo and Thomas, 2001; Thomas et al., 2001; Walton et al., 2003).

In addition to the GSH, the cellular redox homeostatic machinery also consists of anti-oxidative enzymes such as SOD, CAT, and GPx. Although the primary function of these enzymes is to protect against metabolically produced ROS (Benzie, 2000), they can also be induced in response to an external perturbation that causes increased production of oxyradical (Gül et al., 2004). Therefore, increase in the activities of these enzymatic antioxidants are also used as effective biomarkers of exposure to environmental pollutants. The enzyme SOD converts toxic superoxide ions ($O_2^{\bullet-}$) into H_2O and H_2O_2, whereas CAT and GPx catalyze the conversion of H_2O_2 into non-toxic H_2O and O_2 (Chelikani et al., 2004; Fukai and Ushio-Fukai, 2011; Mills, 1957). In the current study, the activities of all 3 antioxidative enzymes (SOD, CAT, and GPx) were upregulated in response to dietary arsenite treatment (Fig. 5.3). These results, in combination with previously discussed increase in lipid peroxidation and decreased GSH:GSSG ratio, suggest a compensatory response to the heightened oxidative stress caused by arsenite only dietary

treatment. Increase in the activities of anti-oxidative enzymes in response to arsenite exposure have also been observed in many other fish species, which is a defensive response to cope with the arsenite-induced oxidative stress (Bhattacharya and Bhattacharya, 2007; Greani et al., 2017a; J. H. Kim and Kang, 2015; Sarkar et al., 2017). Although the cellular compensatory responses may enable an organism to survive a chronic stress, upregulation of antioxidative enzymes is also energetically expensive and may become unsustainable over a long term (Sokolova et al., 2012). An increase in the energetic burden of such compensatory response is also reflected as a significant reduction of HSI and *K*-factor, in the present study with fish exposed to arsenite alone. Consistent with our findings from thiol ratio and lipid peroxidation assays, the combination of arsenite with selenomethionine also resulted in significant upregulation of the activities of the enzymes, in comparison to the control. Therefore, a marked increase in the hepatic lipid peroxidation, despite the upregulated activities of anti-oxidative enzymes suggest that the combination of arsenite and selenomethionine could have overwhelmed the hepatic anti-oxidative response mechanism. It should also be noted that the activities of SOD and GPx, following the treatment with arsenite + high selenomethionine was even higher than the arsenite only group, further suggesting an additive interaction between arsenic and selenomethionine at the dose levels used in the present study.

Biochemical and physiological responses to an environmental pollutant is highly dependent on its uptake and tissue burden, which is influenced by factors such as chemical speciation, water quality, exposure route, and temperature. Therefore, tissue-level accumulation of toxic element, and not merely its environmental presence, is a better indicator of hazardous exposure (McGeer et al., 2003). Presently, there is no consensus on how arsenite and selenomethionine may interact within a biological system, and a lack of understanding of the tissue-level accumulation of arsenic and selenium in their toxicological studies could be one of the

major reasons behind it. In the present study, we observed that chronic exposure to dietary arsenite, alone or in combination with selenomethionine, resulted in maximum accumulation of arsenic in the liver, followed by the kidney and muscle (Fig. 5.4). Similar tissue-specific pattern of arsenic accumulation was also reported in previous studies where fish were chronically exposed to arsenite (Jankong et al., 2007; J. H. Kim and Kang, 2015). Interestingly, supplementing the diet with selenomethionine also facilitated accumulation of arsenic in tissues even from the diets with only background level of arsenic (diets not supplemented with arsenite). In contrast, supplementation of arsenite in the diet dimnished the tissues-level accumulation of Se (Fig. 5). Selenium has been demonstrated to diminish the depuration rate of arsenic by interfering with As(III)-methyltransferase, inhibting the complete metabolic methylation of arsenic in rats, and thus exacerbating the overall toxicity of arsenic (Levander, 1977; Styblo and Thomas, 2001; Thomas et al., 2001; Walton et al., 2003). In the present study, we also observed that the accumulation of arsenic was highly correlated with increased oxidative stress and its detrimental effects (Table 5.2). Thus, it is likely that presence of selenomethionine increased the accumulation of arsenic and its toxicity *via* similar processes. This is the first study to demonstrate the modulatory effects of selenomethionine on tissue-specific accumulation of arsenic, and further investigations to determine the chemical speciation of arsenic and selenium in the tissues will be required to unravel the underlying mechanisms. It is important to note that even though the tissue-specific concentration of selenium was reduced in the presence of dietary arsenite, it was notably higher than the control levels. Therefore, deficiency of selenium cannot be attributed to the increased lipid peroxidation observed following dietary treatment with arsenite and selenomethionine.

Brain is the primary organ that regulates the functions of all other vital organs in vertebrates, including fish. Although the complexity and size may differ, the vertebrate brain is

composed of distinct anatomical regions with highly specialized functions, and accumulation of toxic metals in specific brain regions can impair their associated roles. Previous research has established that metals and metalloids can have preferential distribution pattern in both mammalian and fish brain (MacDonald et al., 2015; Tarohda et al., 2004). In the current study, we used X-ray fluorescence imaging (XFI) to understand the distribution pattern of arsenic and selenium in the telencephalon of the rainbow trout brain (Fig. 5.6 A). Telencephalon of a fish is part of frontal cerebellum, and is involved in a large variety of critical behavioural and learning functions (Gómez et al., 2011; Rodriguez et al., 2005). In the present study, we recorded arsenic deposition in fish brain following chronic exposure to arsenic, which is consistent with previous findings (Bonnineau et al., 2016; Li et al., 2017). Arsenic is known to cross blood-brain barrier through aquaglyceroporins and glucose transporter (GLUT1) (Hamdi et al., 2009; Liu et al., 2006a), and has detrimental effects on neuronal development, locomotion and behavioural functions (Baldissarelli et al., 2012; Li et al., 2009). Interestingly, supplementation of diets with medium or high doses of selenomethionine resulted in increased uptake of both arsenic and selenium in fish brain, even when the diets did not contain supplemented arsenite (Fig 5.7). Moreover, arsenic and selenium also demonstrated high degree of spatial co-localization and correlation among their concentrations in the telencephalic ventricle region (Fig 5.7 – 5.9), which is a region of high neuronal proliferation in vertebrate brain (Barbosa et al., 2016). Intravenous injections of arsenite and selenite were also shown to result in increased co-localization of arsenic and selenium in multiple organs including liver and brain of hamster (Ponomarenko et al., 2017). However, this co-localization of arsenic and selenium was found to occur due to the formation of seleno bis-(*S*-glutathionyl) arsenium ion, which was rapidly excreted out of the hamster's body through bile. In contrast, our results suggest that the presence of selenomethionine in diet above the physiological

requirement of fish (> 3 μg g^{-1} diet) does not facilitate arsenic depuration, and instead leads to increased bioavailability and retention of arsenite in the fish brain. The apparent discrepancy between these two studies might have occurred due to the differences in chemical speciation of selenium (selenite vs. selenomethoinine), exposure route (intravenous injection vs. dietary exposure), exposure duration (30 minutes vs. 30 days) and/or test organisms (rat vs. fish). Nevertheless, our results from the brain tissue are also consistent with increased accumulation of arsenic in the liver, kidney, and muscle tissue in rainbow trout in the presence of dietary selenomethionine.

Since we observed a high correlation between hepatic arsenic deposition and oxidative stress, it is possible that higher accumulation of arsenic in brain during co-exposure with selenium, can cause adverse effects on fish behaviour and cognitive functions. Our results also provide a *prima facie* evidence that use of selenomethionine as a therapeutic agent against environmental arsenic toxicity should be exercised with high caution, especially because of accumulation of arsenic and selenium in the regions of high neuronal proliferation. Further investigation are also needed to understand the mechanisms responsible for this complex arsenic-selenium inter-relationship.

5.5. Conclusion

The present study demonstrated that supplementation of diet with selenomethionine, above the physiological requirements (> 3 μg g^{-1}) , resulted in increased retention of arsenic in liver, kidney, brain, and muscle tissues. Exposure to a dietary combination of arsenite (~ 80 μg g^{-1} diet) with high selenomethionine (~40 μg g^{-1} diet) overwhelmed the hepatic anti-oxidative machinery and caused higher toxic effects. Selenomethionine was also able to increase the uptake and retention of arsenic in the neuronal proliferation zones of the telencephalon, which could also have

detrimental effects on the behavioural and cognitive functions of the fish. Although mammalian studies have demonstrated antagonistic effects between arsenic and selenium previously, synergistic or additive interaction between these two elements, as demonstrated in this study and few other mammalian studies, suggest that methylated forms of selenium can potentiate the toxicity of arsenite, rather than ameliorating it.

Chapter 6: General discussion

6.1. Introduction

Since the industrial revolution, anthropogenic activities have increased the rate of soil erosion and removal of minerals from natural deposits. The rate of deposition of metals and minerals into aquatic habitats in many parts of the world has exceeded their natural ability to recycle, hence causing the concentration of metals to reach toxic levels. For example, selenium, which is otherwise an essential metalloid in low doses, has reached toxic concentrations in many parts of the world because of effluents from coal and metal mining industry. In the recent years, there has been an increasing concern about the health and ecological consequences of environmental pollution from metals. Organisms living in natural habitats are often exposed to a combination of metals and metalloids which modulate each other's toxic effect. Selenium is one such element that is known to co-exist with a variety of other elements and influence their toxicity.

The overall objective of this study was to address a major knowledge gap in the field of environmental toxicology and understand the mechanistic underpinnings of how selenium modulates arsenite or cadmium-induced oxidative stress in freshwater fish, rainbow trout (*Oncorhynchus mykiss*). *In vitro* and *in vivo* experiments were conducted to understand the interactions at both cellular (Chapters 2 & 3) and organismal (Chapters 4 & 5) levels. For investigation at the cellular level, the hepatocytes of rainbow trout in primary culture were used to investigate the interactions between selenium and arsenite or cadmium. In the *in vitro* experiments,

I used selenite and selenomethionine as inorganic and organic forms of selenium to understand the chemical species-dependent effects. The investigations at the organismal level were conducted by exposing the fish to *Artemia* based diet supplemented with selenomethionine in combination with cadmium or arsenic for 30 days. The significant findings from the present research project are summarized in Figure 6.1.

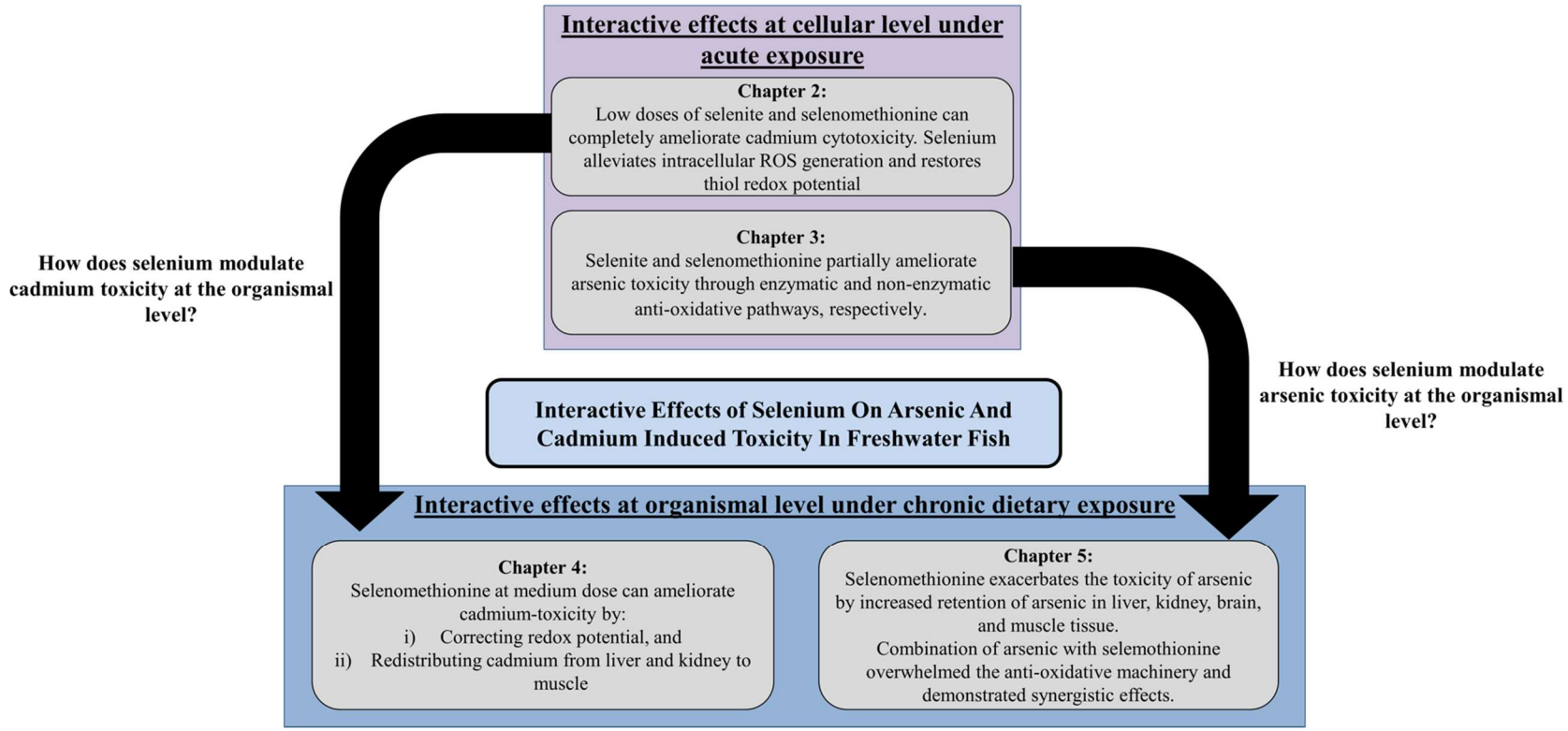


Figure 6.1: An overview of the significant findings of the present research project.

6.2. Interactions between selenium and cadmium (Chapters 2 and 4)

6.2.1. Interactive effects of selenium on cadmium-induced oxidative stress

A series of *in vitro* and *in vivo* experiments were conducted to characterize the hepatic pathways by which different chemical species [inorganic (selenite) *vs.* organic (selenomethionine)] and exposure doses of selenium modulated cadmium-induced oxidative stress at the cellular and organismal level. The results from *in vitro* experiments with hepatocytes of rainbow trout have shown that both, selenite and selenomethionine can antagonize cadmium-induced oxidative stress by enzymatic and non-enzymatic antioxidative pathways which include increased cellular thiol status (GSH:GSSH) and upregulated activities of anti-oxidative enzymes (CAT, SOD, and GPx), respectively. A combination of enzymatic and non-enzymatic pathways resulted in an overall reduction of intracellular ROS production and increased viability of hepatocytes (Chapter 2). However, the protective effects of selenium against cadmium-toxicity were observed only at relatively low to medium concentrations (25 – 50 μM), and a combination of 500 μM selenium with 100 μM cadmium elicited additive toxic effects.

Similar to the cadmium-selenium mechanistic interactions in the hepatocytes, my study has also demonstrated that selenium-cadmium antagonistic interaction also occurs at the organismal level (Chapter 4). Exposure to only cadmium (~ 40 μg g^{-1} diet) supplemented diet resulted in reduced hepatic thiol ratio and increased activities of antioxidative enzymes in rainbow trout. However, medium dose of dietary selenomethionine (~10 μg g^{-1} diet) in combination with cadmium restored thiol ratio and alleviated the heightened response of enzymatic antioxidative machinery. Increase in the activities of antioxidative enzymes in response to sub-lethal exposure to cadmium was likely a compensatory cellular mechanism to cope with the oxidative stress, which

is consistent with previous studies (Almeida et al., 2002; Basha and Rani, 2003; Cuypers et al., 2010; Nair et al., 2015). Hepatosomatic index (HSI) and condition (*K*) factor were used as the indirect measures of the energy status and general well-being of the fish (Chellappa et al., 1995). Upregulated activities of hepatic antioxidative enzymes in response to dietary cadmium was concomitant with reduced HSI and *K*-factor, suggesting that the exposure to cadmium was energetically taxing to the fish, and compromised the growth rate. On the contrary, a dietary combination of a medium dose of selenomethionine and cadmium improved HSI and *K*-factor, and therefore reduced the energetic burden of cadmium toxicity. No antagonistic interactions with cadmium were observed with the higher dose of dietary selenomethionine (~40 μg g^{-1} diet). In addition to the biochemical mechanisms, I also investigated the modulatory effect of dietary selenomethionine on the tissue-level accumulation of cadmium.

6.2.2. Modulation of tissue-specific distribution of cadmium by selenium (Chapter 4)

Exposure to dietary cadmium increased cadmium levels in kidney, followed by liver and muscle (Chapter 4). Although a dietary combination of cadmium and selenomethionine (both medium and high doses) reduced the overall cadmium burden in liver and kidney, the accumulation of cadmium in hepatic tissue of rainbow trout was still significantly higher in comparison to the control. Interestingly, despite the reduced hepatic cadmium levels due to selenomethionine treatment, cadmium-induced oxidative stress was ameliorated only by a medium dose of selenomethionine. This suggests that the hepatic oxidative stress was not dependent only on the cadmium accumulation. A dietary combination of cadmium with high selenomethionine resulted in higher hepatic selenium accumulation in comparison to the cadmium and medium selenomethionine treatment. Since selenium beyond a threshold level can also cause oxidative stress (Kupsco and Schlenk, 2014; Thomas and Janz, 2016), it is possible that higher accumulation

of both cadmium and selenium in the liver following the dietary treatment with cadmium and high selenomethionine could have contributed towards hepatic oxidative stress in an addtive manner. These results are consistent with the findings from my *in vitro* experiments where a high concentration of selenium had additive effects with cadmium and resulted in higher cytotoxicity. Therefore, the tissue levels of both selenium and cadmium appeared to have contributed towards oxidative stress.

Overall, the results support my original hypothesis that selenium will modulate cadmium induced toxicity by ameliorating oxidative stress in a dose specific manner, with antagonistic interactions at low to intermediate exposure levels and additive/synergistic effects at higher doses.

6.3. Interactions between selenium and arsenite (Chapters 3 and 5)

6.3.1. Interactive effects of selenium on arsenite-induced oxidative stress

Experiments on hepatocytes of rainbow trout in primary culture and a 30-days feeding trial were conducted to unravel the mechanisms by which selenium modulates the arsenite-induced oxidative stress in freshwater fish (Chapters 3 and 5). Using a series of *in vitro* experiments, which also involved a suite of pharmacological exposures, I have shown that selenite and selenomethionine mediate their protective effects against arsenite-induced oxidative stress through separate biochemical pathways (Chapter 3); however, the protective effects were only partial. Selenite ameliorated arsenite-induced oxidative stress primarily by upregulating the enzymatic antioxidants (especially SOD), whereas selenomethionine exerted its antioxidative response essentially by upregulating the non-enzymatic antioxidative pathway that involves GSH. The primary function of enzymatic and non-enzymatic antioxidative pathways was to prevent oxidative stress by scavenging of arsenite-induced ROS. In addition to chemical species-specific effects, both selenite and selenomethionine also showed a dose-specific effect against arsenite (100 μM)

toxicity. Selenite alleviated arsenite-induced reduction in cell viability at 10 – 20 µM concentration level, while selenomethionine was most effective at 10 µM against arsenite. Moreover, selenite was more protective than selenomethionine against arsenite toxicity.

While the *in vitro* experiments revealed an antagonistic interaction between arsenite and selenium in fish hepatocytes, a dietary combination of medium (~10 µg g^{-1} diet) or high dose (40 µg g^{-1} diet) of selenomethionine with arsenite (~80 µg g^{-1} diet) resulted in increased toxic effects and overwhelmed the hepatic anti-oxidative machinery (Chapter 5). Diet supplemented with medium selenomethionine and arsenite marginally improved the hepatic redox potential, and it was not sufficient to alleviate the degree of arsenite-induced lipid peroxidation which was still high in comparison to the control treatment group. The lipid peroxidation assay also revealed that chronic exposure to arsenite in combination with high selenomethionine was more toxic than treatment with arsenite or high selenomethionine exclusively. The combination of arsenite and selenomethionine also elicited a compensatory response in the form of upregulated the activities of antioxidative enzymes (CAT, SOD, GPx) to cope with the increased oxidative stress (Bhattacharya and Bhattacharya, 2007; Greani et al., 2017b; J.-H. Kim and Kang, 2015; Sarkar et al., 2017). Similar to the results from selenium and cadmium experiments, the compensatory upregulation of enzymes was also correlated with reduced HSI and *K*-factor, suggesting poor energy status and compromised growth performance of the fish.

6.3.2. Modulation of tissue-specific distribution of arsenic by selenium (Chapter 5)

In my study, I observed that supplementing the diet with selenomethionine increased the accumulation of arsenic in tissues. In contrast, the presence of arsenite in the diet reduced tissues-level accumulation of selenium (Chapter 5). These results are in agreement with previous mammalian studies that demonstrated that selenium could reduce the depuration rate of arsenic

through metabolic interactions (Levander, 1977; Styblo and Thomas, 2001; Thomas et al., 2001; Walton et al., 2003). Interestingly, hepatic arsenic (not selenium) concentration was also highly correlated with oxidative stress and lipid peroxidation (Table 5.2). Therefore, it is possible that the increased oxidative insult in the hepatic tissues of the fish fed with a combination of arsenite and selenomethionine is due to a selenium mediated increase in arsenic accumulation.

I have also used synchrotron-based X-ray fluorescence imaging (XFI) to understand the distribution pattern of arsenic and selenium in the telencephalon of the rainbow trout brain (Chapter 5). Arsenic is known to cross the blood-brain barrier (Hamdi et al., 2009; Liu et al., 2006b); however, the effects of dietary selenium on the accumulation pattern of arsenic in the brain have not been investigated previously. I did not observe any measurable quantities of selenium or arsenic in the brains of the fish fed with control diet (no supplementation with arsenic or selenomethionine). However, supplementation of the diet with selenomethionine above the physiologically essential dose of selenium (> 3µg g^{-1} diet) increased the retention of arsenic in the fish telencephalon. Moreover, arsenic and selenium in the brain were also highly co-localized in the ventricle region of the telencephalon, which is the region of high neuronal proliferation. Interestingly, when the feed was supplemented with medium selenomethionine, arsenic and selenium were present in an equimolar ratio at the accumulation hotspots of the brain. Also, supplementing the feed with high selenomethionine increased the arsenic concentration in the brain, which exceeded selenium by 3 – 5 folds. These results are consistent with the selenium-dependent increased accumulation of arsenic in liver, kidney, and muscle tissues.

Overall, arsenite toxicity in fish occurs by disruption of redox homeostasis, which can be partially ameliorated by selenite and selenomethionine *via* enzymatic and non-enzymatic antioxidative pathways, respectively (Chapter 3). However, the interaction between arsenite and

selenium can be influenced by the metabolic interactions at systemic level, and selenium may actually exacerbate the toxic effects of arsenite under environmentally relevant exposure scenarios (Chapter 5). In conclusion, my research provides novel insights into the biochemical basis of how selenium can influence the biological effects of toxic trace elements, such as cadmium and arsenic, by interacting with them at cellular and systemic levels in a dose and chemical species dependent manner.

6.4. Future research perspectives and recommendations

Arsenic, cadmium, and selenium are all priority aquatic pollutants and often co-exist in aquatic habitats. Therefore, the understanding of their interactive effects on the health of aquatic animals is important and can be used to develop better regulatory guidelines. My research has provided novel and important mechanistic information on the interactive effects of selenium on cadmium and arsenic toxicity in freshwater fish. However, the molecular mechanisms involved in uptake of arsenic or cadmium in the presence of selenium still remain unresolved. For example, selenium was found to antagonize tissue level accumulation of cadmium which was also concomitant with reduced oxidative stress. Since, uptake of cadmium is highly dependent on ion transport channels such as DMT1 and L-type Ca^{+2} channel (Kwong and Niyogi, 2009), the effects of selenium on ion-transport channel mediated uptake of cadmium can be investigated. Similarly, intestinal uptake of arsenic in mammals has been shown to depend on glucose transporters, aquaporins, and phosphate transporters (Calatayud et al., 2012). Investigations are also required to understand how selenium, beyond a physiologically essential concentration, causes increased uptake of arsenic in various tissues.

In my research, co-localization of selenium and arsenic in brain tissues (Chapter 5) suggested formation of biological complex. It has been shown that arsenic and selenium, in the

presence of GSH, can form metabolic intermediates in the blood (Gailer, 2007; Korbas et al., 2008). It is possible that similar metabolic intermediates are formed in fish which need further investigation. Techniques such as synchrotron based X-ray absorption spectroscopy can detect novel biological complexation in the biological samples (Gailer et al., 2000). Also, selenium is being proposed as a therapeutic agent against arsenic toxicity. However, I have provided evidence suggesting that selenium may accentuate arsenic toxicity. Therefore, more research is needed to understand the metabolic relationship between arsenic and selenium.

Exposure to toxic metals and metalloids is known to cause genotoxicity in fish (Barbosa et al., 2010). However, the mutagenic assays have revealed that most of these metals and metalloids are not redox reactive at environmentally relevant concentrations and therefore cannot cause genetic mutations (Bertin and Averbeck, 2006; Valverde et al., 2001). More recently, the deleterious effects of environmentally toxic trace elements have been linked with the epigenetic changes and not with genetic mutations (Nye et al., 2014). On the other hand, selenium is known to modulate one-carbon metabolism and DNA methylation pattern, and prevent epigenomic aberrations in mammals (Speckmann et al., 2017). Therefore, evaluation of the protective role of selenium against epigenetic interferences from arsenic and cadmium could be an interesting prospect which has not been studied in any aquatic animal so far.

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APPENDIX[1]

Synchrotron-based X-ray fluorescence imaging (XFI) and its biological applications

The X-ray fluorescence imaging (XFI) is one of the most popular synchrotron-based techniques that is used to quantify and determine the distribution of multiple elements simultaneously in a biological sample with a high rate of precision (Fahrni, 2007; Paunesku et al., 2006). X-ray fluorescence imaging involves the use of high energy photons in the hard X-ray region (>1 keV) to remove the electrons of metals, as photoelectrons, from their inner core electronic states (Fig. 1.4 a). An electron from the higher energy shell then loses energy in the form of a photon to replace the space in the core-shell that was vacated due to the removal of the photoelectron (Fig. 1.4 b). The energy of the photon emitted during this process is equal to the difference in binding energies of the two shells involved in the transition (Fig. 1.4 b) (Fahrni, 2007; Jones, 1988; Paunesku et al., 2006). This energy is characteristic for each element and is also known as the 'fluorescence' (Jones, 1988). The synchrotron-based XAS provides an excellent alternative to other analytical techniques that require extensive extraction and processing of tissues (B'Hymer and Caruso, 2006; Lakshmi Priya and Geetha, 2011). Due to minimal handling and processing requirements, the structural and histological integrity of the tissue can be maintained, and the spatial distribution of elements can be determined. This is particularly useful for determination of the distribution of multiple elements in delicate and structurally heterogeneous tissues, such as the brain (Surowka et al., 2015). The XFI has also been successfully used to determine the spatial distribution of elements in structurally heterogeneous tissues of fish. For example, distribution of mercury in the lens and photoreceptors of eye was determined in the eye

[1] This chapter includes supplementary data and general introduction to X-ray fluorescence imaging technique. The figure number is presented as Cx.Sy format, where 'Cx' is the chapter number and 'Sy' indicates individual figure. Tables are presented as Cx.Ay format where 'Cx' indicates the chapter number and 'Ay' is the individual table number.

of the fish exposed to methylmercury (0.5 μM) for 48 h (Korbas et al., 2013). Similarly, the effect of maternal transfer of selenium was studied by visualizing the deposition of selenium in various regions of larvae obtained from zebrafish females fed with selenomethionine spiked diet for 25 days (Choudhury et al., 2015). In my research, I have used XFI to determine the spatial deposition of arsenic and selenium in the telencephalic region of the fish brain in response to the interactions between dietary arsenite and selenomethionine. Telencephalon is a highly specialized region of brain that is responsible for various behavioral and cognitive functions (Gómez et al., 2011; Rodriguez et al., 2005), and the spatial distribution of arsenic or selenium in the telencephalon has not been investigated before.

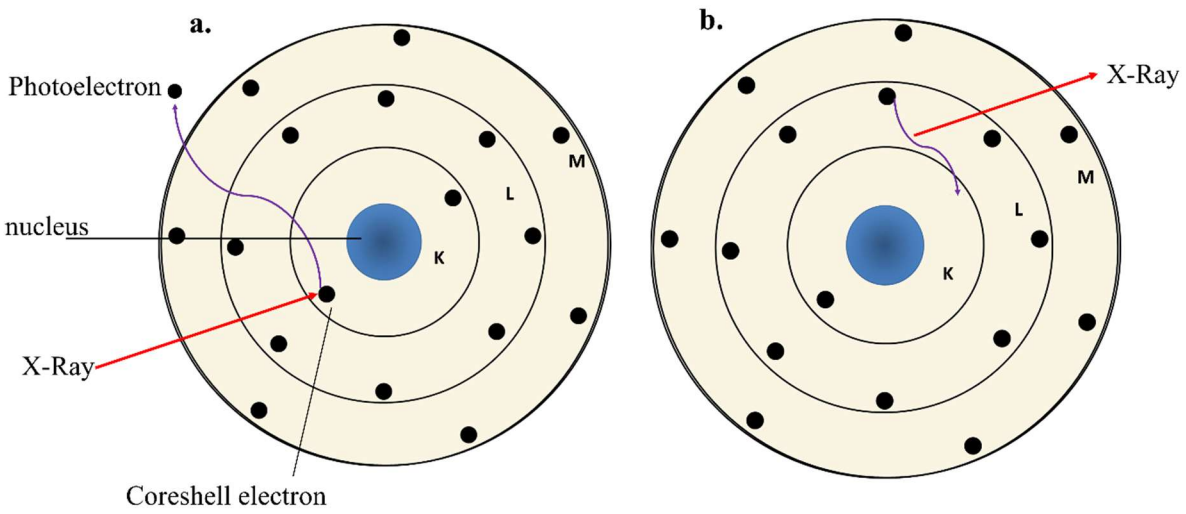

Figure C1 S1: A schematic diagram using Bohr's atomic model to illustrate the principle of X-ray fluorescence. (a.) High energy X-ray beam excites the electron from the core-shell; (b.) an electron from the higher-shell replaces the place vacated by the ejected core-shell electron and emits an X-ray of a characteristic wavelength whose energy is equal to the difference in binding energies of the two shells involved in the transition (Figure modified from Fahrni, 2007).

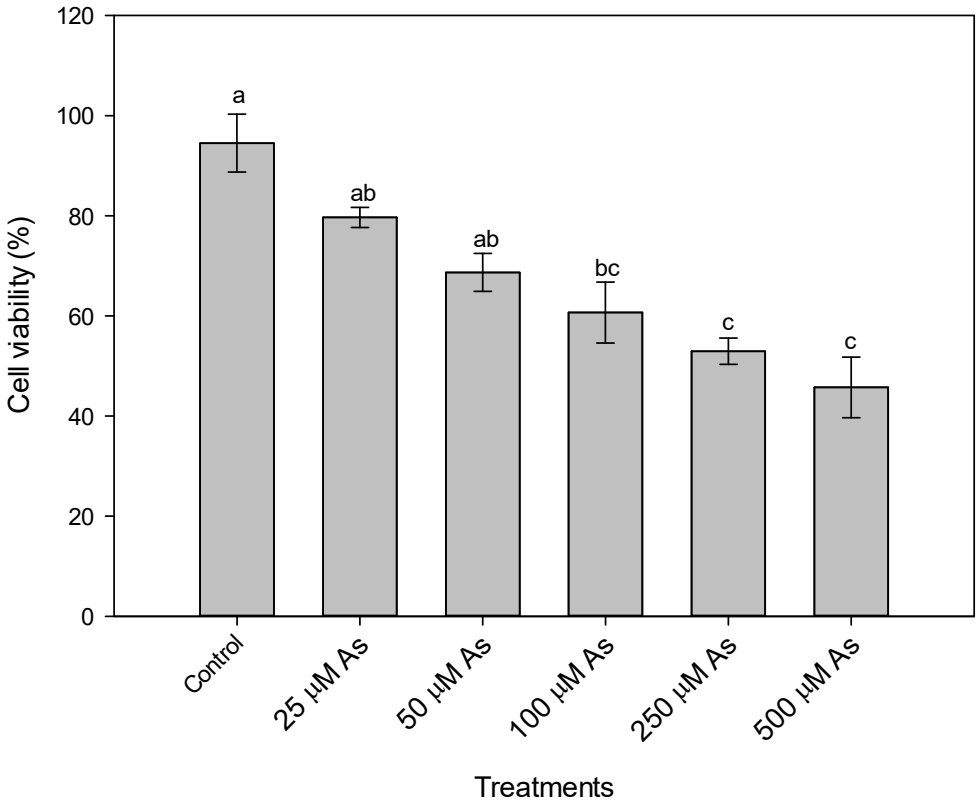

Figure C3.S1: Changes in the viability of rainbow trout hepatocytes when exposed to 25 µM – 500 µM arsenite (As). Data are presented as mean ± S.E.M. (n = 4), where n represents the number of true independent measurements, each performed with cells isolated from a different fish. Mean values with different letters are statistically significant ($p<0.05$).

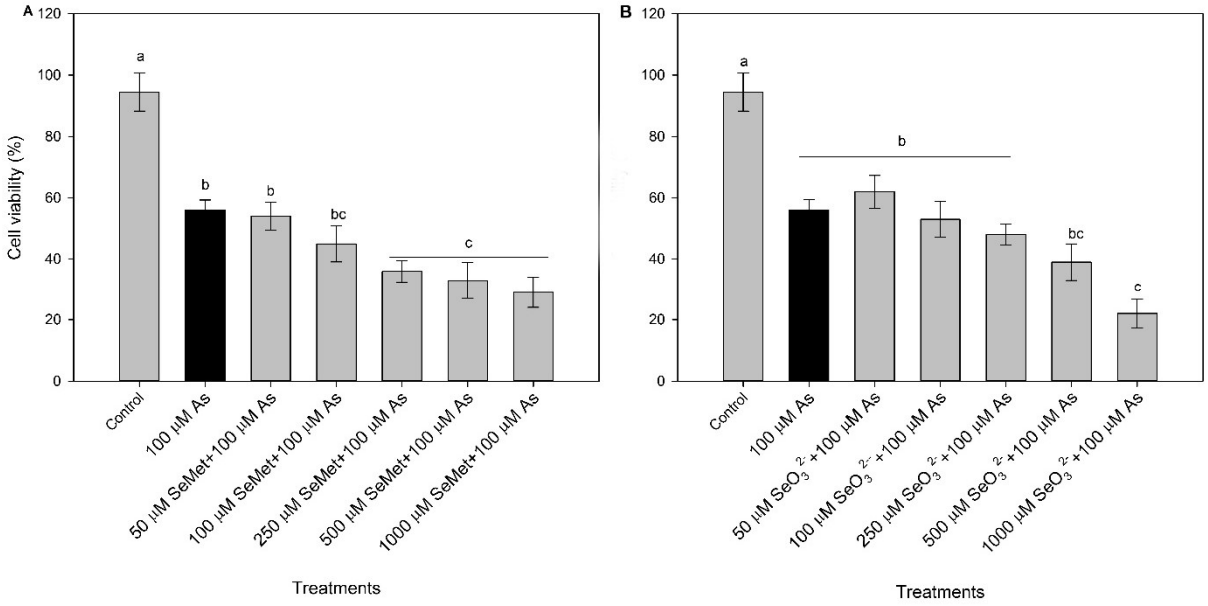

Figure C3.S2: Changes in the viability of rainbow trout hepatocytes when exposed to 100µM arsenite (As), alone or in combination with different concentrations (50µM – 1000µM) of (A) selenomethionine, or (B) selenite. Data are presented as mean ± S.E.M. (n = 4), where n represents the number of true independent measurements, each performed with cells isolated from a different fish. Mean values with different letters are statistically significant ($p<0.05$).

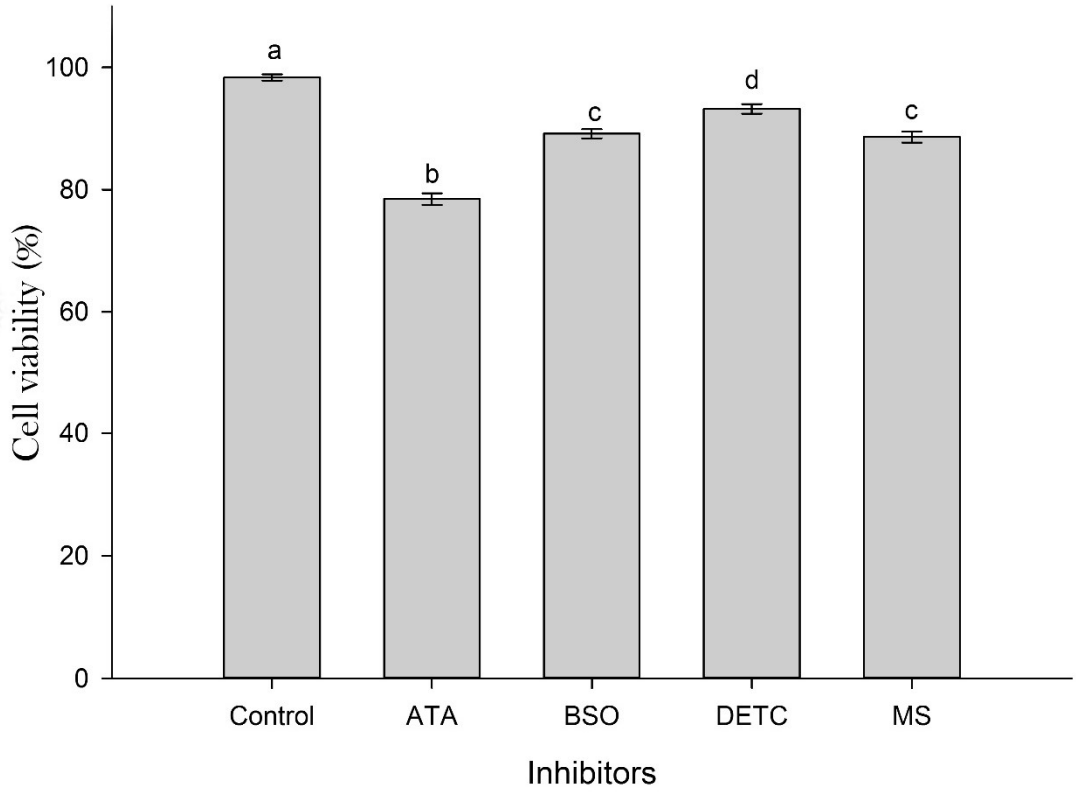

Figure C3.S3: Changes in the viability of rainbow trout hepatocytes when exposed to 0.5 mM concentration of ATA (CAT inhibitor), BSO (GSH inhibitor), DETC (SOD inhibitor), or MS (GPx inhibitor) for 24 h. Data are presented as mean ± S.E.M. (n = 5), where n represents the number of true independent measurements, each performed with cells isolated from a different fish. Mean values with different letters are statistically significant ($p<0.05$).

Table C3.A1: Changes in activities of anti-oxidative enzymes in rainbow trout hepatocytes upon exposure to 0.5 mM concentration of ATA (CAT inhibitor), DETC (SOD inhibitor), or MS (GPx inhibitor) for 24 h. Data are presented as mean ± S.E.M. (n = 5), where n represents the number of true independent measurements, each performed with cells isolated from a different fish. The effects of the pharmacological inhibitors on the treated cells, with respect to the control treatments, were evaluated by t-test. The mean values with asterisk (*) differ significantly from the control treatments ($p<0.05$).

Treatment	Change in the activity of enzymes (% of control)
ATA (0.5 mM)	63.34 ± 6.2*
DETC (0.5 mM)	52.7 ± 9.0*
MS (0.5 mM)	57.3 ± 7.6*