Testing British Columbia's water quality guidelines as a mixture of four important contaminants, hardness, exposure time, and species effects

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Abstract

Provincial water quality guidelines are established in order to prevent detrimental effects of a single toxicant from affecting the health of resident aquatic life. However, the elevation of pollutants in freshwater can occur from many sources simultaneously and interact to form mixtures. In this study, three common freshwater species, *Daphnia magna, Hyalella azteca* and *Oncorhynchus mykiss*, were exposed to cadmium, selenium, nitrates and sulphates as a mixture at concentrations the same as British Columbia's provincial water quality guidelines (BC WQG) for the protection of aquatic life with hard (250 mg/L as $CaCO_3$) and soft (50 mg/L as $CaCO_3$) water conditions. For all three organisms, both acute (48 hour) and chronic (21 day) exposures were used to examine the four contaminants and their mixture at maximum and average BC WQG concentrations. In the short term exposures, the only treatment that was harmful was cadmium, which had a 43% ($p = 0.115$, $n = 3$) and 64% ($p < 0.0001$, $n = 5$) mortality for *D. magna* in soft and hard water respectively. The toxicity of the four part mixture (including cadmium) was reduced, due to the antagonistic effect of selenium on the toxicity of cadmium. During a chronic exposure, the mixture was more (to *D. magna*) or less hazardous (to *H. azteca* and *O. mykiss*) than single contaminants; leading to the conclusion that pollutants can have a different overall effect when simultaneously exposed for longer periods of time. Overall, the interactions between pollutants in a complex mixture should be considered when deriving water quality guidelines. To provide appropriate protection of the environment, these complex interactions should be further investigated with representative species in the BC ecosystem.

Lay Summary

Faculty and students in the Department of Biology are bound together by a common interest in explaining the diversity of life, the fit between form and function, and the distribution and abundance of organisms. Aquatic ecotoxicology is the study of lethal or sublethal effects of substances on aquatic organisms in an ecosystem. It is important for government agencies to establish guidelines for industries, since industrial activity, such as mining, can introduce contaminants into freshwater systems. My aim was to identify the effects of a simultaneous exposure of contaminating substances on freshwater species. As opposed to traditional toxicity testing, which relies upon a single substance exposure, performing these tests using a mixture of contaminants is more realistic to actual freshwater environments. I used three common freshwater species, the water flea (*Daphnia magna),* scud (*Hyalella azteca*)*,* and rainbow trout (*Oncorhynchus mykiss*), to study the effects of hardness on a mixture of British Columbia's short and long term water quality guidelines for four substances: cadmium, selenium, nitrate, and sulphate. Under acute exposure conditions, *Hyalella azteca* and *Daphnia magna* are protected from a mixture of these four contaminants at the acute water quality guideline, in hard and soft water. However, chronic conditions (especially with *D. magna*) reveal that interactions, either harmful or protective, do occur between contaminants, influencing the overall toxicity of a mixture to aquatic organisms. These findings concerning mixture toxicity and hardness can add perspective on making appropriate water quality guidelines to protect our freshwater ecosystems. These results should not be used directly for the development of official water quality objectives, as these experiments require further replication and trials with relevant species to the jurisdiction.

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List of Abbreviations

BC	British Columbia
CA	concentration addition
$CaCO_3$	calcium carbonate
CEPA	Canadian Environmental Protection Act
Cd	cadmium
$CdCl_2$	cadmium chloride
CdO	cadmium oxide
CdS	cadmium sulphide or greenockite
CCME	Canadian Council of Ministers of Environment
D. magna	*Daphnia magna*
EC50	concentration of a substance to cause a 50% effect on a population of organisms
EPS	environmental protection series
H. azteca	*Hyalella azteca*
IA	independent action
LC50	concentration of a substance to cause 50% mortality on a population of organisms
Na_2SeO_4	sodium selenate
Na_2Se	sodium selenide
Na_2SeO_3	sodium selenite
NH_4^+	ammonium ion
NO_2^-	nitrite ion

NO_3^-	nitrate ion
MNR	Ministry of Natural Resources
OMNR	Ontario Ministry of Natural Resources
O. mykiss	*Oncorhynchus mykiss*
P. subcapitata	*Pseudokirchneriella subcapitata*
SAM	standard artificial media formulated for culturing *Hyalella azteca*
Se	selenium
SO_4^{-2}	sulphate ion
TDS	total dissolved solids
US EPA	United States Environmental Protection Agency
WQG	water quality guideline

1.0 Introduction

Contemporary water quality guidelines were created to protect the entirety of local aquatic communities from being exposed to harmful pollutants that could cause detrimental ecological effects. To establish these water quality objectives, supporting data were compiled, which entailed freshwater species being exposed to a single substance of concern. Due to the increased input of pollutants from various sources into aquatic environments, a multiple contaminant exposure is considered a more realistic scenario. In a study by Carvalho et al. (2014), it is suggested that ecosystem level imbalances could be caused by an exposure to a mixture of substances at environmental quality standard concentrations, based a water protection strategy drafted by the European Union. Effective control of ecological impacts including mixture effects are an important issue, especially for water quality guidelines that are being reviewed for highly industrious areas, such as in the Canadian province of British Columbia (BC). In order to maintain effective protection for the aquatic ecosystems of BC, water quality guidelines intended for areas subject to high contamination should be as effective when enforced during a simultaneous exposure as they are when presented individually.

Freshwater quality considers a variety of elements (eg. pollutants, pH, temperature, nutrient content, and microorganisms) that impact the persistence of sensitive inhabitant aquatic species populations. The physical parameters of water quality considers the measurement of chemical elements found in freshwater such as dissolved sodium, calcium, and potassium ions, which are required for vital biological processes affecting the survival of aquatic organisms (Mount et al. 1997; Rand 2001). Toxicity, the

disruption of the vital biological processes in an organism, can occur upon exposure to a substance (usually referred to as a toxicant) and will result in either impairment to function or death. The input of toxic substances, such as cadmium, selenium, nitrates or sulphates, released into a freshwater environment affects aquatic organisms inhabiting the area through direct exposure and accumulation in their tissues by a contaminated diet (Azcue et al. 1995; Paquin et al. 2002; Wiramanaden et al. 2010). Local geological processes and human activities determine the concentrations of toxic substances, which may be ultimately detrimental to aquatic health (Schindler 1987).

Natural geological processes can introduce metals and other ions to a freshwater system depending on a number of environmental factors. The presence of geological formations in contact with a freshwater body can, by the process of erosion, alter dissolved mineral composition and concentrations (Golterman 2011). Geological formations like the Rocky Mountains in BC can increase the hardness of freshwater lakes through weathering of limestone rock (Ford 1971; Renaut 1990). Limestone bedrock (containing calcium and magnesium ions) is located underneath water bodies, which contributes to the high water hardness of the Fraser River in BC (Shaw and Tuominen 1998).

The province of BC is known for its industry for processing coal and metallic-mineral based commodities, surpassed only by Quebec and Ontario in Canada (Natural Resources Canada 2013). The industrial processing of these commodities can introduce inorganic and organic contaminants into the environment. The province of BC has many industrial and agricultural sources of water pollution including coal mining, agriculture,

and forestry projects, which elevate the concentration of dangerous substances in water (BC Ministry of Environment 2001). British Columbia is a leading producer of metallurgical coal in Canada (Natural Resources Canada 2013). A coal mine can produce harmful effluent discharge through four processes: acid mine drainage, heavy metal contamination and leaching, chemical processing, and erosion and sedimentation (Lottermoser 2010). Acidification and contamination of freshwater ecosystems causes stress and decreased survival of local biota inhabiting areas close to these mining sites (Zocche et al. 2014; Kunz et al. 2013). To protect resident aquatic life, the Ministry of Environment of BC regulates the contamination of freshwater bodies in BC through environmental monitoring and research under the guidance of environmental legislature, mainly through the Canadian Environmental Protection Act (CEPA 1999).

To represent potential toxicological effects to the ecosystem laboratory studies use biological indicator species, which have little tolerance to water contaminants, in toxicity assays (Niemi and McDonald 2004). Under stressful or lethal conditions caused by toxicants, biological indicators used in toxicity assays will demonstrate poor physiological function or mortality, indicating that there is a significant decline in animal health. Studies in toxicity focus on organisms that are ubiquitous in nature and can be cultured easily in a controlled laboratory setting. Three freshwater species sensitive to water contamination, *Daphnia magna*, *Hyalella azteca* and *Oncorhynchus mykiss,* are commonly found among aquatic ecosystems throughout Canada and the province of BC (Borgmann and Norwood 1995; Bos et al. 1999; Environment Canada 1998). These organisms are considered standard test organisms in environmental toxicity assays for

detecting changes in freshwater quality, where results of these assays are pertinent for the derivation of environmental quality objectives (Rand 1998).

1.1 Water quality guidelines (WQG)

In compliance with CEPA, water quality guidelines (WQG) were established and implemented at the federal and provincial levels, and are currently used to effectively manage the concentrations of contaminants and changes to aquatic environments affected by human activity. Water quality guidelines are defined by the BC Ministry of Environment in Canada as "a maximum and/or minimum value for a physical, chemical or biological characteristic of water, and applicable province-wide. They should not be exceeded to prevent specified detrimental effects from occurring to a water use, including aquatic life, under specified environmental conditions" (Meays 2012). In Tables 1 to 4, the WQG of provincial, federal, and international WQG are listed for the four contaminants included in this study: cadmium (Table 1), selenium (Table 2), sulphates (Table 3), and Nitrates (Table 4). These WQG are used to responsibly and effectively manage environmental changes in freshwater quality (eg. pH, hardness, conductivity, etc.) beyond acceptable regional or national levels in order to protect human health and aquatic life.

The concentrations that are proposed for the BC guidelines depend on the most recent and relevant scientific information of toxicants in an aquatic ecosystem, while also considering the background water quality of water bodies within the area of concern (CCME 2007; Meays 2012). According to the CCME (Canadian Council of Ministers of Environment), pre-existing guidelines were used or scientific data were evaluated based

on their determinations of lethality to sensitive aquatic organisms and adjusted to meet the water quality conditions of Canadian water bodies. It may also be necessary to incorporate toxicological data referring to humans, especially if the consumption of water is being considered for the derivation of water quality standards, such as those defined by the United States Environmental Protection Agency (US EPA) (US EPA 2013). The concentration of a substance determined by this scientific information and background data provides an appropriate basis for WQG concentrations that will protect aquatic organisms at all trophic levels (including fish, invertebrates, and plants) and potentially humans. Despite the use of scientific data to support the derived WQG, the concentration limit for a lethal substance is then decreased by an uncertainty factor, to account for variability within the data and possibly undiscovered effects. These guidelines are set at the limit for which all resident biota at all life cycle stages are protected from detrimental changes to water quality. In specific areas within BC, where the aquatic community assemblage and background water quality parameters (e.g., pH, dissolved oxygen, and salinity) are atypical from those provincially, the MOE will determine if a modified WQG or a site-specific water quality objective can be appropriately applied (Meays 2012). Overall, the goal of BC provincial WQGs is to prevent toxic effects in residential biota by controlling concentrations of lethal substances from exceeding a safe concentration in the environment (Meays 2012).

This study investigates the duration for maximum and average BC WQGs, which are safe concentrations for short-term and long-term exposures to pollutants and should have no effect on the health of freshwater species. Short-term maximum WQGs or objectives in BC, which are intended to protect freshwater aquatic biota from lethal

toxicants within a zone of initial contamination for a short period (48 to 96 hours) are investigated for three freshwater species. These WQG are purposely constructed for controlling the effects from extremely contaminated waters (such as within the dilution zone of a mining site), where significant harm may occur to aquatic life if the guidelines are exceeded. A longer exposure, in contrast to acute WQG, are called chronic or continuous, or average WQGs, which are designed to protect aquatic organisms over an extended exposure period usually over the course of a complete life cycle (Meays 2012). Acute and chronic WQGs are generated for many individual contaminants such as cadmium, selenium, nitrates and sulphates.

1.1.1 Cadmium

Cadmium is a metallic element found at very low (trace) dissolved concentrations in freshwater lakes, averaging from less than 0.1 to 8.6 µg/L in BC lakes (Environment Canada 1994; CCME 2014). Naturally occurring cadmium is found in compounds such as greenockite (CdS), cadmium chloride ($CdCl_2$), and cadmium oxide (CdO) (Morrow 2001). Although this element is found at trace concentrations, there are several routes by which cadmium mineral particulates are transported: rivers carrying contaminated sediment, atmospheric processes that carry dust sized contaminated particles in the air, and volcanic debris released into the atmosphere can transport cadmium minerals to the surface environment. Cadmium compounds can also be found in areas where there are shale rocks containing sulphide minerals (like pyrite rocks) and where decomposed organic matter is present (Garrett 1995). Geologically, British Columbia has many areas of shale rock, which are natural sources of cadmium (Morford et al. 2001; McGeer et al. 2011).

Industrial waste is a major source of cadmium, as it is a by-product created during the processing of copper, sulphide, lead, and zinc ores (Ravera 1984; Schmidt et al. 2012). Cadmium contamination is caused by a discharge of water from a mining site, especially when the project involves extracting zinc from copper ores (Schmidt et al. 2012). For example, at the site of an active coal mine in BC, the dissolved concentrations of cadmium and zinc exceeded the maximum WQG in a creek, which is downstream of the mine (Quamme et al. 2006). High concentrations of cadmium in freshwater, up to 0.8 µg/L, is primarily the result of anthropogenic emissions rather than from natural particulate dispersal (Cullen and Maldonado 2013).

Depending on the pH, hardness, dissolved oxygen, the duration of the exposure, or species used in the study, an aquatic organism's sensitivity to cadmium in freshwater can change, due to these factors interfering with Cd^{2+} ions binding to the surface membrane of the organism (Suedel et al. 1997; Tan and Wang 2011; McGeer et al. 2011). For sensitive species like *Daphnia* sp., lethal effects can be caused by a cadmium concentration as low as 5 µg/L in moderately hard water (120 mg/L as $CaCO_3$ hardness) (Attar and Maly 1984). Toxic effects of metals like cadmium are dependent on the speciation of free Cd^{2+} ions and binding of these ions to the epithelial surface (or the gill, for freshwater fish) of an aquatic organism (Ravera 1984; McGeer et al. 2011). The accumulation of cadmium has been shown to inhibit normal ion regulation, ultimately leading to a toxic effect, by competing with Ca^{2+} for similar binding sites (McGeer et al. 2011).

The BC WQG for cadmium is used to prevent the conditions of freshwater containing aquatic life and drinking water from having highly dangerous concentrations of cadmium. The BC WQG for cadmium is adjusted depending on the local hardness of the water using an adjustment equation. The WQG for maintaining water quality that will protect freshwater life in British Columbia from cadmium requires a maximum concentration of 0.018 μg/L in soft water (at 50 mg/L as $CaCO_3$). The water quality guidelines for cadmium are different for freshwater in the United States and other regions, as listed in Table 1.

Table 1. Cadmium WQG at 50 mg/L as $CaCO_3$ hardness according to jurisdiction.

Reference	Jurisdiction	Short term WQS dissolved Cd (µg/L)	Long term WQS dissolved Cd (µg/L)
CCME 2014	Canada	1.00[A]	0.09[B]
BC MOE 2006	Site specific to British Columbia	0.018[C]	0.09[D]
US EPA 2014	United States	1.00[E]	0.15[F]
MPCA (Minnesota Pollution Control Agency) 2012	Site specific to Minnesota	Class 2A: 0.51[G] Class 2B: 14.83[H]	0.015[I]
IDAPA (Idaho Department of Environmental Quality) 2012	Site specific to Idaho	0.42[J]	0.13[K]
ANZECC and ARMCANZ 2000	Australia and New Zealand	-	0.32[L]
Environmental Agency (EA) 2008	United Kingdom	-	0.09[M]

Notes:

[A] CWQG hardness adjustment equation (µg/L) = $10^{\{1.016(\log[\text{hardness}]) - 1.71\}}$

[B] CWQG hardness adjustment equation (µg/L) = $10^{\{0.83(\log[\text{hardness}]) - 2.46\}}$

[C] BC WQG hardness adjustment equation = $10^{\{0.86(\log[\text{hardness}]) - 3.2\}}$

[D] BC has no specific long term guideline, follows long term CWQG

[E] CMC hardness adjustment equation = $10^{1.136672-[(\ln \text{hardness})(0.041838)]}$

[F] CCC hardness adjustment equation = $10^{1.101672-[(\ln \text{hardness})(0.041838)]}$

[G] MS Class 2A (excludes salmonid species) equation = $10^{[0.8403(\ln[\text{hardness}]) - 3.575]}$

[H] MS Class 2B (all fish species included) equation = $10^{[0.8403(\ln[\text{hardness}]) - 2.116]}$

[I] Class 2 (all fish species included) equation = $10^{[0.7409(\ln [\text{hardness}]) - 4.719]}$

[J] CMC hardness adjusted equation = $10^{\{0.8367 [\ln(\text{hardness})]+ -3.560\}*}$1.136672-[(ln hardness)(0.041838)]

[K] CCC hardness adjusted equation = $10^{\{0.6247 [\ln(\text{hardness})]+ -3.344\}*}$1.101672-[(ln hardness)(0.041838)]

[L] TV hardness adjusted equation for 95% protection = $0.2(\text{hardness}/30)^{0.89}$

[M] PNEC hardness adjusted equation = $0.09(\text{hardness}/50)^{0.7409}$

1.1.2 Selenium

Selenium enters aquatic systems via soil drainage in areas containing elemental selenium, which can be produced from the erosion of bedrock containing sulphide minerals. Selenium is found in its inorganic form, selenium (IV) or as its various oxidized states such as sodium selenide (Na_2Se), sodium selenite (Na_2SeO_3), or sodium selenate (Na_2SeO_4) (Barceloux 1999). An important part of the biogeochemical cycling of selenium is the presence of microorganisms in either the water column or sediment (Bowie et al. 1996). When inorganic selenium is introduced into an ecosystem, it is assimilated from the aqueous environment by bacteria and phytoplankton and converted into organoselenides (Janz 2012). Surface waters that are not influenced by industrial activity will have a natural background concentration up to 1.2 µg selenium/L (Canton et al. 2008). Although the concentration of selenium can be quite low in surface water, the concern with contamination of freshwater with selenium is its bioaccumulation within the local aquatic ecosystem and its impact on resident fish populations (Janz 2012).

Industrial activities such as the combustion of fossil fuels, leachate from coal fly ash, and an overflow of leachate can allow selenium to flow into rivers and streams (Lemly 1993). If seleniferous minerals are released during the process of agricultural and industrial activities, an elevation of selenium is observed in local freshwater systems. As an example, in the Kemess South Mine northwest of Mackenzie, BC, mining discharge leaching from the waste rock dump site elevated selenium concentrations within a nearby river (Davidson and Chapman 2006). With the increase in the number of mining projects in BC, there is potential that increased concentrations of selenium in surface waters could cause detrimental effects to aquatic ecosystems.

Studies on selenium reveal that it is easily accumulated in the tissues of fish after the consumption of contaminated freshwater planktivorous insects and crustaceans (Lemly 1992; Ponton and Hare 2013). The harm posed by high selenium concentrations in the tissues of aquatic organisms is mainly due to the replacement of sulphur containing amino acids, therefore disrupting the function of essential proteins (Janz 2012). Information on the concentrations of selenium measured in the tissues of fish was reviewed and incorporated into the derivation of BC's provincial WQG as part of the process to establish the sublethal and reproductive effects of selenium. This metalloid is not naturally abundant in British Columbia (Nagpal 1991). The average BC WQG for selenium is 2 μg/L (refer to Table 2) to prevent the bioaccumulation of selenium sufficient to cause lethal toxicity in secondary consumers (such as freshwater fish).

Table 2. Selenium WQG according to jurisdiction.

Reference	**Jurisdiction**	**Short term WQS Se (µg/L)**	**Long term WQS Se (µg/L)**
CCME 2014	Canada	-	1.00
BC MOE 2006	Site specific to British Columbia	-	2.00
US EPA 2014	United States	$1/[(^{A}/185.9) + (^{B}/12.82)]$	5.00
MPCA (Minnesota Pollution Control Agency) 2012	Site specific to Minnesota	20.0	5.00
ANZECC and ARMCANZ 2000	Australia and New Zealand	-	5.00^{C}

Notes:
[A] The proportion of selenite present in water
[B] The proportion of selenate present in water
[C] For 99% protection of aquatic species

1.1.3 Sulphates

There are numerous geochemical sources of sulphate such as atmospheric deposition, stream runoff from rain and snow melting, and from sulphuric acid. One of the natural sources of sulphate is pyrite, a mineral that contains sulphides. Through a process of oxidation, the minerals containing sulphides produce sulphite and eventually sulphate anions in water (Nordstrom et al. 2007). The elemental form of sulphur in water undergoes a biogeochemical cycling process of oxidization by bacteria into stable anionic forms such as sulphate (as shown in Figure 1). Sulphur compounds can be assimilated into organic matter and stored in the sediment layer. The breakdown of the organic layer in the sediment can introduce more sulphate into the system, therefore re-establishing concentrations of sulphur (Cook and Schindler 1983).

In areas of industrial activity such as coal mining, sulphate ions (SO_4^{-2}) are present in high concentrations as the result of water runoff carrying trace amounts from rock blasting and waste dump sites. Runoff from mine effluent can be very acidic and due to this acidity pyrite is eroded to expose sulphate (Sams III and Beer 2000). In freshwater lakes receiving drainage from mining areas, the concentration of sulphate could be five times higher than background concentrations (Herlihy and Mills 1985).

Excess sulphate anions impede osmoregulation in aquatic organisms, causing an imbalance of ions such as sodium (Davies 2007; Soucek and Kennedy 2005). Recent studies indicated that the water hardness had a protective effect on sulphate toxicity to freshwater invertebrates and rainbow trout *(*Elphick et al. 2011; Soucek and Kennedy 2005). To accommodate for freshwater species that are less sensitive to sulphate toxicity

in softer water, the sulphate WQG concentration limit was increased from 100 mg/L to 218 mg sulphate/L for soft water (31 to 75 mg/L as $CaCO_3$) (refer to Table 3).

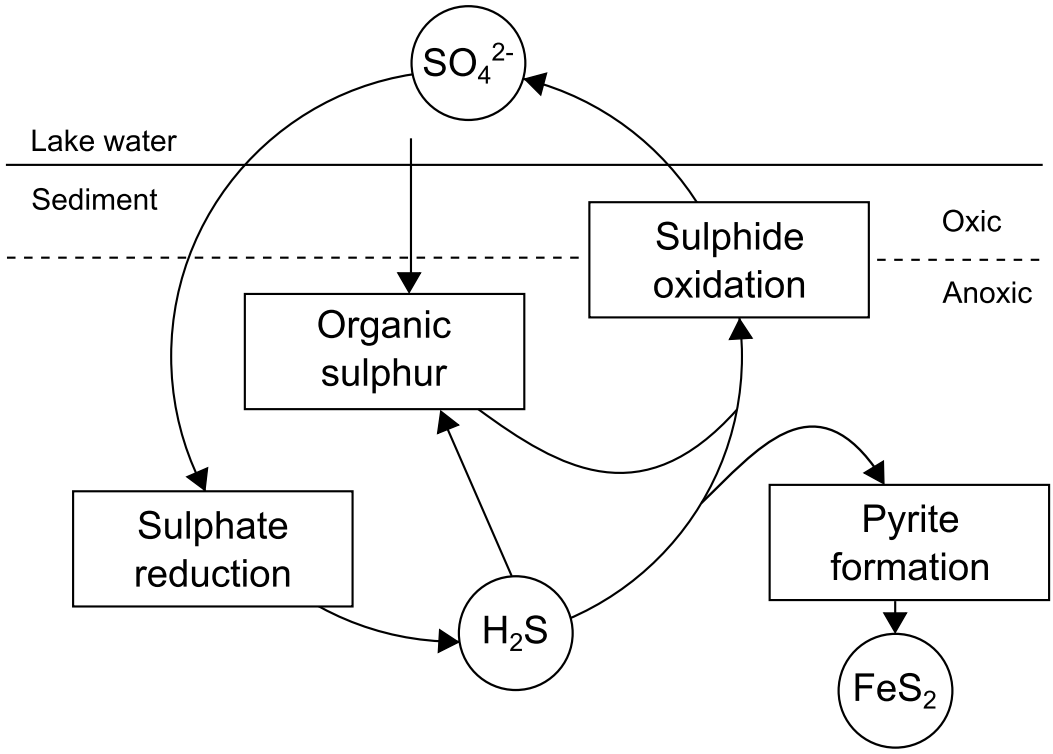

Figure 1. The process of sulphur cycling in freshwater lakes. Sulphate can be deposited into the lake through the atmosphere and through contamination. However, a high percentage of sulphate naturally found in lake water is the result of reduced sulphur products being oxidized within the sediment of the lake (with permission from Holmer and Storkholm 2001).

Table 3. Sulphate WQG at 50 mg/L as $CaCO_3$ hardness according to jurisdiction.

Reference	**Jurisdiction**	**Short term WQS SO_4^{2-} (mg/L)**	**Long term WQS SO_4^{2-} (mg/L)**
CCME 2014	Canada	-	500[A] 1000[B]
BC MOE 2013	Site specific to British Columbia	-	218[C]
US EPA 2014	United States	-	250[D]
MPCA (Minnesota Pollution Control Agency) 2012	Site specific to Minnesota	10[E]	250[D]
IDNR (Iowa Department of Natural Resources) 2009 IEPA (Illinois Environmental Protection Agency) 2009	Site specific to Iowa and Illinois	-	500[F]
ANZECC and ARMCANZ 2000	Australia and New Zealand	-	1000[G]
Environmental Agency (EA) 2008	United Kingdom	-	250

Notes:
[A] Drinking WQG to protect human heath from high sulphate concentrations
[B] Agricultural WQG to protect livestock health
[C] Sulphate WQG according to water hardness category "Soft to moderately soft (31-75)"
[D] Recommended concentration for public water systems, secondary drinking water standard
[E] This standard is applicable from April to August, where wild rice populations are most susceptible to high sulphate concentrations
[F] Sulphate WQG concentrations increases if hardness ≥100 mg/L and chloride concentration ≥ 5 mg/L
[G] Recommended for drinking water and agricultural protection for livestock

1.1.4 Nitrates

Nitrification is a natural process (Figure 2) mediated by aerobic chemoautotrophic bacteria that oxidize one form, the ammonium (NH_4^+) ion to another form, the nitrite ion (NO_2^-) and then converts the nitrite into nitrate (NO_3^-). Nitrogen is found in runoff of water exposed to igneous rocks or excrement from wild or domestic animals into streams resulting in elevated nitrate concentrations in water bodies (Domingues et al. 2011). There are multiple forms of nitrogen, including nitrate (NO_3^-), which are toxic to aquatic life.

The concentration of nitrogenous ions is usually elevated from background in an aquatic environment due to the proximity of agricultural processes and pollution of nearby water sources (Camargo and Alonso 2006). Completely anthropogenic influences on the elevation of nitrate concentration can come from the use of ammonium nitrate explosives for mining, nitrogen fertilizers, and nitrogen-fixing crops (Pommen 1983).

Freshwater invertebrates, fish, amphibians, and bivalves are very sensitive to the effects of nitrate toxicity compared to marine species (Camargo and Alonso 2006). A recent study on the acute toxicity of nitrate showed that freshwater invertebrates have a range of lethal concentrations (LC50) from 357 to 957 mg nitrate/L (Soucek et al. 2011). The harmful effect of nitrate toxicity is due to inhibition of haemoglobin (specifically, its function to carry oxygen) (Camargo and Alonso 2006). Unlike cadmium or sulphate toxicity, the derivation of the nitrate BC WQG was based on studies that did not show hardness effects on nitrate toxicity (Soucek et al. 2011). In countries other than Canada,

WQG have been used primarily to protect humans from the consumption of water with high levels of nitrate (Table 4).

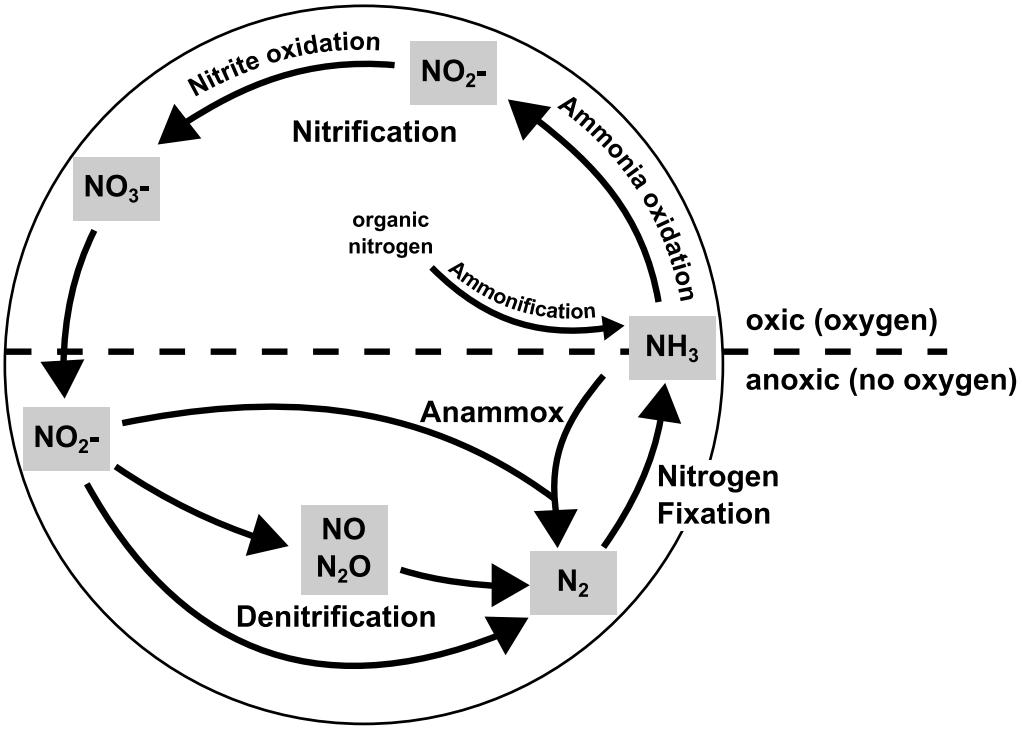

Figure 2. The process of nitrogen cycling in the environment. Nitrate (NO_4^-) is oxidized from nitrite (NO_3^-) by nitrite-oxidizing bacteria genera such as *Nitrospira, Nitrobacter, Nitrococcus,* and *Nitrospina* (with permission, adapted from Bernhard 2010).

Table 4. Nitrate water quality standards according to area jurisdiction.

Reference	**Jurisdiction**	**Short term WQS NO_3^- (mg/L)**	**Long term WQS NO_3^- (mg/L)**
CCME 2012	Canada	550	13.0
BC MOE 2013	Site specific to British Columbia	32.8	3.0
US EPA 2014	United States	-	10[A]
MPCA (Minnesota Pollution Control Agency) 2012	Site specific to Minnesota	41	3.1 4.9
ANZECC and ARMCANZ 2000	Australia and New Zealand	-	0.70
WHO (World Health Organization)	Drinking water parameters for all countries world-wide	-	50

Notes:
[A] Maximum contaminant level goal for drinking water
[B] For protection of 95% aquatic species

1.2 Water hardness and toxicity

Water hardness is an aspect of water quality expressed as milligrams per litre of calcium carbonate ($CaCO_3$) and it measures the general concentration of dissolved cations or divalent salts (Wetzel 2001). As the hardness of freshwater increases (or becomes 'harder'), the concentration of free calcium and magnesium ions increase; water is considered softer as these concentrations decrease. In British Columbia's lakes and rivers, the water hardness can naturally range from 3 (extremely soft) to 180 (moderately hard) mg/L as $CaCO_3$. The rapid input of calcium through the erosion of limestone rock and drainage water from coal mine waste dumps can raise the hardness in freshwater rivers and streams (Adibee et al. 2013; Ford 1971). The calcium concentrations are declining in Canadian lakes, making the water softer, which has an impact on *Daphnia* populations (Jeziorski et al, 2008). Hardness is known to be a limiting factor for the distribution of zooplankton, amphipods, and fish species in lakes.

Calcium is an essential nutrient for many freshwater species and is primarily absorbed from their diet or surrounding environment (Muyessen et al. 2009). Calcium concentrations are also known to affect the tolerance of freshwater organisms to toxic substances (e.g., cadmium and sulphate), as demonstrated in several studies, where higher concentrations of these substances are tolerated under higher hardness conditions (Barata et al. 1998; Paquin et al. 2002; Soucek 2007). The BC WQG for sulphate was also recently updated to incorporate studies of multiple species, in which there was a protective effect of hardness from the toxicity of sulphate. Since the tolerance of freshwater species to these toxicants was increased by hardness, BC WQGs are at a

concentration converted with a hardness adjustment equation to allow higher concentrations in water (Meays 2012).

1.3 Mixture toxicity

Currently, federal and provincial WQGs are created for the purpose of ameliorating the toxic effects of single contaminants. Water quality guidelines rely on results from single-contaminant exposures under controlled laboratory conditions, which may not accurately reflect the multiple-contaminants available in most environments facing contamination. Increasing environmental concentrations of metals, non-metals, and organic substances from anthropogenic sources give rise to simultaneous exposure for aquatic life in the environment of British Columbia. Recent examples of toxic mixtures that are more toxic than their individual components include salmonids exposed to multiple pesticides (Laetz et al. 2009), freshwater amphipods exposed to metal mixtures (Charles et al. 2013), and the additive effects of insecticides on *Ceriodaphnia dubia* (Choung et al. 2011). In these studies, it was revealed that freshwater species can have a lower tolerance to certain pollutants in a mixture-type setting. New Zealand and Australia (ANZECC and ARMCANZ 2000) have formed environment management strategies (through their site-specific WQGs) that incorporate the likelihood that there are harmful mixtures from industrial receiving waters. Recent updates to the Canadian and BC WQGs have considered the role of water hardness in terms of ameliorating the toxicity of certain contaminants (e.g., metals), but they do not directly address the effects from a simultaneous exposure of two or more contaminants (CCME 1999; Meays 2012).

Organisms within aquatic ecosystems will likely encounter mixtures of substances and these substances could interact to cause a unique overall toxic effect. The toxic mechanism is triggered by an external or internal effect concentration. For some dissolved metal ions, the importance of the external effect concentration includes the ability to bind to a receptor, forming a bioligand. However, alternate modes of action may include a previous interaction between components of the mixture to cause a more toxic compound to be present (Escher and Hermens 2002). Identifying the mode of action for substances can lead to more appropriate predictive models that can be applied to environmental risk strategies.

The variety of outcomes that a mixture of substances can have on toxic responses can be modeled by either concentration addition or independent action (also known as response addition). Concentration addition (CA) is a commonly used model for determining the toxicity of a mixture of toxicants that have the same mode of toxic action (Backhaus et al. 2004). It assumes that chemical species will behave similarly to cause toxicological effects but each component will contribute as fractions of the final effect. The response addition approach or independent action model (which also makes a prediction of mixture effects) will assume that each substance within the mixture has a separate toxic effect, that the mixture components do not interact, and that concentrations of substances that produce no individual effect will have no effect as a mixture (Bliss 1939; Rodney et al. 2012). The independent action approach can not be universally applied, as the observational outcomes of a combination of metals with the same toxic action can result in synergism (where the non-toxic components demonstrate a toxic effect as a mixture), potentiation (when a mixture is more toxic than the sum total toxicity

of the toxicants combined) or antagonism (there is a lesser toxic effect than the added effect due to an interaction between components) (Norwood et al. 2003).

Although the potential effects of multiple contaminants within an environment are acknowledged when conducting environmental risk management, there is a still a knowledge gap for directly addressing the effects. To prevent harmful exposures to substances (like mixtures) that influence the health of freshwater species, the current practice is to apply an uncertainty or "safety" factor to the guideline concentration, usually involving an increase or decrease to the concentration recommended by the WQG (Chapman 1998; Meays 2012). It is not clearly established whether current risk assessment strategies, based on single contaminant exposures, are sufficient to protect against the toxicity of a mixture of contaminants (Barata et al. 2006).

1.4 Research objectives

My overall objective of this study was to determine whether a complex mixture of four contaminants (Cd^{2+}, Se (IV), SO_4^{2-}, NO_3^-) at concentrations consistent with the provincial WQGs for British Columbia are sufficiently protective to *Daphnia magna, Hyalella azteca*, and *Oncorhynchus mykiss*. This study will also address whether the duration of the mixture exposure, as short (48 hours) and long term (21 days) experiments, would influence protection of freshwater species. Due to the variability of hardness across habitats in BC, my study will also determine if the extremes of water hardness (50 and 250 mg/L as $CaCO_3$) has an effect on the toxicity of this complex mixture of contaminants at BC's proposed provincial WQGs.

2.0 Methods

2.1 Summary of experimental design

In this study, three standard test species (*Daphnia magna*, *Hyalella azteca*, and *Oncorhynchus mykiss*) at an early life stage were exposed to cadmium, selenium, nitrates and sulphates at concentrations relevant to BC WQG in short-term (acute or 24 hours) and long-term (chronic or 21 day) exposure experiments. Each test species was introduced to either soft (50 mg/L as $CaCO_3$) or hard (250 and 300 mg/L as $CaCO_3$) water media to examine their responses to the BC WQG under variable hardness conditions. Short term and long term exposure experiments were conducted by following a modified version of a relevant Canadian Environment Protection Series protocol when necessary. The standardized EPS protocols used in this study are also used by both private and government laboratories that generate information used in the derivation of BC WQG. The details of these experiments are given below.

2.2 Study Species

2.2.1 Daphnia magna

Daphnia spp. are freshwater planktonic crustaceans that inhabit freshwater ecosystems located worldwide (Ebert 2005). *Daphnia magna* have been chosen as subjects for testing the effects of lethal and sublethal toxicants in Canadian laboratories for their wide distribution, sensitivity to toxicants, and important role in the aquatic food chain (Environment Canada 1996). *Daphnia magna* are found in shallow lakes or ponds and are widely distributed across Canada, including southern BC (Carl 1940; Koivisto

1995; Bos et al. 1999). They are extremely sensitive to the presence of freshwater contamination such as copper and cadmium (Borgmann et al. 1989). Overall, *Daphnia magna* is a common freshwater invertebrate species used in standardized toxicity testing to determine the toxicity of a wide range of substances and was appropriate for use in my study (Hebert 1978; Environment Canada 1996).

The methods for culturing and testing *Daphnia magna* were optimized by following the Environmental Protection Series (EPS) guidelines "Biological Test Method: Acute Lethality Test Using *Daphnia* spp. EPS 1/RM/11" and "Biological Test Method: Reference Method for Determining Acute Lethality of Effluents to *Daphnia magna* EPS 1/RM/14" (Environment Canada 1996; Environment Canada 2000). *Daphnia magna* adults were purchased and shipped from Aquatic Biosystems Inc., Fort Collins, CO, USA. This stock *Daphnia* culture was transferred from soft dechlorinated water to reconstituted water, which was prepared as either 50 (soft) or 300 (hard) mg/L as $CaCO_3$ as per an adjusted EPA guideline recipe (Table 5; Environment Canada 1996). Reconstituted water was prepared with Millipore Milli-Q Type 1 water and the following salts: $CaCl_2$-$2H_2O$, $MgSO_4$-$7H_2O$, $NaHCO_3$, and KCl from Fisher Scientific. As per EPS1/RM/11, selenium (as sodium selenate) and vitamin B12 were added at 2 µg/L as nutritive supplements to reconstituted water.

Table 5. Amount of salt (mg) added per litre of Millipore Milli-Q Type 1 water to create reconstituted water (adjusted from EPA recipe).

Added salts (mg/L)	**$NaHCO_3$**	**$CaSO_4$**	**$MgCl_2$**	**KCl**
Soft water (50 mg/L as $CaCO_3$)	65.8	41.0	41.0	2.8
Hard water (250 mg/L as $CaCO_3$)	454	284	284	19.8
Extremely hard water (300 mg/L as $CaCO_3$)	520	324	324	22.6

After being exposed to either hard or soft reconstituted media for a minimum of two weeks, the acclimated gravid females were isolated into 50 mL tubes with hardness-matched medium and fed 25 µL of *Pseudokirchneriella subcapitata* (previously named *Selenastrum capricornutum*) algae and yeast-cereal grass-trout chow (YCT) premixed feeding solution. This algal food concentrate was grown to a cell density of 3.6 x 10^7 cells/mL and then 10 mL added per litre of culture water. The YCT solution is a feeding solution that consists of yeast, fermented trout chow and cereal grass sprouts, and was prepared as defined in the EPS guideline. After 24 hours, neonates were collected and randomly assigned to the 48 hour acute exposure test vessels. Enough neonates plus 10% of the number needed to obtain 10 neonates per replicate in the exposure was set aside for 24 hours in their respective culture medium and fed with 1 mL per litre of culture. If there was movement by the daphnia's antennae within 10 seconds following careful water movement, it was determined that daphniids were in good health and still surviving, which could be noted for survival. A failure to pass this health test indicated that the organism underwent mortality (or death).

2.2.2 Hyalella azteca

Like *D. magna*, *Hyalella azteca* are used as standard test organisms for toxicity testing because of their sensitivity to changes in water quality and their role in the food chain. *Hyalella* spp. are epibenthic detritivores, distributed in ponds and lakes across North America, in coastal freshwater areas, living primarily within the sediment. These freshwater amphipods have been chosen specifically because of their sensitivity to metal toxicity in both sediment and water (Environment Canada 1997; Wogram and Liess 2001). *Hyalella azteca* is recognized as a species complex (where there is potentially

more than one genetically distinct species) that demonstrates genetic differences based on region (Hogg et al. 1998; Wellborn et al. 2005). They are found in waters of a diverse range of salinity and hardness (above 7 mg/L as $CaCO_3$) and with various substrates such as freshwater, estuarine, and marine ecosystems. As with *Daphnia* spp.*, Hyalella azteca* are used in standardized toxicity testing because they are able to propagate in laboratory conditions and have ubiquitous distribution, but are also very sensitive to water contamination (Environment Canada 2013).

The stock cultures of *Hyalella azteca* were provided by the Canadian Centre for Inland Waters (CCIW) in Burlington, ON. The methods of Borgmann et al. (1989) were used for testing and culturing *H. azteca* in a non-sediment context, given that there was no standard EPS protocol available to test *H. azteca* for acute toxicity of a dissolved toxicant (although, a protocol [Environment Canada 2013] was developed and released while this study was being executed, based on Borgmann et al. [1989]). Adults used for culturing were stored in 2 L polypropylene containers, which contained a 25 cm^2 square (5 cm x 5 cm) piece of gauze and approximately 1 L of standard artificial media (SAM) (Table 6). These adults were kept in SAM due to their sensitivity to extreme fluctuation in water hardness, where a higher mortality and subsequently reduced reproduction was observed under immediate exposure to reconstituted medium at either 50 (soft) or 300 (hard) mg/L as $CaCO_3$. These culture vessels containing initially 40 adult *H. azteca* each were stored in a water bath heated to 24°C and put under a light with a 16 hour light to 8 hour dark photoperiod. Adult *H. azteca* were taken from solution after being captured in a 600 µm Nitex mesh. Young *H. azteca* were captured by filtering culture water with 200 µm Nitex mesh and separated from culture once per week. Before initiation of the acute

test, the young were held in the exposure test media for 48 hours and fed 24 hours before the exposure. If there was movement by the *Hyalella sp.* within 10 seconds following careful water movement, it was determined that they were in good health and still surviving, which could be noted for survival. Similarly to *Daphnia sp.*, failure to pass this health test indicated that the organism underwent mortality (or death).

Table 6. Amount of salt (mg) added per litre of Millipore Milli-Q Type 1 water to create Standard Artificial Media (SAM) recipe for *H. azteca* culturing.

Added salts (mg/L)	**$NaHCO_3$**	**$CaSO_4$**	**$MgCl_2$**	**KCl**
SAM	84	147	616	3.7

2.2.3 Oncorhynchus mykiss

In addition to invertebrate test organisms, vertebrate species are also used for measuring toxicity in freshwater. Fish are used in toxicity assays as indicators of high trophic level effects, especially if a substance is able to biomagnify through the food chain (Environment Canada 1998). As a native species to lakes and rivers of British Columbia and the rest of Canada, rainbow trout (*Oncorhynchus mykiss*) are commonly found and are sensitive to environmental contaminants. As an important member of the food chain, adult rainbow trout feed on aquatic invertebrates, molluscs, and other fishes (Klinkenberg 2012). Rainbow trout is a representative organism for freshwater fish found in cold Canadian lakes, as well as a standard test species cultured by many fish hatcheries and government laboratories for regulatory use due to their sensitivity (Rand 1998).

The procedure for testing rainbow trout (*Oncorhynchus mykiss*) was based on the standardized method "Biological Test Method: Toxicity Tests Using Early Life Stages of Salmonid Fish (Rainbow Trout) 1/RM/28" (Environment Canada 1998). With the assistance of the Peterborough Ministry of Natural Resources (MNR) fish culture station, rainbow trout eggs were extracted from a brood originating from the Ganaraska River region, Ontario. Eggs and milt were shipped using specialized shipping trays with ice, and flown from Sault Ste Marie, Ontario to Thunder Bay, Ontario.

Upon arrival, the unfertilized *Oncorhynchus mykiss* eggs and milt were checked for mortality (where mortality was indicated by white colour) and temperature. Any dead eggs were immediately removed and discarded. Dry fertilization was carried out as per early life stage EPS protocol (Environment Canada 1998) by slowly mixing eggs and milt

prior to water-activating the mixed gametes. Activation of eggs was initiated by the addition of dechlorinated water tempered to the same temperature of eggs upon arrival. The temperature of eggs upon arrival was measured using a glass thermometer, which showed that they were at 10 ºC. Fertilized eggs were held at a temperature of 10 ºC (±1 ºC); acclimation to laboratory conditions required a holding time of 36 hours in control water by adding temperate dechlorinated water to raise the temperature to 14 ºC (±1 ºC) (Beattie et al 2006). Test organisms were maintained at 14 ºC (±1 ºC) aerated dechlorinated water, using a water bath, in constant low light <100 lux, and on a photoperiod of 16 hours light to 8 hours in the dark.

The test organisms were only used for exposures if there was no sudden increase in control mortality above 70% of eggs within 24 hours upon arrival. This was to ensure they were at the correct life stage and healthy enough for the exposure. At a constant temperature of 14 ºC (±1 ºC), rainbow trout are expected to hatch by the 21st day after fertilization (Velsen 1987). If signs of opaque colour, excessive white growths, and premature hatching were observed during the exposure, they were considered as a mortality of the eggs, but were not removed. Egg health and development was observed daily during the exposure, and intensively at the start of hatching, where eyed eggs transitioned into the alevin stage (Environment Canada 1998).

2.3 Water quality

Water samples were taken at the beginning and end of every test to confirm water quality parameters remained constant throughout the exposure. The pH was measured using a Fisher Scientific Accumet AB15 pH meter and probe. This pH meter was

calibrated using standard 4, 7 and 10 solutions from Fisher Scientific. Hardness was determined by a colorimetric titration (Rand 1998). For long-term exposures, water quality measurements (temperature, pH, and hardness) were measured daily and concentrations were confirmed prior to and during the start of the exposure, after the first 24 hours and for each week during the exposure and at the termination of the experiment. New solutions were made 24 hours in advance and water quality measurements were recorded for each renewal solution.

2.4 Acute exposure testing

Acute exposure solutions were prepared 24 hours prior to the initiation of the test and were made as per the following: either soft or hard control water treatment, four separate treatment solutions each representing a single contaminant (cadmium, selenium, nitrate, or sulphate) concentrations consistent with the BC WQG, and a mixture of all four contaminants (cadmium, selenium, nitrate, sulphates) at concentrations representing the BC WQG listed in Table 7. For both hard and soft water treatments during the exposure, each treatment solution was made from concentrated stock solutions of $CdCl_2$, $NaSO_4$, $NaSeO_4$, and $NaNO_3$, where an aliquot of stock was diluted with fresh, hardness-adjusted media. The control treatment was made from the same recipe as the water used for culturing, for both hard and soft conditions (refer to Table 5). A follow-up experiment involving selenium and sulphate with cadmium was conducted to test cadmium alone, and cadmium in binary mixtures with selenium and sulphate, against an appropriate control in order to reveal the nature of the interaction. The detailed procedure for this subsequent exposure followed those established for the four-way mixture described.

For conducting an acute 24 hour toxicity exposure, the test organisms (either 24-48 hour old *D. magna* or 1-7 day old *H. azteca*) were collected and randomly assigned to the test vessel. This two-step transfer process ensured the same initiation of the exposure and the number of organisms introduced to the test vessels was correct. In order to ensure the randomization and interspersion of the organisms, each individual organism was randomly placed into all tubes until there were 10 organisms in each test vessel. In the acute studies, there were three replicates used for each treatment (control, cadmium, selenium, nitrate, sulphate and mixture). An exception was made with acute exposures conducted in hard (300 mg/L as $CaCO_3$) water in that five replicates were used instead of three due to the increased production of neonates. After the test organisms were randomly assigned to experimental treatments, the contents of each tube were transferred gently into each test vessel, thereby initiating the exposure.

Table 7: Proposed BC WQG Maximum (acute) exposure test concentrations for 2013.

These concentrations were used to prepare acute toxicity test solutions.

Concentration of toxicant (mg/L)	Soft water (50 mg/L as $CaCO_3$)	Hard water (300 mg/L as $CaCO_3$)
Cadmium *	1.04×10^{-3}	6.40×10^{-3}
Sulphate	210.00	450.00
Nitrates as N	32.80	32.80
Selenium	0.01	0.01

Notes:
*Cadmium guideline concentrations determined using the equation:
$\mu g/L$, total cadmium = $10^{\{1.016[\log(\text{hardness})] - 1.71\}}$

2.5 Chronic exposure testing

Chronic exposure testing is more appropriate for understanding effects of a toxicant on the entire life cycle of a freshwater organism (US EPA 1996). Unlike acute toxicity studies, in chronic or long-term exposures the organism undergoes various stages of development while being exposed to the toxicant. The solutions for chronic exposure experiments were prepared at least 24 hours prior to being used in the experiment following concentrations from Table 8. The test solutions were renewed every 48 hours to prevent the water quality deviating from the conditions of the initial test solution. Duration of the test exposure and the frequency of water sampling were increased to 21 days and 24 hours, respectively, in contrast to acute studies. The number of surviving organisms was tracked for 21 days, beyond the juvenile stages and into the mature adult stage of the test organisms. The endpoint of these chronic studies was the percent survival at the end of the twenty-first day.

Table 8. Proposed average BC WQGs for 2012-2013. These concentrations were used in the chronic toxicity tests.

Concentration of toxicant (mg/L)	Soft water (50 mg/L as $CaCO_3$)	SAM (120 mg/L as $CaCO_3$)	Hard water (250 mg/L as $CaCO_3$)
Cadmium *	1.8×10^{-5}	3.8×10^{-5}	7.2×10^{-5}
Sulphate	195.0	309.0	450.0
Nitrates as N	3.0	3.0	3.0
Selenium	2.0×10^{-3}	2.0×10^{-3}	2.0×10^{-3}

Notes:
*Cadmium guideline concentrations determined using the equation:
µg/L, total cadmium $= 10^{\{0.86[\log\{\text{hardness}\}]-3.2\}}$

2.5.1 Daphnia magna

The method for conducting a 21 day chronic exposure to *Daphnia magna* was adapted from the US EPA (1996) procedures and the Environmental Protection Series guidelines: Environment Canada (1996) and (2000). There is no standardized method for determining chronic toxicity to *D. magna* in the Canadian Environmental Protection Series. The 21 day exposure with *D. magna* was carried out using a static renewal system, where 80% of the 250 mL solution was renewed every 48 hours until the end of the test (US EPA 1996). In order to renew the test solutions safely, the exposures were conducted in modified polypropylene test vessels. An outer and inner vessel formed the test vessel, where the inner cup had a 2.5 cm x 13 cm piece of 200 μm Nitex mesh attached with aquarium grade silicone (demonstrated in Figure 3). The purpose of the outer cup was to facilitate replacement of the solutions, while the inner cup kept daphniids in 20 mL of solution at all times. During renewal of the solutions, 200 μL of YCT solution and *P. subcapitata* alga concentrate (at cell density described previously) was added to each test vessel to provide a consistent diet over the exposure duration.

2.5.2 Hyalella azteca

The methods for chronic testing with *Hyalella azteca* are described in the Environmental Protection Series guidelines "Biological Test Method: Test for Survival and Growth in Sediment Using the Freshwater Amphipod *Hyalella azteca* EPS1/RM/33" (Environment Canada 1997) and "Biological Test Method: Test for Survival and Growth in Sediment and Water Using the Freshwater Amphipod *Hyalella azteca* EPS 1/RM/33" (Environment Canada 2013). This experiment had an exposure that ran for a period of 21 days in a similar test vessel as presented as Figure 3, but with the modification of an

Figure 3. Modified test vessel for chronic exposure testing with invertebrates. A) The inner cup with a 1 cm by 5 cm strip of 200 micron mesh affixed by aquarium silica B) The outer cup holds both the inner cup and test solution C) Both components of the test vessel as used during the chronic experiments.

opaque polypropylene plastic and light conditions to simulate similar conditions used in routine culturing.

Conducting the 21-day *H. azteca* chronic toxicity tests required that test animals be acclimated to the high and low hardness conditions required by the experimental design. Although test animals were gradually acclimated to higher or lower hardness conditions from their original culture water (hardness of 120 mg/L as $CaCO_3$), by the time hardness was increased to 250 mg/L as $CaCO_3$ or softened to 50 mg/L as $CaCO_3$, 50% and 70% mortality was observed, respectively. Consequently, the 21-d toxicity trials in hard and soft water were not conducted because test animals could not be acclimated to the required experimental conditions. This result necessitated two new experiments to determine the effect of the toxicants in the absence of changing water hardness, and of changing water hardness on *H. azteca*, independent of exposure to toxicants. The first experiment was conducted using exactly the same procedures and experimental design as the high and low hardness exposures, but at one fixed hardness concentration equivalent to the original culturing conditions (i.e., 120 mg/L as $CaCO_3$). The second experiment focused on water hardness in the absence of other toxicants, such that test animals were exposed to several hardness concentrations ranging from 50 – 250 mg/L as $CaCO_3$.

However, despite attempts to run chronic exposures in both hard and soft reconstituted water, the test was instead conducted in SAM culture media (Table 9) at 120 mg/L as $CaCO_3$ to observe if there were effects at the most optimal hardness condition (Borgmann et al. 1989). Unlike *D. magna*, adult *H. azteca* cultures showed a reduced production of young in reconstituted hard and soft water. A separate exposure was designed to test *H. azteca* and their long-term mortality in five different water

hardness conditions, including the SAM recipe by Borgmann et al. (1989). Each treatment used in this experiment was created using Millipore Milli-Q Type 1 water and the addition of five salts: $CaCl_2$-$2H_2O$, $MgSO_4$-$7H_2O$, $NaHCO_3$, and KCl to measure one of five water hardness conditions: 50, 80, 120, 180 and 250 mg/L as $CaCO_3$. *Hyalella azteca* young were taken from culture and the experimental procedure was carried out the same way as per the previous *H. azteca* chronic exposure experiment.

Table 9. Amount of salt (mg) added per litre of Millipore Milli-Q Type 1 water to create SAM for five different hardness levels.

Added salts (mg/L)	**$NaHCO_3$**	**$CaSO_4$**	**$MgCl_2$**	**KCl**
Soft water (50 mg/L as $CaCO_3$)	65.8	41.0	41.0	2.80
Moderately hard water (80 mg/L as $CaCO_3$)	42	73.5	30.8	2.35
SAM (120 mg/L as $CaCO_3$)	84	147	616	3.7
Moderately Hard (180 mg/L as $CaCO_3$)	168	294	123	7.4
Hard water (250 mg/L as $CaCO_3$)	454	284	284	19.8

2.5.3 Rainbow trout (Oncorhynchus mykiss) - Early life stage

The method for conducting a 21 day chronic exposure to *Oncorhynchus mykiss* was adapted from the Environmental Protection Series guidelines (Environment Canada 1996 and Environment Canada 2000). The exposure treatments include control, the four contaminants, and mixture, which were prepared as specified previously to the other chronic exposures *D. magna* and *H. azteca*, with the exception that soft (50 mg/L as $CaCO_3$) was from municipal dechlorinated water. The hard water used in the exposure experiment was prepared as 250 mg/L as $CaCO_3$ using dechlorinated municipal water and the addition of five salts: $CaCl_2$-$2H_2O$, $MgSO_4$-$7H_2O$, $NaHCO_3$, and KCl at the amounts listed in Table 10, as per an adjusted EPA guideline recipe. This exposure used a flow though system, where 100% of the 250 mL solution was renewed gradually every 24 hours (US EPA 1996). The temperature could not deviate by more than 1 ºC, so the flow through system added new solution to the test vessel at a rate of 3 mL per minute.

Table 10. Amount of salt (mg) added per litre of dechlorinated water to create hard reconstituted water (250 mg/L as $CaCO_3$) for chronic *O. mykiss* exposure.

Added salt (mg/L)	**$NaHCO_3$**	**$CaSO_4$**	**$MgCl_2$**	**KCl**
Hard water (250 mg/L as $CaCO_3$)	388	243	243	17.0

2.6 Statistical analysis of data

All statistical analyses were performed using the statistical programming language R (version 3.0.1, R Core Team 2013) with the significance level set at $\alpha = 0.05$. Acute endpoint data were tested for normality using frequency distributions and the Shapiro-Wilks testing. To test for homogeneity of variance, data were analyzed using boxplots and the Bartlett's test. The dependant variable for all acute exposure experiments was percent mortality (the percentage of organisms counted as dead out of 10 individuals after 48 hours) response of test organisms. The independent variable for acute exposures including *D. magna* (with the exception of an additional binary exposure test) and *H. azteca* were tested as follows: control, cadmium, selenium, sulphates, nitrates, and a mixture of the four contaminants. In the case of the additional acute *D. magna* binary exposure test, the treatment variable was composed of four levels: control, cadmium, cadmium with selenium, and cadmium with sulphate. If the acute data satisfied parametric assumptions, a one way analysis of variance (ANOVA) was used, then if necessary, a modified Tukey's HSD post-hoc analysis was applied to identify specific differences between any of the four individual contaminant responses from the control or mixture treatment responses.

Survival analysis by the Cox proportional hazards regression model (Cox 1972; Fox 2003) was employed to compare the responses of the aquatic species to the chronic exposure experiments. The independent variable for chronic exposures testing mixture effects were composed of control, cadmium, selenium, sulphates, nitrates, and a mixture of the four contaminants. However, for chronic testing with *D. magna,* the selenium

treatment was omitted, since it is needed in every treatment as a dietary supplement. In addition to mixture effect, the effect of media hardness was tested, and so for this chronic exposure experiment the independent variable was a range of media hardness including: 50, 80, 120, 180, and 250 mg/L as $CaCO_3$. The survival data (whether individual organisms were alive at a given time-point) was collected for both freshwater invertebrate species, every 48 hours after the first 24 hours into the exposure and for *O. mykiss,* every 24 hours after the test was initiated. To find significant differences between treatments, the relative hazard ratio compared chronic exposure treatments to the control (or 120 mg/L as $CaCO_3$ for the hardness range experiment) using a Cox proportional hazards regression model. Data was right-censored for individuals still alive at the termination of the study at 21 days, as permitted by a proportional hazards regression.

3.0 Results

3.1 Acute exposure testing

3.1.1 Daphnia magna

In the acute exposure test with *D. magna* in both soft (50 mg/L as $CaCO_3$) and hard water (300 mg/L as $CaCO_3$), treatments had a significant effect on mortality, as determined by one-way ANOVA (soft: $p = 0.0257$, $n = 3$ and hard: 2.29×10^{-7}, $n = 5$). Under soft water (50 mg/L as $CaCO_3$) conditions, there was a mean mortality of 43% for *D. magna* treated with cadmium at concentrations relevant to the BC WQG (Figure 4A), but this was not significantly higher from the control mortality using a modified Tukey's post hoc ($p = 0.115$, $n = 3$). In an acute exposure conducted under hard water conditions (300 mg/L as $CaCO_3$), the percent mortality of *D. magna* exposed to cadmium at BC WQG concentrations was significantly higher ($p < 0.0001$, $n = 5$) than all treatments; 64% higher than the control and 56% higher than the mixture (Figure 4B). The proposed maximum cadmium BC WQG concentrations were not safe to *D. magna* according to the high mortality response.

In soft water conditions, *D. magna* exposed to the mixture treatment did not have a significantly higher percent mortality than the control, as confirmed by the post hoc analysis ($p = 1.00$, $n = 3$), despite the presence of toxic cadmium concentrations (Figure 4A). In Figure 4B, it was also observed that in hard water (300 mg/L as $CaCO_3$), *D. magna* had a high percent mortality when exposed to cadmium singly, but a lower mortality in the mixture treatment ($p < 0.0001$, $n = 5$).

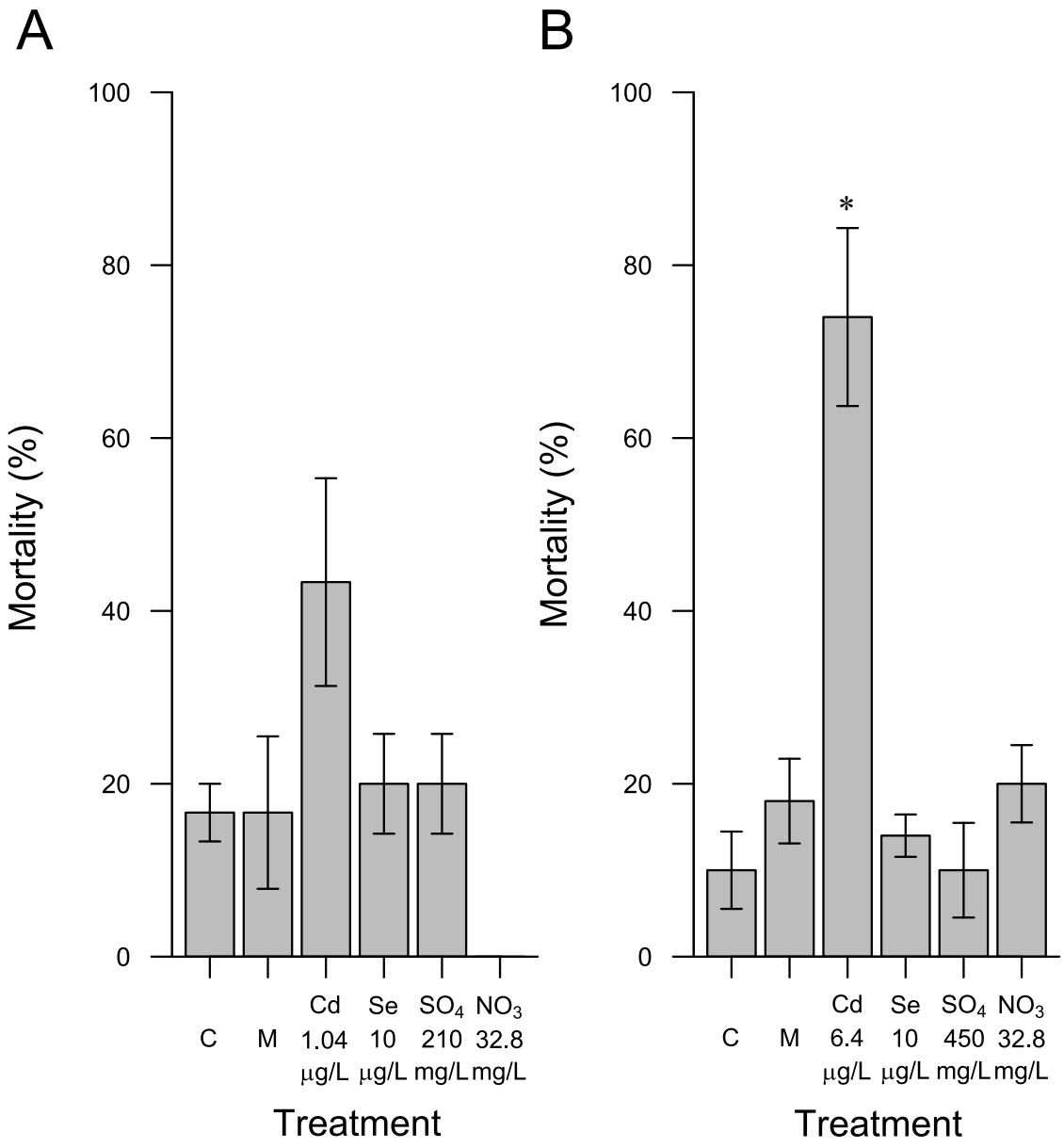

Figure 4. The means (+/- SEM) of *Daphnia magna* percent mortality after a 48 hour exposure to four individual contaminants: control (C), cadmium (Cd), selenium (Se), sulphates (SO_4), and nitrates (NO_3), in addition to their mixture (M) at concentrations listed in Table 7. This experiment was conducted in soft (A) with $n = 3$ or hard (B) water with $n = 5$. The asterisk indicates a significant difference from control ($p < 0.05$).

A follow-up experiment was conducted to understand the binary interaction of cadmium with selenium or sulphate at concentrations representative of BC WQG (Table 7) as treatments in a mixture setting. In this experiment (Figure 5), *D. magna* in hard water (250 mg/L as $CaCO_3$) had the same percent mortality in response to cadmium as observed in previous experiments (Figure 4B). Unlike the high percent mortality of *D. magna* in the individual cadmium treatment, the percent mortality for the treatment containing 10.0 µg selenium/L combined with 6.4 µg cadmium/L was not significantly different from the control ($p = 0.985$, $n = 5$). *Daphnia magna* exposed to cadmium and a cadmium-sulphate mixture treatment had a higher mortality than the control ($p < 0.0001$ and 0.0002, respectively, $n = 5$), but cadmium alone was not different from the cadmium-sulphate binary exposure ($p = 0.095$, $n = 5$). This experiment showed that a simultaneous exposure to selenium and cadmium concentrations antagonizes the effect of cadmium toxicity to *D. magna*.

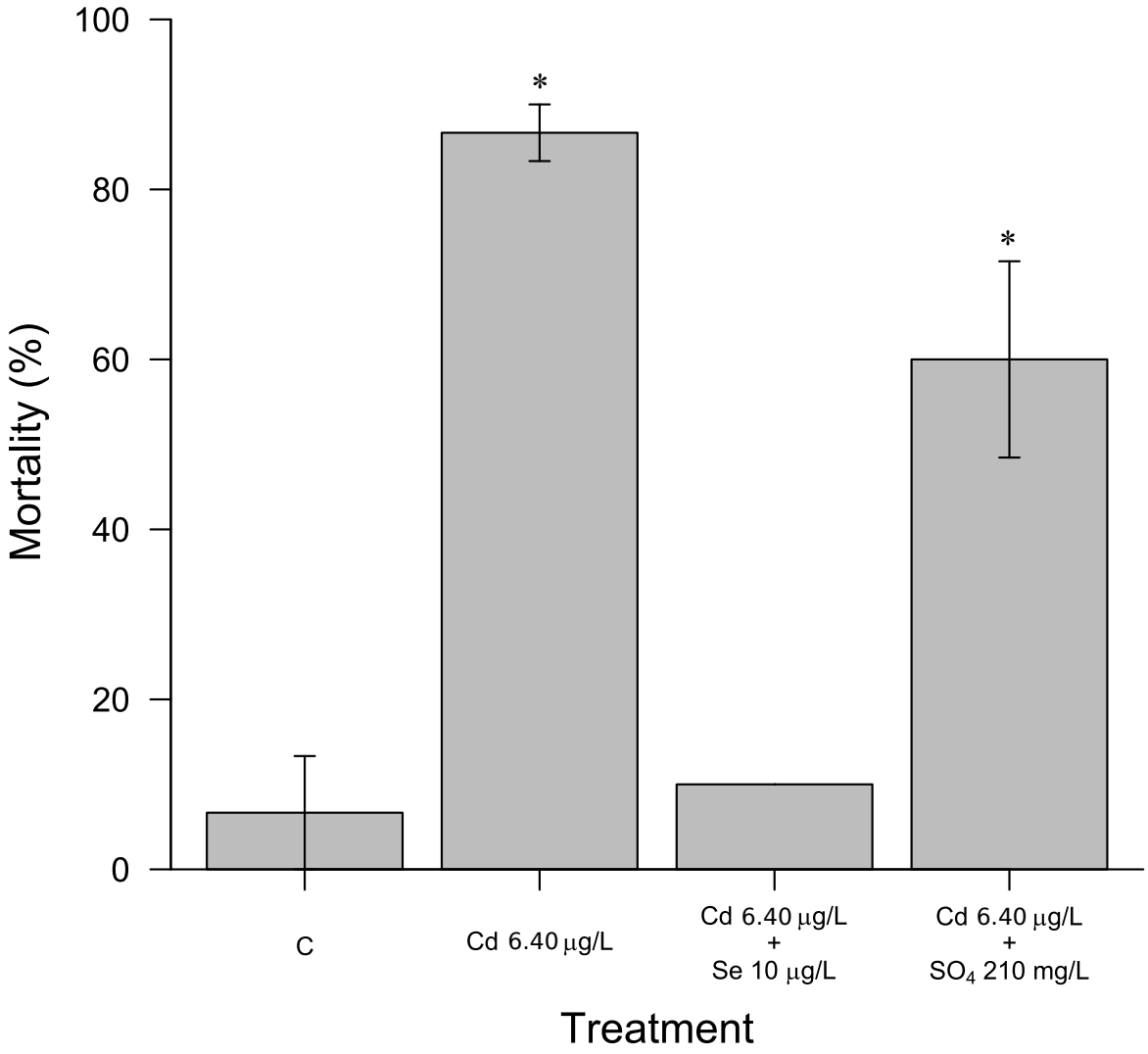

Figure 5. The mean (+/- SEM) percent mortality of *Daphnia magna* at the end of 48 hour exposure to cadmium (Cd) alone, with selenium (Cd + Se), or with sulphate (Cd + SO_4) in hard water (300 mg/L as $CaCO_3$). Each contaminant treatment and their mixture at proposed BC WQG concentrations and n = 5. The asterisk indicates a significant difference from control (C) ($p < 0.05$).

3.1.2 Hyalella azteca

In both soft (50 mg/L as $CaCO_3$) water conditions and hard (300 mg/L as $CaCO_3$), *H. azteca* showed no significantly higher percent mortality in any treatments, as tested by a one-way ANOVA ($p = 0.512$, $n = 3$ and 0.987, $n = 5$ respectively), which includes both individual contaminants and mixture treatments (Figure 6). Cadmium, selenium, sulphate, and nitrate maximum BC WQG are protective for *H. azteca* despite the difference in hardness. In the same hardness extremes, *H. azteca* does not respond differently to a mixture of these four contaminants.

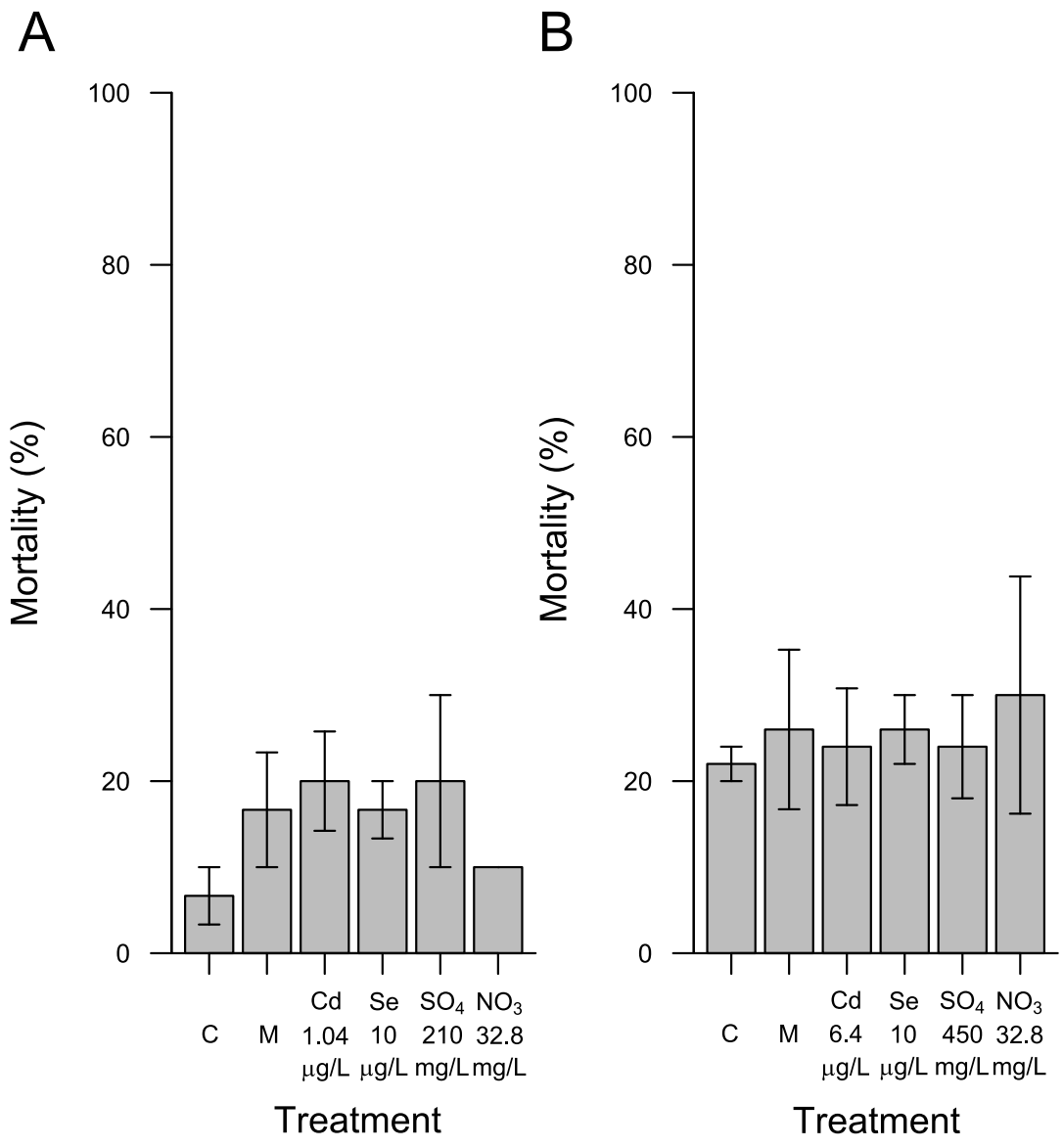

Figure 6. The mean (+/- SEM) percent mortality of *Hyalella azteca* after a 48 hour exposure to four individual contaminants: control (C), cadmium (Cd), selenium (Se), sulphates (SO_4), and nitrates (NO_3), in addition to their mixture at environmental guideline concentrations. No significant differences between the treatments were detected for both soft (A) water ($p = 0.512$, $n = 3$) and hard (B) water conditions ($p = 0.987$, $n = 5$).

3.2 Chronic exposure testing

3.2.1 Daphnia magna

Daphnia magna were continually exposed to a mixture, and the four contaminants, for 21 days in both soft (50 mg/L as $CaCO_3$) and hard (300 mg/L as $CaCO_3$) water conditions. Survivorship of organisms was observed every 48 hours (n = 40 organisms per treatment). In soft water conditions, survival of *D. magna* declined gradually as shown in Figure 7, but individuals had survived by end of the 21 day exposure for all treatments. Survival analysis, by Cox proportional hazard model, compared each treatment to the control survivorship, revealing that the declination of survival for the mixture (hazard ratio = 3.0186, $p < 0.0001$) and sulphate (hazard ratio 2.2101, $p = 0.00428$) treatments was significantly more hazardous than the other contaminants.

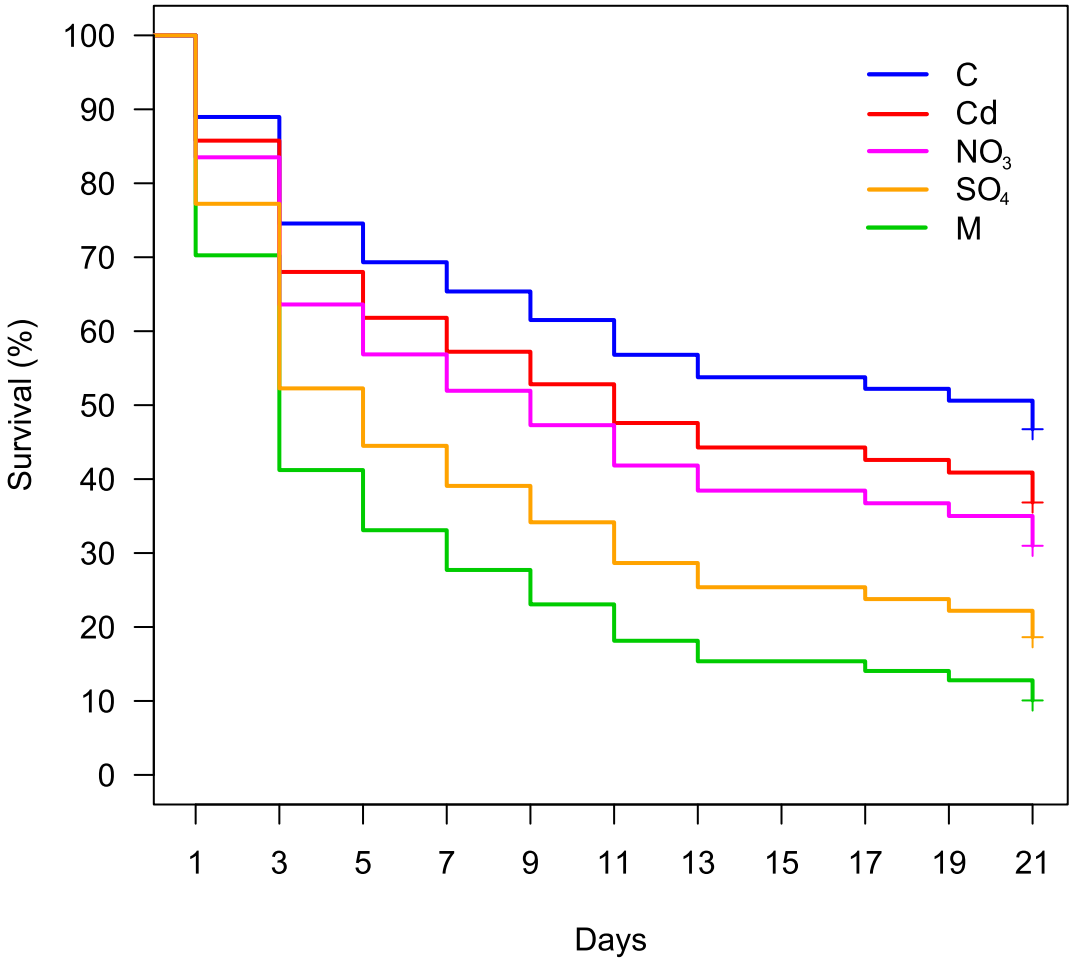

Figure 7. Survivorship of *Daphnia magna* exposed for 21 days to contaminants in soft water (50 mg/L as $CaCO_3$) (n = 40). The treatments were compared to control (C) using a relative hazard ratio; including cadmium (Cd) at 1.3132 (p = 0.35614), sulphates (SO_4) at 2.2101 (p = 0.00428), and nitrates (NO_3) at 1.5409 (p = 0.13244), in addition to their mixture (M) at 3.0186 (p < 0.0001). Treatments are relevant to proposed provincial WQG, as described in Table 8.

In hard water conditions (refer to Figure 8), only the mixture (hazard ratio = 3.0186, $p < 0.0001$) treatment compared to the regression of control survivorship was significantly more hazardous than the single contaminant treatments. In contrast to the soft water conditions, the hard water chronic exposure showed that the sulphate treatment (hazard ratio = 1.16327, $p = 0.59123$) did not cause a significant decline in survivorship.

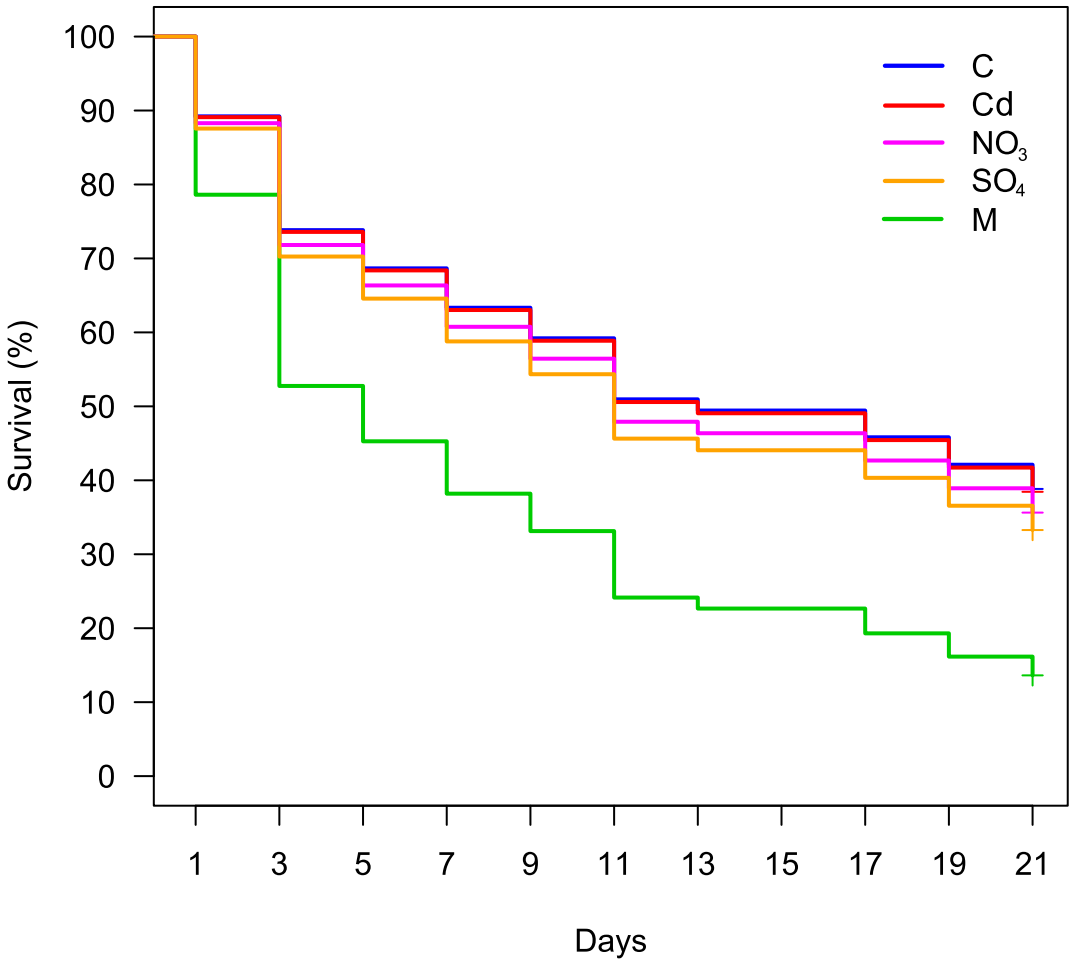

Figure 8. Survivorship of *Daphnia magna* exposed for 21 days to contaminants in hard water (250 mg/L as $CaCO_3$) (n = 40). The treatments were compared to control (C) using a relative hazard ratio; including cadmium (Cd) at 1.0106 (p = 0.97148), sulphates (SO_4) at 1.16327 (p = 0.59123), and nitrates (NO_3) at 1.09092 (p = 0.76117), in addition to their mixture (M) at 2.10721 (p = 0.00535). Treatments are relevant to proposed provincial WQG, as described in Table 8.

3.2.2 Hyalella azteca

Hyalella azteca were exposed to four individual contaminants and their mixture in only SAM medium (120 mg/L as $CaCO_3$) for 21 days (n = 30). All treatments show that *H. azteca* survivorship declined until the end of the study, but some individuals remained alive by the end of the 21st day. In Figure 9, it was observed that compared to control survivorship, cadmium treatment (hazard ratio = 3.7732, p = 0.00145) was significantly more hazardous. However, the mixture treatment (containing cadmium) was found to not be any more harmful than the control treatment (hazard ratio = 1.1842, p = 0.72791).

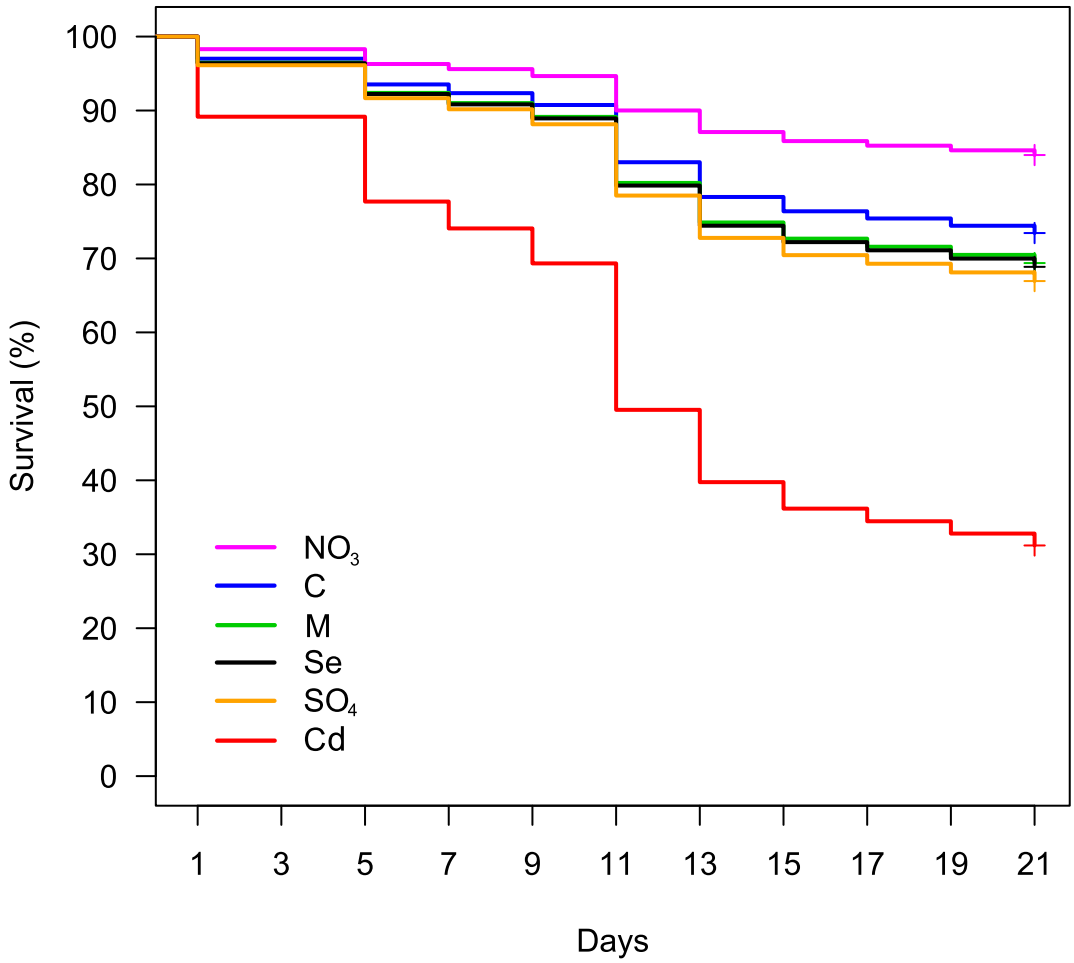

Figure 9. Survivorship of *Hyalella azteca* for 21 days exposed to contaminants in a standard artificial medium (120 mg/L as $CaCO_3$) (n = 30). The treatments were compared to control (C) using a relative hazard ratio; including cadmium (Cd) at 3.7732 (p = 0.00145), sulphates (SO_4) at 1.2998 (p = 0.58042), selenium (Se) at 1.2082 (p = 0.69718) and nitrates (NO_3) at 0.5653 (p = 0.31714), in addition to their mixture (M) at 1.1842 (p = 0.72791). Treatments are relevant to proposed provincial WQG, as described in Table 8.

Due to culturing difficulties, *H. azteca* could not be similarly exposed in 50 and 250 mg/L as $CaCO_3$. The following experiment observed the survivorship of juvenile *H. azteca* in five different media, which vary from 50 to 250 mg/L as $CaCO_3$ water hardness without any additional contaminants (n = 30). *Hyalella azteca* are cultured at 120 mg/L as $CaCO_3$, and so as a control treatment, a greater than 80% survival was expected. However, *H. azteca* observed for 21 days in softer media prepared at 50 mg/L (hazard ratio = 2.9999, p = 0.0165) and 80 mg/L as $CaCO_3$ hardness (hazard ratio = 3.12217, p = 0.0121) treatment was found to be significantly more hazardous than the control medium.

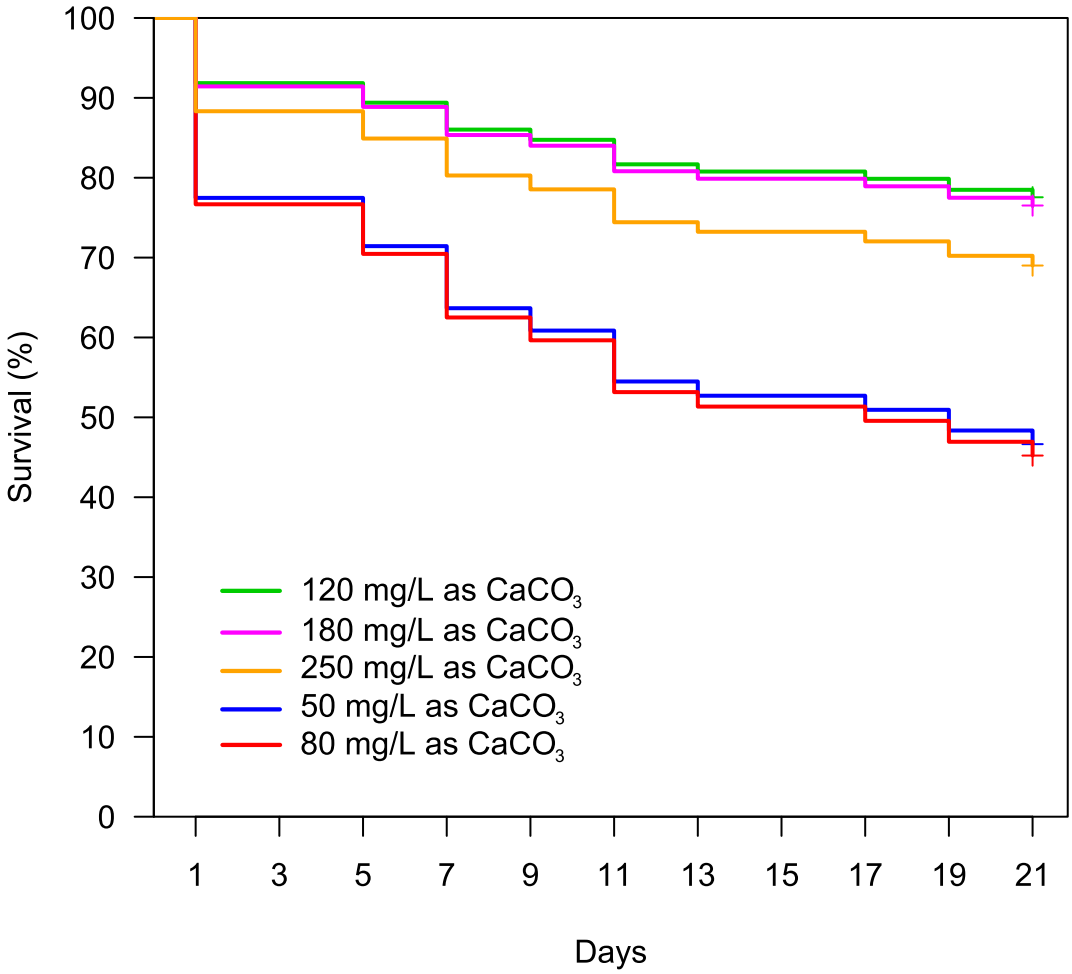

Figure 10. Survivorship of *Hyalella azteca* for 21 days in five media types, ranging from 50 to 250 mg/L as $CaCO_3$ water hardness (n = 30). No contaminants were added to treatments. The treatments were compared to 120 mg/L as $CaCO_3$ (control) using a relative hazard ratio; including 50 mg/L as $CaCO_3$ at 2.9999 (p = 0.0165), 80 mg/L as $CaCO_3$ at 3.12217 (p = 0.0121), 180 mg/L as $CaCO_3$ at 1.05263 (p = 0.9236) and 250 mg/L as $CaCO_3$ at 1.45926 (p = 0.4432).

3.2.3 Rainbow trout (Oncorhynchus mykiss) - Early life stage

Immediately after egg fertilization, rainbow trout were observed every 24 hours for 21 days in both soft (50 mg/L as $CaCO_3$) and hard (250 mg/L as $CaCO_3$) water conditions (n = 45). In soft media (50 mg/L as $CaCO_3$), rainbow trout survival compared to the control survivorship declined significantly for all treatments. In increasing order of hazard, the treatments that were significantly more hazardous than the control were: cadmium (hazard ratio = 3.9457, $p < 0.0001$), sulphates (hazard ratio = 5.0347, $p < 0.0001$), nitrates (hazard ratio = 7.7292, $p < 0.0001$), selenium (hazard ratio = 8.4907, $p < 0.0001$), and the mixture (hazard ratio = 8.9158, $p < 0.0001$). On day 16 (refer to Figure 11), the survivorship of *D. magna* succumbed to a major decline, which corresponds to the life cycle transition from eyed egg stage to alevin stage.

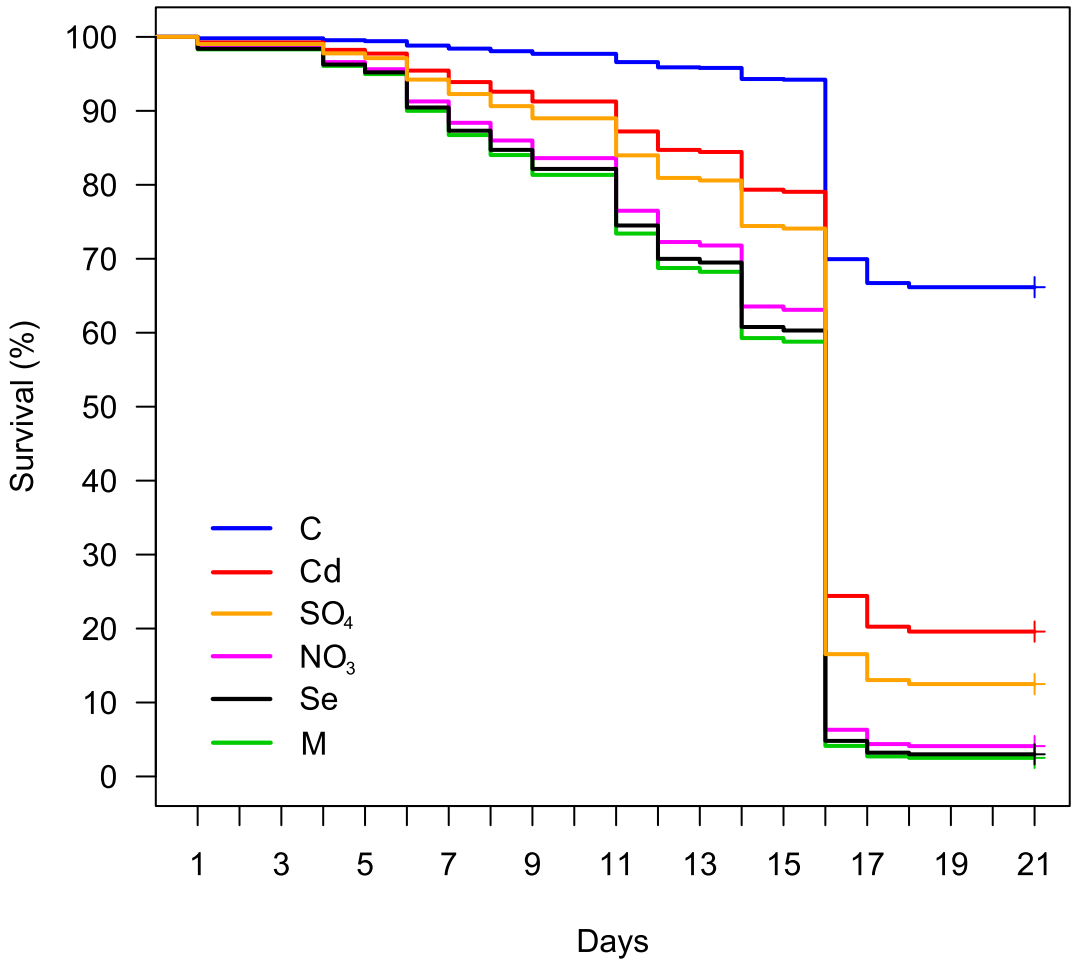

Figure 11. Survivorship of rainbow trout eggs exposed for 21 days to contaminants at proposed BC WQG in soft (50 mg/L as $CaCO_3$) dechlorinated water (n = 45). The treatments were compared to control (C) using a relative hazard ratio; including cadmium (Cd) at 3.9457 ($p < 0.0001$), sulphates (SO4) at 5.0347 ($p < 0.0001$), selenium (Se) at 8.4907 ($p < 0.0001$) and nitrates (NO3) at 7.7292 ($p < 0.0001$), in addition to their mixture (M) at 8.9158 ($p < 0.0001$). Treatments are relevant to proposed provincial WQG, as described in Table 7.

In hard media (250 mg/L as $CaCO_3$), all single contaminant treatments were significantly more hazardous to rainbow trout than the control, except for their mixture (hazard ratio = 1.9128, $p = 0.0639$). In increasing order of hazard, single contaminant treatments that were significantly more hazardous than the control were: sulphates (hazard ratio = 6.1098, $p < 0.0001$), cadmium (hazard ratio = 6.5249, $p < 0.0001$), selenium (hazard ratio = 6.9051, $p < 0.0001$), and nitrates treatment (hazard ratio = 12.8549, $p < 0.0001$).

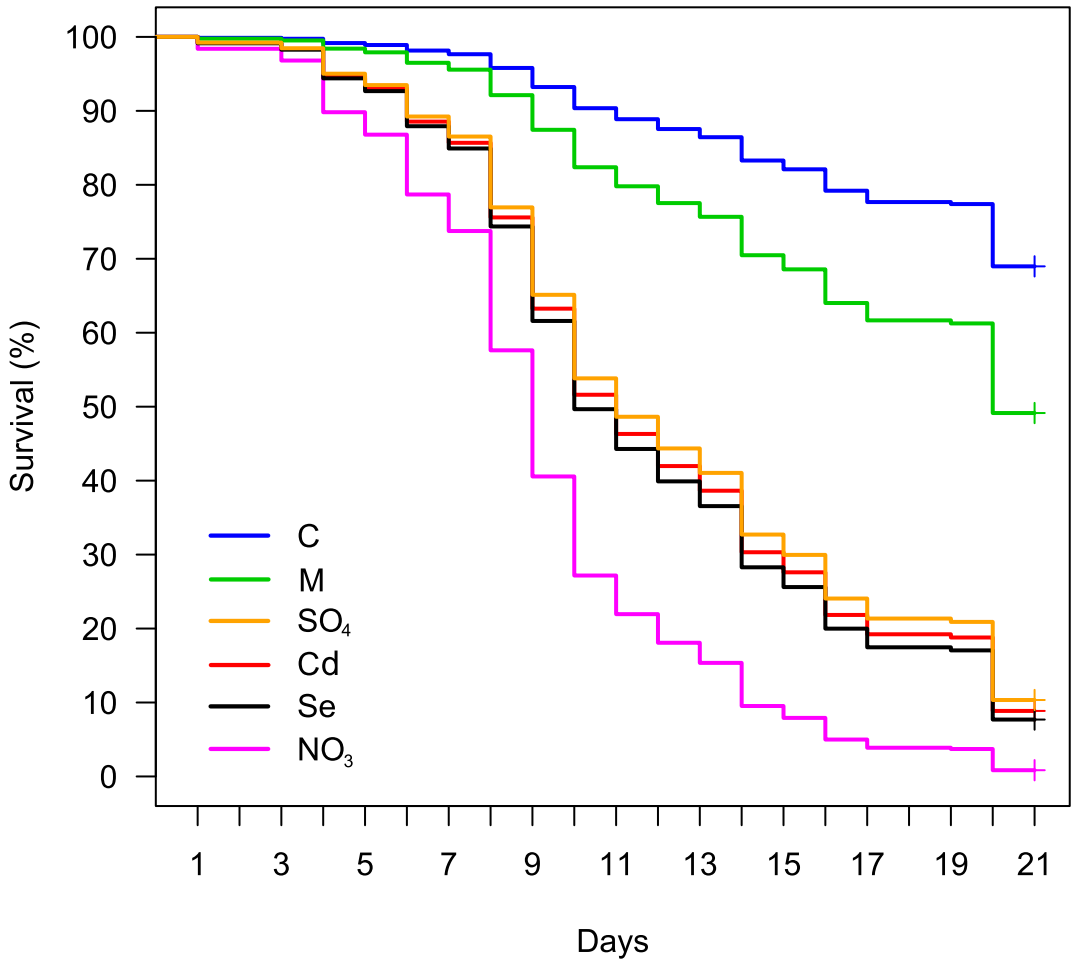

Figure 12. Survivorship of rainbow trout eggs exposed for 21 days to contaminants at proposed BC WQG in hard (250 mg/L as $CaCO_3$) dechlorinated water (n = 45). The treatments were compared to control (C) using a relative hazard ratio; including cadmium (Cd) at 6.5249 ($p < 0.0001$), sulphates (SO4) at 6.1098 ($p < 0.0001$), selenium (Se) at 6.9051 ($p < 0.0001$) and nitrates (NO3) at 12.8549 ($p < 0.0001$), in addition to their mixture (M) at 1.9128 ($p = 0.0639$). Treatments are relevant to proposed provincial WQG, as described in Table 8.

4.0 Discussion

4.1 Complex mixtures of contaminants

Under both hard and soft water exposure conditions, a mixture of four contaminants (Cd^{2+}, SeO_4^{2-}, SO_4^{2+}, NO_3^-) was either safe or unsafe depending on the species being exposed to the treatment. The specific toxicological response of freshwater invertebrates (*D. magna* and *H. azteca*) and vertebrates (rainbow trout), when exposed to a mixture of cadmium, selenium, nitrate, and sulphate at safe concentrations for a short and long term, was not explored previously. As reviewed in Tables 1 to 4, no local or international WQGs incorporate mixture data, or implement adjustments in the guideline limit for potential mixture effects, other than with the application of uncertainty factors such as those used in the BC WQGs. As a mixture, the toxicity of individual substances can impose a different overall effect on aquatic life in a natural setting.

The exposure of multiple substances polluting freshwater can result in a reduced toxicity, due to antagonistic effects between substances, thereby reducing the overall toxicity of a mixture of contaminants. In the acute binary exposure experiment shown in Figure 5, we see an interaction between cadmium and selenium that could be responsible for a reduced toxicity of cadmium in *D. magna*. A study with marine dinoflagellates showed that with low concentrations of both cadmium and selenium present, the toxicity was also reduced due to an antagonism effect (Prevot and Soyer-Gobillard 1986). The toxicity of both these substances has been associated with the interference of calcium uptake; both cadmium and selenium is known to share common binding sites with calcium on the epithelia of *D. magna* (Winner and Whiteford 1987; Guan and Wang 2004). The selenium concentration for the BC WQG is expected to be safe for *D. magna*

because it is an essential nutrient for healthy development and reproduction (Environment Canada 1996; Elendt and Bias 1990). The reduced mortality of an acute exposure to a mixture of four contaminants (seen in Figure 4) to *D. magna* could be the result of a short term antagonistic effect of selenium on cadmium toxicity. The model for predicting the toxicity of combined selenium and cadmium mixtures to *D. magna* could utilize the concentration addition approach.

Although the mixture of contaminants showed no toxic response in the short term, a chronic exposure to a mixture of long term WQGs demonstrated that there is a decreased survival in our test organisms. In contrast to acute WQG, the chronic exposures with *D. magna* (Figure 7 and 8) demonstrated a hazardous effect of the mixture of contaminants over the course of 21 days. Since the individual exposure treatment with sulphate caused a more hazardous response than the other individual contaminant treatments, it can be assumed that the higher mixture toxicity relies primarily on the toxicity of this contaminant. While exploring the mechanism of selenate toxicity in the study by Ogle and Knight (1995), it was indicated that higher concentrations of sulphate could diminish the uptake of selenium. Considering there could be a decreased selenium uptake due to these increased sulphate concentrations, the overall toxicity of the mixture is likely a result of reduced selenium allowing a higher sensitivity to cadmium, and the sulphate toxicity alone (as the sulphate treatment was also toxic). In order to provide effective environmental risk management, further studies on the complexity of contaminant interactions should be explored, especially when the synergistic or potentiated interactions (where the toxicity is increased to resident organisms) between substances

could cause ecological harm despite being within the bounds of water quality standards (Jonker et al. 2005).

Hyalella azteca may be less vulnerable to a mixture than other species in water only exposures, as is adapted to living near the sediment level (an area with possibly different interactions) (Gust 2005). For exposures with *H. azteca,* there was no detectable difference between the mixture and the individual four contaminants, indicating that the contaminants might be below the concentrations for components to interact in acute studies. However, a potential mixture effect, which is the reduced toxicity of cadmium (antagonism) was found between cadmium and another contaminant within the mixture for the chronic exposure to *Hyalella azteca*. The availability of free cadmium ions is directly related to its toxicity (Borgmann et al. 1991), and can be reduced by dissociated sulphate and nitrate anions binding to available cadmium cations. Further studies could investigate the effects of increasing hardness on *H. azteca*, which has a protective effect on cadmium toxicity, in the presence of increased concentrations of other contaminants like selenium, nitrates and sulphates.

The influence of hardness has been found to have an effect on the sensitivity of eyed eggs to a mixture of contaminants. Although it was not shown with *H. azteca,* hardness had a protective effect on the toxicity of the mixture with *O. mykiss*, where the mixture was less hazardous than contaminants in soft water (50 mg/L as $CaCO_3$). All contaminants were hazardous to eggs when exposed individually, and most hazardous when combined in a mixture. Therefore, hardness plays a dominant role in the range of sensitivity of early stage rainbow trout to a combination of pollutants.

4.2 Hardness effects on toxicity

In standardized toxicity testing protocols, culturing of *Hyalella azteca* and *Daphnia magna* is recommended to follow optimal culture media conditions. In studies that investigate an exposure to a test medium with a different hardness, the standardized protocol require *D. magna* to be cultured in water that is within 20% of the test medium hardness for at least 7 days (Environment Canada 1996). This is a particularly stressful period for the organisms, as mentioned by Borgmann et al. (1989): *H. azteca* had increased uptake of calcium and cadmium ions as acclimation to soft water conditions occurred. In my study, when exposed to different chemical ionic compositions with only two days acclimation, softer medium was more hazardous to *H. azteca* during the 21 day exposure (Figure 10), within the first 24 hours. The attempts to acclimate adult *H. azteca* for longer than a period of two days resulted in an inadequate amount of young for chronic testing. Further studies that are concerned with long-term exposures should incorporate this sensitivity to hardness, in addition to the toxicity of substances, as it has an influence on the development of *H. azteca*.

Although the cadmium concentration in my study was adjusted according to the hardness equation from the proposed BC WQG to prevent lethal cadmium toxicity, water hardness surprisingly offered little protection against cadmium toxicity as demonstrated by the high *D. magna* mortality observed under short term hard water exposure conditions (Figure 4). Calcification occurs during the first 48 hours after molting of the invertebrate species (there is a restricted time window for calcification, despite a prolonged exposure to calcium). The presence of calcium ions (or increased hardness of water) is known to reduce the uptake of cadmium in *D. magna*, and therefore reduce its

toxicity. The protection offered by water hardness from cadmium is suspected to be due to calcium ion interference in cadmium ion uptake (Tan and Wang 2011; Tan and Wang 2008). The hardness equation concept was largely based on studies that assumed that a linear relationship between decreasing cadmium toxicity and increasing calcium ions exists for all species (CCME 1999). In the acute soft water exposure to cadmium, *D. magna* neonates did not demonstrate the same response as in the hard water exposure, in that cadmium caused more toxicity in hard water (Figure 4). Although hard and soft water acute experiments were conducted with a different number of replicates, there is potentially a non-linear relationship between cadmium and calcium for this species in contradiction to what is assumed by the hardness adjustment factor on the BC WQG. A non-linear and non-protective relationship between cadmium and increased water hardness would require further investigation (in additional species as well), as cadmium toxicity could vary greatly in waters with high hardness.

4.3 Influence of duration on toxicity

Chronic responses for *D. magna* were used in the BC WQG derivation, and these data contribute to the derivation of a much lower concentration for the BC WQG for cadmium during the long-term exposure in this study. Although the invertebrates did not respond to the contaminants within the acute 24 hour period, the chronic exposure period showed an increased toxicity of all contaminants. However, for *H. azteca*, the lack of an acute toxic effect in these exposures (in Figure 6) to the mixture under culture conditions it can be assumed that most likely there are no interaction between the four contaminants. However, the survivorship in Figure 9 demonstrates a possible interaction between cadmium and other contaminants could be offering some protection from cadmium

toxicity in moderate hardness conditions. It can be inferred that *H. azteca* is more tolerant of high cadmium concentrations at the BC WQG for a short period. The steady decrease in survivorship in my results of the chronic experiment with *H. azteca* and the study by Suedel (1997) demonstrates that there is a sensitivity of *H. azteca* to cadmium. In chronic exposures with *H. azteca*, decreased survivorship was observed during a prolonged exposure beyond 14 days, which may be missed in the results of the recommended 13 day standardized exposure period for standardized toxicity testing that is outlined in the EPS guidelines (Environment Canada 2013).

There are two explanations for this delay in response for chronic exposures. In long-term exposure results, we see that the initial days of exposure to cadmium could have allowed for less tolerant individuals within the treatment to die, but after this initial exposure period, the mortality remained relatively stable compared to the other treatments in the study. This stable response has been seen in another exposure with *D. magna*, where cadmium toxicity did not increase after 7 days into the exposure (Suedel et al. 1997). The other explanation may be due to a *Daphnia magna* cadmium detoxification mechanism (Fraysse et al. 2006), where metallothionein-like proteins bind to non-essential Cd^{2+} and it is possible that this mechanism is initiated after a week of development and exposure. Future studies with long term exposure periods could shed light on the detoxification mechanism of contaminants for less conservative and more appropriate environment management strategies.

Rainbow trout was exposed to contaminants and their mixture for 21 days, initiating from fertilization of an egg. This life stage is used in toxicity studies because of

its high sensitivity to changing water conditions, which would have influenced the mortalities seen in the 21 day exposure. Certain stressors like extreme fluctuations in temperature, physical shock, and water quality all can interfere with a healthy progression of early development and allows the development of a depressed immune system in rainbow trout eggs (Environment Canada 1998). Due to the disruption of this development, it could be assumed that structures involved in preventing the permeability of toxic compounds (such as the chorion) are compromised, allowing for continual exposure and increased susceptibility to lethal concentrations of contaminants later in the exposure (Van Leeuwen et al. 1985). The active use of recently developed gills is another contributor to the survivorship declining vastly during the alevin stage; because of this maturation, susceptibility to contamination can occur (Middaugh and Dean 1977).

Experimental variables that impact toxicity testing were explained in the US EPA protocol for chronic testing (1996) and include the physical shock from rough handling and stress from temperature fluctuation and ionic shock from the renewal solutions. Although steps were taken to prevent this type of stress on the test organism (such as gentle pipetting and pouring), the compounding frequency of stressful effects could have an impact on the long-term survival and therefore increase beyond natural mortality of the species.

4.4 Species responses

The federal and BC's provincial WQG is expected to protect freshwater species like *D. magna* from the lethal acute toxic effects of dissolved contaminants such as cadmium. In the results of the acute exposure studies, *D. magna* mortality was elevated in

media containing cadmium at proposed BC WQG concentrations in both hard and soft water conditions. In comparison to the acute responses of other freshwater species, *D. magna* can only tolerate very low concentrations of cadmium. In derivation of the BC and CCME WQG for cadmium, a linear regression model involving 40 freshwater species (including *Hyalella azteca*) was created to incorporate the relationship between hardness and cadmium toxicity. This approach used for other WQG for the US, Australia and New Zealand, is based on a species sensitivity distribution (SSD), of which its objective is to obtain the concentration limit that protects 95% of local species. Among the species used in my study, *D. magna* was the most sensitive within the range of species susceptible to contaminants (such as cadmium). Overall, both invertebrates and rainbow trout species showed sensitivity to freshwater contamination, but the nature of the sensitivity was species-specific. Selection of toxicity data for various species can effectively influence what the guideline concentration is and which species may actually be protected.

For the purposes of identifying only the effects of exposures to multiple contaminants, the species used in this study were those reared a laboratory. However, since variables in these experiments are controlled (pH, hardness, temperature, etc.), the stressors in the environment could have an impact on local biota that would not be detected here. Ideally, these studies should be conducted using species and conditions from the field, where not only is it more likely to have multiple pollutants present, but there may be changes in environmental factors (temperature, pH, mixing zones, etc). Also, the genetic background of these species may allow a tolerance, as in the sensitivity of species to contaminants may influence the response. Considering this, the *H. azteca*

that was used in my study and for other toxicological studies may not represent the same responses, and may be more sensitive than organisms found in the environment (Major et al. 2013; Duan et al. 2001). Genetic differences have also been seen to influence toxic responses, especially in the case of local adaptations to increasing pollutant risk within some localized populations of *D. magna* (Coors et al. 2009),

The control treatment is used to represent the natural mortality of the population (where a portion of the population may have weak or highly sensitive individuals) in the presence of no additional contamination (Rand 2001). Standardized procedures for toxicity testing like the USEPA will also use control response for validation, and require the mortality be ≤ 20% for invertebrates or ≤ 40% for early stage rainbow trout trials (USEPA 2002; Environment Canada 1998). To determine if a treatment is harmful, the experiments in my study compared the results to that of the survivorship of the control. Although these did not meet the criteria of Environment Canada's standardized test protocols in every experiment, the interpretations reported here are still useful because of the relative comparisons to the control treatment within each experiment.

4.5 Conclusion/ Future Directions

It was determined in this study that *Daphnia magna, Hyalella azteca* and *Oncorhynchus mykiss* were tolerant to a mixture of four contaminants (cadmium, selenium, sulphate and nitrate), depending on the duration and the hardness conditions of the exposure. Duration was a factor in the response of species, such as the mixture of four contaminant exposed *D. magna,* which was safe in the short term, likely due to an antagonistic effect of selenium on cadmium in the mixture. However, a long term

exposure showed that contaminants within a mixture could interact to be harmful to certain species like *D. magna*. As well, hardness is known to have a protective effect on some contaminants, but this may not prevent the toxicity of other substances present in freshwater and may even cause some mortality without contamination. Although the interaction between the contaminants was not identified definitively, the mixture of contaminants was toxic during longer exposures, even compared to the short term experiments.

More studies on other sensitive species exploring the effect of a toxic mixture would aid in the development of better environmental quality standards. Although the toxicity of individual components is important, more information on the response of local and potentially endangered freshwater species to a mixture of pollutants from the environment can be studied further through the modeling of mixture toxicity. Due to a small sample size and a high mortality in controls, these experiments do not fit the criteria required to support water quality guideline derivation. These experiments should be repeated for an increased statistical power and to confirm the interpretations that were discussed.

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