

DISSERTATION

SELENIUM SPECIATION DETERMINED BY ICPMS: EFFECTS ON FISH DIVERSITY,  
SOLUBILITY, AND BIOAVAILABILITY TO THE BRYOPHYTE HYGROHYPNUM  
OCHRACEUM IN FOUNTAIN CREEK, COLORADO

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## ABSTRACT

### SELENIUM SPECIATION DETERMINED BY ICPMS: EFFECTS ON FISH DIVERSITY, SOLUBILITY, AND BIOAVAILABILITY TO THE BRYOPHYTE *HYGROHYPNUM* *OCHRACEUM* IN FOUNTAIN CREEK, COLORADO

Selenium (Se) is a micronutrient that can be present in high levels in aquatic environments which may result in toxic effects observed in aquatic wildlife. The levels in Fountain Creek Colorado are of special concern as these levels are above the EPA limit of 5 µg/L. The high Se levels are a result of the exposure of the water to Pierre Shale deposits that underlie parts of the creek. The effects of this creek water on fish diversity were examined at different locations along the creek. The hypothesis tested was that high Se present in the water and bryophytes should be an indicator of fish species diversity. In addition, the possibility of low toxicity resulting from Se species was explored. The speciation analysis determined the levels of Se(IV) and Se(VI) at 12 sites and the statistical results show that sites with higher Se(IV)/Se total exhibit lower fish diversity and fish number than the other sites.

There is a statistically significant difference in Ca, Mg, and Se levels in each of the 3 main tributaries in the Fountain Creek Watershed. The tributaries are Monument Creek and Upper Fountain Creek, which join at a confluence near Eighth Street in the city of Colorado Springs, Colorado, to form the Lower Fountain Creek which empties into the Arkansas River in eastern Pueblo, Colorado. The following factors were considered in determining the forms of Se that could exist:  $\text{Ca}^{2+}$ ,  $\text{Mg}^{2+}$ ,  $\text{SeO}_4^{2-}$ ,  $\text{SeO}_3^{2-}$ ,

and carbonates in addition to the reported thermodynamic relationships. There is a correlation that exists between water hardness and Se level. This correlation can be described in terms of the formation of a soluble  $\text{CaSeO}_4$ . The formation of  $\text{CaSeO}_4$  is assisted by the increase in  $\text{Ca}^{2+}$  by the presence of  $\text{Mg}^{2+}$  regardless of the equilibria with the  $\text{Ca}^{2+}$  level reducing carbonate.

The bryophyte *Hygrohypnum ochraceum* has been shown to accumulate zinc, cadmium, and lead is often found growing near acid mine drainages. This natural ability to accumulate metals makes *H. ochraceum* an good organism to use in the study of heavy metals and metalloids in the environment. In a previous work the bryophytes were shown to differentially uptake Se based on season. In this study the seasonality of the uptake of Se was examined and it is suggested to be related to an Iron (Fe) cofactor. The *H. ochraceum* cultures were placed in the creek for 10 days, harvested, dried and digested according to EPA Method 3052. The resulting digestates were analyzed using EPA Method 6020a for ICPMS metal determination. The results show that Fe and Se uptake are correlated. The hypothesis that was developed from these results is that Fe is needed by the plant for Se uptake from the creek water. Indeed *H. ochraceum* demonstrated statistically significant log-linear uptake of Se in the presence of dissolved Fe ( $R^2=0.8488$ ,  $p=0.002$ ). Se uptake was negatively sloped in the fall compared to the linear relationship in the spring. It was determined that the Fe in the water went from a soluble form to an insoluble form. We failed to reject the null hypothesis that Fe is not required in a soluble form for the bryophyte *H. ochraceum* to uptake Se. Further examination did show that a significantly different slope exists

between the Se(IV) uptake and the Se(VI) uptake. There was not a significant difference between the total dissolved Se and Se(VI).

In the future directions, this work could be extended to look at specific markers for Se induced stress in the fish populations that may aid in determining the cause for a lack of diversity in some areas as the habitat along the reaches is similar for that reach. Targeted research of water chemistry could investigate the interesting solubility phenomenon giving rise to the seasonal variation of the Fe in Fountain Creek. Finally, the use of plants as bioremediation in the high Se areas could be further investigated with the knowledge that plant nutritional needs must be accounted for when using plants such as *H. ochraceum* as biological indicators or biological remediators.

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## CHAPTER 1 THE EFFECTS OF SELENIUM IN THE BIOSPHERE AND IN FOUNTAIN CREEK COLORADO

The examination of Selenium (Se) in this introductory chapter will illustrate the multifaceted role it plays in nutrition and toxicology. Selenium chemistry and speciation is a topic that is necessary for the fundamental understanding of Se as a nutrient and toxin. It is also a topic that plays a role in Se uptake, environmental stability and availability. Since plants are the primary producers in an ecosystem the native bryophyte *Hygrohypnum ochraceum* was used as a model plant to study the bioavailability of Se found in Fountain Creek and its two main tributaries. These tributaries and specifically lower Fountain Creek are known to have high levels of Se. The availability of Se in the Fountain Creek watershed as determined by the bryophytes and fish are the topic of this thesis.

### **SELENIUM AND HEALTH: NUTRIENT AND TOXIN**

Selenium is essential micronutrient for all mammals including humans and is important for many cellular processes (Bird, Ge et al. 1997, Bird and Tyson 1997, Combs and Gray 1998, Mousa, O'Connor et al. 2007, Reyes, Mar et al. 2009, Weekley and Harris 2013, Block, Booker et al. 2016). However, at elevated levels, Se is also toxic and can cause increased risk of cancer and death due to Se poisoning (Combs and Gray 1998). Selenium toxicity manifests itself in two forms; acute and chronic. In the acute form Se is absorbed in large doses whereas as in the chronic form the lower dose is accumulative over a longer period of time (MacFarquhar, Broussard et al. 2010). An example of acute toxicity was presented in a case study involving the consumption of a dietary supplement that had Se levels of 40,800 µg/1 oz (MacFarquhar, Broussard

et al. 2010). In this case, 201 people in 10 states reported having signs of Se toxicity which include: vomiting, nausea, nail discoloration, nail brittleness and loss, fatigue, hair loss, irritability, and foul breath odor, also called “garlic breath” (Yang, Wang et al. 1983, Fan and Kizer 1990, United States Department of Health and Human Services 2003, Nuttall 2006). In the aforementioned case the symptoms of toxicity existed for more than 90 days due to the differential perfusion of Se into tissues (MacFarquhar, Broussard et al. 2010). The reason for the long recovery period is that the human body does not have an efficient mechanism for detoxification and elimination of high levels of Se (MacFarquhar, Broussard et al. 2010). Toxicity from chronic exposure is more difficult to determine and depends on the soils and geological characteristics of a region (Gore, Fawell et al. 2010). The evidence of chronic exposure to high Se levels can range from no clinical signs to fatigue, lesions of the skin, loss of hair and nails, loss of appetite, gastrointestinal disturbances, cardiac insufficiency and heart failure (Gore, Fawell et al. 2010). Lowered levels of glutathione peroxidase have been measured in individuals with high Se exposure originating from ground water (Gore, Fawell et al. 2010). While the evidence is not concrete Se exposure has also been linked to an increased risk for type 2 diabetes (Bleys, Navas-Acien et al. 2007).

The Se levels required to sustain health exist in a narrow window, where too much or too little can be deleterious to health and where toxicity depends on the Se species. As a result, Se environmental levels are regulated and strictly enforced by the EPA in the USA (Edelmann, Ferguson et al. 2005, Divine and Gates 2006, Colorado Department of Public Health and the Environment Water Quality Control Commission 2013, United States Environmental Protection Agency 2016). Moreover, global dietary

guidelines for human daily Se intake vary widely with most countries recommending 60-70 µg/day (Kim and Mahan 2003). The current United States Recommended Daily Allowance for intake of selenium is 55 µg/day for a healthy adult person (National Institute of Health 2013) with a tolerable upper limit of 400 µg/day in a healthy adult (United States Department of Health and Human Services 2003).

The duality of Se as a toxin and nutrient was developed during the 20<sup>th</sup> century. Initially Se was considered an undesirable element for higher organisms in the first half of the 20th century, due to its toxicity at high levels (Oldfield 1987). The toxic effects were first confirmed in the western United States in 1933. This finding was determined in livestock that consumed selenium hyperaccumulating plants of the genus *Astragalus*, *Xylorrhiza*, *Oonopsis* and *Stanleya* (Oldfield 1987). A new perspective developed in the last half of the 20th century which recognized the need for selenium as a nutritional element (Oldfield 1987). Selenium was shown to prevent liver necrosis in rats being fed a Torula – yeast and vitamin E deficient diet (Schwarz and Foltz 1957). Additional support for the benefits of Se was shown in the discovery of its role in the formation of glutathione peroxidase, thioredoxin reductase, and other enzymes that protect against oxidative stress (Rotruck, Pope et al. 1973). Indeed a lack of dietary selenium can result in deficiency diseases that may include reproductive impairment growth depression and white muscle disease, which is a myopathy of heart and skeletal muscle primarily affecting calves (Reilly 1996). Human selenium deficiency diseases have also been found to include endemic cardiomyopathy and Kashin – Beck disease which is a type of osteochondropathy that is believed be caused by Se deficiency allowing mycotoxins to cause oxidative damage to the joints (Rayman 2000, Yao, Pei et al. 2011). In

mammalian systems, selenium is known to substitute for sulfur in key pathways resulting in the formation of selenomethionine (Sem), selenocysteine (Sec), selenodiglutathione, and glutathione selenopersulfide. In addition, more than 30 selenoproteins have been discovered to date and contain Sec and/or Sem residues (Rayman 2000, Tinggi 2008). Limiting dietary Se has serious health consequences as Se is often essential for the biological functions of these proteins.

There has been a great increase in knowledge of antioxidant selenoenzymes and the role of these enzymes in protecting cells from oxidative stress as well as the role of these enzymes and various diseases (Tinggi 2008). Gaps in knowledge extend to the mechanism of action by which Se aids in the protection of tissues and cells from damage due to oxidative stress (Tinggi 2008). Oxidative stress is linked to a host of previously mentioned diseases such as heart disease that result in deaths worldwide (Tinggi 2008, Yao, Pei et al. 2011) Furthermore the presence of these Se based diseases seems to be linked to environmental factors such as the geographical distribution of Se and the environmental availability of Se (Reilly 1996, Rayman 2000, Yao, Pei et al. 2011).

## **SELENIUM CHEMISTRY AND SPECIATION.**

Se exists in several oxidation states II, IV and VI and, in natural environments, the oxides  $\text{SeO}_2$  and  $\text{SeO}_3$  containing Se(IV) and Se(VI), respectively, are most common (Sors, Ellis et al. 2005). The most common species existing in aqueous systems is selenate ( $\text{SeO}_4^{2-}$ ) which is Se(VI) and is the stable form in the presence of oxygen (Sors, Ellis et al. 2005). The more reduced form selenite ( $\text{SeO}_3^{2-}$ ) is Se(IV) and is stable in more reducing environments (Sors, Ellis et al. 2005). Selenite in the presence of oxygen will oxidize to form selenate unless it is in a reducing environment.

Although there are significant differences in these two anions with respect to their redox reactivity, electronically and structurally they have many similarities. Se(II) is commonly observed in organoselenium compounds. Se(II) bound to C is particularly stable and major forms of Se that exist in biological systems are organometallic Se derivatives (Sors, Ellis et al. 2005), which are analogous to similar S compounds.

### **SELENIUM UPTAKE**

The uptake of Se varies with the Se source, anthropogenic or natural, and whether the Se is in the anionic inorganic forms, selenate or selenite, or one of the organic alkylated selenium compounds such as selenocysteine (Sors, Ellis et al. 2005). Selenocysteine and other amino acid derivatives are known to enter cells using amino acid transporters (Pilon-Smits and Quinn 2010) whereas selenate and selenite are believed to enter cells through the same transporters that support cell uptake of sulfate, sulfite and other oxoanions (Burk and Levander 1999, Rosen and Liu 2009, Pilon-Smits and Quinn 2010). Counterions are known to impact uptake and bioprocessing of (Chatkon, Chatterjee et al. 2013) other drugs, however little is known about the role of counterions for the uptake and processing of the inorganic selenium oxoanions in bryophytes or plants (Boudy, Voute et al. 2002).

### **SELENIUM AND THE ENVIRONMENT**

Selenium is readily available in many soils in North America resulting from the breakdown of selenium containing sedimentary deposits like shales (Dhillon and Dhillon 2014). Water quality issues exist from the contamination of rivers and streams by seleniferous agricultural runoff and exposure to selenium containing shales (Presser, Sylvester et al. 1994). The accumulation of high selenium waters due to runoff can have detrimental consequences on wildlife species. A well-known selenium contaminated site

is the Kesterson Reservoir in Merced County, California. Studies at Kesterson point to an increase in selenium as a cause for low egg hatching success, also called nest failure (Ohlendorf, Hothem et al. 1989). One of the most notable impacts of low hatching success was in Eared Grebes (*Podiceps nigricollis*) whose diet consists of insects and crustaceans, which likely were a source of dietary Se (Ohlendorf, Hothem et al. 1989). This finding illustrates how Se can get magnified in a food chain with resulting detrimental impacts.

On the other hand, there are organisms that do not appear to be negatively affected by high environmental levels of Se (Oldfield 1987). Bryophytes or aquatic plants related to mosses have been found to be indicators of environmentally biologically available Se and have been used in toxicology studies involving metals found in acid mine drainages (Nimmo, Herrmann et al. 2006, Herrmann, Turner et al. 2012, Nimmo, Herrmann et al. 2016). These organisms seem to have adaptive mechanisms for dealing with elevated Se exposure (Nimmo, Herrmann et al. 2006). Since, plants are the primary biomass producers and the beginning of the food chain of all ecosystems and contribute significantly to uptake of selenium from the environment (Pilon-Smits and Quinn 2010). *Hygrohypnum ochraceum* has a demonstrated ability to uptake metals from streams and rivers that have been impacted by anthropogenic sources (Claveri, Guerold et al. 1995, Nelson and Campbell 1995, M.A.S. and P.J. 1997, Samecka-Cymerman and Kempers 2001). Therefore, due to this ability, *H. ochraceum* was specifically used to study Se accumulation in a watershed and a river (Nelson and Campbell 1995, Herring, Castle et al. 2001). Selenium has become a major concern in Fountain Creek, due to its contact with Se



rich Pierre Shale (Edelmann, Ferguson et al. 2005). Fountain Creek is one of the larger tributaries of the Arkansas River Figure 1.1. This creek drains an area of 2,398 km<sup>2</sup> and includes areas of Pikes Peak, the cities of Colorado Springs, Monument, Fountain and Security (Bruce 2002). The Colorado Springs metro area is rapidly growing area and in 2010 contained 668,353 people (Census Bureau (United States Census Bureau 2011). Fountain Creek is a sandy bottom creek that has highly variable flows and is prone to flooding from increased storm flows due to expanding urbanization along the creek (Edelmann, Ferguson et al. 2005). In 2006, selenium was listed as a special concern along with non-point source pollution in The Fountain Creek Crown Jewel Project (Salazar 2006). Several water quality sampling efforts have been made on this tributary and all have shown levels above in-stream regulatory guidelines of >5.0 µg/L as total Se (Mueller, De Weese et al. 1991, Van Derveer and Canton 1997, Bossong 2001, Divine and Gates 2006).

## SELENIUM IN FOUNTAIN CREEK, COLORADO (STUDY SITES)

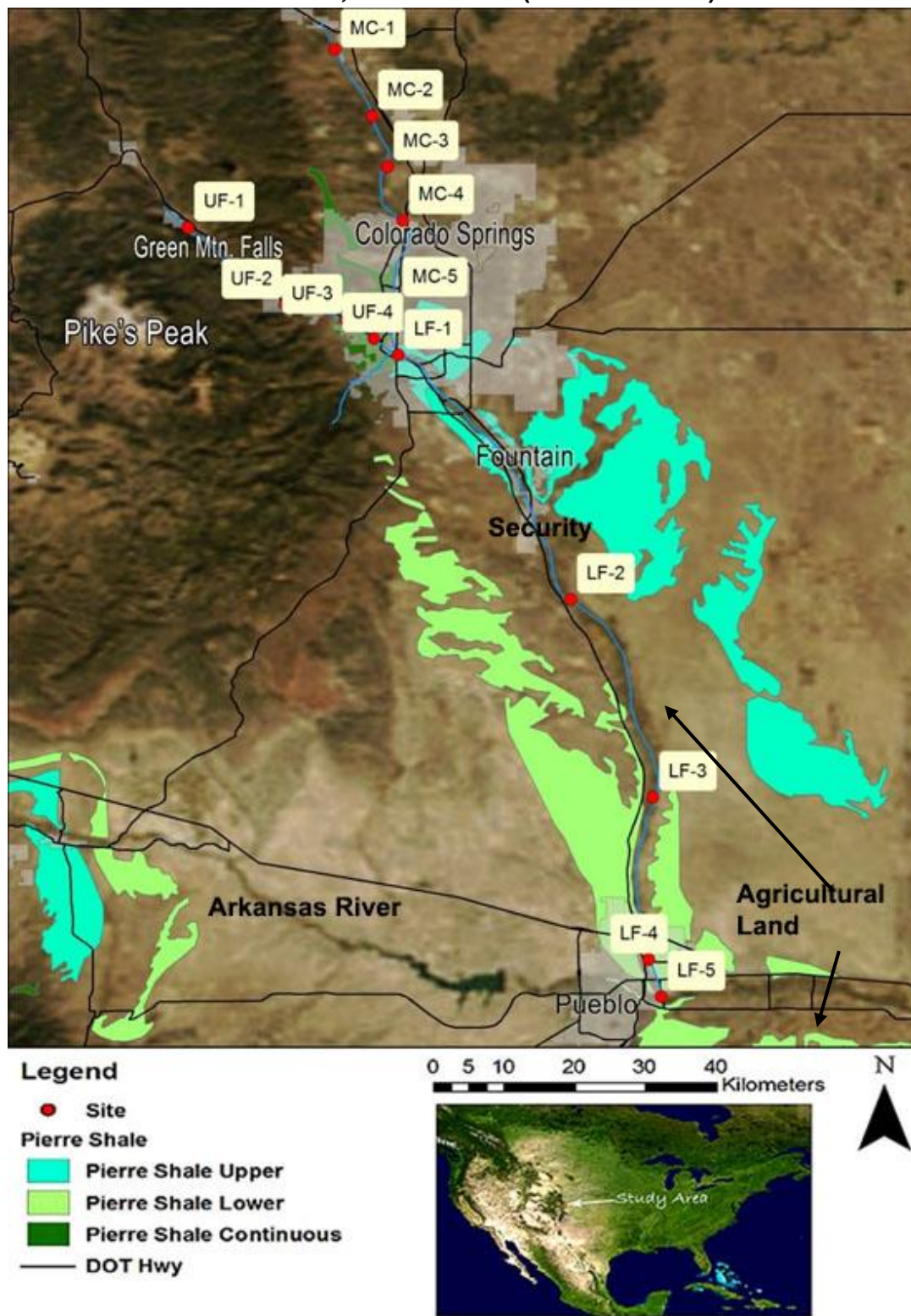


Figure 1.1 Map of study area with sample sites (Herrmann, Turner et al. 2012).

The Se load is carried by the Fountain Creek south as it enters the Arkansas River in the eastern part of the city of Pueblo (Herrmann, Turner et al. 2012). The area east of Pueblo is an agricultural area that raises several important food crops including chilies, melons, corn, tomatoes, pumpkins, winter squash and alfalfa(Howe, Lazo et al. 1990). There is also a large amount of pastureland in the Arkansas River basin east of Pueblo, Colorado. These areas are at risk of developing elevated levels of selenium released by Fountain Creek into the Arkansas River. The area of interest is managed by two water districts the Southeastern Colorado Water Conservation District and the Lower Arkansas Water Conservation District (Abbott and Southeastern Colorado Water Conservancy District 1985).

The coexistence of high levels of Se with agricultural land east of Pueblo, Colorado, makes it critical that studies examine the complex relationships between this element and the environment and its corresponding biota.

The Fountain Creek study area is shown in Figure 1.1. The study area consists of 14 collection sites with 4 sites distributed along the Upper Fountain Creek (UF1 – UF4). There are 5 collection sites along Monument Creek (MC1-MC5) and 5 collection sites along Lower Fountain Creek (LF1 – LF5). These sites were chosen according to the selenium distribution and potential for human impact on water chemistry in the Fountain Creek study area.

Upper Fountain Creek is a mountain creek that starts at a higher altitude in the watershed and is well above the exposed Pierre Shale that is present in the lower Fountain Creek. The Upper Fountain Creek passes through the town of Manitou and has an intermittent flow in the late season in drought years. Upper Fountain Creek is

also prone to flooding as a result of storm runoff from burn scars remaining from the Waldo Canyon fire.

Monument Creek begins near the top of Monument Hill and passes through the town of Monument, the United States Airforce Academy (USAFA) and from the north to the south of the city of Colorado Springs. Monument Creek has a continual flow and is a carrier of treated wastewater from the previously named cities and the USAFA. MC-5 on Monument Creek is the first site with exposed Pierre Shale. Lower Fountain Creek is formed from the confluence of Upper Fountain Creek and Monument Creek.

Lower Fountain is a wastewater impacted stream (Gautam, Carsella et al. 2014) and is the reach with the largest extent of exposed Pierre Shale (Figure 1.1). This reach has the collection sites with the highest Se levels and discharges into the Arkansas River in the eastern part of the city of Pueblo, Colorado. Lower Fountain Creek is prone to flooding and large amounts of sediment redistribution (Edelmann, Ferguson et al. 2005).

Indeed, others have shown, and we show that Se speciation is important to resulting effect of Se levels and therefore it is not enough to simply monitor the levels of selenium present in the water system (Torres, Pintos et al. 2010, Torres, Pintos et al. 2011, Mast, Mills et al. 2014, Carsella, Melnykov et al. 2017, Carsella, Sánchez-Lombardo et al. 2017). Bioavailability of the selenium is critical to the effects of the selenium and must also be considered in any analysis (Lemly 1997, Herrmann, Turner et al. 2012, Lemly 2014, Carsella, Melnykov et al. 2017). on Se levels in the water, Se speciation levels to be measured in the water and correlations to uptake by primary producers (plants) and animals in higher positions in the food chain. This approach

required environmental, chemical and biological studies to determine the selenium content in the environment, the chemistry that impacts selenium levels in fish, water, plants, and the mechanisms of selenium transport or influx and metabolism in plants. This thesis describes work relevant to environmental issues associated with selenium, specifically, the first two chapters of this thesis have resulted in two published manuscripts; Chapter 2 concerns Se effects on fish diversity and Chapter 3 concerns the Se speciation in the Fountain Creek water system. In Chapter 4 the issue of Se bioavailability is addressed. Since environmental studies have often used *Hygrohypnum ochraceum*, a native species (Nimmo, Herrmann et al. 2006), in its natural setting, *H. ochraceum* was chosen as the appropriate organism to determine the Se availability to plants in the Fountain Creek water (Chapter 4).

## CHAPTER 2 SELENIUM SPECIATION IN THE FOUNTAIN CREEK WATERSHED AND ITS EFFECTS ON FISH DIVERSITY<sup>1</sup>

### SYNOPSIS

Se is an environmental concern as it can be toxic if present in high concentrations even though it is a dietary requirement for all animals. Se levels are a special concern in the Fountain Creek Watershed located in southeastern Colorado whose geological source is the Se rich Pierre Shale. Segments of Fountain Creek have Se water levels that exceed the current EPA limit of 5 µg/L. In the studies described here, the effects of river water containing selenium were examined on fish populations at different sites along the Fountain Creek Watershed. Based on the hypothesis that high levels of Se present in the Creek and resident bryophytes should be an indicator of diversity in the river fish we explored the possibility that the low toxicity of the selenium could be due to speciation. A speciation analysis was conducted to determine the selenium(IV) and selenium(VI). Our results show that sites with higher ratios of the more toxic Se(IV) relative to total selenium exhibit lower fish diversity and numbers. Our results indicate that, factors, other than total Se, such as Se-speciation may be involved in controlling the bioavailability and toxicity of this element to aquatic organisms in Fountain Creek

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<sup>1</sup> This chapter is published in Journal of Biological Inorganic Chemistry and is referenced as (Carsella, Melnykov et al. 2017). Table and figure numbers have been modified to reflect that they are specific to this chapter, e.g. figure 1 is now figure 2.1. and the reference were moved to the end of this document. This article is reproduced with permission, and only minimal modifications were made to meet formatting requirements. No other modifications were made.

## INTRODUCTION

Selenium (Se) is an element that has both beneficial and toxic effects (Bird, Ge et al. 1997, Bird and Tyson 1997, Combs and Gray 1998, Mousa, O'Connor et al. 2007, Weekley and Harris 2013, Block, Booker et al. 2016) and as a result its environmental levels are regulated and strictly enforced by the EPA in the USA (Edelmann, Ferguson et al. 2005, Divine and Gates 2006, Colorado Department of Public Health and the Environment Water Quality Control Commission 2013, United States Environmental Protection Agency 2016). Selenium toxicity in fish is primarily acquired through the consumed diet (Janz, DeForest et al. 2010) and is not the result of passive absorption from water. However, Se is known to be transferred from adults to the eggs (Hamilton, Holley et al. 2005, Hamilton, Holley et al. 2005, Hamilton, Holley et al. 2005, Janz, DeForest et al. 2010) and thus can act as a reproductive toxicant (Janz, DeForest et al. 2010). This toxicity on fish reproduction has caused the EPA to develop a chronic exposure criterion for aquatic life (United States Environmental Protection Agency 2015). The elevated levels of Se in the Lower Fountain Creek Watershed (LFCW) in southeastern Colorado have a significant environmental impact as the Creek empties into the Arkansas River, on the east side of the city of Pueblo, Colorado, and is a major source of the Se in the Arkansas River (Divine and Gates 2006). The geological sources of the Se are the shale deposits that underlie the waterways in various areas in Colorado (Divine and Gates 2006). We have carried out studies, characterizing and monitoring sites along the Lower Fountain Creek for Se content arriving from the Pierre Shale in the LFCW (Nimmo, Herrmann et al. 2016) during 2007-2009. The reported Se levels in Fountain Creek for total and dissolved Se ranged from 1.3 to 64.4 µg/L, with mean values of 5.4 µg/L (Divine and Gates 2006). The mean value exceeds the EPA's

past (5.0 µg/L) and new recommended values of 1.2 µg/L for Se in water (Divine and Gates 2006, United States Environmental Protection Agency 2016) and also exceeds the State of Colorado's recommended water level of 4.6 µg/L (Colorado Department of Public Health and the Environment Water Quality Control Commission 2013).

Se bioaccumulation has been reported in birds in the Kesterson National Wildlife Refuge (KWF) in California underlining the importance of reports of elevated levels of Se in fish consumed by resident birds (Presser, Sylvester et al. 1994). The EPA's current fish tissue exposure limits are given as 15.8 mg Se/Kg for egg/ovary, 8.0 mg Se/Kg whole body and 1.2 µg Se/L for water (United States Environmental Protection Agency 2015). The Arkansas River in southeastern part of the State of Colorado has been identified as an area of concern to the Central Flyway (Presser, Sylvester et al. 1994). For example, the eastern Colorado Lower Arkansas River portion of the Central Flyway contains approximately 400 species of birds (Andersson, Davis et al. 2015, Wildlife 2016). The Se levels vary dramatically and, accordingly, reports have been prepared to describe some of the inhospitable environments in these rivers. Indeed, these reports detail the teratogenic deformities or birth defects in fish as a result of Se in the eggs or chronic exposure to high Se-levels (e.g. Kesterson, Presser 1994 paper on Ark River and Fish) (Lemly 1997, Kennedy, McDonald et al. 2000, Hamilton, Holley et al. 2005, Hamilton, Holley et al. 2005, Hamilton, Holley et al. 2005, Mathews, Fortner et al. 2014). Observed deformities in these studies and others typically include lordosis, scoliosis, kyphosis, missing or deformed fins, missing or deformed gills or opercula, abnormally shaped head, missing or deformed eyes, and deformed mouths (Lemly 1997, Kennedy, McDonald et al. 2000, Hamilton, Holley et al. 2005, Hamilton, Holley et



al. 2005, Hamilton, Holley et al. 2005, Mathews, Fortner et al. 2014). Acute toxic Se exposure in fish results in edema, exophthalmos, and cataracts, which was observed in Red Shiners (*Notropis lutrensis*) (Lemly 1997). However, other fish species are found to tolerate higher levels of Se and show no adverse effects with a key example being the Cutthroat Trout (*Oncorhynchus clarki lewisi*) (Kennedy, McDonald et al. 2000). The reported literature speaks to different chemical forms being toxic with species-dependent effects (Bird, Ge et al. 1997, Kennedy, McDonald et al. 2000, Hamilton, Holley et al. 2005, Hamilton, Holley et al. 2005, Hamilton, Holley et al. 2005, Mathews, Fortner et al. 2014). Furthermore, high levels of Se accumulation have been reported in apparently healthy fish species (Kennedy, McDonald et al. 2000, da Silva, Mataveli et al. 2013, Maneetong, Chookhampaeng et al. 2013, Maseko, Callahan et al. 2013, Thosaikham, Jitmanee et al. 2014).

Se is directly below sulfur in the periodic table and as a result many of its properties are similar to sulfur's (Baes and Mesmer 1976, Block 2013). The oxidized forms of Se, selenite ( $\text{SeO}_3^{2-}$ ) and selenate ( $\text{SeO}_4^{2-}$ ), are the most common forms of Se in environmental settings although the reduced form  $\text{H}_2\text{Se}$  exists in the earth's crust and is co-located with sulfur (Combs and Gray 1998). Se levels in aqueous environments are generally between 1 nmol/L to 5000 nmol/L. Se is most soluble in aqueous solution under oxidizing conditions and such conditions can enhance its solubilization from rock (Holland and Turekian 2004). A number of methods have been reported describing the analytical methods available to carry out speciation studies (Bird, Ge et al. 1997, Bird and Tyson 1997, Narasaki and Mayumi 2000, Weekley and Harris 2013, Jagtap and Maher 2016). Pourbaix diagrams suggest that selenous acid ( $\text{H}_2\text{SeO}_3$ ) is favored at the

conditions that are normally found in oxidizing natural stream waters ( $pE \approx 13.5$ ) at a  $pH < 3.0$  (Baes and Mesmer 1976) Hydrogen selenite ( $HSeO_3^-$ ) is favored in the same oxidizing conditions up to about  $pH 5.0$ . Above  $pH 5.0$  selenate ( $SeO_4^{2-}$ ) is the most favored Se species. Using the information in Pourbaix diagrams, it would follow that in less aerated waters ( $pE \approx 13.5$ ) at  $pH$  values between 3 and 8, hydrogen selenite,  $HSeO_3^-$  is the favored species (Torres, Pintos et al. 2010). Because the redox potential determines whether  $HSeO_3^-$  or  $SeO_4^{2-}$  forms, which species predominates between  $pH 3-8$  depends on the  $pE$ . The  $pE$  measurement reported often assumes the water is aerated because the water is in contact with air. However, in waters that are not surface waters the amount of aeration is less therefore, the assignment of Se in this  $pH$  range should be in the form as Se(IV). In natural aerated waters the conversion of Se(IV) (selenite,  $SeO_3^{2-}$ ) to Se(VI) (selenate,  $SeO_4^{2-}$ ) is kinetically slow and non-equilibrium conditions predominate (Bodek, Lyman et al. 1988). Other dissolved metals can also affect the process of oxidation and highlight the need to experimentally measure the Se species present in environmental samples under consideration (Torres, Pintos et al. 2010).

The Simpson Species Richness Index (SSRI) (Simpson 1949, Lande 1996, Smith and Wilson 1996, Gray 2000, Korb, Daniels et al. 2007) is used to characterize the sites sampled in Fountain Creek. The SSRI is an index measure based on the abundance of fish species present at a given site as well as the number of individuals of a given fish species at a site compared to the probability of encountering the species at any site in the study area (Lande 1996, Smith and Wilson 1996, Gray 2000, Korb, Daniels et al. 2007). Se(II), Se(IV) and Se(VI) are known to facilitate several modes of

action including forming adducts with cysteine residues, selenoproteins, and serving as cofactors for the reduction of antioxidant enzymes (Doyotte, Cossu et al. 1997, Ip, Birringer et al. 2000, Glass, Berry et al. 2008, Zhang, Rocourt et al. 2010, Moon, Ko et al. 2012) and function of other critical enzymes such as MAP kinase (Hei, Farahbakhshian et al. 1998) and protein phosphatases (Shelton and Capel 1994, Mueller, Bosse et al. 2009). Indeed, differential action of selenite and selenate have been reported with regard to cellular uptake by phosphate transporters (Zhang, Hu et al. 2014), effect on tumor progression (Yoon, Kim et al. 2001) and in general impact on fresh water organisms [38]. Knowing the oxidation state of Se is very important in beginning to elucidate the mode of action of the Se compound.

In this manuscript we analyze the effects of Se on the diversity of fish populations in a series of sites along the Fountain Creek. The analysis shows that Se content can be important for diversity in fish species although other variables play a role as well. The analysis underlines the importance of measuring the oxidation states of the environmental Se if a full understanding of the watershed sites is desired. When speciation measurements were done a distinct variance in Se(IV) and Se(VI) levels in the different sites was observed. Importantly, the low Se(IV) levels are consistent with low toxicity at sites where total Se content was much higher than the EPA recommended level. These studies demonstrate the importance of determining both Se(IV) and Se(VI) in the aqueous samples and illustrate the need for such analysis should a complete understanding of the system be desired.

## EXPERIMENTAL

The chemicals were purchased from Sigma-Aldrich unless specified otherwise. The HPLC solvents were HPLC grade and purchased from Fisher Scientific. The chemicals were also purchased ultra-pure grade and used without purifications.

### *Materials*

### *Sample Collection*

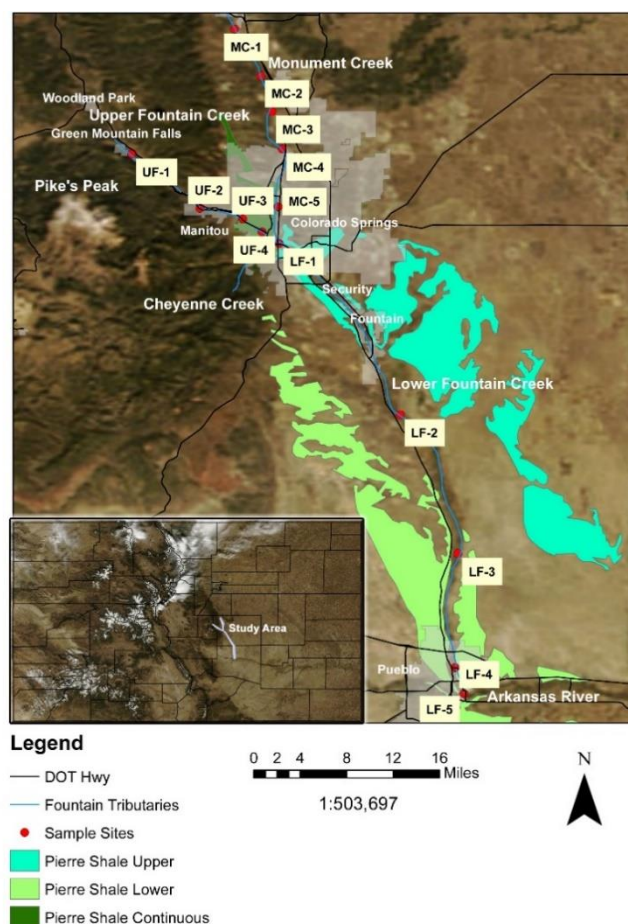


Figure 2.1 Map of study area and sample sites showing areas of Se rich Pierre Shale deposits (Green 1992). The inset shows the location of the study area in the State of Colorado (National Aeronautics and Space Administration Goddard Space Flight Center Rapid Response 2013).

Water samples were collected from Lower Fountain Creek, near Pueblo, Colorado see Figure 2.1. Sites for sample collections were selected based on map locations and information reported previously on the sites UF-1 to UF-3, MC-1 to MC-5

and LF-1 to LF-5 (Nimmo, Herrmann et al. 2016). Water samples were collected according to United States Geological Survey (USGS) water sampling protocols (Shelton and Capel 1994) with some modifications (Bednar, Chappell et al. 2010). The samples were placed on ice in the field to prevent selenite oxidation to selenate. After returning from the field the samples were split into 5 ml aliquots with the first aliquot analyzed immediately upon returning to the lab. The remaining aliquots were stored at -20 °C under nitrogen for future use.

#### *Methods for Sample Analysis*

Total water Se was measured by ICPMS using EPA Method 200.8 on an Agilent 7500ce ICPMS (United States Environmental Protection Agency 1994). The chromatographic separation of the selenium species was carried out on a Thermo–Dionex ICS-5000 coupled to an Agilent 7500ce ICPMS. The analytical column used was a Dionex AS-7 anion exchange column. Separation of hydrogen selenite (Se(IV)) and selenate (Se(VI)) was adapted from Ge et al. (Ge, Cai et al. 1996) and Bednar et al. (Bednar, Kirgan et al. 2009). Improved chromatographic separation was accomplished using a 17.5 mM citrate buffer at pH 5.2 as the mobile phase. Furthermore, methanol (2% v/v) was used in the mobile phase to enhance the Se signal (Larsen and Stürup 1994). Mobile phase was isocratically delivered at 300 µl/min during the HPLC separation. After separation the species was identified using Agilent 7500ce ICPMS.

#### *Electrochemistry*

Redox (pE) measurements of collected water samples were carried out on a Microlab F-522 system using the electrochemical interface and pH probe reference. The redox data obtained from the Microlab interface were normalized for temperature and to the  $E_0$  hydrogen electrode standard since the Microlab uses a silver electrode. The  $E_0$

data were then converted to pE (Suslow 2004, Duffy 2011). pH measurements were obtained using the Microlab F-522 pH interface with a Microlab electrode.

### *Statistical analysis*

All samples were prepared in triplicate and averages are reported. These averages are reported with their standard errors shown. This was done both for the data previously reported and the new data obtained as a result of the analysis carried out here. The Simpson Species Richness Index (SSRI) was used to compare the diversity of fish and relative number of each species at each site (Simpson 1949).

Simple Canonical Correlation Analysis (SCCA) (Hotelling 1936) was used to examine the possibility of a relationship between dissolved Se, biologically available Se as bryophyte Se, fish species number and fish diversity (Knapp 1978, Härdle and Simar 2007, Huang, Lee et al. 2009). The variable silt/clay percentage was also added as the silt/clay content could have a significant effect on fish reproduction.

## **RESULTS AND DISCUSSION**

### *Data Analysis*

In Table 2.1 we list the Se content in Fountain Creek Watershed (Nimmo, Herrmann et al. 2016) and compare the Se content with the number of fish species at these sites, the number of fish species found at a site divided by the probability of finding a fish at this site (PI) and The SSRI factor  $PI^2$ . It is the objective to use the SSRI index and the SSRI factor  $PI^2$  to derive conclusions regarding fish diversity. The data from the previous studies (Nimmo, Herrmann et al. 2016) along the Fountain Creek in Colorado was subjected to this analysis. The calculated  $PI^2$  values are listed in Table 1 with the data used for these calculations (Nimmo, Herrmann et al. 2016). The use of the

SSRI index for the three reaches of Fountain Creek shown below Table 1 indicate that conclusions regarding fish diversity cannot be made for the data in Table 1 with respect to dissolved Se water levels. The idea that high Se levels should be detrimental to fish diversity and health is not reflected in the SSRI values for each reach as evidenced by the medial value of SSRI in the high Se reach of Lower Fountain Creek. The higher SSRI values of Monument Creek and Lower Fountain Creek suggest the need for additional information that is speciation analysis, which was therefore carried out in this manuscript.

#### *Simpson Species Richness Index (SSRI)*

Our previous study identified some sites to contain high concentrations of Se in the Fountain Creek, Table 1 (Nimmo, Herrmann et al. 2016). High levels of Se in creek water generally correspond to high level of Se in the aquatic (bryophyte) plant (Nimmo, Herrmann et al. 2016). However, there is a surprising difference in water Se content between some sites with similar Se in the plants (UF-4, LF-1 and LF-5). These results support the interpretation that the varying Se levels are real and point to the need for additional data analysis and possibly a new analytical approach. The Simpson Species Richness Index (SSRI) [37] is a weighted index measure based on the abundance of fish species present at a given site as well as the number of individuals of a given fish species at a site compared to the probability of encountering the species at any site in the study area. The SSRI value increases when both species number and population of a given species increase (Simpson 1949). In the following Table 2.1 we will include the fish diversity in the Fountain Creek data in the analysis.

The SSRI for the sites sampled in Fountain Creek provide a new aspect of the observations regarding Se content in aqueous samples and in the plants. The table

indicates what is expected at the Monument Creek sites (MC) which are among the lower containing Se sites in the aqueous sample series. The Se content in the water and in the native bryophyte species *Hygrohypnum ochraceum* is relatively low. The lower Se levels would suggest an area of favorable fish health in a reach of stream where fish habitat is similar. The SSRI values for MC (SSRI=0.054), LF(SSRI=0.025) and UF (SSRI=0.010) demonstrate that the greatest diversity of fish is in Monument Creek followed by Lower Fountain Creek.

The  $PI^2$  values were used as a predictor of diversity at each site for statistical analysis. The MC 1- MC-3  $PI^2$  values of 0.011, 0.015, and 0.022 respectively are accurate predictors of the higher SSRI value of MC.

The Lower Fountain Creek sites (LF) also share similar habitats and are more sand-based than Monument Creek and Upper Fountain Creek (Edelmann, Ferguson et al. 2005). The unexpected high  $PI^2$  value at LF-4 ( $PI^2=0.011$ ) is interesting since it is the highest value in the LF reach and it is equivalent to the 3rd highest  $PI^2$  value in the entire Fountain Creek Watershed. This high  $PI^2$  level is unexpected in an area where the water Se is dramatically above EPA and CDPHE water standards.



Table 2.1 Se content and fish sampled in Fountain Creek Watershed reported previously (Nimmo, Herrmann et al. 2016)  
Simpson Species Richness Index Factor (PI<sup>2</sup>). <sup>a-c</sup>

Sample Sites	Water Se (µg/L)	Standard Error Water Se	Plant: <i>H. ochraceum</i> Se (mg/Kg)	Standard Error Plant Se	Number of Fish Species	PI <sup>a</sup>	PI <sup>2b</sup>
UF-1	0.193	0.011	400.940	43.071	1.000	0.043	0.002
UF-2	0.140	0.011	528.660	33.366	1.000	0.052	0.003
UF-3	0.673	0.004	867.520	75.233	1.000	0.043	0.002
UF-4	1.323	0.021	934.680	61.917	2.000	0.052	0.003
MC-1	0.207	0.011	738.260	43.288	2.000	0.104	0.011
MC-2	0.277	0.048	746.400	52.137	3.000	0.122	0.015
MC-3	0.343	0.017	426.300	56.580	2.000	0.148	0.022
MC-4	0.443	0.029	736.760	82.547	3.000	0.052	0.003
MC-5	1.863	0.028	410.200	11.400	1.000	0.052	0.003
LF-1	2.050	0.142	907.460	119.82	3.000	0.052	0.003
LF-2	2.780	0.011	1305.800	108.17	2.000	0.061	0.004
LF-3	3.290	0.011	1096.680	58.88	2.000	0.043	0.002
LF-4	9.687	0.004	2033.880	510.77	3.000	0.104	0.011
LF-5	7.910	0.021	937.620	60.431	2.000	0.070	0.005
Reference	(Nimmo, Herrmann et al. 2016)		(Nimmo, Herrmann et al. 2016)		(Nimmo, Herrmann et al. 2016)	This work	This work

<sup>a</sup> PI is the probability of finding a given species at a given location and is based on observed data.

<sup>b</sup> PI<sup>2</sup> is the square of PI the sum of which is equal to the SSRI for a given reach of the creek.

<sup>c</sup> SSRI values for each reach: UF SSRI=0.010, LF SSRI=0.025, MC SSRI=0.054.

These observations do have precedent in a report in 2009, where the highest fish whole body Se values of 3,393 µg/Kg dry weight and 906 µg/Kg wet weight were found and there was no evidence of teratogenic effects on any fish sampled (Nimmo, Herrmann et al. 2016). These observations point to an interesting paradox between water and fish Se levels and the expected outcome on fish health and diversity. Further analysis was thus carried out to include the fish diversity in the SSCA analysis.

Table 2.2 The simple canonical correlation analysis on dissolved selenium and fish diversity of the data reported previously (Nimmo, Herrmann et al. 2016).

Name	Quality	Mass	Inertia	Component 1 (Water-Bryophyte)			Component 2 (Number of Fish Species)		
				Coord.	Corr.	Contribution	Coord.	Corr.	Contribution
<b>Number of Fish Species</b>	1	0.002	0.168	0.191	0.202	0.040	-0.379	0.798	0.946
<b>Se in Water</b>	1	0.003	0.825	0.888	0.993	0.958	-0.073	0.007	0.039
<b>Se in Bryophyte</b>	1	0.995	0.002	0.002	0.748	0.002	0.001	0.252	0.003
<b>PI<sup>2</sup></b>	0.403	0.000	0.005	0.385	0.081	0.000	-0.767	0.322	0.012

A Simple Canonical Correlation Analysis (SCCA) (Hotelling 1936) was used to examine the possibility of a relationship between dissolved Se, biologically available Se as bryophyte Se, fish species number and fish diversity. The variable silt/clay percentage was also added as the silt/clay content could have a significant effect on fish reproduction. The results are summarized in Table 2.2. These results, based on previous data, statistically support the conclusion that two main components contribute to fish diversity in Fountain Creek. Component 1 is described as the available selenium in water and is indicated by the high correlation of Se in water and Se in bryophyte shown in Table 2.2 (Corr. 0.993, 0.748). The second component (Component 2)

describes the fish species diversity and is indicated by the strong correlation between fish species number and diversity index in Table 2.2 (Corr. 0.798, 0.322). This SCCA analysis provides two parameters, Component 1 and Component 2, which can be used to further understand the nature of the Se levels and the Fountain Creek sites.

Table 2.3 The simple canonical correlation analysis on dissolved selenium species and fish diversity of the data reported previously (Nimmo, Herrmann et al. 2016).

Name	Quality	Mass	Inertia	Component 1			Component 2		
				Coord	Correlation	Contribution	Coord	Correlation	Contribution
<b>Number of Fish Species</b>	0.849	0.002	0.064	-0.086	0.051	0.004	0.343	0.798	0.739
<b>Dissolved Se Water</b>	0.987	0.003	0.367	0.759	0.986	0.41	-0.022	0.001	0.004
<b>Se in Bryophyte</b>	0.978	0.992	0.004	-0.005	0.966	0.005	0.000	0.012	0.001
<b>PI<sup>2</sup></b>	0.339	0.000	0.000	0.198	0.282	0.000	0.089	0.056	0.000
<b>Silt/Clay</b>	0.59	0.000	0.063	-0.56	0.32	0.023	-0.515	0.27	0.247
<b>Se(IV)</b>	0.692	0.000	0.015	0.483	0.658	0.012	-0.11	0.034	0.008
<b>Se(VI)</b>	0.997	0.003	0.485	0.914	0.997	0.547	0.012	0.000	0.001

The SCCA results presented in Table 2.3 indicate that Component 1 in these results is similar to the results presented in Table 2.2. There is a high degree of correlation between dissolved Se in water, Se in bryophyte and the Se species Se(IV) and Se(VI). There is also a small correlation with the silt/clay content which is probably related to the silt/clay content affecting the dissolution of the Se forms into the water. The major contributions to Component 1 come from dissolved Se in water (0.41) and

the predominant Se(VI) (0.547). Component 1 in this table can be thought of as Se in water.

Component 2 in these results shows a high correlation of fish species number (0.798) to silt/clay content (0.27) in the creek bed and provide the greatest contributions to this component.

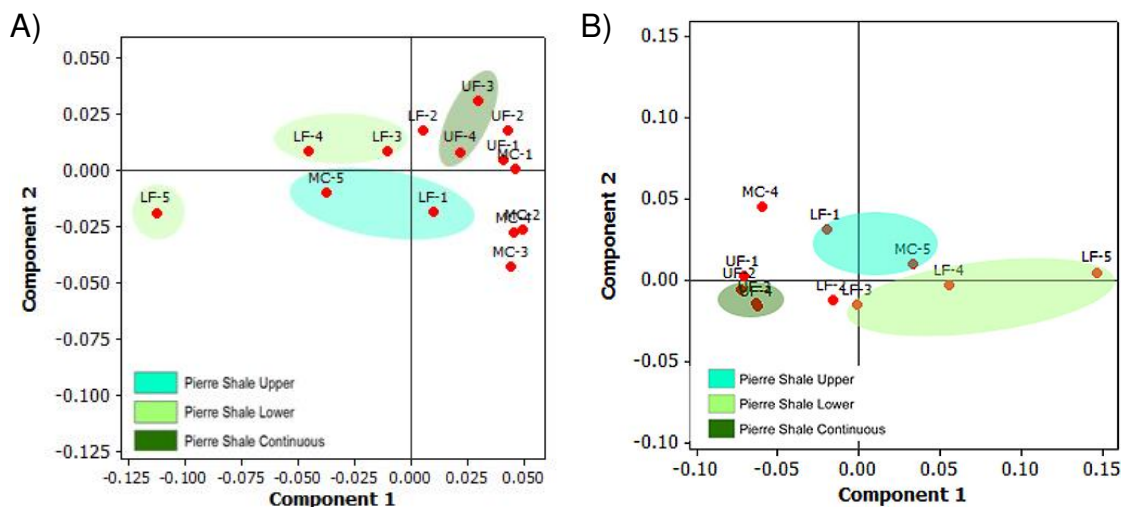


Figure 2.2 A) Row plot showing the Se content in water and bryophyte (Component 1) compared to fish diversity (Component 2) highlight the relationship between the different sites; B) Row plot showing the Se(IV) species content in water and Se content in bryophyte (Component 1) compared to fish diversity and Se(VI) content in the water (Component 2) highlights the relationship between the different sites.

The Row plot shown in Fig. 2.2a has Component 1 and Component 2 on the X and Y axis. This plot splits the sites into 4 distinct quadrants each reflecting the similarity of the diversity in fish species by site. The 4 regions also correspond to the 3 areas of Pierre shale found in the Fountain Creek Watershed (FCW). There exists hydrological and geochemical similarities between the Lower Arkansas River (LAR) and the Fountain Creek (FC) (Presser, Sylvester et al. 1994). The source of the LAR Se is Se rich tributaries with high Se effluent such as FC(Presser, Sylvester et al. 1994). The Se source in FC is Pierre Shale, a Se-rich shale that was deposited in the late

Cretaceous period (Tourtelot 1962). This shale is also exposed in the Lower Fountain Creek Watershed (LFCW) in southeastern Colorado and results in elevated levels of Se in Lower Fountain Creek water and in natural springs that feed the Creek (Edelmann, Ferguson et al. 2005). The two left quadrants describe sites with generally higher levels of Se content in the aqueous samples and in the biosamples. The two right quadrants separate out the samples with the greater  $PI^2$  and are predominantly populated by the samples from the Upper Fountain Creek. The elevated levels of Se in the LFCW have a significant environmental impact as the creek empties into the Arkansas River, on the east side of the city of Pueblo, Colorado, and is a major source of Se in the Arkansas River (Divine and Gates 2006)

Figure 2.2b separates the Se species from total dissolved Se in the water in the FCW. The figure clearly indicates that predominant Se species found in the Lower Pierre Shale have little to no effect on fish species diversity while the Upper Pierre Shale has a positive effect on fish species diversity. The continuous Pierre Shale has a negative effect on fish species diversity. These effects on fish species diversity could be due to the form of Se dissolving from the parent rock material and the dissolved Se interactions that depend on local environment and water chemistry.

Plotting the results of the SCCA in a Loading Plot (Figure 2.3a) with Component 1 on one axis and Component 2 on the other axis the relationship between different

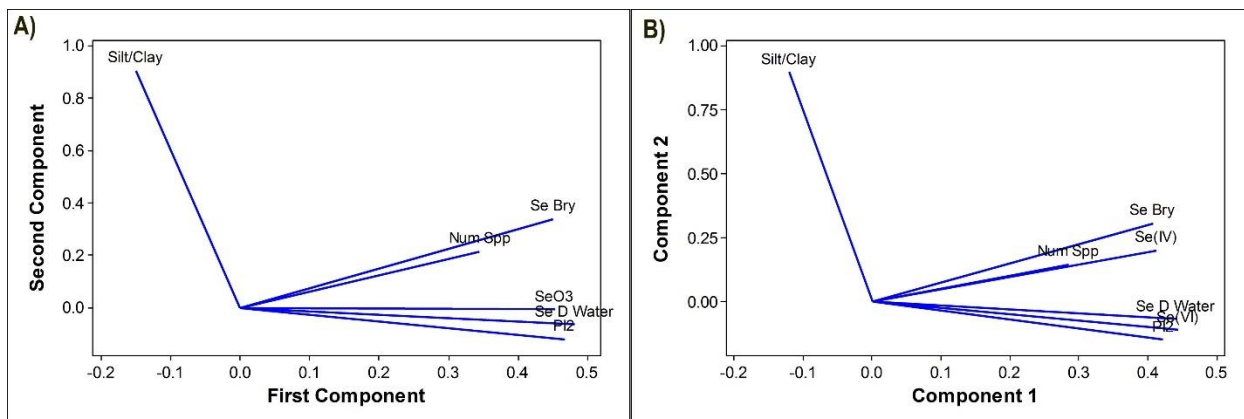


Figure 2.3 A) Loading plot illustrating the relationship between First and Second Components describing the relationship between the different variables at the Fountain Creek sites; B) Loading plot, including Se(IV) and (VI), illustrating the relationship between First and Second Components describing the relationship between the different variables at the Fountain Creek sites

parameters used in the simple canonical correlation analysis is illustrated. Group 1 is Se found in *H. ochraceum* (abbreviated Se Bry) and number of fish species (Num Spp). Group 2 is a correlation between  $PI^2$ , Se (IV) ( $SeO_3$ ) and total dissolved Se (Se D Water). The results show that the variable of silt/clay percentage which was thought to be a major factor in fish diversity in Fountain Creek is nearly orthogonal in relation to the other lines. The interpretation of this result is that the silt/clay variable is independent of fish diversity while the other variables are correlated in two groups.

In contrast, the Se levels in the *H. ochraceum* (Se Bry) are responding similarly as the number of fish species (Num. Spp.) at each site. These two variables are different from the correlation between  $PI^2$ , Se (IV) levels ( $SeO_3^{-2}$ ) and total dissolved Se (Se D Water), which all respond similarly. Together, analysis of this data demonstrates that fish species diversity is related to Se levels with the Se levels in the bryophytes related more closely to fish species number, the Se(IV) in the aqueous phase and the total dissolved Se. This correlation follows the mapping of Pierre Shale deposits found

in this watershed. The source of the Se in the watershed comes from three Pierre Shale deposits known as upper, lower and continuous. Each of these deposits is represented in the row plot (Figure 2.2) along with the associated level of fish species diversity. The Se does increase in the fish with increasing dissolved Se water levels. No teratogenic effects were found in these high Se areas (LF-4 and LF-5) even though the Se levels are more than double the EPA-recommended water limit of 5.0 ppb.

Plotting the results of the SCCA Se speciation analysis in a Loading Plot (Figure 2.3b) with Component 1 on one axis and Component 2 on the other axis the relationship between different parameters used in the simple canonical correlation analysis is illustrated. Group 1 is Se found in *H. ochraceum* (abbreviated Se Bry), the number of fish species and Se(IV). Group 2 is a correlation between  $PI^2$ , Se (VI) and total dissolved Se (Se D Water). The results still indicate that the variable of silt/clay percentage is independent of the other groups shown by nearly orthogonal position in relation to the other lines. The interpretation of this plot infers that Se species are important in determining fish health and diversity in the watershed. This statement is supported by the grouping of Se(IV) with fish species number and Se(VI) with the SSRI predictor ( $PI^2$ ).

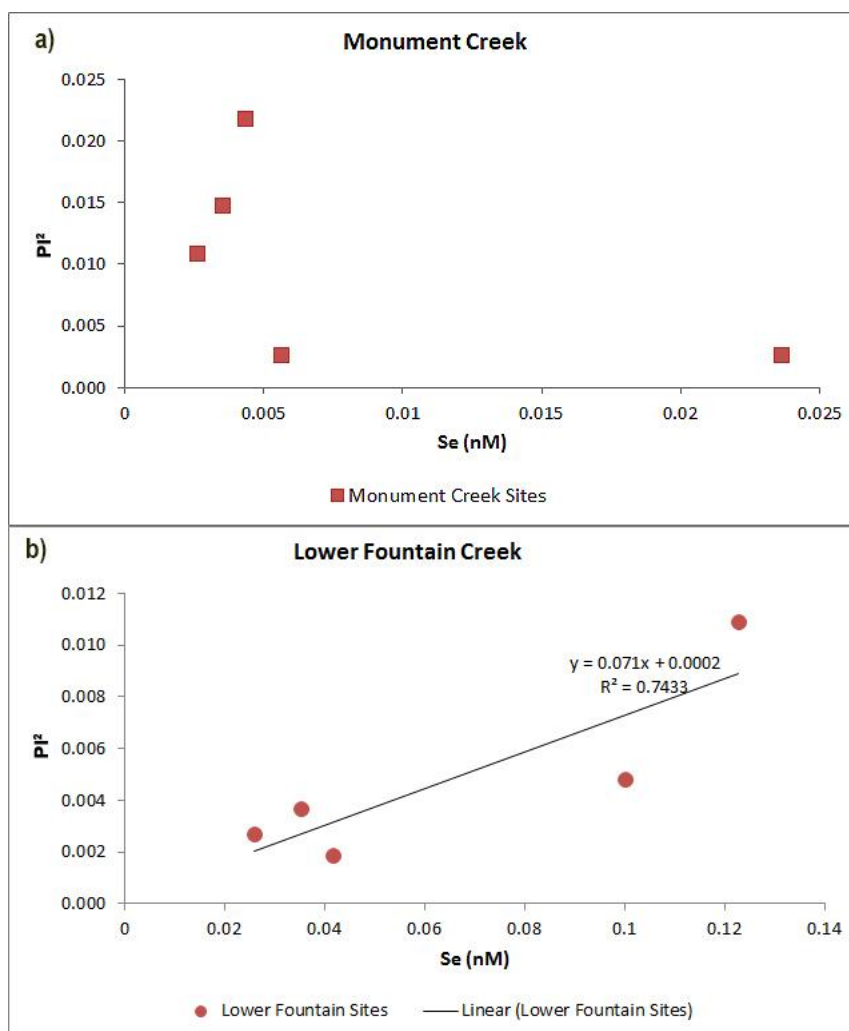


Figure 2.4 a) and b) show the SSRI factor  $PI^2$  is plotted as a function of nM Se for Monument Creek and Lower Fountain Creek. The symbol for Monument Creek is a solid square and the symbol for Lower Fountain is a solid circle.

In Figure 2.4 the  $PI^2$  factor is shown as a function of the Se concentration for Monument Creek and Lower Fountain Creek sites. As shown in Table 2.1 the Upper Fountain Creek sites show little change. Monument Creek shows an upward trend in diversity except for the sites with  $Se > 0.005$  nM, Figure 2.4a. When the Se concentration is higher than 0.005 nM, the sites have higher Se(IV) concentrations and fish diversity is lower. In contrast the sites along the Lower Fountain Creek show a



different pattern with diversity increasing as a function of total Se concentration. As shown in Figure 2.4b there is a linear relationship with an  $R^2 = 0.743$ . At these sites there is a higher Se(VI) concentration.

Speciation has been reported to be important in interactions with other elements and influences not only uptake but also biological responses to different species that may differ in geometry, charge and oxidation state for elements (Willsky, Chi et al. 2011, Crans 2015, Doucette, Hassell et al. 2016). It has been reported that the toxicity effects of Se are dependent on a compound's oxidation state and presumably on the bioavailability of the chemical species (Rosenfeld and Beath 2013). That is, the Se(IV) has been reported to be more toxic than Se(VI), which may be surprising since many beneficial effects particularly related to the antioxidant effects are mainly observed with Se(IV) (Doyotte, Cossu et al. 1997, Ip, Birringer et al. 2000, Glass, Berry et al. 2008, Zhang, Rocourt et al. 2010, Moon, Ko et al. 2012) . The Se availability from the aqueous samples varies with the pH and concentration according to the speciation diagrams [28]. Thus, the observed toxicity is likely site-specific and may be related to the specific Se speciation chemistry occurring at each site. Because some of the differences in fish diversity may be related to the uptake and nature of the internalized species, more information on Se speciation at each sample collection site is desirable. Given the potential relationship between Se species, uptake, and toxicity, we subsequently investigated the oxidation state of the Se in water samples recently collected at each site to determine whether site-specific speciation of Se in the Fountain Creek samples factors in the observed variation in fish diversity between sites.

*Sample collection from Fountain Creek:*

Water samples were collected at each of the sites labeled in Figure 1 except for MC-1- MC-3. The reason these sites were not collected was the inability to access the private property at MC-1 and the closure of the restricted areas of the United States Air Force Academy (MC-2 and MC-3). The water sample were collected according to USGS surface water sampling protocols (United States Geological Survey 2006). Samples were collected in clean 250 ml high density polyethylene containers. The water samples were obtained by rinsing the containers at the site with creek water to equilibrate the container and the samples were taken with non-isokinetic sampling methods. The containers were immediately placed on ice for transport. In addition, pH, specific conductance and dissolved oxygen were measured on site. In the lab the samples were fractionated into total, dissolved fractions. The dissolved fractions were filtered using a 0.45-micron syringe filter. The dissolved fractions were separated into 5 ml speciation aliquots. 1 aliquot was used for total dissolved analysis and 1 was used for Se speciation. The remainder of aliquots was frozen for stability and future use. The total and dissolved fractions used for EPA 200.8 analysis were acidified with optima grade nitric acid. Hardness was calculated from ICPMS data.

*Method for determining both Se(IV) and Se(VI) Oxidation States.*

To explore Se speciation, a new study that would allow speciation to be determined was designed. Water samples containing Se were collected from the Lower Fountain Creek, near Pueblo, Colorado. The sites sampled have the highest levels of Se recorded in the Creek and have a high bryophyte Se concentration as shown in a previous study (Herrmann, Turner et al. 2012). Although the samples were collected according to USGS water sampling protocols (Shelton and Capel 1994), recent reports

suggest that these samples may not be as stable as previously anticipated (Mast, Mills et al. 2014, Nimmo, Herrmann et al. 2016).

Due to the high levels of Se in the water and the fact that fish living in these waters seems to be unaffected, it is important to determine the speciation of the Se at each site. The chromatographic separation of the selenium species is readily accomplished using ICPMS after chromatographic separation of the Se(IV) and Se(VI). Using a Dionex AS-7 anion exchange column, the separation was readily accomplished using a mobile phase of 17.5 mM citrate buffer at pH 5.2. The buffer improved the separation presumably through the interaction between Se and citrate, and  $^1\text{H}$  NMR studies confirmed that complexes do form between selenate and citrate (unpublished). Furthermore, the Se signals were enhanced using 2% aqueous methanol in the mobile phase as reported previously for arsenic (Larsen and Stürup 1994, Suzuki and Ogra 2002).

Table 2.4 Se-oxidation state for water samples from the sites on Monument Creek (MC) Upper Fountain Creek (UC) and Lower Fountain Creek (LF)<sup>a,b,c</sup>

Site	Total Se (ICPMS) (µg/l) <sup>b</sup>	Standard Error Total Se (ICPMS)	Total Se (sum of Se(IV) & Se(VI)) (µg/l)	SeO <sub>3</sub> <sup>2-</sup> Se(IV) (µg/l) <sup>b</sup>	Standard Error SeO <sub>3</sub> <sup>2-</sup>	SeO <sub>4</sub> <sup>2-</sup> Se(VI) (µg/l) <sup>b</sup>	Standard Error SeO <sub>4</sub> <sup>2-</sup>	pH	Se(IV)/ tot Se (%)	Se(VI)/ tot Se (%)
UF-1	0.193	0.011	0.055	0.023 <sup>c</sup>	0.018	0.032 <sup>c</sup>	0.075	8.04	41.82 <sup>c</sup>	58.18 <sup>c</sup>
UF-2	0.102	0.011	0.229	0.226 <sup>c</sup>	0.02	0.0003 <sup>c</sup>	0.031	7.66	99.87 <sup>c</sup>	0.13 <sup>c</sup>
UF-3	0.218	0.004	0.099	0.0646 <sup>c</sup>	0.021	0.034 <sup>c</sup>	0.048	8.03	65.52 <sup>c</sup>	34.48 <sup>c</sup>
UF-4	0.556	0.021	0.127	0.073	0.046	0.054	0.058	7.94	57.48	42.52
MC-4	0.618	0.011	0.555	0.123	0.047	0.432	0.138	8.07	22.16	77.84
MC-5	1.874	0.048	1.648	0.112	0.026	1.536	0.116	8.08	6.8	93.2
LF-1	2.1	0.017	1.836	0.044	0.096	1.792	0.026	8.22	2.4	97.6
LF-2	3.602	0.029	3.321	0.365	0.046	2.956	0.026	8.13	10.99	89.01
LF-3	3.627	0.028	3.429	0.358	0.074	3.071	0.008	8.23	10.44	89.56
LF-4	10.778	0.142	10.585	0.51	0.115	10.075	0.11	8.33	4.82	95.18
LF-5	9.252	0.029	8.354	0.413	0.06	7.941	0.13	8.12	4.94	95.06

<sup>a</sup> Samples were collected in October 2016

<sup>b</sup> Detection Limits are as follows: ICPMS Total Se 0.073 µg/l, SeO<sub>3</sub><sup>2-</sup> Se(IV) 0.023(µg/l), SeO<sub>4</sub><sup>2-</sup> Se(VI) 0.034(µg/l)

<sup>c</sup> Transformed data used for statistical analysis. The process for data transformation is described in the paragraph below.

*Se(IV) and Se(VI) Speciation of Se in Fountain Creek Water Samples.*

Samples were collected from 11 different sites along the Fountain Creek.

Initially, we measured the speciation of samples along the entire Creek and in Table 2.4 we show the data for measurement of not only total Se, but the distribution of the Se as selenite, Se(IV), and selenate, Se(VI), and the pH of the respective samples. The Se-content in the samples is very similar in the sites examined except for the MC-4 site; the amounts are higher in this recent study. Because the Se-content varies with seasonal and weather/ precipitation patterns, variations would have been anticipated from the previous study, however, very few are observed (Nimmo, Herrmann et al. 2016).

For the purposes of the SCCA, the readings for Se(IV) and Se(VI) at sites UF-1, UF-2, and UF-3 that went below the instrument detection limits were estimated using the regression with the Total Se as a single predictor. Due to the uneven distribution of data in the range of available values, the readings had to be transformed to the logarithmic scale in the case of Se(IV) and square root scale for Se(VI). In two cases such estimates yielded values above the detection limit and were trimmed at the limit values: 0.023 and 0.034  $\mu\text{g/l}$  for Se(IV) and Se(VI), respectively.

The Upper Fountain Creek sites were reported to have the lowest total Se, Se(IV) and Se(VI) and collectively the highest percentage of Se(IV). As seen from the data in Table 2.1, the Upper Fountain Creek sites also had a less diverse fish habitat (except for the UF-4). Once the water flows down to the Monument and Lower Fountain sites, the majority of the Se had oxidized to selenate. Since selenite at neutral pH readily oxidizes to selenate, and the oxidation is more likely to happen at lower concentrations and at higher salinity levels in the presence of nitrate, conversion of Se(IV) to Se(VI) is expected (Torres, Pintos et al. 2010, Torres, Pintos et al. 2011). The

presence of a higher concentration of Se(VI) at the Lower Fountain sites was therefore anticipated although this could be due to additional oxidation of Se(IV) or dissolution of more Se(VI) from the shale or a combination of both oxidation and dissolution processes to form more Se(VI). Indeed, the data in Table 2.4 confirm that in all but one Lower Fountain sites more than 90% of the Se was in the form of selenate, Se(VI).

The results for the LF-4 and UF-4 sites were a little different than that of the others in that the standard errors were a little higher for these sites. This increase in standard error at higher concentration is most likely due to the increase in dissolved solids and is the result of a matrix effect caused by a combination of easily ionized elements that are abundantly present and the more difficult to ionize Se. The Se species are measured after a column separation resulting in a cleaner sample entering the plasma of the ICPMS.

Using speciation analysis, we considered the speciation using the known constants reported for the distribution of species in aqueous solution (Baes and Mesmer 1976). In Figure 2.5 we calculated the equilibrium concentrations of solutions at the concentrations measured at the sites we were investigating (UF, LF-1 and LF-4). As shown in Figure 2.6 the equilibrium concentrations favor selenate as the major form because the pH was near 8 in the sites. As the concentration of the Se increased although more Se(IV) was present, the difference in the concentration between the Se(IV) and Se(VI) species was less. At the neutral to basic concentrations observed in the Fountain Creek sites the deprotonated selenate and selenite were the major species. A small amount of monoprotonated selenate was found at the site with the higher Se(VI) concentration. This is interesting because the pKa values for selenite are

higher, however, because the concentration of Se(IV) is 100-fold less there would be more  $\text{HSeO}_4^-$  than  $\text{HSeO}_3^-$  at this site if equilibrium conditions existed. Although the conversion of Se(IV) to Se(VI) may be slow, it is of interest to investigate the system further, and at least establish what is expected should the system be governed by the thermodynamic stability.

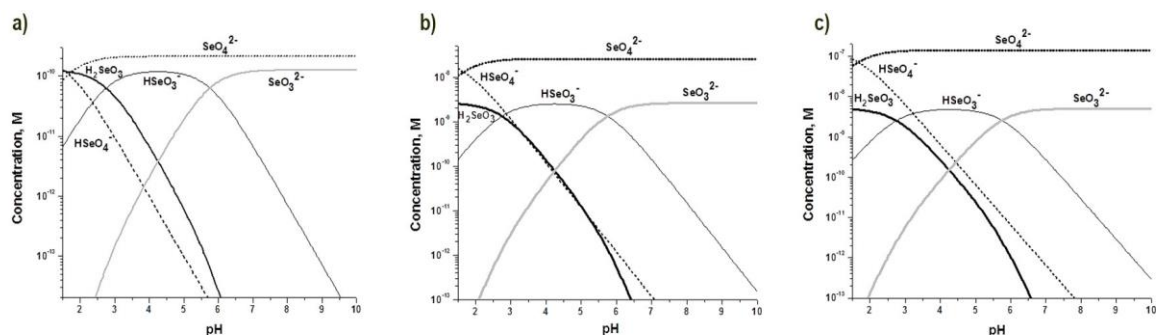


Figure 2.5 Speciation diagrams were calculated for three different sites along the Fountain River. a) The low concentration UF sites with  $[\text{Se}]_{\text{tot}} 1.27 \cdot 10^{-10} \text{ M}$ , with b) LF and MC sites with  $[\text{Se}]_{\text{tot}} 2.65 \cdot 10^{-9} \text{ M}$  and with c) LF-4 with  $[\text{Se}]_{\text{tot}} 5.05 \cdot 10^{-7} \text{ M}$ .

The Pourbaix diagram describes the thermodynamically stable forms of Se in a graphical form shown in Figure 2.6. In this Figure it is shown that the conditions controlling the major species of Se at neutral pH are complicated and depending on the redox potential the thermodynamic stable form which may be Se(IV) or Se(VI). In order to investigate the nature of the aqueous redox environment, the electrochemical potential of samples, LF-4 and LF-5, which contained the highest observed levels of oxidized Se(VI), were measured. These studies were performed to determine whether the high levels of observed Se(VI) are due to oxidation reactions in the water, or to unchanged Se (VI) from the shale.

Table 2.5 Se content and redox potential for Lower Fountain Creek sites with the highest Se levels

Site	pH	Temperature (C)	pE	Se(IV) $\mu\text{g/l}$	Se(VI) $\mu\text{g/l}$	Se(IV) percentage of total Se	Se(VI) percentage of total Se
LF-4	7.47	8	6.35	0.89	11.30	7.3	93
LF-5	7.52	8	6.37	0.79	9.88	7.4	93

<sup>a</sup> Samples were collected in April 2016

The  $E_0$  data were converted to pE and these values are shown in Table 5. pH data were also obtained using the Microlab F-522 pH interface with a Microlab electrode. As shown in Table 2.5, sampling the Lower Fountain Creek sites LF-4 and LF-5 in April gave a pH value that was slightly above neutral. Determining the  $E_0$  / pE and pH data allows us to reference sample data with a Pourbaix diagram which describes the thermodynamically state species of the Se. This allows us to determine whether the sample contains Se(VI) or Se(IV). For low concentrations of Se at neutral pH and pE below 7 / 8, the Pourbaix diagram predicts that the thermodynamically stable form is Se(IV). It was surprising that the pE for the LF samples collected in the spring of 2016 had values of 6.35 / 6.37. This suggests that in the creek water samples the stable form of the Se in LF-4 and LF-5 sites is Se(IV). However, because it was experimentally determined that Se(VI) was the major component, it is likely that the predominance of the Se(VI) form is dependent on other environmental factors and supports the findings of Mast et. al (Mast, Mills et al. 2014).



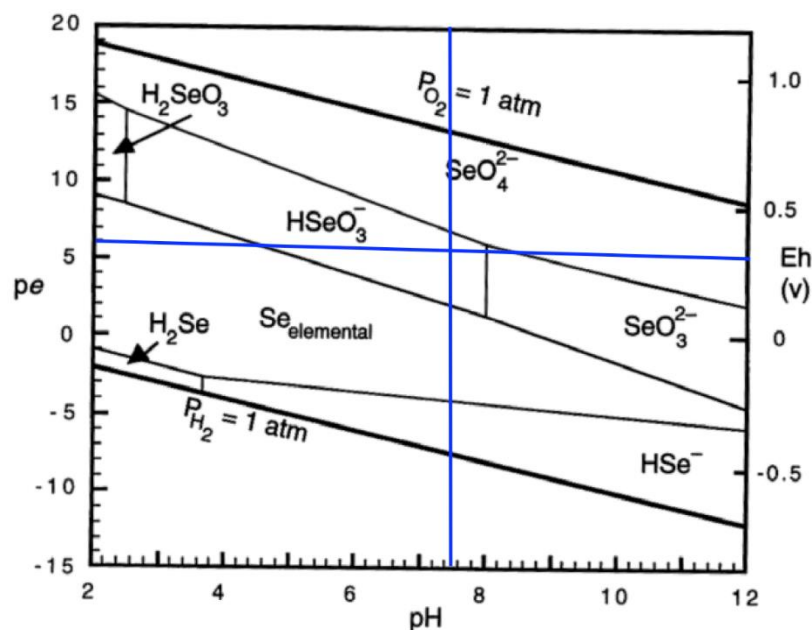


Figure 2.6 The Se Pourbaix diagram of Se in water reported previously (Drever 1997) was added the specific conditions of the LF samples investigated in the Spring of 2016 where the crosshair intercept. The Pourbaix diagram was reproduced with permission

Using the SSRI predictor data ( $PI^2$ ) data in Table 2.3 SCCA was carried out and the results show that a Se species effect on diversity exists and that effect is independent of the silt/clay content of the creek bed material present at the sites. The Se species effect can be positive or negative depending on the origin of the creek bed parent material. This is most notable between the LF-3, LF-4, and LF-5 sites compared to the UF-3 and UF-4 sites, the latter of which has a higher percentage composition of Se(IV). Thus this analysis confirms that there is less observed toxicity at the LF-3 - LF-5 sites despite the higher total Se levels. Although this analysis does not prove that the Se(VI) is less toxic to the fish, the analysis does provide a statistical basis to suggest that the toxicity effect is correlated with observed oxidation state.

Previously a report on Se-levels in the Fountain Creek fish study did indicate increased levels of Se in ovarian and liver tissue of the fish (Nimmo, Herrmann et al.

2016). The Se level increase in these tissues may be a result of increasing levels of Se containing enzymes. The Se-level increase in ovarian tissue may explain teratogenic effects in new hatchlings if the Se-levels continue to rise. The most common mode of toxicity of Se is oxidative damage (Simpson 1949). This type of toxicity requires that Se be internalized and assimilated into the organs and the fish. Se is generally non-toxic when it is stored in a “safe” form, and either because it cannot be absorbed or transported, it is rapidly excreted or is not metabolized. However, some transporters prefer Se(IV) over Se(VI). (Zhang, Hu et al. 2014) The high Se water levels at several sites and the lack of observation of teratogenic levels in the fish raise the question of how Se levels in this biota can be so high without causing toxic effects in fish. The data provided here show that there are significant differences in the speciation in the creek water and the lack of teratogenic response to the Se level may be because the Se is in the Se(VI) form which is less toxic than Se(IV).

In summary, current speciation studies with Se generally investigate only one oxidation state; however, as described in this manuscript, speciation studies of the water at representative Fountain Creek sites demonstrate that total Se levels cannot fully describe the interactions of Se in the complex environmental system and will require additional analyses in the future. Our experimental data show that a correlation exists between fish species diversity and dissolved Se in Fountain Creek water and this is backed up by statistical analyses. The increase in fish diversity with the dissolved Se may be a result of a limited amount of total Se or particular Se species in other areas of the watershed (Baes and Mesmer 1976, Nimmo, Herrmann et al. 2016). Se is known to have protective effects such as reducing oxidative stress and is a required component

of some enzymes (Kennedy, McDonald et al. 2000, Hamilton, Holley et al. 2005, Hamilton, Holley et al. 2005, Hamilton, Holley et al. 2005, Mathews, Fortner et al. 2014). The high diversity at the LF-4 site where the dissolved Se concentration is well above EPA limits may be consistent with a low amount of Se(IV) and high amount of Se(VI) observed at these sites. EPA and other studies have indicated that Se(IV) is the more toxic of the two forms [38].

## **CONCLUSION**

Based on previous data on the Se levels along the Fountain Creek (Nimmo, Herrmann et al. 2016) a Canonical Correlation Analysis was done to examine the possibility of a relationship between dissolved Se, biologically available Se as bryophyte Se, fish species number and fish diversity. The analysis revealed that two different components were involved and that diversity in fish species was related to the total dissolved Se levels in the creek water samples. Because some sites were found to contain high levels of Se and high fish diversity when the opposite was expected for some sites, we investigated the possibility that such differences between sites could arise due to Se-speciation differences. As a result, we designed and performed studies where we measured the amounts of two different Se species (that is Se(IV) and Se(VI)) as well as total dissolved Se content at selected sites along the Fountain Creek, and determined the oxidation state of the Se. The speciation studies showed a distinct variance in Se(IV) and Se(VI) levels in the different sites consistent with the observations that low toxicity is observed when low levels of Se(IV) are present at the sites. Although these studies do not prove that the level of selenite in the water is the principal factor for toxicity, they do provide the data consistent with this interpretation. These studies underline the importance of determining both Se(IV) and Se(VI) in the

aqueous samples, and the need for such speciation analyses should a complete understanding of the complex system be desired.

## CHAPTER 3 SELENIUM SPECIATION IN THE FOUNTAIN CREEK WATERSHED CORRELATES WITH WATER HARDNESS, CA AND MG LEVELS <sup>2</sup>

### SYNOPSIS

The environmental levels of selenium (Se) are regulated and strictly enforced by the Environmental Protection Agency (EPA) because of the toxicity that Se can exert at high levels. However, speciation plays an important role in the overall toxicity of Se, and only when speciation analysis has been conducted will a detailed understanding of the system be possible. In the following, we carried out the speciation analysis of the creek waters in three of the main tributaries—Upper Fountain Creek, Monument Creek and Lower Fountain Creek—located in the Fountain Creek Watershed (Colorado, USA). There are statistically significant differences between the Se, Ca and Mg, levels in each of the tributaries and seasonal swings in Se, Ca and Mg levels have been observed. There are also statistically significant differences between the Se levels when grouped by Pierre Shale type. These factors are considered when determining the forms of Se present and analyzing their chemistry using the reported thermodynamic relationships considering  $\text{Ca}^{2+}$ ,  $\text{Mg}^{2+}$ ,  $\text{SeO}_4^{2-}$ ,  $\text{SeO}_3^{2-}$  and carbonates. This analysis demonstrated that the correlation between Se and water hardness can be explained in terms of formation of soluble  $\text{CaSeO}_4$ . The speciation analysis demonstrated that for the Fountain Creek waters, the  $\text{Ca}^{2+}$  ion may be mainly responsible for the observed

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<sup>2</sup> This chapter is published in *Molecules* and is referenced as (Carsella, J.S., Sanchez-Lombardo, I., et al. 2017). Table and figure numbers have been modified to reflect that they are specific to this chapter, e.g. figure 1 is now figure 3.1. and the reference were moved to the end of this document. This article is reproduced with permission, and only minimal modifications were made to meet formatting requirements. No other modifications were made.

correlation with the Se level. Considering that the  $Mg^{2+}$  level is also correlating linearly with the Se levels it is important to recognize that without  $Mg^{2+}$  the  $Ca^{2+}$  would be significantly reduced. The major role of  $Mg^{2+}$  is thus to raise the  $Ca^{2+}$  levels despite the equilibria with carbonate and other anions that would otherwise decrease  $Ca^{2+}$  levels.

## **INTRODUCTION**

The environmental levels of Se are regulated and strictly enforced by the Environmental Protection Agency (EPA) in the USA (Edelmann, Ferguson et al. 2005, Divine and Gates 2006, Colorado Department of Public Health and Environment 2013, United States Environmental Protection Agency 2016) because even though low levels are beneficial high levels are toxic (Bird, Ge et al. 1997, Bird and Tyson 1997, Combs and Gray 1998, Mousa, O'Connor et al. 2007, Weekley and Harris 2013, Block, Booker et al. 2016). High Se toxicity on aquatic life and particularly fish reproduction (Hamilton, Holley et al. 2005, Hamilton, Holley et al. 2005, Hamilton, Holley et al. 2005, United States Environmental Protection Agency 2015) has led to the development of a chronic exposure criterion (United States Environmental Protection Agency 2015). High Se-levels are often found when the waterways run over the Se rich shale deposits (Divine and Gates 2006) or when agricultural runoff carries Se into waterways from Se rich soils (Presser, Sylvester et al. 1994, Lemly 1997, Kennedy, McDonald et al. 2000, Mast, Mills et al. 2014). Specifically, the Lower Fountain Creek (LF) in southeastern Colorado is a known and major contributor to the Se levels downstream from its confluence with the Arkansas River (AR); this confluence is in the eastern part of the city of Pueblo, Colorado (for a map see Refs. (Divine and Gates 2006, Herrmann, Turner et al. 2012)). These high Se levels in the Fountain Creek and in the Arkansas River increase the potential of damaging environmental effects downstream in the Arkansas River basin

(Divine and Gates 2006). While Se toxicity is linked to particular species (Bird, Ge et al. 1997, Kennedy, McDonald et al. 2000, Hamilton, Holley et al. 2005, Hamilton, Holley et al. 2005, Hamilton, Holley et al. 2005, Mathews, Fortner et al. 2014) and the presence of selenite ( $\text{SeO}_3^{2-}$ ) (Rosenfeld and Beath 1964, Carsella, Melnykov et al. 2017), many environmental studies only measure and report the total amount of elemental Se. When analyzing and investigating Se speciation, a better understanding of the interaction of Se species in a natural environment is gained (Bird, Ge et al. 1997, Kiss and Odani 2007, Kiss, Jakusch et al. 2008, Block 2013, Crans, Woll et al. 2013, Crans 2015, Block, Booker et al. 2016, Doucette, Hassell et al. 2016, Carsella, Melnykov et al. 2017). Recent studies of fish in the Fountain Creek Watershed demonstrated that high numbers of fish species can be observed at sites with high levels of Se (Carsella, Melnykov et al. 2017). This work led to the hypothesis that the toxicity is dependent on the presence of toxic Se species and not the total Se level (Carsella, Melnykov et al. 2017) and support the need for additional studies considering the effects of Se species on different types of fish. The following manuscript analyzes the chemistry of Se speciation in these waters and that of two major cations,  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$ , using the thermodynamic parameters.

Total Se concentrations in aqueous environments generally vary between 0.06  $\mu\text{L}$  to 400  $\mu\text{L}$  (Smith and Westfall 1937, Scott and Voegeli 1961, Lindberg and Bingefors 1970, Fawell and Combs 2011). The most common forms of Se in surface waters are Se(IV) and Se(VI) with some reports also including Se(II) in either organic or inorganic form (Combs and Gray 1998, Kulp and Pratt 2004, Torres, Pintos et al. 2010, Torres, Pintos et al. 2011, Mast, Mills et al. 2014). Selenium is most soluble in aqueous

solution under oxidizing conditions and the distribution of the oxidized forms of Se, selenite ( $\text{SeO}_3^{2-}$ ) and selenate ( $\text{SeO}_4^{2-}$ ) depend on specific conditions (Combs and Gray 1998, Torres, Pintos et al. 2010, Torres, Pintos et al. 2011, Mast, Mills et al. 2014). The less soluble and reduced form of Se,  $\text{H}_2\text{Se}$ , exists complexed in the earth's crust, and may also be found at low levels in reducing water environments (Combs and Gray 1998). Pourbaix diagrams are important to describe the speciation of Se in idealized systems (Combs and Gray 1998, Torres, Pintos et al. 2010, Torres, Pintos et al. 2011, Mast, Mills et al. 2014, Carsella, Melnykov et al. 2017). However, considering that the Pourbaix diagrams are calculated assuming specific conditions, for example, the concentration and ionic strength do not affect the speciation; such representations are at best, a first approximation (Torres, Pintos et al. 2010, Torres, Pintos et al. 2011, Mast, Mills et al. 2014, Carsella, Melnykov et al. 2017). From the reported Pourbaix diagrams, it is clear that selenous acid ( $\text{H}_2\text{SeO}_3$ ) is favored at the conditions that are normally found in oxidizing natural stream waters ( $\text{pE} \approx 13.5$ ) at a  $\text{pH} < 3.0$  (Baes and Mesmer 1976). Hydrogen selenite ( $\text{HSeO}_3^-$ ) is favored in the same oxidizing conditions up to about  $\text{pH} 5.0$ . Above  $\text{pH} 5.0$  selenate ( $\text{SeO}_4^{2-}$ ) is the most favored Se species. Using this information, it follows that in less aerated waters ( $\text{pE} \approx 13.5$ ) at  $\text{pH}$  values between 3 and 8, hydrogen selenite,  $\text{HSeO}_3^-$  is the favored species (Torres, Pintos et al. 2010, Carsella, Melnykov et al. 2017). However, recently we demonstrated that for Fountain Creek the speciation was about 90% Se(VI) and 10% Se(IV) even through the Pourbaix diagrams predicted more Se(IV) (Carsella, Melnykov et al. 2017). In the following, we explore *the hypothesis that classical speciation plots are effective in describing the Se speciation when considering two influential cations,  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$ .*



The conversions between the different oxidation states of Se are reported to be slow (Torres, Pintos et al. 2011) and it is possible that the speciation in the watershed does not represent equilibrium conditions. Previously a correlation between total elemental Se and with water hardness (Herrmann, Turner et al. 2012) was reported. However, the detailed chemistry of this correlation involving a combination of  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  was not investigated (Herrmann, Turner et al. 2012). Specifically, the possibility of the formation of the soluble and insoluble forms of  $\text{CaSeO}_4$  correlate with the observed aqueous Se levels and equilibrium conditions.

In the following, the Se speciation chemistry of waters in the Fountain Creek Watershed sites was explored. Since the two most common cations determining water hardness are calcium ( $\text{Ca}^{2+}$ ) and magnesium ( $\text{Mg}^{2+}$ ), Se, Ca, and Mg levels were measured at the Fountain Creek sites. Furthermore, the speciation chemistry and its relationship to Se content with  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  contents were explored. Samples were collected from three different Fountain Creek reaches Upper Fountain Creek (UF), Monument Creek (MC) and the previously mentioned LF portion of Fountain Creek. The Se concentrations, the pH, and the concentration of  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  were determined. Since correlations have been reported between Se levels and water hardness, the current manuscript explores the chemical speciation of Se, Ca, and Mg. The goal was to examine if specific chemical relationships between Se and  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  combined can explain the reported correlation with water hardness. Speciation diagrams show that the Se content indeed correlates with the soluble  $\text{CaSeO}_4$  levels even though the thermodynamic equilibria of Se speciation alone are not sufficient to describe all the properties of this system.

## EXPERIMENTAL

### *Materials.*

The standards for EPA method 200.8 were purchased as a NIST traceable multielement custom made standard from Inorganic Ventures, Christiansburg, Virginia, USA (IV, CSTU-STD-1) and from SPEX CertiPrep, Metuchen, NJ, USA (part# CL-CAL-2A). The IV standard was made to contain 1000 $\mu$ g/ml of Ca, Mg, Na, Fe, K. Furthermore, it contains 10 $\mu$ g/ml Al, Sb, As, Ba, Cd, Se, Ag, Pb, Be, Co, Cr, Zn, Cu, Mn, Mo, Ni, V, U, Th, Tl. The SPEX standard was diluted to 50 ppb and used as a check standard to verify calibration. The Internal standards used were (Inorganic Ventures 2008ISS-125,  $^6$ Li, Sc, Y, In, Tb, Bi, Ho) and Germanium(Ge) (Inorganic Ventures MSGE-10PPM-125ML). The nitric acid (Fisher A467-500) and hydrochloric acid (Fisher A466-500) used in these procedures were purchased as Optima grade from Fisher Scientific, USA.

### *Fountain Creek Sampling.*

Water quality measurements of surface waters included measuring temperature, pH, specific conductance, and dissolved oxygen per the protocols described by the United States Geological Survey (USGS) protocols (Fishman 1993, Herrmann, Turner et al. 2012). Each parameter was measured three times at each site over a 10-day period. The measurements were made at 0, 5 and 10-day intervals. Water samples were taken at equivalent distance points on a line across the creek at each sampling site. The samples were collected in 250 ml LDPE plastic bottles and stored on ice for transport. The collection was performed per limnological sampling protocol by holding the bottle with the mouth of the container facing downstream at an angle near 45°. The container was rinsed with creek water 3 times prior to taking the sample. Triplicate samples were collected at each site during each sampling interval and filtered through a

0.45 micron reconstituted cellulose membrane syringe filter purchased from Phenomenex (Phenex AF0-8103-12)(Fishman 1993). After filtering, water samples including field blanks for each trip were preserved with 1% Optima grade HNO<sub>3</sub>. The sample was divided in two, one was analyzed and the other stored at 4°C. Six milliliters were used for cation determination by ICPMS.

*Analysis for Se, Ca and Mg.*

Water samples were analyzed for Se, Ca and Mg on an Agilent 7500ce ICP-MS following EPA Method 200.8 (United States Environmental Protection Agency 1994). Since these samples were not intended for evaluation for municipal drinking water, the ICPMS was run with the Octopole Reaction System (ORS). Hydrogen was used as a reaction gas for <sup>40</sup>Ca and <sup>78</sup>Se measurements. The elements of <sup>24</sup>Mg and <sup>44</sup>Ca were measured using helium as a collision gas, to reduce interferences. Multi-element environmental external calibration standard (CSTU-STD-1) and internal standards were diluted in 1 % nitric and 0.5 % hydrochloric acid prior to analysis. The internal standard was diluted to a final concentration of 20 ppb from a 10ppm stock. Germanium was added to the final internal standard mix before dilution to make a final concentration of 20ppb. The Ge was used as an internal standard for Se because of Ge having a closer ionization potential to Se. Scandium was used as the internal standard for Ca and Mg.

*Alkalinity.*

Alkalinity was measured by titration on all samples collected using the protocols listed in the book Standard Methods for the Examination of Water and Wastewater for alkalinity (American Public Health Association 2005).

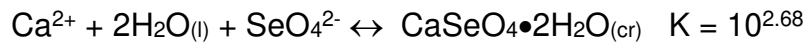
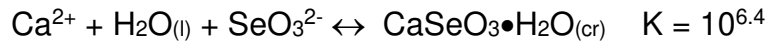
### *Statistical Calculations.*

Analysis of Variance was used to determine if a statistically significant difference exists between the water components (Ca, Mg, and Se) exposed to Pierre Shale type, creek reach, and sites. Creek discharge rates were obtained from USGS monitoring sites via web interfaces (Fishman 1993, Herrmann, Turner et al. 2012). The sites nearest the monitoring stations (UF-1, UF-4, MC-1, MC-2, MC-4, LF-1, LF-2, and LF-4) used the data directly from the monitoring stations. The sites between the stations (UF-2, UF-3, MC-3, MC-5, LF-3, and LF-5) used a weighted average of the flow rates measured at the USGS stations above and below the sampling site used in this study.

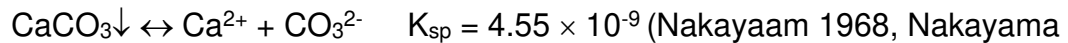
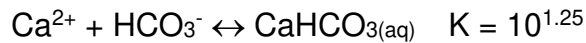
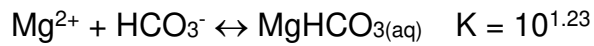
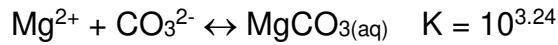
### *Speciation Calculations.*

Species distribution diagrams were calculated by using HYSS 2003 software (Alderlghi, Gans et al. 1999). The concentration used for the consideration described here for the UF-2 site was  $[\text{Se}]_{\text{tot}} 3.36 \cdot 10^{-10} \text{ M}$ , and for LF-4 site was  $[\text{Se}]_{\text{tot}} 1.22 \cdot 10^{-7} \text{ M}$ . When the speciation diagrams with Ca and Mg cations were constructed the concentrations were  $[\text{Se(IV)}] 1.2 \cdot 10^{-8} \text{ M}$ ,  $[\text{Se(VI)}] 1.1 \cdot 10^{-7} \text{ M}$ ,  $[\text{Ca}]_{\text{tot}} 1.7 \cdot 10^{-3} \text{ M}$  and  $[\text{Mg}]_{\text{tot}} 9.4 \cdot 10^{-4} \text{ M}$ .

The speciation diagrams were constructed using the following equilibrium reactions and constants:



\*Cr is crystalline phase, constants for 25 °C and  $I=0$  (Olin, Noläng et al. 2005)



1971)

Table 3.1 Se levels in the water along the Fountain Creek Watershed (Nimmo, Herrmann et al. 2016, Carsella, Melnykov et al. 2017).<sup>3</sup>

	Spring						Fall					
Sampl Sites	T (°C)	pH	Water Se (µg/L)	Water Ca (µg/L)	Water Mg (µg/L)	Alkalinity (mg CaCO <sub>3</sub> /L)	T (°C)	pH	Water Se (µg/L)	Water Ca (µg/L)	Water Mg (µg/L)	Alkalinity (mg CaCO <sub>3</sub> /L)
UF-1	4.6	7.2	0.193	28440	5354	72	5.8	8.2	0.063	19307	5481	76
UF-2	4.9	7.2	0.14	28867	5474	50	5.7	8.1	0	13003	3481	63
UF-3	5.7	7.6	0.673	34863	6890	81	6	8.1	0.043	13897	4041	146
UF-4	6.6	7.6	1.323	39017	10134	83	7	8.1	0.34	15303	5490	154
MC-1	4.2	7.4	0.207	12227	1742	66	5.5	7.9	0.177	15757	4772	86
MC-2	3.9	7.5	0.277	19380	3400	74	5.3	8	0.33	21547	7599	89
MC-3	4.1	7.2	0.343	21623	3818	73	4.5	8.1	0.597	22450	8043	88
MC-4	4.1	7.5	0.443	26327	4365	78	5	8	0.86	27047	8450	86
MC-5	4.8	7.4	1.863	38503	6712	110	6.4	8.1	5.283	41080	13217	105
LF-1	7.7	7.5	2.05	42303	8135	104	9.9	8.1	2.97	29947	10960	108
LF-2	12.2	7.2	2.78	53465	14540	141	15.2	8.1	3.773	38537	18177	179
LF-3	10.6	7.6	3.29	64447	17703	138	15.1	8.1	3.8	41837	19770	180
LF-4	11.3	7.7	9.687	69083	22943	140	15.2	8.1	18.59	46683	29830	183
LF-5	11.8	7.8	7.91	69373	23183	-	15.8	8.2	14.05	47137	29900	-
Ref.		<sup>4</sup>	<sup>2</sup>			<sup>5</sup>		<sup>2</sup>	<sup>2</sup>			<sup>3</sup>

<sup>3</sup> Measurements are averages of three as indicated in the experimental section.

<sup>4</sup> (Nimmo, Herrmann et al. 2016)

<sup>5</sup> (Survey 2017, Survey 2017, Survey 2017, United States Geological Survey 2017, United States Geological Survey 2017, United States Geological Survey 2017)

## RESULTS AND DISCUSSION

### *Characterization of the Se-levels and speciation in the Fountain Creek.*

Fountain and Monument Creeks were sampled for dissolved Se in the past (Presser, Sylvester et al. 1994, United States Environmental Protection Agency 2002, Edelmann, Ferguson et al. 2005, Divine and Gates 2006, Herrmann, Turner et al. 2012, Herrmann, Nimmo et al. 2016, Herrmann, Sublette et al. 2016, Nimmo, Herrmann et al. 2016, Carsella, Melnykov et al. 2017). High levels were reported of the Se (3.4 – 12 µg/L) (Divine and Gates 2006) up to 64 µg/L (Divine and Gates 2006) between Colorado Springs and Pueblo, Colorado. Selenium originates from the Pierre Shale located in southeastern Colorado (Colorado Department of Public Health and Environment 2013) which is underlying both Fountain and Monument Creeks. Fourteen sites were monitored in the spring and fall of 2007 (Table 3.1, Figure 3.1) and part of this data was reported previously (Nimmo, Herrmann et al. 2016, Carsella, Melnykov et al. 2017). Se concentrations and cation concentrations were measured using ICP-MS and the results with Ca<sup>2+</sup> and Mg<sup>2+</sup> are now reported in this manuscript. In Table 1 the Se, pH, and alkalinity reported previously are listed for all water samples. The Ca and Mg concentrations and temperature are reported here. As previously shown, the temperature and pH influence speciation of different elements and these parameters were investigated here.

Several Analyses of Variances (ANOVA) were conducted on the Se levels from all reaches to consider which Se measurements are statistically different between site, reach, the type of shale and Ca, and Mg levels. In Figure 1, the observed Se concentrations are plotted at each site, for both, spring and fall. Due to the exponential increase in the Se, Ca, and Mg concentrations in the reaches of the sampled creeks,

natural log transformations were used to fit the data to a normal distribution to carry out the ANOVA analysis. An ANOVA was also conducted on the Se, Ca, and Mg levels from the Pierre shales (Pierre Shale (PS), Upper Pierre Shale (UPS), Lower Pierre Shale (LPS), Continuous Pierre Shale (CPS) and No Pierre Shale (NPS)). A statistically significant difference in Se levels exists between LF and both the UF and MC reaches ( $p < 0.0005$ ). No statistically significant difference exists between the UF and MC reaches of the Fountain Creek Study area. The ANOVA with Tukey's pairwise comparisons results also show a statistically significant difference between the Se levels and the Shale type ( $p < 0.0005$ ). The results indicate that a statistically significant difference exists between the Se levels in the water exposed to LPS and the rest of the PS formations as well as NPS area of the study area. In addition, there is a statistically significant difference between the Se levels in the water in the UPS and the area of NPS. No statistically significant differences were found between the NPS and CPS shale. A statistically significant difference exists between all reaches for Ca and Mg levels ( $p < 0.0005$ ) with the lowest levels in MC. The ANOVA between shale type and Mg indicates a statistically significant difference exists between LPS and UPS and NPS. There is an overlap between CPS and all shale types with respect to Mg levels. The Ca levels are statistically significantly different in all shale types except for the CPS in the spring data set. In the fall Ca dataset, there is an overlap between the levels in the CPS and NPS as well as LPS and UPS. A statistically significant difference exists between CPS-NPS and UPS-LPS ( $p < 0.0005$ ).

The highest Se concentrations were observed at the MC-5 site that is in Colorado Springs and LF-4 and LF-5 sites. These sites are in the city of Pueblo (during both,



spring and fall), see Figure 3.2, and for a map see Refs. (Divine and Gates 2006, Herrmann, Turner et al. 2012). These sites with very high Se levels are located where the human use is becoming more prevalent and thus include both contributions from natural and human waste sources (Nimmo, Herrmann et al. 2016, Carsella, Melnykov et al. 2017). The highest Se concentration in Monument Creek at MC-5 could be attributed to the presence of UPS under Monument Creek. While the elevated Se concentrations in the LF-4 and LF-5 sites are due to the LPS formation under the creek (Herrmann, Turner et al. 2012).

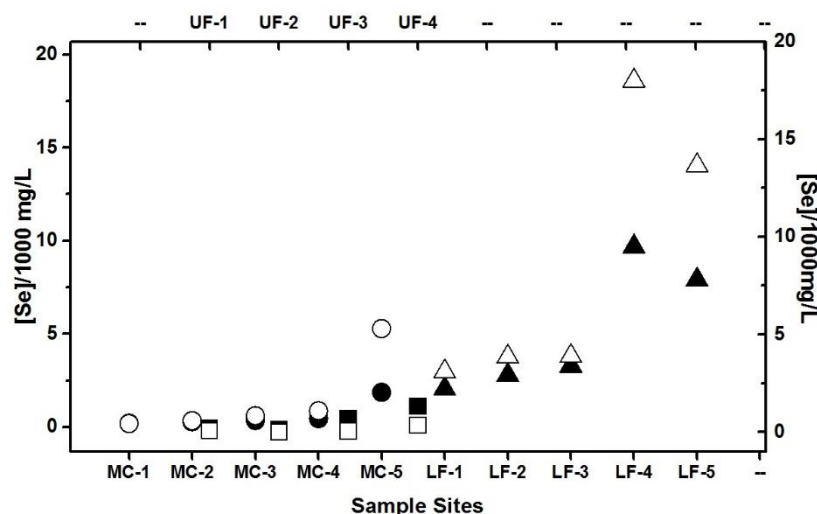


Figure 3.1 Total Se concentrations in the stream water (■- solid symbol) spring and (□- open symbol) fall 2007 in  $\mu\text{g/L}$ . UF stands for Upper Fountain Creek (squares), MC Monument Creek (circles) and LF, Lower Fountain Creek (triangles) (Carsella, Melnykov et al. 2017). Data points were measured in triplicates.

#### *Se, Ca and Mg levels and hardness in the Fountain Creek water.*

Because correlations were reported between Se levels and water hardness by Herrmann and coworkers (Herrmann, Turner et al. 2012), the current manuscript explores the chemical speciation of Se, Ca and Mg to examine if specific chemical relationships between Se and Ca or Mg can explain the reported correlation with

water hardness. In Figure 3.2, the Se concentrations are plotted as a function of total hardness, and a correlation was suggested for the final sites at the Fountain Creek Watershed. Because water hardness is generally attributed to the amount of calcium ( $\text{Ca}^{2+}$ ) and magnesium ions ( $\text{Mg}^{2+}$ ) dissolved in the water, we investigated the relationships by plotting Se level as a function of the hardness (in Figure 3.2A), the concentrations of  $\text{Ca}^{2+}$  (Figure 3.2B) and the concentration of  $\text{Mg}^{2+}$  (Figure 3.2C). The plots show that more Ca was measured in the water in spring as compared to the fall and that this difference correlates with the difference observed in the hardness.

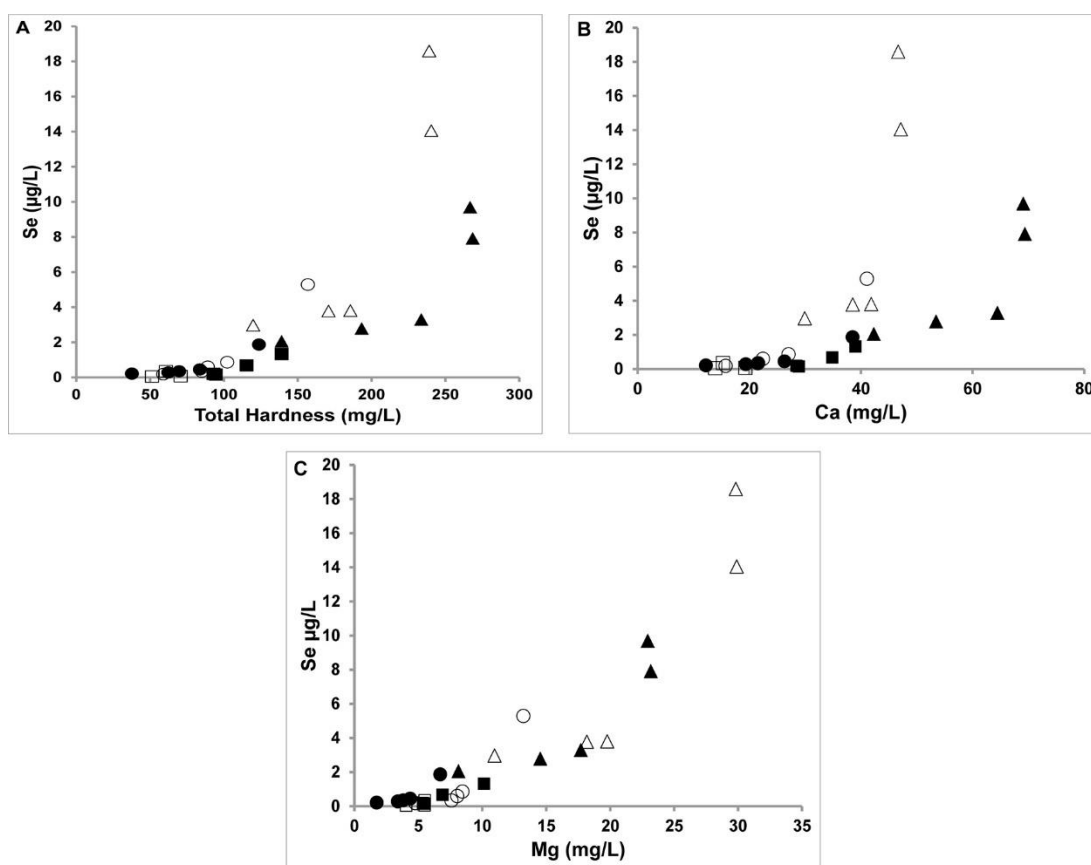


Figure 3.2 Total Se level ( $[\text{Se}]/1000 \text{ mg/L}$ ) vs Total hardness (a), Total Ca level (b) and Total Mg level (c) in the stream water (■- solid symbol) spring and (□- open symbol) fall 2007 in mg/L. UF is the Upper Fountain Creek (squares), MC Monument Creek (circles)

and LF Lower Fountain Creek (triangles) (Carsella, Melnykov et al. 2017). Data points were measured in triplicates.

Based on Figure 3.2 plots of Se versus  $\text{Ca}^{2+}$  and Se versus  $\text{Mg}^{2+}$  as well as plots of each cation versus collection site shown in Figure 3.2, the Se levels increase as the creek runs downstream and across the PS formation. Variations in Se levels exist for each cation and water sample location and some different patterns emerge between the three parameters. Specifically, in the fall the Se concentrations are higher than in the spring for most samples. Linear relationships can be suggested between Se levels and hardness, between Se and  $\text{Mg}^{2+}$  levels for both fall and spring. Linearity is also observed for Se levels and  $\text{Ca}^{2+}$  -levels measured in the spring, but not in the fall. This change in the pattern is of interest because it could be related to uptake of Se by plants in the creek waters, and this will be the topic of a future investigation as well as considering the measurements of Se(IV) and Se(VI) [51]. Most UF and MC sites that have not been exposed to the PS formation, exhibit characteristically low Se concentrations in the water. The Se levels begin to rise at the UF-4 and MC-5 sites which are where the UF reach crosses the CPS formation and the MC reach crosses the UPS formation. The water from both reaches confluence with the LF reach and into the LF-1 site. The most interesting pattern shown here is the correlation of the Se level with  $\text{Ca}^{2+}$  concentrations in the spring measurements and with the  $\text{Mg}^{2+}$  concentrations in both spring and fall seasons.

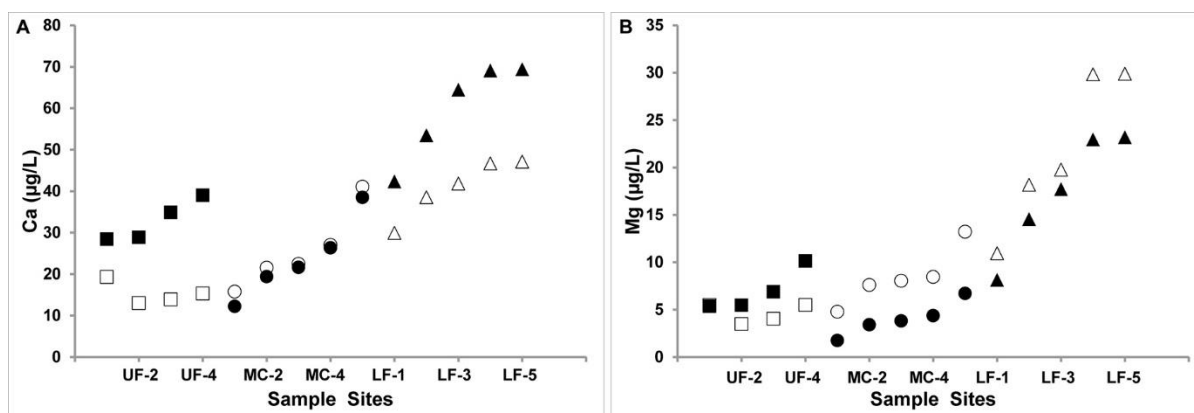


Figure 3.3 A) Total calcium concentrations (presumed  $\text{Ca}^{2+}$ ) in the creek water (■- solid symbols) spring and (□- open symbols) fall year in mg/L. and B) total magnesium concentration (presumed  $\text{Mg}^{2+}$ ) in the stream water (■- solid symbols) spring and (□- open symbols) fall year in mg/L. UF is the Upper Fountain Creek (symbol squares), MC Monument Creek (symbol triangles) and LF Lower Fountain Creek (symbol circles). Data points were measured in triplicates.

In the case of the  $\text{Mg}^{2+}$ , there is less of a variation with changing seasons (Figure 3.2C). In Figure 3.3B the concentrations of Mg at the different sample sites were plotted, and the same trend for the sample locations was found for the Mg levels in samples collected in the fall or spring (see Figures 3.2 and 3.3). The UF-4, MC-5, LF-4 and LF-5 sites contain the highest levels of Ca in spring. Therefore, the fact that the Se levels correlate differently with the hardness in the fall versus the spring streams can be attributed to the seasonal variations in  $\text{Ca}^{2+}$  levels shown in Figure 3.2B. We suggest that although  $\text{Mg}^{2+}$  is important for defining the environment in these systems because the concentrations of  $\text{Mg}^{2+}$  remain constant, it influences hardness by facilitating dissolution of insoluble  $\text{Ca}^{2+}$  species as has been reported previously for  $\text{MgCO}_3\text{-CO}_2$  systems [52].

#### *Speciation Profile for sites with low and high levels of Se.*

In Figure 3.4 we show the distribution of Se species that would be anticipated if the most of the oxidized Se species present are in a thermodynamic equilibrium using the

reported formation constants (see experimental for formation constants). The figures show that under oxidizing conditions the major species is predicted to be  $\text{SeO}_4^{2-}$  from pH 5 to 8, but that at higher concentrations the contribution of Se(IV) to Se(VI) is smaller. However, it must be noted, that these calculations are done at oxidizing conditions, and as reported previously using Pourbaix diagrams, when the pE measured at these sites along the Fountain Creek is used, the predicted major Se component was Se(IV) (Carsella, Melnykov et al. 2017). However, speciation studies showed that regardless, about 90% of the Se was found to be in oxidation state VI (Carsella, Melnykov et al. 2017) and using these results we calculated the speciation observed for the UF-2 and LF-4 sites.

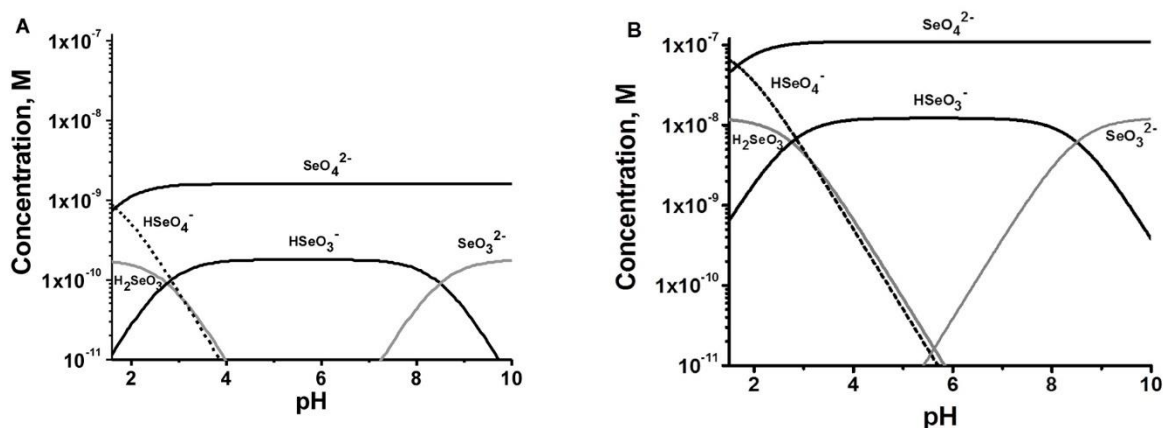


Figure 3.4 Speciation diagrams were calculated for two different sites along the Fountain Creek in the stream water. A) The low concentration UF-2 sites with  $[\text{Se}]_{\text{tot}} 3.36 \cdot 10^{-10} \text{ M}$ , and B) LF-4 with  $[\text{Se}]_{\text{tot}} 1.22 \cdot 10^{-7} \text{ M}$ . Recently the speciation was determined to around 90% Se(VI) for several of the sites studied in this work [25].

$\text{Ca}^{2+}$  is known to form both soluble and insoluble  $\text{Ca}^{2+}\text{-SeO}_4^{2-}$  species and the distribution of these species were investigated using the LF-4 site concentrations of Se, Figure 5A. The levels of the soluble form of  $\text{CaSeO}_4$  were determined previously in

other waters in studies using isotopically pure materials. These studies confirmed the presence of a significant amount of oxidized Se(VI) in such waters (Nakayaam 1968, Nakayama 1971). Studies with the Fountain Creek waters have previously shown that the formation of  $\text{CaSeO}_4$  is competing with the formation of  $\text{Ca}^{2+}$  complexes with  $\text{CaSeO}_3$ ,  $\text{CO}_3^{2-}$ , and with  $\text{HCO}_3^-$ ; and the latter will be discussed below. Figure 5A shows the speciation of Se(IV) and Se(VI) in the presence of  $\text{Ca}^{2+}$  showing more  $\text{CaSeO}_4$  than  $\text{CaSeO}_3$ . As in the case of the  $\text{Ca}^{2+}$ , the  $\text{Mg}^{2+}$  speciation was considered in Figure 5. The speciation diagram was constructed for the Mg and Se species using the LF-4 site concentrations, showing that the most favorable species in the water under these conditions were  $\text{MgSeO}_4$  and the insoluble  $\text{MgSeO}_3$ .

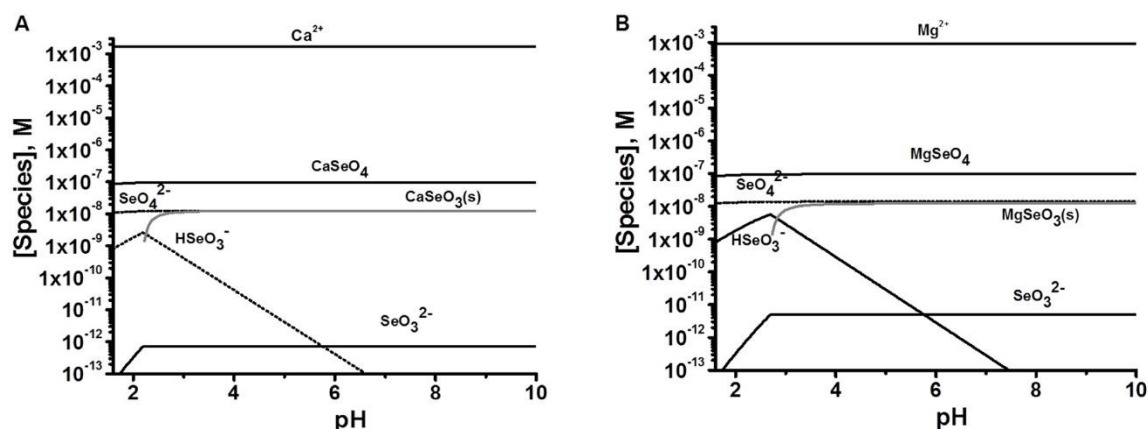


Figure 3.5 Speciation diagram was calculated for LF-4 site along the Fountain Creek in the stream water with concentrations A) of  $[\text{Se(IV)}] 1.2 \cdot 10^{-8}$  M,  $[\text{Se(VI)}] 1.1 \cdot 10^{-7}$  M and  $[\text{Ca}]_{\text{tot}} 1.7 \cdot 10^{-3}$  M and B)  $[\text{Se(IV)}] 1.2 \cdot 10^{-8}$  M,  $[\text{Se(VI)}] 1.1 \cdot 10^{-7}$  M and  $[\text{Mg}]_{\text{tot}} 9.4 \cdot 10^{-4}$  M.

As elevated concentrations of  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  were measured, the speciation diagram for both cations was plotted in Figure 5A and by comparison, we include the speciation diagram for the Se,  $\text{HCO}_3^-$  and  $\text{CO}_3^{2-}$  species with both  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$

together in Figure 6. As expected, the carbonate species with both cations are the most abundant since their equilibrium constants are the highest. The carbonates thus shift the selenite equilibrium for the  $\text{Mg}^{2+}$  since no insoluble  $\text{MgSeO}_3$  is present; however, this is not the case for the  $\text{CaSeO}_3$  that continues to have insoluble material. The presence of carbonates with their large equilibrium constants and the higher concentration of  $\text{Ca}^{2+}$  versus  $\text{Mg}^{2+}$  would support the formation of insoluble calcium selenite. This behavior could be a consequence of a cycle whereby carbonates are consumed by organisms present in the water leading to more release of  $\text{Ca}^{2+}$ . This is a logical explanation given that the pH of the Fountain Creek water (Table 3.1) is slightly alkaline and the alkalinity measurements indicate that under these conditions the waters are oversaturated with  $\text{Ca}^{2+}$  according to the Langelier saturation index L.S.I. (Panthi 2003) The L.S.I.

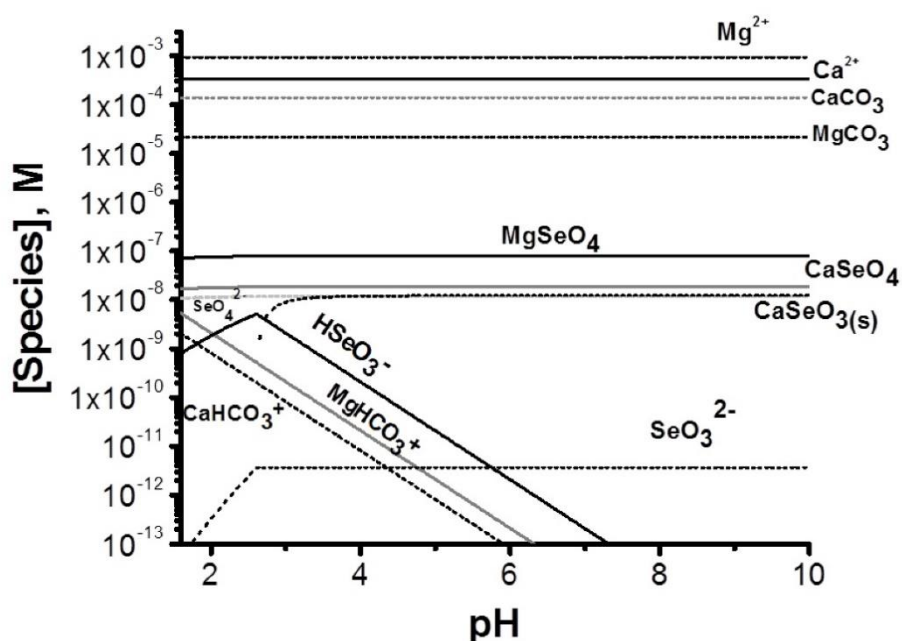


Figure 3.6 Speciation diagram was calculated for the LF-4 site along the Fountain Creek in the stream water with concentrations of [Se(IV)]  $1.2 \cdot 10^{-8}$  M, [Se(VI)]  $1.1 \cdot 10^{-7}$  M,  $[Ca]_{tot}$   $1.7 \cdot 10^{-3}$  M and  $[Mg]_{tot}$   $9.4 \cdot 10^{-4}$  M.

was calculated for the LF-4 site in the summer using the alkalinity value of 140 mg/L of  $CaCO_3$  and the  $K_{sp}$  for  $CaCO_3$  ( $6.92 \cdot 10^{-9}$ ) where  $L.S.I. = \log([Ca^{2+}][CO_3^{2-}]/K_{sp}) = 2.45$  indicating that the water is saturated with  $CaCO_3$  which would allow precipitation of some  $CaCO_3$  particles in the water.

Although the formation constant for  $CaCO_3$  is large, at pH values near neutral the concentration of  $CO_3^{2-}$  in the creek waters is very low. The protonated form of  $HCO_3^-$  is present, but the complex that it forms with  $Ca^{2+}$  is much less stable. The methods used in this study specifically preclude measurements of carbonate and the values are thus not routinely measured. However, carbonate levels in river waters have been reported previously and are in the range of 20-200 mg/L  $CaCO_3$  (Wurts and Durborow 1992,



Panthi 2003). To illustrate the levels of calcium carbonates in these systems, we assume a carbonate level of 140mg/L in the speciation diagram calculated in Figure 3.6. Since the formation constant for the soluble  $\text{CaSeO}_4$  is high ( $K = 10^{2.68}$ ) a significant portion of the soluble Se presumably exists as a soluble form of  $\text{CaSeO}_4$ , however, the solubility product for the insoluble  $\text{CaSeO}_4$  may be reached at some of the sites with high Se concentrations. The possibility that the  $\text{CaSeO}_4$  species is a major species involved in the correlation between Se levels and hardness was considered, and the speciation diagrams including the  $\text{Ca}^{2+}$  cations were examined at the LF-4 site concentrations and are shown in Figure 6. As seen from Figure 3.6 the major species in solution are indeed  $\text{CaSeO}_4$  and  $\text{Ca}(\text{HCO}_3)_2$ .

At the pH values ( $5 < \text{pH} < 8$ ) of the water that has been investigated, most of the carbonate would be protonated and not as prone to precipitation. In addition, Castanier and coworkers show that production of carbonate particles by heterotrophic bacteria follows different paths and these induce a pH increase and an accumulation of  $\text{CO}_3^{2-}$  and  $\text{HCO}_3^-$  ions in the system (Castanier, Le Métayer-Levrel et al. 1999). This is important because the high concentration of  $\text{Ca}^{2+}$  found in the water correlates with the total Se and because in our previous work (Carsella, Melnykov et al. 2017) we found that the major Se species is present in the nontoxic form of Se(VI) as  $\text{SeO}_4^{2-}$ . However, additional analysis is needed and will be the topic of future communications.

Particularly the subject of other cations will be considered particularly when the processing of the Se when taken up by the plants in the creek bed are to be considered. Thus, speciation of iron and other metal ions become important and their corresponding speciation (Ip, Birringer et al. 2000, Kotrebai, Birringer et al. 2000, Hamada, Bayakly et

al. 2006, Crans, Woll et al. 2013, Choudhury, Thomas et al. 2015, Fleming, Groves et al. 2015, MacDonald, Korbass et al. 2015)

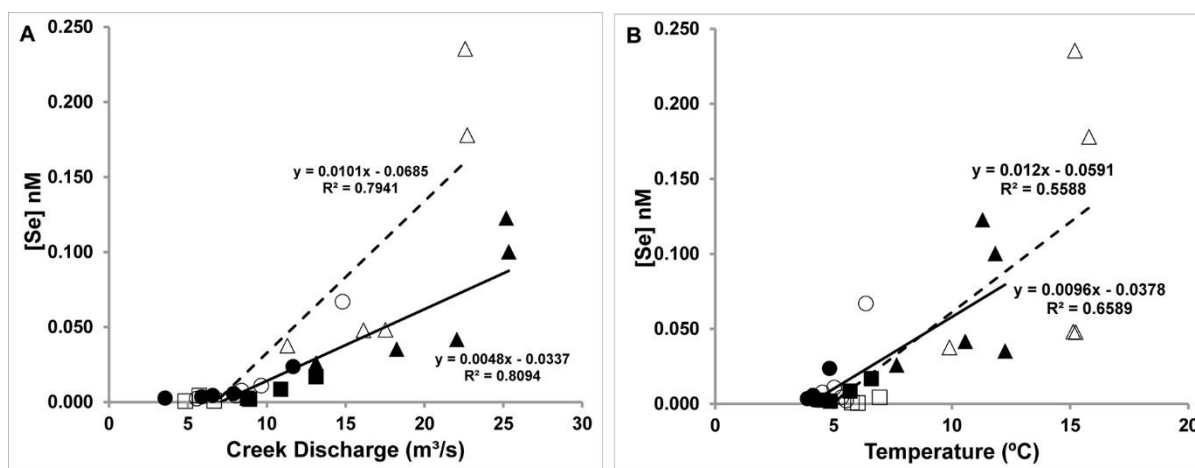


Figure 3.7 Total Se concentration in nM v. Creek discharge in m<sup>3</sup>/second (3.7a) and Se concentration in nM v. water temperature (°C) in the creek water (■- solid symbols) spring and (□- open symbols) fall year and UF is the Upper Fountain Creek (symbol squares), MC Monument Creek (symbol circles) and LF Lower Fountain Creek (symbol triangles). Regression lines are shown as solid for spring and dashed for fall.

*Exploration of other physical parameters and their impact on the speciation.*

To further explore the effects of other physical parameters the Se, Ca and Mg levels were measured in addition to pH, temperature, and discharge. The fourteen sites were monitored in spring and fall of 2007 in this study (Table 1) and we found that the highest Se concentrations in each reach were observed at the UF-4 and MC-5 site in Colorado Springs and LF-4 and LF-5 sites in the city of Pueblo in both seasons as shown in Figure 3.2. The water temperature values are more consistent within one season (Table 3.1) and the water temperatures are colder during the spring. The discharge rates are higher during the spring runoff and the lower discharge during the fall resulted in higher Se concentrations (Figure 3.7a). This is consistent with the observation of higher Se levels when there are higher water temperatures and smaller discharge rates; this allows for more dissolution of Se into the creek water. The total Se

content in the water during spring and fall correlates with the water temperature and the creek discharge rate as shown from the plot in Figure 7. The best relationship to dissolved Se in the creek exists with flow rate (Figure 3.7a,  $R^2_{\text{spring}}=0.8094$ ,  $R^2_{\text{Fall}}=0.7941$ ). Temperature is also correlated with dissolved Se, but the correlation is not as strong as the flow rate (Figure 3.7b). Together the data for temperature and flow rate suggest that there are symbiotic effects on dissolved Se and that these parameters assist in increasing the amount of dissolved Se, and because these parameters affect the  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  in a similar manner, they contribute to increasing the  $\text{Ca}^{2+}$  level above that expected for the solubility product (Figure 3.8).

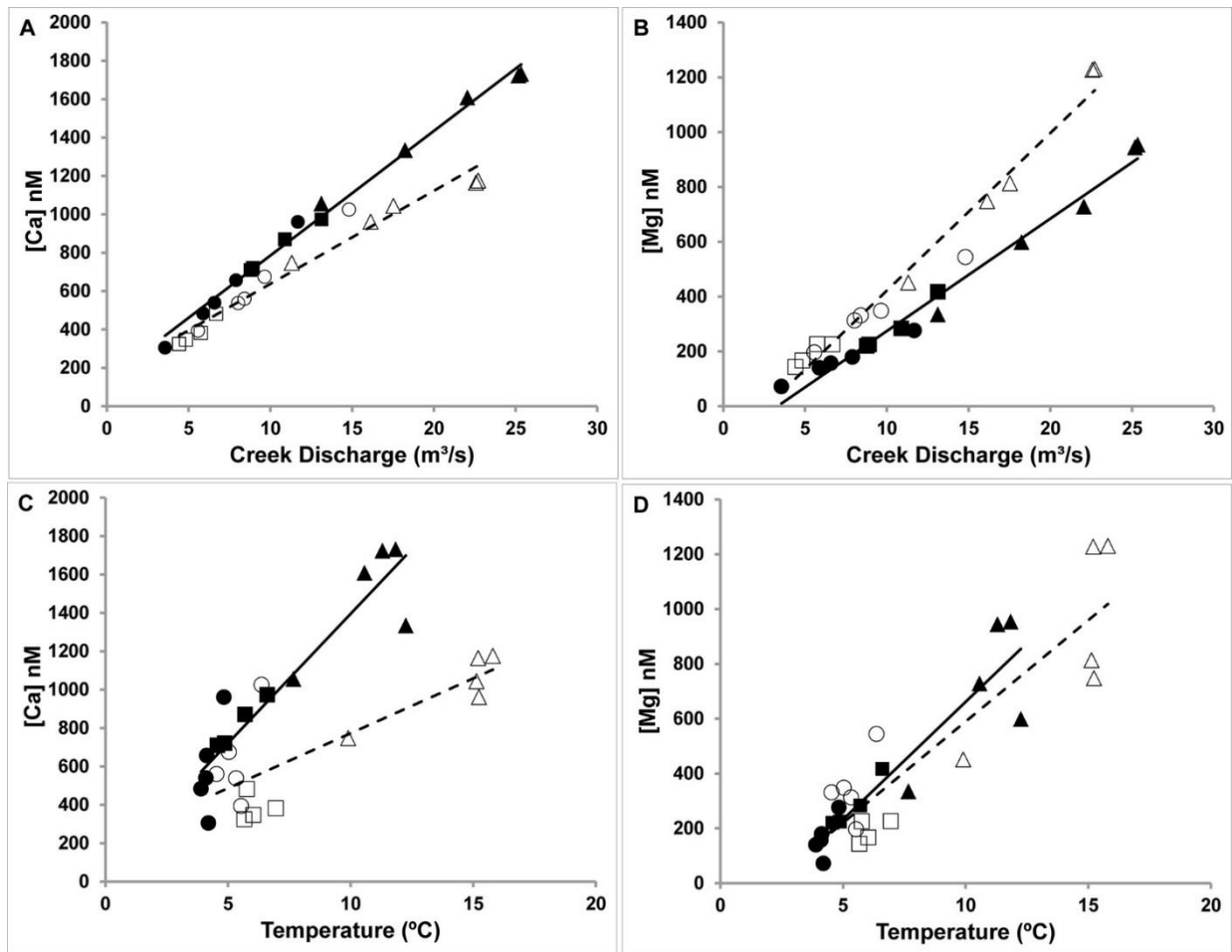


Figure 3.8. Total Ca concentration in nanomoles plotted v. creek discharge in m³/s (3.8a). For comparison, total Mg concentration in nanomoles plotted v. creek discharge in m³/s (3.8b). Total Ca concentration in nanomoles plotted v. water temperature (°C) (3.8c). Total Mg concentration in nanomoles plotted v. water temperature (°C) (3.8d). In all graphs spring, Ca and Mg concentrations are plotted as solid colors with shapes for each segment. UF is the Upper Fountain Creek (symbol squares), MC Monument Creek (symbol circles) and LF Lower Fountain Creek (symbol triangles). Regression lines are shown as solid for spring and dashed for fall.

## CONCLUSIONS

A speciation analysis was carried out on Se and pH data on creek samples from 14 sites in the Fountain Creek Watershed which were supplemented with new measurements of Ca, Mg and temperature on these same sites. The subsequent

analysis supports the interpretation that the  $\text{Ca}^{2+}$  ion is mainly responsible for the observed high Se levels in these waters presumably by the formation of the soluble and insoluble  $\text{CaSeO}_4$ . Although the  $\text{Mg}^{2+}$  levels also correlate with the Se level, this cation is not present at the same high level and thus serves mainly to increase the  $\text{Ca}^{2+}$  levels beyond the solubility limit through ion-pair formation [52]. These findings are important because several of these sites are found to have high levels of Se. However, the fish population has demonstrated that there are no apparent toxicity effects associated with these high Se levels [19] (Ip, Birringer et al. 2000, Kotrebai, Birringer et al. 2000, Hamada, Bayakly et al. 2006, Crans, Woll et al. 2013, Choudhury, Thomas et al. 2015, Fleming, Groves et al. 2015, MacDonald, Korbas et al. 2015). The data presented here thus further characterize the Se-rich waters in which most of the Se is in the Se(VI) oxidation state. The studies here suggest that the observation that Se toxicity is reduced because the major part of the Se is Se(VI) and the studies presented here suggest that a major part of the Se(VI) is in the form of  $\text{CaSeO}_4$ .

The detailed analysis of the Se-species and its ability to explain observations underline the importance of consideration of the speciation chemistry in these environmental systems. The studies demonstrate that measurement of Se species allows for additional insights into the processes in a hydrological system. As illustrated in this manuscript the use of speciation analysis significantly enhanced the understanding of previously reported results for other systems (Ip, Birringer et al. 2000, Kotrebai, Birringer et al. 2000, Hamada, Bayakly et al. 2006, Crans, Woll et al. 2013, Choudhury, Thomas et al. 2015, Fleming, Groves et al. 2015, MacDonald, Korbas et al. 2015). The detailed considerations including  $\text{Mg}^{2+}$  and  $\text{Ca}^{2+}$  allowed for an insight into

the form of Se and the environmental system at hand. These considerations do require measurements of the oxidation state of the Se-species and do need for continued development of advanced methods for detection and measurements of components in complex matrices.

## CHAPTER 4 SELENIUM UPTAKE IN THE BRYOPHYTE HYGROHYPNUM OCHRACEUM AND THE RELATIONSHIP TO IRON AVAILABILITY

### SYNOPSIS

Plants, specifically bryophytes, have been used to study the impacts of trace level contamination of heavy metals in aquatic ecosystems. Bryophytes placed in mine drainages have been shown to accumulate zinc, cadmium, and lead. In North America, the bryophyte *Hygrohypnum ochraceum* has been widely used to study heavy metals in aquatic environments. In a previous study on Fountain Creek, CO water quality, we reported *H. ochraceum* to uptake selenium from Se-rich water found in the Fountain Creek Watershed. In this study the seasonal *H. ochraceum* concentration dependent uptake is examined. All cultures were maintained for 10 days, harvested, dried, and digested according to EPA Method 3052. ICPMS was used to determine metals, Se and Fe in digest solutions. Data analyses suggested that selenium uptake is correlated with Se and Fe concentrations in the water, which led to the hypothesis that Se transport into bryophytes is mediated by soluble Fe. *H. ochraceum* showed a statistically significant, log-linear uptake of Se in the presence of dissolved Fe ( $R^2=0.8488$ ,  $p=0.002$ ). Se uptake was negatively correlated in the absence of dissolved Fe, which supports the hypothesis that soluble iron is required for Se uptake by *H. ochraceum*. This result indicates the importance of considering nutritional factors when determining bioavailability of Se in aquatic systems. The presence of micro-nutrients and the form of these nutrients has far reaching consequences in the use of bryophytes to determine the availability of elements such as Se

## INTRODUCTION

Selenium (Se) is an unequally distributed element in the earth's crust that has nutritionally beneficial and toxic effects.(Presser, Sylvester et al. 1994, Bird, Ge et al. 1997, Bird and Tyson 1997, Combs and Gray 1998, Mousa, O'Connor et al. 2007, Weekley and Harris 2013, Block, Booker et al. 2016) Previous research has indicated that Se enrichment in food crops is needed to combat deficiencies of Se in the diets of people living in Se depleted areas.(White and Broadley 2009, Norton, Deacon et al. 2010, Briat, Dubos et al. 2015, Briat, Rouached et al. 2015, Connorton, Balk et al. 2017, de Figueiredo, Boldrin et al. 2017, White 2017) In contrast areas with high Se often exhibit increased toxicity in aquatic life.(Presser, Sylvester et al. 1994, Carsella, Melnykov et al. 2017). This toxicity often depends on the species of Se present, the environment in which the Se is deposited as well as the bioavailability of the Se to plants and the diets of the fish and aquatic birds present.(Presser, Sylvester et al. 1994, Hamilton, Holley et al. 2005, Hamilton, Holley et al. 2005, Hamilton, Holley et al. 2005, Janz, DeForest et al. 2010, Pettine, Gennari et al. 2012, Cesa, Baldisseri et al. 2013, Debén, Aboal et al. 2015, Debén, Aboal et al. 2017) This justifies the need for biomonitoring of Se in aquatic systems.(Cesa, Baldisseri et al. 2013, Debén, Aboal et al. 2015, Debén, Aboal et al. 2017) This toxicity has lead the United States Environmental Protection Agency (EPA) to produce and enforce strict guidelines for Se in aquatic systems .(United States Environmental Protection Agency 2015) However, the guidelines fall short because they do not consider the various factors such as speciation of Se and associated micronutrients that may affect bioavailability of Se in specific cases.(Cesa, Baldisseri et al. 2013, Debén, Aboal et al. 2015, Carsella, Melnykov et al. 2017, Debén, Aboal et al. 2017). Since most effects of Se in aquatic systems are seen



in fish and birds as a result of dietary exposure, the best way to monitor these complex systems is to use plants, which are primary producers in ecosystems and are at the beginning of the food chain. Simple plants like bryophytes are often used for biomonitoring freshwater systems.(Herrmann, Turner et al. 2012, Cesa, Baldisseri et al. 2013, Whitton 2013, Gecheva and Yurukova 2014, Debén, Aboal et al. 2015, Debén, Aboal et al. 2017) Frequently, bryophytes are placed in freshwater aquatic systems and sampled to look at uptake without regard to other physiological needs of the plant.(Nelson and Campbell 1995, Whitton 2013, Gecheva and Yurukova 2014, Debén, Aboal et al. 2015)

There are documented instances of interactions between Sulfur (S) and Iron (Fe) homeostasis in plants.(Zuchi, Cesco et al. 2009, Couturier, Touraine et al. 2013, Forieri, Wirtz et al. 2013) Fe-S clusters are essential in many plant metabolic processes including photosynthesis, respiration and many enzymatic reactions.(Couturier, Touraine et al. 2013, Vigani and Briat 2016) It has been demonstrated that leaf Fe decreases in S starved tomatoes.(Zuchi, Cesco et al. 2009) In addition, Fe starvation effects S metabolism in *Arabidopsis*.(Forieri, Wirtz et al. 2013) There has been some work showing that gene regulation is involved in the homeostasis of Fe and S when one or the other is significantly reduced from the plants environment.(Astolfi, Cesco et al. 2006, Schuler, Keller et al. 2011, Kobayashi and Nishizawa 2012, Forieri, Wirtz et al. 2013, Paolacci, Celletti et al. 2014) These factors have overlap with mitochondrial function and may have internal feedback loops that aid in the regulation and homeostasis of Fe and S.(Vigani and Briat 2016)

Due to Se atomic similarity with S, it is believed that Se is transported by a high-affinity sulfate transporter which is found in the roots of most plants.(Li, McGrath et al. 2008, Pilon-Smits and LeDuc 2009, Pilon-Smits and Quinn 2010) Therefore, sulfate is a competitive inhibitor to Se uptake.(Li, McGrath et al. 2008, Pilon-Smits and LeDuc 2009, Pilon-Smits and Quinn 2010) The inhibition of Se transport by S is seldom recognized in most Se biomonitoring studies but is referenced in Se crop enrichment research.(White and Broadley 2009, Norton, Deacon et al. 2010, Herrmann, Turner et al. 2012, Whitton 2013, Gecheva and Yurukova 2014, Debén, Aboal et al. 2015, de Figueiredo, Boldrin et al. 2017, White 2017) There are chemical similarities between sulfates and selenates, likewise between sulfites and selenites.(Baes and Mesmer 1976, Block 2013)

The data presented this study is a continuation of a previous study in which environmental Se in the Fountain Creek Watershed was biomonitoring with the bryophyte *Hygrohypnum ochraceum*, which indicated seasonality of Se uptake.(Herrmann, Turner et al. 2012) The hypothesis examined is that the nutritional factor dissolved Fe must be present as a dissolved nutrient for Se to be uptaken by *H. ochraceum*.

## **EXPERIMENTAL.**

Living sections of *H. ochraceum* were collected from Nate Creek ditch near Owl Pass in Ouray County, Colorado. Plants were removed from the banks, logs, and rocks by hand, washed in native water and placed in plastic coolers and transported to the lab. Plants were washed vigorously with pressurized deionized water at Colorado State University-Pueblo (CSU-P) to remove sand, sediment, leaves, and macroinvertebrates. The plants were mechanically separated into strands and washed with pressurized deionized water. The plants were formed into 10cm square sections and placed in

culture trays where they were grown and maintained hydroponically in reconstituted water. (United States Environmental Protection Agency 1985, G.R. Rehe Jr. and Nimmo 2001, Herrmann, Turner et al. 2012) While in culture over the winter the bryophytes increased in biomass by about 30% before being placed into nylon mesh bags for deployment into the Fountain and Monument Creeks, CO.

*Field Measurements and Sampling of Plant and Water  
Experimental Concept.*

Two control groups were created for each sample deployment in spring and fall. Control group 1 consisted of five randomly chosen samples that were kept in an incubator in hydroponic conditions.(Herrmann, Turner et al. 2012) Control group 2 consisted of five randomly chosen samples that were bagged, placed in coolers, transported and raised in the reconstituted water in the lab.(Herrmann, Turner et al. 2012) These two groups were used to quantify sample contamination due to deployment methods.

*Sampling of Bryophytes.*

Seventy 30-gram samples of plants were placed in nylon mesh bags tied with jute drawstrings and deployed in the Fountain Creek watershed utilizing the same 14 sites as previous studies.(United States Environmental Protection Agency 2002, Herrmann, Turner et al. 2012, Herrmann, Nimmo et al. 2016, Herrmann, Sublette et al. 2016, Carsella, Melnykov et al. 2017) Deployed bags, 5 per site, were attached to existing rocks on stream banks or tied to bricks in Monument Creek and Fountain Creek for 10 days.(Herrmann, Turner et al. 2012) After removal from the streams, plants were washed vigorously on site with native water then returned to the laboratory and frozen.(Herrmann, Turner et al. 2012) After thawing, the samples were washed with pressurized deionized water to remove sand, sediment and other debris, dried for 48 hours at 60oC in an oven,

ground to a powder using a mortar and pestle and stored in clean Ziploc® bags.(Herrmann, Turner et al. 2012)

#### *Sampling of water in the stream around the Bryophytes.*

Water quality measurements consisting of temperature, pH, specific conductance, and dissolved oxygen were gathered at deployment (day 1), mid-point (day 5), and retrieval (day 10) of the bryophytes.(Herrmann, Turner et al. 2012) Three water samples per site were also gathered during the study at the same times. Field blanks were also created at 3 sample sites one on each reach of the watershed (Upper and Lower Fountain and Monument Creeks) and used as controls during the collection of the samples. The field blanks were created by pouring deionized water (DIW) that was transported into the field from the lab and sampled using the same sampling apparatus as the samples collected in the field and poured into sampling containers and treated the same way as field water samples. The water samples were returned to the lab in coolers. Each sample was subdivided into 3 parts. One part of 30ml was filtered through a 0.45 µm filter (Phenex AF0-8103-12) becoming the dissolved fraction.(Fishman 1993) The second part of 30 ml was left unaltered and labeled total fraction. The fractions were then acidified to a pH of 2.0 with concentrated nitric acid and stored at 4°C until analyzed.(Fishman 1993) The remaining third part was used for the determination of alkalinity, and hardness by titration.(Fishman 1993)

#### *Analysis of Water and Plant Samples*

Water and plant digestate samples were analyzed on an Agilent 7500ce ICP-MS following EPA Methods 200.8 (United States Environmental Protection Agency 1994) and 6020a.(United States Environmental Protection Agency 2007) Multi-element environmental external calibration standards (CSTU-STD-1) and internal standards

(2008ISS-125) were purchased from Inorganic Ventures and diluted in 2% nitric acid ( $\text{HNO}_3$ ) and 0.5% hydrochloric acid ( $\text{HCl}$ ) prior to analysis.

#### *Analysis of Water.*

Acidified water samples were transferred into 15ml screw capped disposable centrifuge tubes (Fisher Scientific 14-959-49B) and analyzed directly by ICP-MS following EPA Method 200.8 (United States Environmental Protection Agency 1994). Since these samples were not drinking water samples, they were analyzed using the Agilent Octopole Reaction System (ORS) for interference removal on Se, Fe, Ca, Zn, Cu, Cr, Co, Mn, Ni, As, and V. The use of the ORS system is valid to use under these circumstances under the method modification section of Code of Federal Regulations Title 40 Part 136.6 (United States Environmental Protection Agency 2007).

#### *Analysis of Bryophyte samples.*

Bryophyte samples were digested in a Milestone Ethos EZ microwave by placing 0.1 g of the homogenized, prepared tissue sample in a quartz insert; 2 ml of concentrated  $\text{HNO}_3$  and 3 ml of 35% hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) and 0.25 ml  $\text{HCl}$  were added to each insert. The insert was placed in a microwave vessel containing 10 ml D.I. water and 2 ml 35%  $\text{H}_2\text{O}_2$ . The vessels were heated to  $180^\circ\text{C}$  and maintained at  $180^\circ\text{C}$  for 10 minutes. After cooling the digested samples were diluted to a volume of 10 ml. Prior to ICP-MS analysis a 1:10 dilution of the sample was made. National Institute of Standards and Technology (NIST) tomato leaves (SRM-1573a) were used to check digestion efficiency and elemental recovery during ICP-MS analysis. ICP-MS analysis was performed following USEPA Method 6020a. (United States Environmental Protection Agency 2007) ICP-MS operating conditions are presented in Table 4.1.

Table 4.1 Agilent 7500ce operating conditions

	<b>USEPA Method 200.8</b>	<b>USEPA Method 6020</b>
<b>Plasma RF power</b>	1500 W	1500 W
<b>Sample Depth</b>	8.0 mm	9.8 mm
<b>Carrier gas flow</b>	0.82 L/min	0.85 L/min
<b>Sample flow rate</b>	400ul/min	400ul/min
<b>Spray chamber temperature</b>	2 °C	2 °C
<b>Nebulizer</b>	Glass Expansion Micromist	Glass Expansion SeaSpray
<b>Interface</b>	Nickel Sample and skimmer cones	Platinum Sample and skimmer cones

*Statistical analysis.*

Principle component analysis (PCA)(Jolliffe 2002) was performed to determine the significant interactions between elements found in the water with the uptake of selenium in the bryophytes. The groups developed by PCA were then tested for significance using statistical correlations, modeling and regression methods. Minitab version 18 statistical analysis software was used to complete the statistical calculations presented in this paper.

## RESULTS AND DISCUSSION

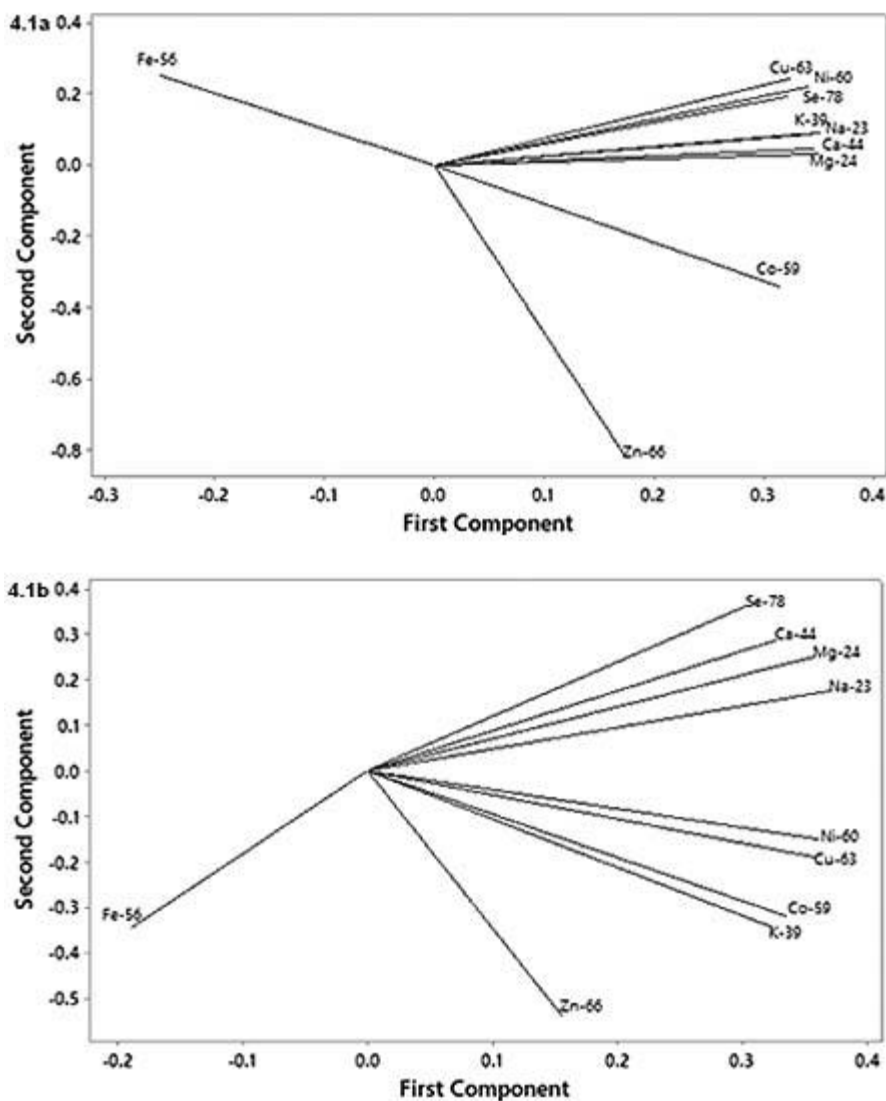


Figure 4.1. Loading plot of correlations of elements in Fountain Creek dissolved fraction of water samples. for spring (4.1a) and fall (4.1b).

The spring Loading Plot (Figure 4.1a) indicates that dissolved Fe is nearly inversely related to Se and related constituents (Ca, K, Na, Mg, and Ni) as indicated by Fe being negative in the first component. The Loading Plot also indicates that in the spring dissolved water fraction Se and Fe are both positive in the second component. Loading Plots also indicates that dissolved Fe is inversely related to dissolved Se in the fall. The

loading plot for the fall dissolved fraction (Figure 4.1b) shows the Fe vector in the negative for both components which. This is a significant change from the positive in the second component in the spring (Figure 4.1a). In contrast the Se vector increases in the positive of both components in the fall (Figure 4.1b) as opposed to the spring (Figure 4.1a) where Se is more weakly positive in both components.

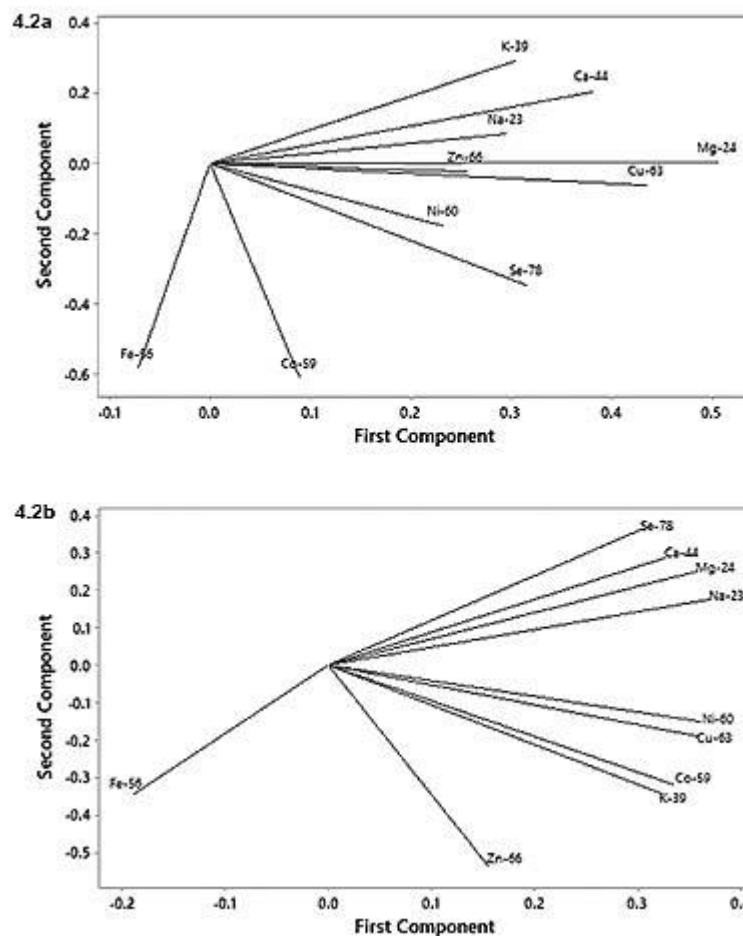


Figure 4.2. Loading Plot of the of *H. ochraceum* exposed to Fountain Creek Water in the Spring (4.2a) and fall (4.2b).

The Fe vector is near orthogonal to the Se vector in both plots indicating that they are independent of each other in the bryophyte (*H. ochraceum*). The Loading Plot for the spring (Figure 4.2a) shows the Fe vector in the negative for both first and second



components while Se is in the positive for the first component and negative in the second component. The fall Loading Plot (Figure 4.2b) is in stark contrast to the spring plot (Figure 4.2a). In the fall (Figure 4.2b) the Fe vector is highly positive in the first component and negative in the second component while Se is largely positive in the second component but not the first. The plots in Figure 4.2 indicate a substantial change in the plants handling of Se and Fe uptake in response to the dissolved fraction of Fountain Creek water.

Table 4.2. PCA of Spring and Fall Fountain Creek Water eigenanalysis correlation matrix.

	Fountain Creek Water			Fountain Creek Water		
	Spring			Fall		
Component	PC1	PC2	PC3	PC1	PC2	PC3
<b>Eigenvalue</b>	7.837	1.109	0.664	6.616	1.847	0.746
<b>Proportion</b>	0.784	0.111	0.066	0.662	0.185	0.075
<b>Cumulative</b>	0.784	0.895	0.961	0.662	0.846	0.921

The PCA results shown in Table 4.2 indicate that 2 components are responsible for the variation seen in the data for spring and fall. According to the Kaiser criteria component, PC3 should be rejected in both spring and fall since these eigenvalues are less than 1.(Yeomans and Golder 1982) The 2 components PC1 and PC2 are responsible for 89.5% of the variation in the spring and 84.6% responsible for the variation in the data in the fall.

Table 4.3. Eigenvectors from PCA of Spring and Fall Fountain Creek Water

<b>Eigenvectors</b>	<b>Fountain Creek Water Spring</b>		<b>Fountain Creek Water Fall</b>	
	<b>PC1</b>	<b>PC2</b>	<b>PC1</b>	<b>PC2</b>
<b>Ca-44</b>	0.346	0.047	0.327	0.288
<b>Mg-24</b>	0.350	0.033	0.324	-0.345
<b>Na-23</b>	0.352	0.094	0.358	0.251
<b>K-39</b>	0.344	0.089	0.371	0.177
<b>Fe-56</b>	-0.251	0.254	-0.189	-0.346
<b>Co-59</b>	0.315	-0.339	0.334	-0.318
<b>Ni-60</b>	0.341	0.222	0.361	-0.152
<b>Cu-63</b>	0.324	0.243	0.358	-0.189
<b>Zn-66</b>	0.171	-0.809	0.155	-0.540
<b>Se-78</b>	0.321	0.195	0.303	0.364

The eigenvectors produced from the PCA shown in Table 3 indicate that the water hardness elements Ca and Mg as well as Na, K, Co, Ni, Cu, and Se are strongly positively correlated while Fe is negatively correlated in PC1 in the spring and fall indicating an inverse relationship between Fe and the other elements listed in Table 2. The results also show that Zn is strongly negatively correlated as well as Co in the spring in PC2. In the fall PCA results Mg, Fe, Ni, and Cu all switch from a positive correlation to a negative relationship to the other elements in PC2 listed in Table 3.

Table 4.4. PCA of *H. ochraceum* exposed to Spring and Fall Fountain Creek Water.

	<i>H. ochraceum</i> Exposed to FC Water Spring					<i>H. ochraceum</i> Exposed to FC Water Fall			
Component	PC1	PC2	PC3	PC4	PC5	PC1	PC2	PC3	PC4
Eigenvalue	3.471	2.071	1.256	1.033	0.798	4.428	1.957	1.248	0.725
Proportion	0.347	0.207	0.126	0.103	0.08	0.443	0.196	0.125	0.072
Cumulative	0.347	0.554	0.68	0.783	0.863	0.443	0.639	0.763	0.836

The PCA results presented in Table 4 indicate that according to the Kaiser criteria 4 components are needed to describe the variation in the spring dataset while 3 components are needed in the fall.(Yeomans and Golder 1982) This is shown by the eigenvalues of PC5 (spring) and PC4 (fall) having values less than 1. The 4 components in the spring are responsible for 78.3% of the variation while the 3 components in the fall are responsible for 76.3% of the variation.

Table 4.5 Eigenvectors from PCA of Spring and Fall Fountain Creek Water

Eigenvectors	<i>H. ochraceum</i> Exposed to FC Water Spring				<i>H. ochraceum</i> Exposed to FC Water Fall		
	PC1	PC2	PC3	PC4	PC1	PC2	PC3
<b>Ca-44</b>	0.380	0.205	0.341	0.154	0.167	0.440	-0.324
<b>Mg-24</b>	0.506	0.005	-0.034	-0.028	0.462	-0.052	-0.016
<b>Na-23</b>	0.295	0.086	-0.624	-0.236	0.346	0.101	-0.517
<b>K-39</b>	0.305	0.292	-0.060	0.082	0.177	0.422	0.414
<b>Fe-56</b>	-0.072	-0.581	-0.069	0.339	0.391	-0.294	0.170
<b>Co-59</b>	0.089	-0.610	0.071	-0.003	0.433	-0.230	0.089
<b>Ni-60</b>	0.232	-0.178	0.525	-0.268	0.298	-0.018	0.242
<b>Cu-63</b>	0.434	-0.060	0.250	0.031	0.418	0.062	-0.078
<b>Zn-66</b>	0.255	-0.024	-0.209	0.761	0.053	0.449	0.531
<b>Se-78</b>	0.315	-0.346	-0.312	-0.383	0.022	0.520	-0.269

The eigenvectors shown in Table 5 indicate a complex balance between elements in the plants with changing water chemistry based on seasons. The weights of the factors are from highest on the left (PC1) to Lowest on the right (PC4; spring PC3; fall). The vectors show a strong correlation between Se with most elements except Fe and Co in the spring (PC1 spring). While Fe has a strong correlation with most elements in the fall except Ca, K, Zn, and Se (PC1 fall). It is interesting to observe that Fe went from a weak negative correlation to a strong positive as the switch in seasons and water chemistry occurred. In component 2 (PC2) the Fe-Se correlation is much stronger and is negative in the spring. However, negative Fe and positive Se in the fall indicate an inverse

correlation between the two elements. The graphical representation of these relationships can be seen in Figure 4.2a and 4.2b.

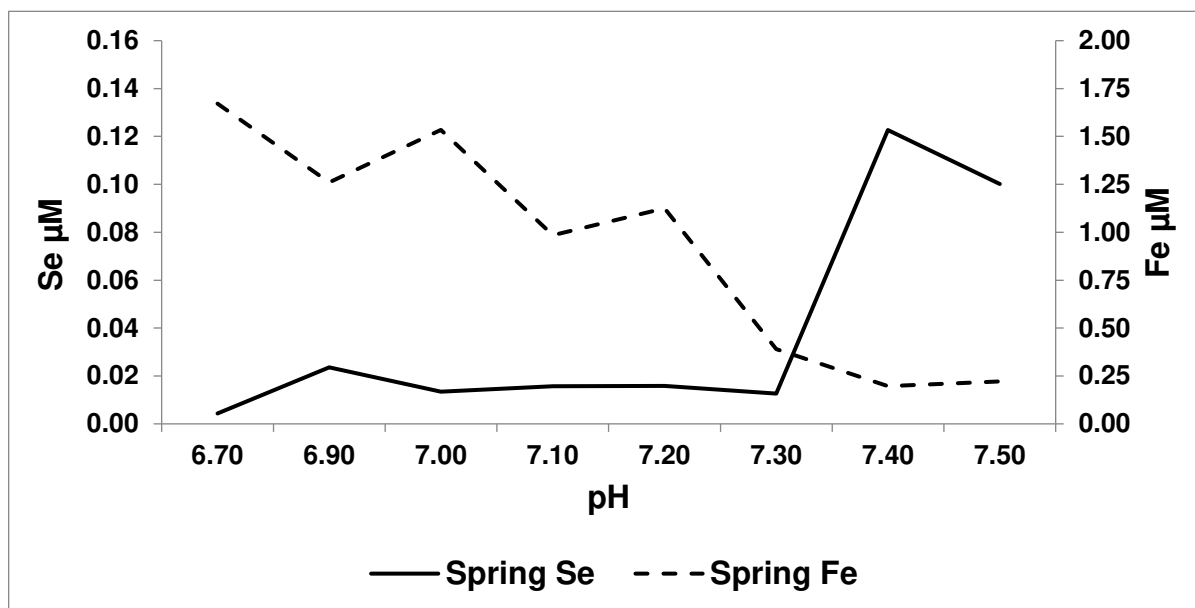


Figure 4.3. Spring Fe-Se-pH interaction plot with pH as the independent variable. Se concentration in the dissolved fraction of Fountain Creek water is on the left axis and the Fe concentration in Fountain Creek Water is on the right axis.

It is evident in Figure 4.3 that the Fe concentration goes down with increasing pH. While the Se concentration rises under the same conditions. Overall the pH rises as the sample sites decrease in elevation. This decrease is caused by the water interaction with the creek bed which contains basic minerals such as calcium and magnesium carbonates which have interesting effects on the solubility of Se species.(Carsella, Sánchez-Lombardo et al. 2017)

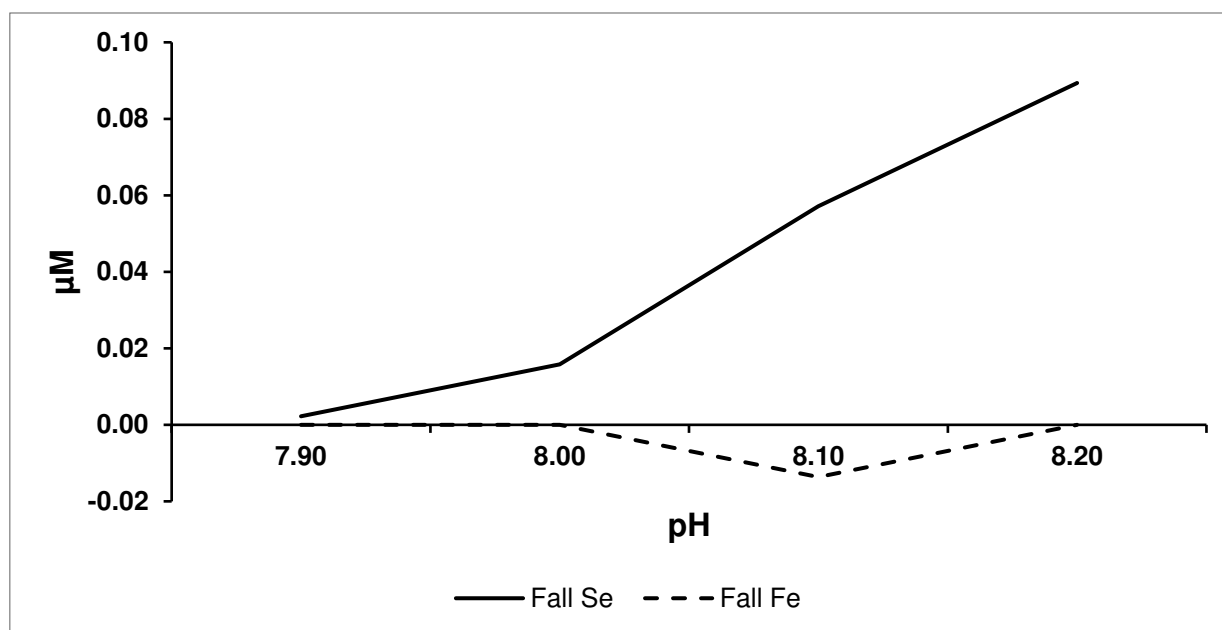


Figure 4.4. Spring Fe-Se-pH interaction plot with pH as the independent variable.

In Figure 4.4 the Fe concentration is very low to nonexistent in the dissolved fraction of the water samples which is likely due to the formation of insoluble forms of Fe as a result of the seasonal change in pH. There is still a significant amount of Fe in the total fraction, which is not shown in the interaction plot in Figure 4.4, supporting the idea that a large insoluble portion of Fe exists.

To develop a simpler working model, the variables with the highest weights in the selenium and iron groups were used in combination to form a model of plant selenium uptake. The variables were fitted to a linear regression model and each variable tested for significance. Variables with a p-value > 0.05 were rejected.

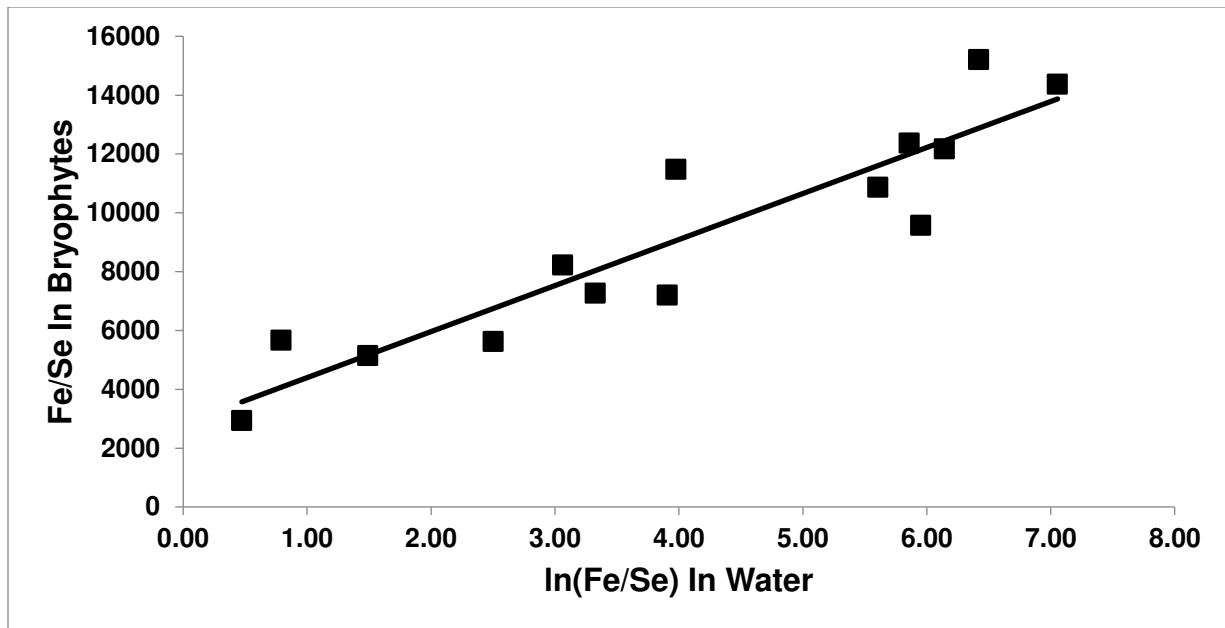


Figure 4.5. Fitted line plot of water  $\ln(\text{Fe}/\text{Se})$  ratio vs. bryophyte  $\text{Fe}/\text{Se}$  ratio .  
( $R^2=0.8488$ )

The first model presented in this study focuses on Fe and Se groups in the spring data. Since the Fe relationship in the Se group is negative, the two elements are inversely related. Se was chosen for the denominator as it is never 0. The natural log was used on the water fraction to normalize the Se and Fe concentrations in the water as these elements increase in a large amount from west to east and north to south over the study area.

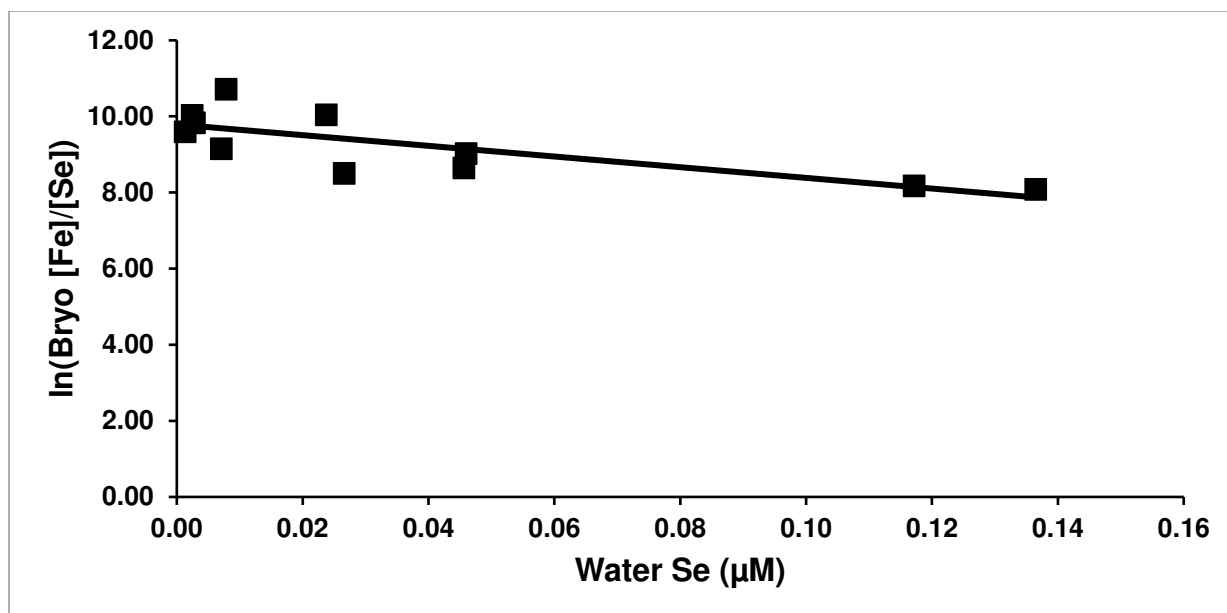


Figure 4.6. Water Se vs. ln (Bryo Fe/Se) Showing the negative linear relationship ( $R^2 = 0.5949$ ,  $y = -14.035x + 9.7887$ ) with Se plant uptake and no dissolved Fe in the water.

In contrast to the spring data, there is no dissolved Fe in the water in the fall. Because of the absence of dissolved Fe, it is not included in the model. The dissolved Se is more linear in the fall and the natural log is not used. The result of the model shows Se uptake in *H. ochraceum* to have a negative slope when the Se concentration increases in the absence of dissolved Fe.



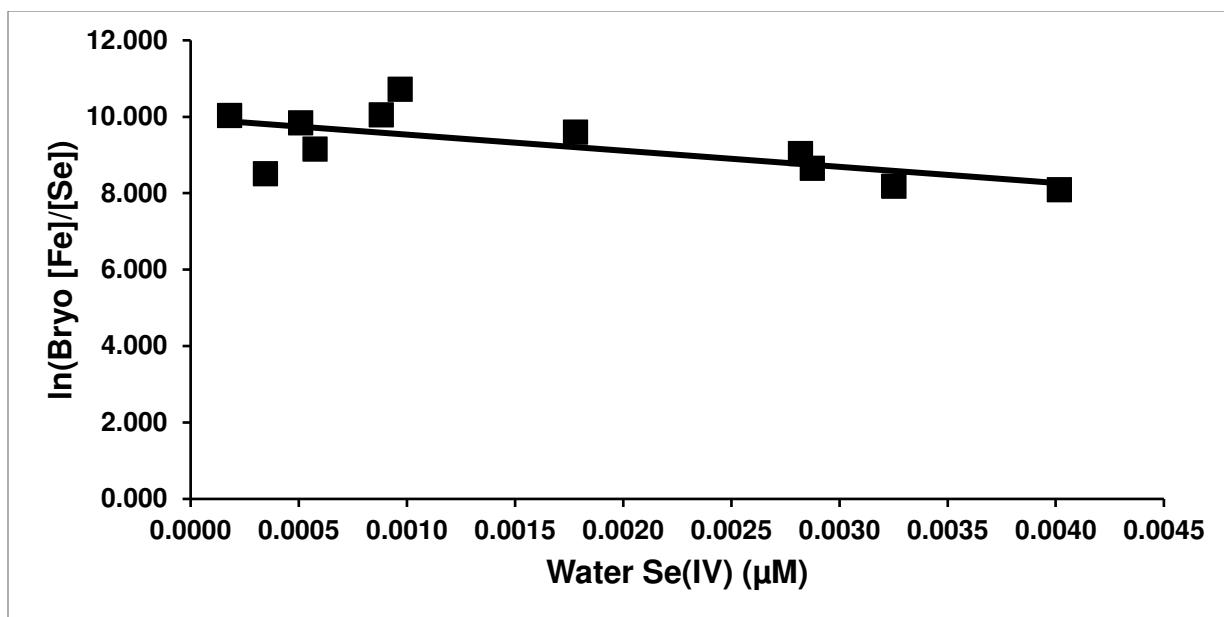


Figure 4.7. Water Se(IV) vs ln (Bryo Fe/Se) Showing the negative linear relationship ( $R^2 = 0.4462$ ,  $y = -421.39x + 9.9543$ ) with Se plant uptake and no dissolved Fe in the water.

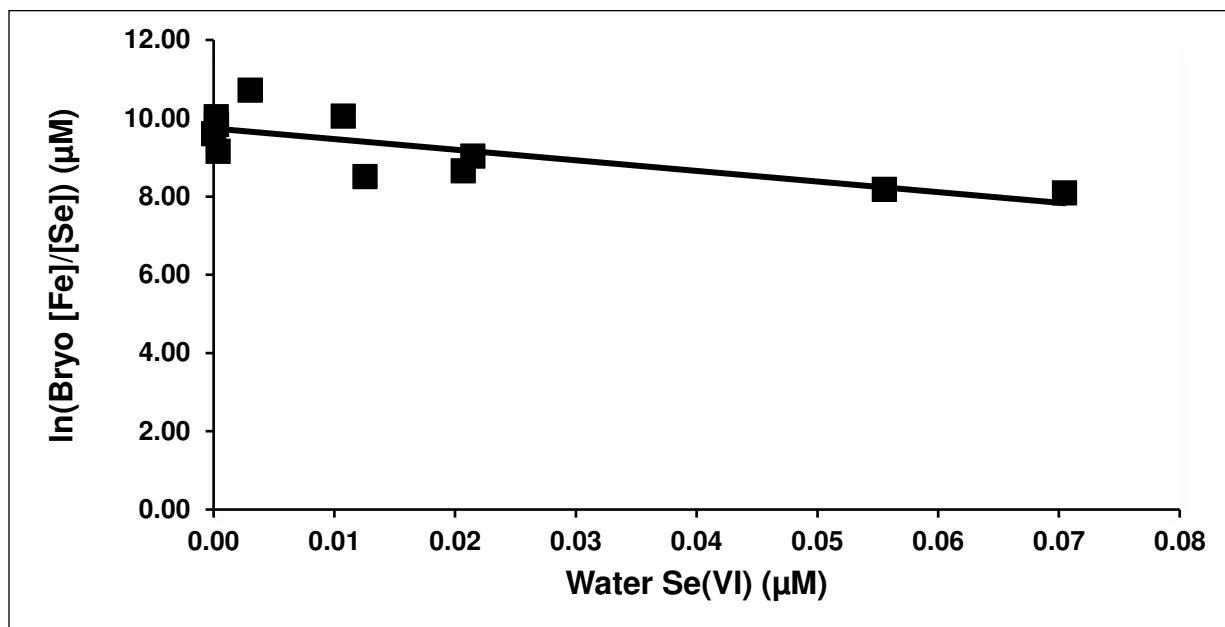


Figure 4.8. Water Se(VI) vs ln (Bryo Fe/Se) Showing the negative linear relationship ( $R^2 = 0.5769$ ,  $y = -27.112x + 9.7382$ ) with Se plant uptake and no dissolved Fe in the water.

Figures 4.7 & 4.8 illustrate the same plot as Figure 6 using selenite (Se(IV)) and selenite (Se(VI)) respectively. Figure 7&8 also show a negative relationship like the Se relationship shown in Figure4.6.

Table 4.6. Statistical analysis of regression lines in figures 6-8 presented above.

<b>Term</b>	<b>Coefficient</b>	<b>SE Coefficient</b>	<b>P-Value</b>
<b>Constant</b>	9.789	0.242	0.000
<b>Water Se</b>	-14.04	4.12	0.002
<b>Species</b>			
<b>Se(IV)</b>	0.166	0.385	0.671
<b>Se(VI)</b>	-0.051	0.336	0.882
<b>Water Se*Species</b>			
<b>Se(IV)</b>	-407	143	0.008
<b>Se(VI)</b>	-13.08	9.06	0.161

The regression lines in Figures 4.6-4.77 were tested for statistical significance using a T-test. The test indicates that no statistically significant difference exists between the fall total dissolved Se model (Figure 4.6) and the selenate model (Figure 8) ( $p=0.161$ , Table 4.3). There is a statistically significant difference between the fall total dissolved Se model (Figure 4.6) and the selenite model (Figure 4.7) ( $p=0.008$ , Table 4.3). Table 4.6 also indicates that there is not a significant difference between the uptake of Se with respect to species however the terms used in the model for dissolved Se are statistically significant.

Experimental evidence supports the hypothesis that Fe is required for uptake of Se by the bryophyte *H. ochraceum*. This evidence is provided in the experimental results that

are showing a positive dose-dependent relationship between Se uptake with increasing Se in water in the presence of Fe (Figure 4.5). This result is consistent with previous studies indicating that Se can be assimilated like S in plants.(Pilon-Smits and LeDuc 2009, Pilon-Smits and Quinn 2010, Briat, Rouached et al. 2015, Vigani and Briat 2016)

The negative slope of the lines in Figures 6-8 shows a trend of less Se uptake in water with higher Se. This trend is likely a result of oversaturation of Se with respect to Fe. The only difference between Figure 5 and Figure 6 is the absence of dissolved Fe. The loss of dissolved Fe is probably caused by a seasonal change in pH that results in the Fe adsorbed to suspended sediments in the water or into plaques. The Fe becomes part of a particle and can be adsorbed to suspended sediments in the water or into plaques(Xin-Bin, Wei-Ming et al. 2007). The Fe becomes part of a particle and is not available to the plant(Xin-Bin, Wei-Ming et al. 2007).(Xin-Bin, Wei-Ming et al. 2007). It is important to realize that bryophytes like *H. ochraceum* do not have any roots, so these plants must absorb all their nutrients by other means and not by conventional roots. The lower pH allows the Fe to stay dissolved in the water in the spring and therefore allows the uptake of Se by *H. ochraceum*. These findings are consistent with several works indicating that S and Fe homeostasis are linked by some gene expression mechanism.(Kobayashi and Nishizawa 2012, Couturier, Touraine et al. 2013, Forieri, Wirtz et al. 2013, Paolacci, Celletti et al. 2014, Briat, Rouached et al. 2015, Vigani and Briat 2016) Since S and Se are related elements it is likely that Se uptake is controlled by the same mechanism as S as Se is thought to be transported by S transporters in plants.(Bird, Ge et al. 1997, Pilon-Smits and LeDuc 2009, Vigani and Briat 2016)

There is no observable effect in the creek water between selenite and total dissolved Se (Table 5). This is most likely due to the fact that the majority of the Se present in Fountain Creek is in the selenate form (Carsella, Melnykov et al. 2017, Carsella, Sánchez-Lombardo et al. 2017).. (Carsella, Melnykov et al. 2017, Carsella, Sánchez-Lombardo et al. 2017) There is a statistically significant difference between the uptake of total dissolved Se and selenite (Table 3). Based on the slope of the line in Figure 4.7 compared to Figure 4.8 the amount of selenite uptaken by the plant should be less than selenate. The findings presented here are important in two areas: 1. The nutritional requirements of the plant being used for biomonitoring must be satisfied and understood. Without this understanding, seasonal variations can confound the model. 2) It is necessary to understand the impact that speciation may play in the plant's ability to uptake the element of interest. This effect may be direct or may be indirect. In the case presented here, we can see that there is a difference between uptake of selenite when Fe is present and selenite in the absence of Fe. The solubility of Fe is also affected by the form that it is in, which is determined by pH, and. Furthermore, the presence of Fe influences how S and therefore likely how Se is uptaken by the plant. This has implications for watershed management if other plants work the same way and if the hydrological management of the watershed changes to allow slightly more acidic water to continue to move down the watershed. This change in pH may come from sources such as increased creek flow volume that results from increased flow from wastewater treatment plants and water added to the creek from other municipal uses.

## CHAPTER 5 IS LIVING WITH SELENIUM CONTAINING RIVER WATERS SUSTAINABLE

### **PERSPECTIVES ON LIFE IN A WASTEWATER IMPACTED SELENIUM CONTAINING CREEK**

The information gained from the studies presented in the previous three chapters has far reaching implications in water management, that enhance understanding of plant nutrient control and bioremediation. The Fountain Creek Watershed is a complex system of creeks, intermittent streams and springs. Most of these water sources drain into one of three reaches of Fountain Creek. The geology and hydrology of this area has a profound impact on the water quality of the lower Fountain Creek (Edelmann, Ferguson et al. 2005, Herrmann, Turner et al. 2012). Human activity, wastewater, and stormwater runoff also affect the water quality in the lower Fountain Creek (Gautam, Carsella et al. 2014). This project has illustrated that this complexity extends to the chemistry of the water and the Se species present in different reaches of Fountain Creek (Carsella, Sánchez-Lombardo et al. 2017). The speciation of Se and the interaction with other cations greatly affect the solubility of Se in the Fountain Creek (Carsella, Sánchez-Lombardo et al. 2017). It is well known that Se speciation is dependent on pH and the redox conditions found in the water column (Drever 1997, Carsella, Sánchez-Lombardo et al. 2017). The work with the bryophytes presented in Chapter 4 illustrate the concept that a plant nutritional requirement, specifically Fe in this case, affects the uptake of Se. This effect is also species dependent for both Fe and Se. This is important to consider as plants as primary producers are a gateway to higher parts of the food chain which can have effects on fish diversity (Carsella, Melnykov et al. 2017).

First, from a management perspective, these studies highlight the need to obtain measurements on aquatic systems including determining the source of elements of concern and the speciation of these elements to set a baseline and identify potential problems. After determining the baseline assessment of existing conditions how future change may impact valuable water resources. In the specific case of Fountain Creek, the flow rate helps govern the pH. Historically seasonality helped govern temperature and therefore spring runoff which increased discharge. This allowed for a seasonal fluctuation in flow and we now know a seasonal fluctuation in Se availability (Chapter 4). Since this historic Se availability is discharge (flow rate) dependent, anything that effects the discharge could affect the Se availability (Mau, Stogner et al. 2007). There are a number of items along Fountain Creek that can increase discharge out of the normal historical seasonal fluctuations (Mau, Stogner et al. 2007). Recent forest fires have left burn scars that readily increase the risk of flooding in the creek (Cole, Friesen et al. 2014, Arnette and Zobel 2016). The increase of development and lack of a citywide storm runoff plan for the Colorado Springs area, also increases the amount of water entering Fountain Creek (Mau, Stogner et al. 2007). This increase in discharge probably causes spikes in available Se as well as scours the creek bed and exposing more Se containing shale (Mau, Stogner et al. 2007). Urban development along Fountain Creek should consider what the consequences of increased discharge from storm and wastewater could mean to the available Se levels in the creek. Such an increase in discharge maybe small but could result in a smaller but sustained discharge into the creek increasing overall discharge, resulting in a pH change that could affect Se speciation. In this case the species of Se should be monitored along with total Se levels.

With increased development the increased need for water brings challenges as well. One such project is a pipeline called the Southern Delivery System (SDS) is used to bring needed water from Pueblo Reservoir to the Colorado Springs area and return processed wastewater to the Arkansas River system via Lower Fountain Creek and was the phase 1 part of a two phase plan (Colorado Springs Utilities 2018). This phase I of the SDS began operation in 2016 and the phase II future plan expands SDS to include two reservoirs to enhance raw water delivery to the Colorado Springs area (Colorado Springs Utilities 2018). Both SDS phases may have significant impacts on the flow rates and therefore affect the solubility of Se in water found in Lower Fountain Creek. Another area of interest might entail agriculture as this was the source for increased Se found in the water entering the Kesterson National Wildlife Refuge (Ohlendorf, Hothem et al. 1989, Presser, Sylvester et al. 1994). If agricultural runoff along Fountain Creek were to significantly increase, it would affect Se in the creek. The effects of fertilizer on Se shales was shown to be significant in the Mancos area (Mast, Mills et al. 2014). Agriculture can increase bacteria found in streams but a recent study did confirm that the bacteria present in Fountain Creek does not come from agriculture but from birds nesting under bridges along the water way (Stoeckel, Stelzer et al. 2011).

Second, in the area of plant nutrients and uptake it is important to know what species of Se is present and which are most available for the plant to incorporate into its biomass. Thus, knowledge of how to control the uptake of these essential elements by plants is vital and requires additional investigations to understand the complex transport mechanisms at work in plants. An extension of this work would be to see how

conserved these traits are genetically and how easily they might be regulated and harnessed to the benefit of the environment and mankind.

Third, the ability to use plants for bioremediation of Se has been studied to some degree (Pilon-Smits and LeDuc 2009, Pilon-Smits and Quinn 2010). Indeed some plants such as *Stanleya pinnata* have been shown to uptake selenate and mitigate Se toxicity by releasing dimethyl selenide (Parker, Feist et al. 2003). This groundwork but needs to be further developed to determine answers to the following questions:

- 1) What are the limits for uptake of Se and related compounds by plant species of interest and how does plant nutrition effect the uptake of Se?
- 2) What should be done with the plant biomass that accumulates in these areas?
- 3) Is it possible to extract useful compounds from plants such as bryophytes that favor acid mine drainages that would allow the passive mining of rare metals from mine effluents?

Fourth, stress makers related to Se exposure could be studied in the fish populations in Fountain Creek to confirm, if the lack of diversity in some areas is due to Se exposure and furthermore if some species have become Se adapted.

Fifth, the biodiversity of the microbiome needs to be evaluated. This could greatly further explain the species of Se present in the reaches of Fountain Creek and help in understanding the geochemistry present in this system (Knack, Wilcox et al. 2015). The microbiome also can have a profound effect on the availability of nutrients to plants and animals with profound effects on the ecosystem.



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