

Impacts of Mountaintop Removal Coal Mining on the Mud River, West Virginia:

Selenium Accumulation, Trophic Transfer, and Toxicity in Fish

by

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Dissertation submitted in partial fulfillment of  
the requirements for the degree of Doctor  
of Philosophy in the  
Environment in the Graduate School  
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2014

ABSTRACT

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## Abstract

Selenium (Se) is a micronutrient necessary for the function of a variety of important enzymes; Se also exhibits a narrow range in concentrations between essentiality and toxicity. Oviparous vertebrates such as birds and fish are especially sensitive to Se toxicity, which causes reproductive impairment and defects in embryo development. Selenium occurs naturally in the Earth's crust, but it can be mobilized by a variety of anthropogenic activities, including agricultural practices, coal burning, and mining. Mountaintop removal/valley fill (MTR/VF) coal mining is a form of surface mining found throughout central Appalachia in the United States that involves blasting off the tops of mountains to access underlying coal seams. Spoil rock from the mountain is placed into adjacent valleys, forming valley fills, which bury stream headwaters and negatively impact surface water quality. This research focused on the biological impacts of Se leached from MTR/VF coal mining operations located around the Mud River, West Virginia.

In order to assess the status of Se in a lotic (flowing) system such as the Mud River, surface water, insects, and fish samples including creek chub (*Semotilus atromaculatus*) and green sunfish (*Lepomis cyanellus*) were collected from a mining impacted site as well as from a reference site not impacted by mining. Analysis of samples from the mined site showed increased conductivity and Se in the surface waters compared to the reference site in addition to increased concentrations of Se in insects and

fish. Histological analysis of mined site fish gills showed a lack of normal parasites, suggesting parasite populations may be disrupted due to poor water quality. X-ray absorption near edge spectroscopy techniques were used to determine the speciation of Se in insect and creek chub samples. Insects contained approximately 40-50% inorganic Se (selenate and selenite) and 50-60% organic Se (Se-methionine and Se-cystine) while fish tissues contained lower proportions of inorganic Se than insects, instead having higher proportions of organic Se in the forms of methyl-Se-cysteine, Se-cystine, and Se-methionine.

Otoliths, calcified inner ear structures, were also collected from Mud River creek chubs and green sunfish and analyzed for Se content using laser ablation inductively couple mass spectrometry (LA-ICP-MS). Significant differences were found between the two species of fish, based on the concentrations of otolith Se. Green sunfish otoliths from all sites contained background or low concentrations of otolith Se ( $< 1 \mu\text{g/g}$ ) that were not significantly different between mined and unmined sites. In contrast creek chub otoliths from the historically mined site contained much higher ( $\geq 5 \mu\text{g/g}$ , up to approximately  $68 \mu\text{g/g}$ ) concentrations of Se than for the same species in the unmined site or for the green sunfish. Otolith Se concentrations were related to muscle Se concentrations for creek chubs ( $R^2 = 0.54$ ,  $p = 0.0002$  for the last 20% of the otolith Se versus muscle Se) while no relationship was observed for green sunfish.

Additional experiments using biofilms grown in the Mud River showed increased Se in mined site biofilms compared to the reference site. When we fed fathead minnows

*Pimephales promelas*) on these biofilms in the laboratory they accumulated higher concentrations of Se in liver and ovary tissues compared to fathead minnows fed on reference site biofilms. No differences in Se accumulation were found in muscle from either treatment group. Biofilms were also centrifuged and separated into filamentous green algae and the remaining diatom fraction. The majority of Se was found in the diatom fraction with only about 1/3<sup>rd</sup> of total biofilm Se concentration present in the filamentous green algae fraction

Finally, zebrafish (*Danio rerio*) embryos were exposed to aqueous Se in the form of selenate, selenite, and L-selenomethionine in an attempt to determine if oxidative stress plays a role in selenium embryo toxicity. Selenate and selenite exposure did not induce embryo deformities (lordosis and craniofacial malformation). L-selenomethionine, however, induced significantly higher deformity rates at 100 µg/L compared to controls. Antioxidant rescue of L-selenomethionine induced deformities was attempted in embryos using N-acetylcysteine (NAC). Pretreatment with NAC significantly reduced deformities in the zebrafish embryos secondarily treated with L-selenomethionine, suggesting that oxidative stress may play a role in Se toxicity. Selenite exposure also induced a 6.6-fold increase in glutathione-S-transferase pi class 2 gene expression, which is involved in xenobiotic transformation. No changes in gene expression were observed for selenate or L-selenomethionine-exposed embryos.

The findings in this dissertation contribute to the understanding of how Se bioaccumulates in a lotic system and is transferred through a simulated foodweb in

addition to further exploring oxidative stress as a potential mechanism for Se-induced embryo toxicity. Future studies should continue to pursue the role of oxidative stress and other mechanisms in Se toxicity and the biotransformation of Se in aquatic ecosystems.

## **Dedication**

This work is dedicated to my friends and family who have loved and supported me through even the toughest times. I would also like to dedicate this work to the hardworking, generous people of West Virginia.

I saw a star...



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# **1. Introduction**

## ***1.1 Selenium***

### **1.1.1 Selenium**

In 1818 Swedish chemist Jöns Jakob Berzelius discovered selenium while preparing sulfuric acid, choosing the name from “Selene” the Greek word for moon because he believed it was similar to tellurium, which was named for the Earth (Wisniak 2000). Selenium (atomic number 34) is a naturally occurring element belonging to the chalcogens on the periodic table, meaning it is similar to sulfur (Lenz et al. 2009). Selenium is often associated with natural sulfides, including high-sulfur coals found in the USA (Yudovich et al. 2006). The world average of selenium in coal ranges from 1.0-1.6 mg/kg, although it can reach up to 43 mg/kg (Yudovich et al. 2006). The distribution of selenium varies considerably throughout the Earth’s crust. For example, soils from some areas in China are highly seleniferous while other areas are considered selenium deficient (Dhillon et al. 2003).

The majority of selenium for industrial use is produced as a byproduct from anode slimes that form during electrolytic copper refining because the low concentrations of selenium in the Earth’s crust does not allow for efficient mining (Jorgenson 2002). Selenium has a variety of uses in several industries, including glass tinting, electronics, dietary supplements for people and livestock, fertilizers, and fungicides (Jorgenson 2002, USHHS 2003). Selenium can be mobilized via several anthropogenic processes including

the combustion of coal, mining, and farming (Presser et al. 1987, Ramirez et al. 2002, Lussier et al. 2003).

Selenium was first identified as a micronutrient essential for life in 1957 (Schwarz et al. 1957). Since its discovery, researchers have identified a variety of enzymes that require selenium for normal function. For example, selenium is an essential component of glutathione peroxidases (GPx), enzymes involved in protection from oxidative stress (Rotruck et al. 1973). Selenium is also important in the function of the thyroid hormones iodothyronine deiodinases, which regulate circulating levels of thyroid hormone (Arthur et al. 1990). Other selenium dependent proteins include selenoproteins (e.g SelP, SelH) selenophosphate synthetase, and thioredoxin reductases (Reddy et al. 1983, Sunde 1984, Schrauzer 2000).

### **1.1.2 Chemistry**

Selenium has six stable isotopes and is chemically similar to sulfur (Muscatello et al. 2009). There are several different oxidation states of selenium (Figure 1), including -II, 0, IV, and VI that include organic selenium compounds such as selenoaminoacids (USEPA 2004). Selenite, elemental selenium, selenate, and selenous acid exist in waters at 6 – 8 pH (Milne 1998). Selenium chemistry is highly complex and the form of selenium can influence the rate of uptake and toxicity to aquatic organisms. Organic forms of selenium are more bioavailable and toxic to organisms compared to inorganic forms (Schrauzer 2000).

Inorganic selenium is readily accumulated by organisms at the base of an aquatic food web (Figure 2) (Baines et al. 2001). For example, selenium is an essential element for some species of algae and is purposefully taken up by algal cells (Price et al. 1987). Once a cell takes up selenium it can be transformed into organoselenium compounds such as selenomethionine (Figure 2), which is known as the 21<sup>st</sup> amino acid (Shrift 1958, Bowie et al. 1996, Riedel et al. 1996). Microphytes living in seleniferous waters were able to biomagnify 1400-fold of selenium from the water but the subsequent transfer of selenium from microphytes to macroinvertebrates was 1.9-fold, highlighting the importance of microbes and algae at the base of the food chain in selenium bioavailability (Fan et al. 2002). Vertebrates, including fish, lack the ability to synthesize selenomethionine and thus must obtain the amino acid through their diets (Schrauzer 2000).

### **1.1.3 Toxicity**

Research into the toxicity and essentiality of selenium has a complicated and interesting history. Selenium was identified in the 1930s as the cause behind alkali disease in livestock in the United States (Anderson et al. 1961). Symptoms of alkali disease in livestock include muscle damage, hair loss, the shedding of hooves, and emaciation (O'Toole et al. 1995). Scientists discovered that plants in the genus *Astragalus* growing on highly seleniferous soil could hyperaccumulate the selenium, which was subsequently toxic to grazing animals (Davis 1972).

Alkali disease revealed that selenium can be toxic in high concentrations, but the discovery of selenoproteins in a variety of plant and animal species indicated that it was also essential for life. One of the most curious features of selenium is that it has a very narrow range of concentrations between nutritionally beneficial and toxicity (Wilber 1980). A lack of selenium can cause cardiomyopathy (Keshan Disease in humans), while too much selenium can be highly detrimental to both humans and other organisms at concentrations only approximately 7-30 times what is required for nutrition (Hodson et al. 1983, Schrauzer 2000).

The dual nature of selenium essentiality and toxicity is also true for non-human vertebrates such as fish and birds. Oviparous vertebrates are vulnerable to selenium toxicity through dietary exposure because selenium is transferred via yolk proteins from mother to developing embryo where it can cause significant developmental defects and subsequent embryo mortality (Figure 3) (Janz et al. 2010). Maternal transfer of selenium has been demonstrated for a variety of species, including birds, fish, invertebrates, lizards, and amphibians (Davis et al. 1996, Hopkins et al. 1998, Hopkins et al. 2004, Roe et al. 2004, Conley et al. 2009). Maternal transfer of selenium and subsequent juvenile toxicity studies conducted on bluegill sunfish (*Lepomis macrochirus*) showed a dietary threshold of 6-12 mg/kg dw for effects on larval development and survival (Cleveland et al. 1993, Lemly 1993, McIntyre et al. 2008).

Increased concentrations of selenium in eggs from female fish exposed to selenium have been correlated with increases in larval deformities (Holm et al. 2005,

Muscatello et al. 2009). Tissue concentrations of 8-12 mg/kg dw in muscle, liver, and ovary can cause reproductive failure in fish (Lemly 2002). Other pathologies associated with selenium exposure in fish include cataracts, gaping mouth, lordosis, and edema (Lemly 2002). Selenium toxicity in fish most often manifests as teratogenesis, edema, and larval mortality (Janz et al. 2010). There have been several studies showing developmental defects in fish larvae from a variety of species exposed to selenium (reviewed by Lemly 2002 and Janz et al. 2010). For example, there are several studies showing the relationship between selenium exposure and pericardial edema in fathead minnows (Pyron et al. 1989, Hermanutz 1992). Teratogenicity and reproductive impairment has also been reported for salmonids (Holm et al. 2005, Hardy et al. 2010), esocids (Muscatello et al. 2006, Muscatello et al. 2009), and centrarchids (Lemly 1982, Coyle et al. 1993).

In recognition of these issues with selenium exposure, the U.S. EPA released a freshwater aquatic life criterion of 5.0 µg/L for waterborne Se in 1987 and in 2004 a proposed whole body fish tissue burden limit of 7.91 mg/kg dry weight (dw) was developed (USEPA 1987, USEPA 2004). A new Aquatic Life Ambient Water Quality Criterion for Selenium (USEPA 2014) in freshwater was recently released in by the U.S. EPA in 2014 for external peer review. The proposed standards include national monthly water criteria for selenium of 1.3 µg/L in lentic water and 4.8 µg/L in lotic water, which are not to be exceeded more than once in three years on average (USEPA 2014). The draft also proposes a fish whole body concentration of 8.1 mg/kg or a muscle



concentration of 11.8 mg/kg in addition to an egg/ovary standard of 15.2 mg/kg (USEPA 2014). Previous research, however, has indicated that lower ( $\leq 4$  mg/kg dw whole body) concentrations of selenium can have significant negative impacts fish development and reproduction (Lemly 2002)

#### **1.1.4 Mechanisms of selenium toxicity**

The original hypothesis for selenium toxicity is that it occurs when selenium is incorrectly substituted for sulfur during protein synthesis. Selenium substitution for sulfur can disrupt the formation of disulfide chemical bonds, impairing protein and enzyme function, which leads to deleterious effects in fish and other vertebrates (Diplock 1976, Reddy et al. 1983). Although selenium can be assimilated into two amino acids—selenomethionine and selenocysteine—there is currently some evidence that the substitution of methionine with selenomethionine does not generally disrupt protein structure and function (Yuan et al. 1998, Mechaly et al. 2000, Schrauzer 2000) and there is also evidence that cysteine and selenocysteine are interchangeable in some organisms (Allan et al. 1999). The selenium-sulfur substitution mechanism is unclear and alternative mechanisms including oxidative stress are now hypothesized to play a role in selenium induced embryo toxicity.

Due to the lack of evidence for the selenium-sulfur substitution mechanism, a second hypothesis based on selenium induced oxidative stress has recently gained more support. Mallard ducks (*Anas platyrhynchos*) fed selenomethionine showed increased

GPx activity as well as a dose-dependent increase in the hepatic ratio of oxidized glutathione (GSSG) to reduced glutathione (GSH) and an increase in hydroperoxides that cause lipid peroxidation (Heinz et al. 1988, Hoffman et al. 1998). Similar responses have been demonstrated in both rats (LeBoeuf et al. 1985) and fish (Atencio et al. 2009).

Research also shows that rainbow trout (*Oncorhynchus mykiss*) embryos are capable of enzymatically cleaving parentally derived organic selenium into metabolites such as methylselenol, which can generate reactive oxygen species, causing oxidative stress (Wang et al. 2002, Palace et al. 2004). Homogenate from rainbow trout embryos was shown to transform L-selenomethionine, which is considered to be a less reactive form of selenium, into methylselenol, which can produce superoxide in the presence of glutathione (Palace et al. 2004). Rainbow trout embryos exposed to selenium experience yolk sac and pericardial edema, and research suggests that these forms of edema may be caused by oxidative stress (Palace et al. 2004, Bauder et al. 2005).

Selenium has also been demonstrated to decrease the activity of antioxidant enzymes, including hepatic superoxide dismutase in Japanese medaka (*Oryzias latipes*) exposed to selenite or nanoparticle selenium (Li et al. 2008). In contrast, aqueous exposure of selenite did not change antioxidant enzyme activity or lipid peroxidation in juvenile rainbow trout (Miller et al. 2007), indicating that the species tested, route of exposure, dose, life stage, and other factors may influence the toxicity of selenium.

A third mechanism of toxicity proposed for selenium is the suppression of the immune system. Selenium is an antioxidant that can enhance the immune system at supplementary levels (Tapiero et al. 2003). Elevated concentrations, however, have been shown to decrease immune response (Fairbrother et al. 1990, Fairbrother et al. 1994). The dual nature of selenium on the immune system has been demonstrated in birds. For example, adult common eiders (*Somateria mollissima*) were immunocompromised due to a lack of thymus glands and reduced cell-mediated immunity after being fed diets containing a high dose of selenium while birds fed a lower dose exhibited increased humoral immunity (Franson et al. 2007). More research is needed to better understand the impacts of selenium on the immune system in wildlife.

### **1.1.5 Historical selenium contamination**

There have been several historical examples of selenium contamination of aquatic environments. Selenium is mobilized by a variety of anthropogenic activities, including agriculture, the burning of coal, and mining (reviewed by Maher et al., 2010). Once an ecosystem is contaminated by selenium, the effects can be long lasting and devastating to the local wildlife. For example, one of the most well-known cases of aquatic ecosystem contamination by selenium occurred in Belews Lake, North Carolina. Belews Lake received wastewater from a selenium-laced fly ash pond (150-200 µg/L) associated with a coal-fired power plant. Selenium accumulated in the lake's food chain, causing extirpation of all but a few resistant fish species (Cumbie 1978, Cumbie 1978). Fish

captured from Belews Lake exhibited severe deformities, tissue pathologies, and reproductive failure (Sorensen et al. 1984, Lemly 2002). Green sunfish (*Lepomis cyanellus*) collected from Belews Lake showed increased tissue concentrations of selenium as well as histopathological changes in gill, hepatopancreas, kidney, heart, and ovarian tissues (Sorensen et al. 1984). Decreased condition-factors of fish were significantly correlated with increasing concentration of selenium in hepatopancreas and skeletal muscle tissue (Sorensen et al. 1984). The plant ceased discharging fly ash wastewater in 1986 and fish populations began to slowly recover, although monitoring studies showed that selenium remained in the sediments at elevated levels over a decade later (Lemly 2002).

There are other examples of lakes receiving coal fly ash effluent that have experienced significant biological impairment from selenium contamination. For example, Lake Sutton has historically been used as a cooling reservoir and disposal site for coal ash wastewater from a coal-fired power plant (Lemly 2013). Up to almost 30% of the bluegill and bass collected recently from Lake Sutton exhibited deformities characteristic of selenium exposure and a total monetized value for fishery losses in 2013 due to selenium poisoning in the lake were estimated to be over \$5.5 million (Lemly 2013). Hyco Lake is another reservoir that has received coal ash wastewater. Largemouth bass (*Micropterus salmoides*) and bluegills collected from Hyco Lake showed significantly higher body burdens of selenium compared to reference site fish with

selenium concentrations 1,000 times higher in bluegill ovaries compared to surface water (Baumann et al. 1986).

Another well-known case of selenium contamination in an aquatic ecosystem occurred in the Kesterson Reservoir, San Joaquin Valley, California. The San Joaquin Valley has been subjected to intensive agricultural development and the Kesterson Reservoir was built to help manage water flow through the area. In 1983 it was discovered that eight species of fish had completely disappeared from the surrounding aquatic environment and the one species remaining, mosquitofish (*Gambusia affinis*), was experiencing significant reproductive impairment (Saiki et al. 1995). It was determined that selenium had leached out of seleniferous soils during irrigation of the agricultural lands, leading to significant contamination of the ecosystem and a loss of both fish and bird species until remediation began in 1988 (Ohlendorf et al. 1986, Saiki et al. 1995).

In addition to agricultural practices and the production of coal ash, mining activities can also mobilize selenium in the aquatic environment. Runoff and leachate from mining operations can contain high concentrations of selenium. For example, uranium mining and milling operations in Saskatchewan, Canada has released significant amounts of selenium into surrounding lake habitats, causing selenium accumulation in lake biota and deformities in fish (Muscatello et al. 2006, Muscatello et al. 2008, Muscatello et al. 2009). Coal and phosphate mining have also been shown to produce selenium-laden effluent (Lemly 1999, Hamilton et al. 2002).

### **1.1.6 Lentic versus lotic selenium contamination**

Lotic systems are systems with flowing waters, such as rivers or streams. In contrast, lentic systems are standing water systems such as ponds or lakes. The flow and movement of water through an ecosystem may have significant effects on contaminant uptake, biotransformation, and toxicity. Considerable attention has been placed on selenium contamination in lentic systems (Cumbie 1978, Cumbie 1978, Sorensen et al. 1984, Lemly 2002). Significant amounts of selenium, however, are also released into lotic systems, including the rivers and streams of Appalachia that are impacted by coal mining.

One of the key properties of selenium is that in anoxic environments, such as those found in lentic systems, inorganic selenium in the form of selenate or selenite is reduced to more biologically available selenides and selenoamino acids such as selenomethionine (reviewed by Maher et al., 2010). In a study comparing riverine and lake sediments in Utah, insects from the lentic system contained 7 times greater concentrations of selenium compared to insects from the lotic site (Hillwalker et al. 2006). These data suggest that selenium bioaccumulation and toxicity may be of greater concern in lentic systems where the long residence time of sediments can be lead to greater levels of accumulation. In contrast to flowing water systems, Se that is bound in sediments has high potential to be transported downstream, leading to a lower concentration of exposure but simultaneously generating exposure over a larger area. New proposed monthly average exposure values for selenium reflect the increased risk of

lentic systems to selenium compared to lotic systems (1.3 µg/L versus 4.8 µg/L), although the criterion for lotic systems has been lowered compared to previous standards (5 µg/L), reflecting the increasing concern for selenium toxicity in lotic systems (USEPA 2014).

Despite evidence of potentially decreased accumulation in lotic systems, streams and rivers may still be at risk for experiencing significant toxicity from selenium contamination. Anoxic conditions exist in lentic habitats (stream sediments, channel eddies, large in channel pools and other areas of low or slow flow) distributed throughout lotic systems, and within microhabitats in stream biofilms even in fast moving sections allowing for the microbial and algal transformation of selenate into organic forms of selenium. Such habitats and microhabitats could provide the entry points for bioavailable forms of selenium into a lotic aquatic food web.

## ***1.2 Mountaintop Removal/Valley Fill Coal Mining***

### **1.2.1 History and Policy**

Coal mining activities began in the central Appalachian Mountains approximately 200 years ago (Merovich et al. 2007). Mountain top removal valley fill (MTR/VF), a form of surface mining (Figure 4), is a more recent development in coal extraction that became more common starting in the 1970s. MTR/VF coal mining involves the removal of peaks and ridges of mountains to access underlying low-sulfur coal (Hartman et al. 2005). During the surface mining process, the mountain is first deforested, the top soil is removed, and explosives are used to blast off the tops of mountains (Palmer et al. 2010,

Bernhardt et al. 2012). Giant excavators known as drag lines are used to scoop out the layers of coal (Peng 2000). In 2012, West Virginia had 104 surface mines and produced approximately 40,000 tons of coal via surface mining (USEIA 2012)

One of the most controversial aspects of MTR/VF is dumping spoil rock materials (overburden), which are created during the blasting off of the mountaintops, into adjacent valleys (Peng 2000, Palmer et al. 2010). The valleys used in MTR/VF coal mining are often home to headwaters for major rivers, and contain ephemeral, intermittent, and even perennial streams that are permanently lost upon filling (Hartman et al. 2005, Pond et al. 2008, Bernhardt et al. 2011). Significant increases in conductivity—an estimate of ion concentrations in the water—as well as increases in the concentrations of inorganic contaminants have been associated with the practices of valley fill (Palmer et al. 2010). High conductivity has been shown to negatively impact macroinvertebrate diversity (Pond et al. 2008) and recently the U.S. EPA has released a new freshwater conductivity benchmark of 300  $\mu\text{S}/\text{cm}$  set to protect freshwater invertebrates (USEPA 2011). The Natural Resources Defense Council estimates that over 470 mountains have been destroyed due to MTR/VF coal mining and almost 2000 km of streams and rivers in Appalachia have been destroyed or damaged by coal mining (NRDC 2010).

Coal and its adjacent strata are known sources of selenium. During the mining process when rock is broken up and exposed to the elements in order to access the underlying coal (Johnson et al. 1999), selenium can be mobilized and released into the aquatic environment as oxidized inorganic anions selenate ( $\text{Se}^{4+}$ ) and selenite ( $\text{Se}^{6+}$ ).



The Clean Water Act (CWA) is a federal law enacted in 1972 with the goals of regulating the release of toxic chemicals into water and safeguarding those surface waters used by people for recreational purposes including fishing and swimming (USEPA 1972). With the introduction of CWA, the National Pollutant Discharge Elimination System (NPDES) was developed. NPDES permits are used by the U.S. EPA to regulate point sources of water pollution, including mining facilities and require the discharger to meet effluent standards and monitor effluent activities (USEPA 1972, Copeland 2010).

Under the CWA, the EPA released a guidance document outlining the role of EPA in reviewing Appalachian surface coal mining (USEPA 2005, Silva et al. 2010). Peer-reviewed science, public input, and implementation experience were utilized to develop this guidance. Under CWA Section 402, Appalachian surface coal mining operations holding NPDES permits must comply by characterizing discharged effluent, conduct analyses on potential harm of effluent, set water quality-based effluent limits, incorporate whole effluent toxicity (WET) tests, as well as comply with standards involving minimizing impacts outlined in Clean Water Act Section 404 (USEPA 1972).

### **1.2.2 Mud River, West Virginia**

The Mud River is a third-order tributary stream of the Guyandotte River, originating in Boone County in southwestern West Virginia (Figure 5). A 9 km section of the Mud River passes through the Hobet 21 surface mine, a MTR/VF coal mining operation active since the 1970's that continues to discharge effluent into the Mud River

(Palmer et al. 2010, Lindberg et al. 2011). The Hobet 21 coal mine is the largest surface mine in West Virginia at over 24 km long. Connelly Branch, a former third order tributary of the Mud was filled to create one of the largest valley fills ever permitted in the United States. Connelly Branch now drains approximately 6.5 square km of fill (Lindberg et al. 2011). Another valley fill called Ballard Fork is located in the upper Mud River basin and it drains approximately  $4.9 \times 10^7$  m<sup>3</sup> of mining spoil (M. Ross, Duke University, Durham, NC, USA, personal communication). The main stem Mud River receives selenate-laden discharge from over 100 MTR/VF coal mining permits issued through the National Pollutant Discharge Elimination system (NPDES) (Vesper et al. 2004, Lindberg et al. 2011).

The West Virginia Department of Environmental Protection Agency (WVDEP) examined larval deformity rates in bluegill sunfish (*Lepomis macrochirus*) living in the Mud River and Upper Mud River Reservoir (WVDEP 2010). They found bluegill ichthyoplankton deformity rates of 0-1.27% in reference locations while deformity rates ranged from 0-47.6% in mining impacted sites. Concentrations of selenium were 64.6 mg/kg dw in largemouth bass eggs (*Micropterus salmoides*) from mining-impacted water (WVDEP 2010). Ferreri et al. (2004) sampled sites along the Guyandotte River drainage in West Virginia. They found that both the number of fish species and benthic macroinvertebrate diversity were greater at reference sites compared to sites impacted by MTR/VF coal mining. Several of the Mud River, WV sites sampled during the study had levels of water-borne selenium ranging from 9.5-31.5 µg/L, which exceeded the current

water quality criteria of 5.0 µg/L (USEPA 2004). A recent study comparing fish assemblages of the Mud River showed fewer species, lower numbers, and decreased biomass of fish compared to sites not impacted by mining (Hitt et al. 2014). The decreased numbers and varieties of fish in the Mud River were associated with increased conductivity and surface water selenium concentrations (Hitt et al. 2014).

### **1.2.3 Biofilms and selenium**

Aquatic biofilms are complex systems made up of a variety of microorganisms including bacteria, filamentous algae, and diatoms (van Hullebusch 2003, Wimpenny 2000) that are found on a variety of surfaces in aquatic environments. Biofilms have been previously shown to interact with and influence their surrounding aquatic environment through a variety of biochemical processes (reviewed by van Hullenbusch 2003). Thus, biofilms can play an important role in the fate and transport of metals in a contaminated stream. As the basis of many stream ecosystems for both aquatic invertebrates and grazing fish, biofilms can be the gatekeepers for inorganic contaminants as higher trophic organisms feed on them (Kimball et al. 1995, Farag et al. 2007, Ancion et al. 2013).

Biofilms are capable of accumulating inorganic forms of selenium and as well as transforming selenium from one species to another (Dowdle et al. 1998, Losi et al. 1998, Conley et al. 2013). Microbes within the biofilms can biotransform inorganic selenium such as selenite into organic forms such as selenoamino acids (Doran et al. 1977, Turner et al. 1998). Selenomethionine is highly bioavailable and toxic to aquatic vertebrates

(Hamilton 2003, Vidal et al. 2005). There is a strong correlation between metals in biofilms and the tissue metal concentrations of aquatic invertebrates that consume these contaminated biofilms (Rhea et al. 2006, Farag et al. 2007, Conley et al. 2009, Conley et al. 2011), suggesting that these contaminants in biofilms maybe be transferred to other higher trophic organisms including fish. Previous work in selenium contaminated systems has shown that microphytes biomagnified 1400-fold the concentration of selenium from water, and the selenium was subsequently transferred to macroinvertebrates (Fan et al. 2002). Biofilms may be important in the accumulation and trophic transfer of selenium released into an ecosystem from surface mining effluent.

### ***1.3 Mud River Fish***

The Mud River (Left Fork and main stem) is home to a multitude of species of fish, including but not limited to the following:

- *Campostoma anomalum* (central stoneroller)
- *Ericymba buccata* (silverjaw minnow)
- *Luxilus chrysocephalus* (striped shiner)
- *Nocomis leptocephalus* (bluehead chub)
- *Semotilus atromaculatus* (creek chub)
- *Lepomis* spp. (e.g. green sunfish, pumpkinseed, redbreast sunfish, warmouth, bluegill, longear, and readear)
- *Micropterus* spp. (e.g. smallmouth and largemouth bass)
- A variety of darter species (e.g. *Percina*, *Etheostoma*)

Two species of fish, the creek chub and the green sunfish collected from the Mud River in an effort to determine the status of selenium in wild fish are discussed below.

Both the creek chub and green sunfish are considered resistant to degraded water quality

(increased conductivity and high selenium) associated with MTR/VF coal mining (Hitt et al. 2014).

### **1.3.1 Creek Chub**

Creek chubs (*Semotilus atromaculatus*) are a Cyprinid fish found throughout eastern North America in small, clear streams and lakes (Lee et al. 1980) (Figure 6A). Creek chubs are often used as bait fish in the United States and Canada (Barber et al. 1971). They are a minnow with a broad head, terminal mouth, a barbell in the mouth groove, with a green and white coloring with a stripe and dorsal fin spot (Stauffer et al. 1995). Male creek chubs will develop tubercles during breeding season (Stauffer et al. 1995), when they guard pit nests with rocks. Female creek chubs produce approximately 500-4,000 eggs during the spawning season (Reighard 1910).

Adult creek chubs range from approximately 100-305 mm total length (Lee et al. 1980). Creek chubs are omnivorous and predominantly feed in the early evening on berries, aquatic insect larvae, small fishes, amphibians, and mollusks (Barber et al. 1971, Moshenko et al. 1973). Fish longer than 81 mm predominantly feed on larger aquatic insect larvae and small fishes (Johnson et al. 1982, Magnan et al. 1984). During the summer months, young creek chubs live in moderately deep pools while older chubs are found in deeper pools, where chubs often move to in the winter (Moshenko et al. 1973). Creek chubs are commonly found in even small streams and have limited in-stream movement, meaning they can be useful as biological monitor for stream health in place of

a community-level assessment such as an Index of Biotic Integrity (IBI) (Fitzgerald et al. 1999).

### **1.3.2 Green Sunfish**

Green sunfish (*Lepomis cyanellus*) is has been introduced throughout the United States and occurs in many habitat types (Figure 6B) (Lee et al. 1980). The green sunfish is a blue-green color with a yellow ventral surface, a large mouth, and a spot on the operculum (personal observation, M. Arnold, Duke University). The prey of green sunfish consists primarily of aquatic invertebrates, especially decapods, and fish (Werner et al. 1977, Stauffer et al. 1995).

The spawning season of the green sunfish generally occurs from June to August (Hubbs 1935), although the timing may depend on location. They become sexually mature at approximately 7.5 cm in length (Hubbs 1935). Green sunfish males build and defend nests during spawning season in spring and early summer (Hunter 1963). Interestingly, green sunfish are able to hybridize with a variety of other fish including bluegills and pumpkinseeds (Avisé et al. 1984, Dawley et al. 1985).

Notably, green sunfish have been shown to suppress native fish populations in stream ecosystems (Lemly 1985). They are considered tolerant of poor water quality and pollution therefore a high prevalence of green sunfish has been used as an indicator of poor water quality in indices of fish assemblage integrity and indices of biotic integrity (Hughes et al. 1998, Sullivan et al. 2004, Lau et al. 2006).

## ***1.4 Small Laboratory Model Fish***

Small model fish are becoming increasingly common in research laboratories due to their relative ease in care, rapid and external development, prolific reproduction, and decreased cost in comparison to traditional mammalian models. Zebrafish (*Danio rerio*) are a particularly powerful model because of their fully sequenced genome and the development of transgenic strains. Fathead minnow (*Pimephales promelas*) are commonly used in drug testing and environmental toxicology research due to their ease in culture compared to other stream minnows.

### **1.4.1 Fathead Minnows**

The fathead minnow (*Pimephales promelas*) is a brownish-green and white minnow found in streams throughout North America (Stauffer et al. 1995). Fathead minnows are sexually dimorphic, with males growing a large fat pad and tubercles on their heads, thus giving rise to their common name (Ramaswami et al. 1955). Breeding males develop dark brownish-black coloration except for a two vertical lightly colored bands (McMillan et al. 1974). The fathead minnow is a common bait fish as well as a species frequently used in toxicological research because it is the standard freshwater fish model for aquatic toxicity as mandated by the U.S. Environmental Protection Agency (U.S. EPA) due to their tolerance of a variety of water quality conditions, easily identifiable developmental stages, transparent chorion, and short time to hatch. (Ankley et al. 2001, Ankley et al. 2006). EPA has developed several fathead minnow embryo-

based toxicity tests, including the acute toxicity Whole Effluent Test (WET), in order to test a variety of potential freshwater contaminants, including those permitted under the NPDES used in MTR/VF coal mining (USEPA 2002).

Fathead minnows typically lay their eggs in late spring to early summer underneath surfaces that are horizontal to the water's surface (Markus 1934). Males are highly territorial and spend a lot of energy defending the nest and caring for eggs (Unger 1983). During egg care, males will oxygenate the eggs using their enlarged dorsal pad and tubercles (Ramaswami et al. 1955, Branson 1962). Territorial behavior including lateral displays, biting, chasing, tail beating, and head butting using tubercles can occur in breeding males to keep away intruders (McMillan et al. 1974, Unger 1983). Embryos hatch within 96-120 hours post fertilization (hpf). The fathead minnow's diet primarily consists of algae and aquatic macroinvertebrates (Scott et al. 1973). In the laboratory, fathead minnows are often maintained on a diet consisting of *Artemia* and commercially available flake food.

Fathead minnows were chosen as a model organism for the research described in Chapter 4 because they are widely distributed in streams throughout the United States, are highly tolerant to a variety of water quality conditions, and will readily consume several types of diets including biofilms. Fathead minnows were used as a substitute for other stream minnows found in West Virginia such as the creek chub, which are often difficult to culture in the laboratory.



### 1.4.2 Zebrafish

Zebrafish (*Danio rerio*) are a small freshwater fish originally from slow moving water in South and East Asia (Perry et al. 2010). The zebrafish genome sequencing project has provided new genetic tools for zebrafish research including mutant strains, transgenic techniques, and targeted gene expression, making it a powerful model organism for biological research (Sprague et al. 2006, Lieschke et al. 2007). Zebrafish are particularly useful as a research organism because they are considerably less costly to maintain compared to rodent models, are prolific breeders, possess transparent chorions that allow for *in vivo* imaging, are easily maintained in the laboratory, and develop quickly with 44 well defined stages of development (Detrich et al. 1999, Sprague et al. 2006). In the laboratory, zebrafish are often fed a diet consisting of *Artemia* and powdered commercially available food.

Zebrafish are somewhat sexually dimorphic. Mature males have yellowish ventral surfaces and are slightly smaller than females. Both sexes have horizontal blue strips, although the female is more silver in color. Zebrafish reach sexual maturity after approximately three to four months, after which females produce eggs that are externally fertilized by males. Embryos develop rapidly and typically hatch within 72 hpf.

Zebrafish were chosen as a laboratory model for the research described in Chapter 5 because they readily produce large amounts of embryos whose development can be easily observed and photographed through transparent chorions after aqueous exposure to selenium. Additionally, genetic tools including primers for a multitude of genes are

available for zebrafish, meaning they are excellent models to investigate potential mechanisms of toxicity for selenium.

### ***1.5 Dissertation Objective and Outline***

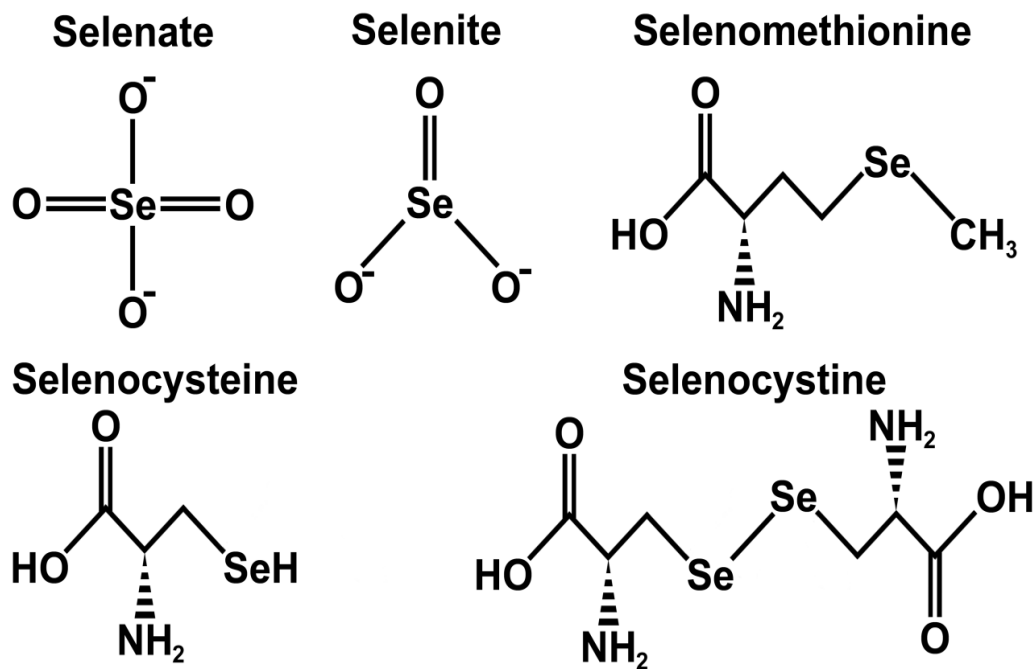
Current research remains lacking on how MTR/VF contaminants such as selenium move through an aquatic ecosystem and how selenium exerts its toxicity in fish. The goal of this study was to determine the accumulation and trophic transfer of MTR/VF stream contaminants in fish. Wild Mud River fish impacted by MTR/VF were collected and analyzed for Se accumulation and associated health effects, providing a comparison to laboratory studies. Gene expression, reproductive success, and quantification of inorganic contaminant burdens were used as tools to understand which components are driving observed toxicity in laboratory and native fish.

This study used a multidisciplinary approach to assess mining impacts on an aquatic ecosystem that is broadly applicable to the Appalachian region and beyond. The data gathered provide valuable insight into how contaminants, with a focus on selenium, can move through an ecosystem and exert toxic effects on vulnerable life stages in aquatic organisms. Additionally the research described here can be used to better refine standards used to manage impacted sites, including contaminant limits set to protect aquatic ecosystems.

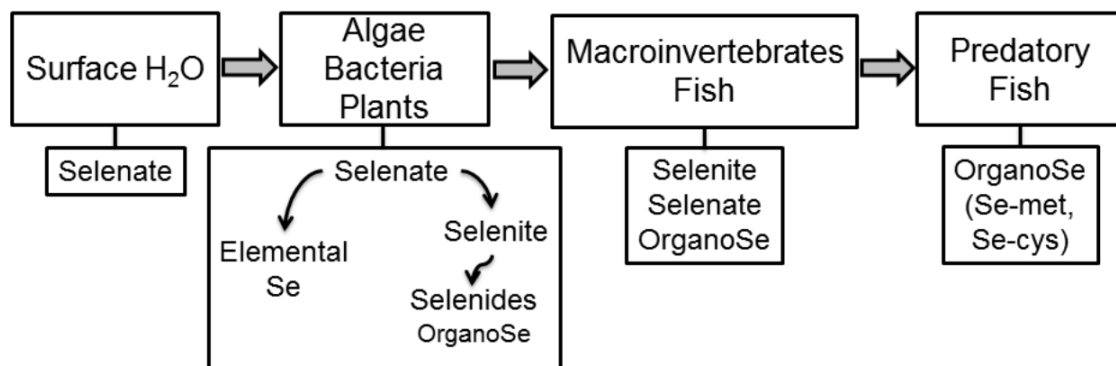
This dissertation is organized into four research chapters that examine various aspects of Se bioaccumulation, fate, trophic transfer, and toxicity. The objectives of each chapter are listed below:

- Chapter 2: To characterize the bioaccumulation and fate of selenium in biota from the Mud River main stem, which is impacted by MTR/VF coal mining effluent
- Chapter 3: To use otoliths to provide a temporal record of selenium exposure in fish from the Mud River in comparison with soft tissue analysis and water chemistry
- Chapter 4: To study the trophic transfer of selenium from biofilms to fathead minnows in a laboratory simulated food chain
- Chapter 5: To explore the possible role of oxidative stress in selenium induced teratogenicity in zebrafish embryos

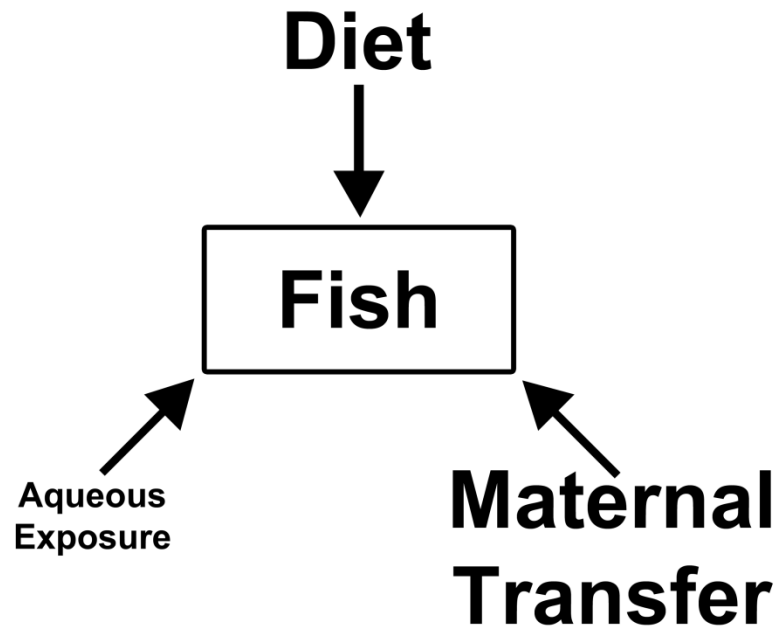
Finally, the findings of this dissertation are summarized in Chapter 6. The implications of these data and future directions are also discussed.



**Figure 1: Chemical structures of various species of selenium**



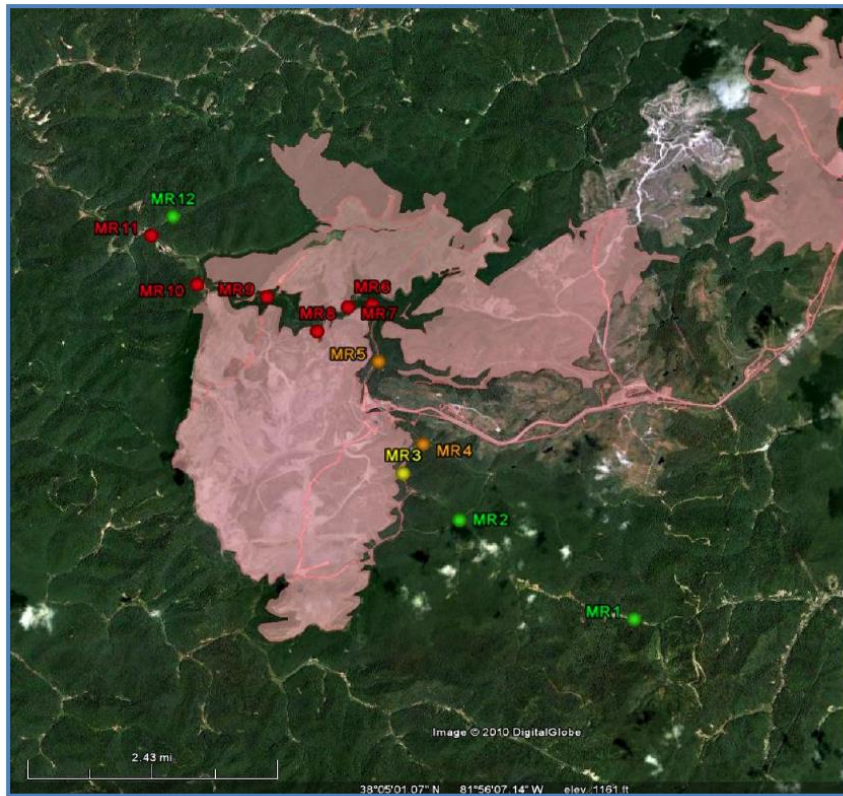
**Figure 2: Selenium cycling in an aquatic ecosystem. Selenium is released into surface waters as selenate, which is taken up by producers and biotransformed into other forms of selenium, including organoselenium (OrganoSe). Predators bioaccumulate organic forms of selenium including selenomethionine (Se-met) and selenocysteine (Se-cys).**



**Figure 3: Routes of selenium exposure in fish. The decreased size of the aqueous exposure route indicates a less important exposure pathways (not to scale).**



**Figure 4: Photograph of mountaintop removal/valley fill coal mining in West Virginia. Photograph by Mariah Arnold.**



**Figure 5: Map of Mud River, West Virginia. Pink area outlines the Hobet 21 surface mine. Dots represent sampling sites along the Mud River. The color of the dots indicates valley fill input where green is little to no input while increasing red indicates the site receives more valley fill effluent. Map created by Ty Lindberg.**





**Figure 6: A. Creek chub (*Semotilus atromaculatus*). B. Green sunfish (*Lepomis cyanellus*)**

## **2. Bioaccumulation and speciation of selenium in fish and insects collected from a mountaintop removal coal mining-impacted stream in West Virginia**

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### ***2.1 Introduction***

Mountaintop removal coal mining is a form of surface mining that involves blasting off mountaintops to access underlying coal seams (Merovich et al. 2007). The practice of mountaintop removal coal mining and valley fill (MTR/VF) is common in the Appalachian coal mining region where US coal mining began over 200 years ago (Merovich et al. 2007). Spoil rock is often placed in an adjacent valley to form what is known as a valley fill or head-of-hollow fill. Valley fills permanently bury stream headwaters and sometimes entire stream systems. MTR/VF coal mining has been practiced broadly in West Virginia since the 1970's (Fox 1999). The Hobet 21 coal mine is one of the largest surface mines in the United States and discharges effluent into the Mud River. Connelly Branch was a third order tributary of the Mud River in West Virginia and now contains one of the largest valley fills ever permitted in the United States (Lindberg et al. 2011). Ballard fork valley fill, located in the upper Mud River basin, drains approximately  $4.9 \times 10^7 \text{ m}^3$  of mining spoil (M. Ross, Duke University,

Durham, NC, USA, personal communication).

These valley fills can have negative consequences for downstream aquatic ecosystems, including significant alterations in stream chemistry and loss of sensitive biota (Merrick et al. 2007, Pond et al. 2008). One of the major concerns related to MTR/VF coal mining is the release of selenium (Se) into aquatic ecosystems. Selenium can be found in coals and adjacent shale—rock formations targeted by MTF/VF coal mining operations in West Virginia—and it can leach into the environment during rock processing and weathering (Vesper et al. 2004, Vesper et al. 2008).

Selenium (Se) is an essential micronutrient because it is necessary for proper functioning of important antioxidant enzymes, including glutathione peroxidases (Eisler 2000). However, Se has a narrow margin between essentiality and toxicity (Janz et al. 2010). Many species of fish, including trout, Sacramento splittail, and salmon are sensitive to elevated concentrations of aqueous or food-borne Se (Hamilton et al. 1990, Teh et al. 2004, Hardy et al. 2010). Selenium toxicity can manifest itself through a wide variety of deformities, including skeletal and jaw malformations, circulatory defects, edema, and cataracts (Lemly 1997, Lemly 2002, Teh et al. 2004). Developmental deformities caused by Se can be lethal and if widespread, can lead to population crashes and loss of fish diversity in Se contaminated environments (Lemly 1985). Currently, the U.S. EPA has a freshwater aquatic life criterion of 5.0 µg/L for waterborne Se and in 2004 they released a proposed whole body fish tissue burden limit for Se of 7.91 mg/kg

dry weight (dw) (USEPA 1987, USEPA 2004).

Selenium chemistry is highly complex and the chemical form of Se influences the uptake rate and ultimate toxicity to aquatic organisms. The weathering of selenium-enriched rock layers exposed during the mining process results in the mobilization and release of selenate ( $\text{SeO}_4^{2-}$ ) and selenite ( $\text{SeO}_3^{2-}$ ) into streams receiving effluent from active and decades old mining operations (USEPA 2004, Palmer et al. 2010). Organic Se compounds are known to be significantly more bioavailable to higher organisms than inorganic forms such as selenate (Kleinow et al. 1986). Selenium metabolism is dependent on its oxidation state and previous research reported that Se in fish is mostly composed of selenomethionine, selenocysteine, and selenocystine (Phibbs et al. 2011, Misra et al. 2012). Characterizing tissue-specific Se speciation is an important step in elucidating possible pathways that drive the differential uptake of bioavailable selenium species in the various tissue compartments (Misra et al. 2012).

Previous water quality analysis on the Mud River system revealed that as the upstream areal percent of MTR/VF-impacted watershed increased, the concentrations of Se, manganese, sulfates, and other inorganic solutes increased proportionately (Lindberg et al. 2011). Dissolved Se concentrations in the Mud River surpassed the EPA's freshwater chronic criterion concentration in 43 out of 52 water samples taken during May to December, 2010. The West Virginia Department of Environmental Protection (WVDEP) examined larval deformity rates in bluegill sunfish (*Lepomis macrochirus*)

living in the Mud River and Upper Mud River Reservoir (WVDEP 2010). They found bluegill ichthyoplankton deformity rates of 0-1.3% in reference locations while deformity rates in mining impacted sites ranged from 0-48% of the embryos sampled (WVDEP 2010). The concentration of Se in the composite sample of fish eggs was less than 0.8 mg/kg dw in reference bluegill eggs while the composite sample from mining-impacted water had a Se concentration of 9.8 mg/kg dw (WVDEP 2010).

In the current study, we collected wild fish from the Mud River in West Virginia with electroshocking. Body tissue burdens of Se were analyzed in skinless fillets, liver, and ovaries of captured fish to determine how Se from MTR/VF coal mining effluent accumulates in higher trophic organisms in a freshwater lotic environment and to provide insight into how Se accumulates in two different species tissue compartments. X-ray absorption near edge structure (XANES) spectroscopy was used to determine Se speciation in fish and insect tissues in an effort to understand how Se biotransforms through a stream food chain. Tissue samples including gills and ovaries were processed for histological analysis in order to gain insight into how mountaintop removal coal mining could impact the health of native stream fish.

## ***2.2 Materials and methods***

### **2.2.1 Field collection**

The Mud River, a tributary of the Guyandotte River, is located primarily in Lincoln and Boone counties in southwestern West Virginia. A 9 km section of the Mud

River passes through the Hobet surface mine, a MTR/VF coal mining operation that has been active since the 1970's and continues to discharge mining effluent into the river (Palmer et al. 2010, Lindberg et al. 2011). The Mud River is composed of two forks, referred to here as the main stem Mud River (MR) and left fork Mud River (LFMR) (Figure 7). Naming conventions are in keeping with previously published research (Lindberg et al. 2011) with the main stem impacted site located at MR7, (River Km 9.4) at the confluence with Laurel Branch. The main stem Mud River receives selenate-laden discharge from multiple MTR/VF coal mining permits issued through the National Pollutant Discharge Elimination system (NPDES) (Vesper et al. 2004). The left fork sampling sites were located on the left fork of the Mud River, approximately 9.2 River km upstream from the confluence of the two forks at the Mud River Reservoir. Both sites show similar hydrology but MR7 has received MTR/VF coal mining effluent for over 40 years while LFMR does not receive input from MTR/VF coal mining.

Water samples for soluble metals determination were taken concurrent with fish collection. Samples were passed through a 0.45  $\mu\text{m}$  pore size filter and then stored in metal-free certified 50 mL polypropylene tubes (VWR International, LLC, Radnor, PA, USA) with the soluble metal samples acidified with trace metal grade  $\text{HNO}_3$  and all kept on ice until they were transported back to Duke University where they were stored at 4°C until analysis. All samples were diluted until total dissolved solids were less than 150 mg/L with a 2%  $\text{HNO}_3$ /0.5%  $\text{HCl}$  solution and analyzed for trace element content using

inductively coupled plasma-mass spectrometry (ICP-MS).

Fish were collected from the Mud River using a backpack electroshocker (HT-2000 Battery Backpack Electro-Fisher, Halltech Aquatic Research Inc., Ontario, Canada) during six sampling trips from March to July, 2011-2013. Animal collection and processing was done under a scientific collecting permit from the West Virginia Division of Natural Resources (#2013.145) and Duke University IACUC approval (protocol A211-10-08). At least five each of reproductively active adult green sunfish (*Lepomis cyanellus*) and creek chubs (*Semotilus atromaculatus*) were targeted for sampling from each site on each trip. All fish were individually labeled and identically processed.

Thirty-seven creek chubs and 6 green sunfish were collected from LFMR and 32 creek chubs and 22 green sunfish were collected from MR7. Three composite stream insect samples were collected from riffle habitat using a kick net at MR7 and LFMR during May 2013. To avoid potentially overwhelming contaminant signals during chemical analysis of these composite samples, members of the Tipulidae and Megaloptera families were excluded due to their disproportionately large mass compared to other insect families. A variety of insects were found in the kick net samples, but the majority of the sample was composed of mayflies (Ephemeroptera), stoneflies (Plecoptera), dragonflies and damselflies (Odonata), beetles (Coleoptera), midges (Diptera), and aquatic worms (Annelida). Insect samples were frozen and processed as described below.

### 2.2.2 Tissue processing for ICP-MS and histology

After euthanizing captured fish with ethyl 3-aminobenzoate methanesulfonate (MS-222, Sigma-Aldrich Co., LLC) wet weight and standard length were measured and fish were inspected for jaw, fin, and skeletal alterations characteristic of Se exposure. Fish were then dissected to remove skinless fillets, ovaries, and the hepatopancreas (name used due to the presence of exocrine pancreas at the hylus of the liver but hereafter referred to as “liver”) if the tissue had sufficient mass. Insects were stored in certified metal-free 50 mL polypropylene tubes. Fish and insect samples were then transported frozen on ice back to Duke University where they were stored at  $-80^{\circ}\text{C}$  until preparation for ICP-MS analysis. Gills and a portion of the ovary from creek chubs were preserved in 4% paraformaldehyde or Davidson’s fluid for histological preparation and analysis.

Selenium content in fish tissue and insects was measured using ICP-MS (Agilent 7700X ICP-MS equipped with an Octopole Reaction System). Samples were freeze-dried for 24 hrs and then ground into a fine powder using a porcelain mortar and pestle and weighed before digesting at least 6 hours in concentrated nitric acid at  $75^{\circ}\text{C}$ . Digested samples were diluted 1:10 with double deionized water ( $\text{ddH}_2\text{O}$ ) and then further diluted 1:5 with a 2%  $\text{HNO}_3$ /0.5%  $\text{HCl}$  mixture. Selenium determination was performed with a hydrogen reaction gas at a  $\text{H}_{2(\text{g})}$  flow rate of  $4 \text{ mL min}^{-1}$ . Other trace elements were analyzed in no gas mode or helium collision mode. A certified reference material (DORM-2 dogfish protein, National Research Council, Canada) containing  $1.40 \pm 0.09$



mg/kg Se dw, was digested in parallel with fish and insect samples, and the recovery of the certified Se values were  $87.5 \pm 8.3\%$  (N=17). Double-deionized water was also digested along with samples to act as a method blank.

Quality control for ICP-MS chemical analysis and instrument calibration were maintained using SRM 1643e (National Institute of Standards and Technology, Gaithersburg, MD) and CRM-TMDW-A (High Purity Standards, Charleston, SC) as an external standard and indium and rhodium as internal standards. Double deionized water was used as a blank to determine contamination during sample preparation and were generally below detection limit or very low in concentration.

Tissues chosen for histological analysis were serially dehydrated using standard procedures and embedded in paraffin blocks that were sections at 5  $\mu\text{m}$  using a Microm HM 355S microtome (Thermo Fisher Scientific Inc., Waltham, MA, USA). Resultant sections were mounted on glass slides following removal of excess paraffin and clearing. Sections were stained with Hematoxylin and Eosin (H&E) and imaged (NIS Elements BR 3.2, Melville, NY, USA) using light microscopy (Nikon Eclipse E600 and Nikon digital camera DXM1200, Melville, NY, USA) at various magnifications to determine cellular level changes in tissue structure and/or parasite load. Slides were analyzed blindly.

### **2.2.3 X-ray absorption near edge spectroscopy**

Skinless fillet, liver, ovary, and composite insect samples from MR7 were freeze dried as described above for X-ray Absorption Near Edge Structure Spectroscopy (XANES) analysis. Only creek chubs were analyzed due to limitations in time and resources. XANES data was gathered from LFMR samples but the Se concentrations were too low to allow accurate quantification of Se species present in the samples. Freeze dried tissues were packed into specially designed copper cuvettes and held in place using Kapton® tape (DuPont™, Research Triangle Park, NC, USA). Samples were then stored in liquid nitrogen or on dry ice to maintain cryogenic temperatures. Selenium K-edge XANES data were collected at Beamline X3A at the National Synchrotron Light Source, Brookhaven National Laboratory in Upton, New York, USA.

All data for standards and samples were collected at 22 K maintained by a liquid helium cryostat in order to avoid changes in Se speciation from thermal and radiation exposure. Spectra for standards were collected using ion-chamber detectors in transmission mode, and spectra for samples were collected using a 13-element germanium detector in fluorescence mode. At the low Se amount in our samples (approximately 6-27 mg/kg), the self-absorption effect (Troger et al. 1992) was considered to be minor at the high X-ray energy used (12.7 keV) (Conley et al. 2013).

The monochromator energy was calibrated to the maximum of the first inflection point in the K-edge derivative spectra from an elemental Se<sup>0</sup> standard. Spectra were

collected at photon energies between -200 to +400 eV relative to the Se K-edge energy at 12658 eV, using a step size of 0.5 eV across the absorption edge region (-50 to +50 eV).

Multiple XAS scans on each sample were aligned, merged, and processed using the Athena program, an interface to IFEFFIT (version 1.2.10) (Sayers et al. 1988, Newville 2001, Ravel et al. 2005). Spectra were baseline corrected using a linear pre-edge function between -200 and -30 eV and normalized using a linear or quadratic function between 150 and 350 eV, including a flattening function in the post-edge region. The speciation of Se in the samples was determined using the linear combination fitting (LCF) across from 30 eV below to 40 eV above the Se absorption edge using the Athena program (Ravel et al. 2005). Commercially-purchased powders of iron selenide (Alfa Aesar), elemental (gray) Se<sup>0</sup> (99.99%, Sigma-Aldrich), selenomethionine (Sigma-Aldrich), and selenocystine (Sigma-Aldrich) were used as reference materials in LCF. The reference spectrum of methyl selenocysteine is a courtesy of Dr. Dean Hesterberg (North Carolina State University, Raleigh, NC) (Conley et al. 2013). Reference materials for selenite and selenate were prepared by sorbing these species to a concentration of 500 mmol kg<sup>-1</sup> on poorly crystalline aluminum hydroxide at pH 7, as described by our previous methods (Liu et al. 2013).

#### **2.2.4 Statistical analysis and quality control**

Fish tissue and whole-body insect Se concentrations from MR7 and LFMR as well as soluble Se water concentrations were compared using the Student's t-test with

$\alpha=0.05$ . All ICP-MS data are reported as mean  $\pm$  standard error. Data was analyzed using GraphPad Prism 4 software (La Jolla, CA). XANES data are presented as mean  $\pm$  standard deviation with  $\chi^2$  being the normalized sum of the squared residuals of the fit ( $R\text{-factor} = \sum(\text{data-fit})^2/\sum\text{data}^2$ ).

## **2.3 Results**

### **2.3.1 Selenium concentrations in water, insects, and fish tissues**

Water sampling efforts conducted during this study showed soluble Se concentrations for LFMR of  $0.33\pm 0.2$   $\mu\text{g/L}$  while Se concentrations were significantly higher ( $p<0.01$ ) in MR7 water at  $4.7\pm 1.1$   $\mu\text{g/L}$  (Figure 8A). Conductivity was also significantly higher for MR7 at  $1041\pm 205.4$   $\mu\text{S/cm}$  ( $p<0.005$ ) compared to  $101.8\pm 26.2$   $\mu\text{S/cm}$  for LFMR (Figure 8B).

Fish tissue Se analysis revealed increased concentrations of Se in main stem MR7 fish fillets, ovaries, and liver compared to LFMR fish of both species (Figure 9A-D). LFMR creek chub fillets averaged  $2.0\pm 0.05$   $\text{mg/kg Se dw}$  while MR7 creek chub fillets had significantly higher concentrations at  $6.3\pm 0.3$   $\text{mg/kg Se dw}$  ( $p<0.0001$ ) (Figure 9A). Green sunfish fillets showed a similar pattern of significantly higher concentrations in MR7 samples ( $8.8\pm 0.4$   $\text{mg/kg Se dw}$ ) compared to LFMR samples ( $3.5 \pm 0.3$   $\text{mg/kg Se dw}$ ) ( $p<0.0001$ ). Average liver and ovary Se concentrations were significantly higher for MR7 creek chub ( $30.4\pm 4.8$  and  $8.8\pm 1.3$   $\text{mg/kg Se dw}$  respectively) compared to liver and ovary tissue samples from LFMR creek chubs ( $12.6\pm 1.5$  and  $4.3\pm 0.4$   $\text{mg/kg Se dw}$

respectively) ( $p < 0.0001$  for creek chub liver,  $p < 0.02$  for creek chub ovary). MR7 green sunfish liver and ovary samples tended to have higher concentrations of Se compared to LFMR green sunfish liver and ovary tissue samples but the difference was not statistically significant with  $p > 0.1$  for both tissues (Figure 9B and C).

ICP-MS analysis of the composite insect samples revealed an average Se concentration of  $4.5 \pm 0.6$  mg/kg dw for LFMR insects while MR7 insects had a significantly higher average Se concentration ( $p < 0.001$ ) of  $10.1 \pm 0.2$  mg/kg dw (Figure 9D).

### **2.3.2 Deformities and histopathology**

Three MR7 green sunfish (two collected in April 2011 and one collected in May 2012) exhibited upper jaw deformities characteristic of Se exposure (Figure 10). The maxillae in these fish were shortened compared to typical jaw length. Selenium concentrations were 6.6 and 7.8 mg/kg dw in the fillets of deformed green sunfish from April 2011 while the deformed fish from the May 2012 collection effort had fillet Se concentrations of 10.3 mg Se/kg dw. In general, LFMR fish were subjectively observed to be in better health than main stem fish in terms of coloration, scale quality, and population numbers.

Ovarian development was analyzed for three creek chubs from MR7. Ovaries from LFMR creek chub females were not available for histological analysis because no

female creek chubs with sufficient ovarian mass were collected during the histological sampling trips. Early, mid, and late stage vitellogenic oocytes were present in most samples. Normal development was observed for oocytes in all samples.

Analysis of gill tissues revealed aneurysms in 50% (2/4) of the creek chub samples from LFMR during the May 2012 sampling period (Figure 11B). 25% of the creek chub gills (2/8) from June 2012 contained aneurysms. Aneurysms were not observed in the five MR7 creek chub gills analyzed from either sampling period. Parasites including trematodes and protozoa were observed in LFMR creek chub gills (4/12) while no parasites were observed in MR7 gill samples (0/13) (Figure 11C and D).

### **2.3.3 XANES analysis**

XANES was used to quantify the major Se species in creek chub tissues and whole-body insect samples from MR7. XANES analysis was limited to these samples due to time and cost restrictions. The data shown in Figure 6a indicated some variation in spectral features between the tissue samples. These variations included the position of the white line peak (between 12662.8 and 12665.6 eV) and the presence of a second peak in some samples after the first white line peak. LCF of the fish tissue sample data with reference spectra indicated that Se was predominantly (>75%) in the form of organoselenium compounds including methyl-Se-cysteine, selenocystine, and selenomethionine (Figure 12, Appendix Table 1). The exception was one skinless fillet sample (MR7 84) that indicated approximately 50% organoselenium compound, with the

remaining comprising of inorganic selenite and selenate species. MR7 creek chub livers were predominantly mixtures of Se-methionine and Se-cystine. The best fit for liver samples MR7 81 and MR7 84 was obtained by including selenite and a small portion (approximately 6% or less) of selenate into the model. In liver MR7 32, 30±4% methyl-Se-cysteine was detected. Analysis of ovary samples showed a mixture of methyl-Se-cysteine, Se-methionine, selenate, and selenite. Composite insect samples from MR7 contained approximately 30% selenite and 10% selenate. The remaining fraction of organo-Se was composed of Se-methionine and Se-cystine.

## ***2.4 Discussion***

West Virginia and other states within the Appalachian Coalfield Region have been extensively mined with an estimated 1200 miles of headwater streams impacted between 1992-2002 and 724 stream miles were covered by valley fills from 1985 to 2001 (USEPA 2005). Altered stream chemistry associated with MTR/VF coal mining, including increased alkalinity and metal contamination has led to the disappearance of sensitive taxa (Bryant et al. 2002, Pond et al. 2008). In an effort to understand how MTR/VF coal mining was impacting wild fish populations in the Mud River, we collected fish over the course of three years and determined Se accumulation and speciation in tissue compartments including skinless fillet, liver, and ovary. Fish collected from the mining-impacted MR7 site exhibited elevated levels of Se in skinless fillets, ovaries, and liver compared to fish from the reference site LFMR. MR7 insects also

contained higher concentrations of Se compared to LFMR insects.

Previous research has shown a 20% increase in total edema, craniofacial and skeletal deformities was seen in larvae from adult northern pike (*Esox lucius*) captured downstream of a uranium mine in Saskatchewan, Canada (Muscatello et al. 2009). The adult pike contained 33.55 and 21.54  $\mu\text{g Se/g}$  dry weight in egg and muscle tissues, respectively (Muscatello et al. 2009). A regression analysis by Holm *et al.* (2005) indicated that egg Se concentrations of 8 to 10  $\mu\text{g/g}$  would be responsible for 15% of all observed skeletal and craniofacial deformities and edema, and at 12  $\mu\text{g/g}$ , Se would account for 30-70% of the skeletal, craniofacial, and edema deformities in larvae. These regressions were similar to those calculated by Lemly 2002.

The data presented in this paper combined with previous research on the biota of the main stem Mud River indicate that the system is significantly disturbed and that Se is readily accumulating in at least two different fish species. A creek chub caught in the Mud River by the WVDEP in 2012 contained egg concentrations of 17.7 mg Se/kg dw (WVDEP 2010), which is slightly higher than the average concentrations found in this study. Concentrations between 8.8 and 10.5  $\mu\text{g Se/g}$  wet egg were calculated to negatively affect 15% of a population of rainbow trout (*Oncorhynchus mykiss*) inhabiting a stream receiving selenium-contaminated effluent from a coal mine in Alberta, Canada (Holm et al. 2005). The fish captured from the main stem of the Mud River in this study had comparable concentrations of Se in their ovaries, indicating creek chubs and green



sunfish from the main stem of the Mud River may be experiencing significant negative consequences to Se exposure.

Green sunfish feed on aquatic invertebrates and small fishes while younger creek chubs feed predominately on invertebrates and switch to small fishes after a few years (Barber et al. 1971, Stauffer et al. 1995) . Both species of fish can be exposed to Se via these diets. A variety of centrarchids were observed in LFMR, including bluegills (*Lepomis macrochirus*) and warmouth (*Lepomis gulosus*). These species were rarely seen in MR7, which was instead dominated by green sunfish, which are known to be ecologically tolerant (Lee et al. 1980), and were not often observed in LFMR during electroshocking efforts compared to MR7. Green sunfish are known to decrease populations of native species by consuming their young (Lemly 1985). This behavior combined with exposure to Se may be contributing to the lack of centrarchid diversity observed at MR7 during electroshocking efforts.

A freshwater chronic criterion concentration of 5.0 µg Se/L was determined to be protective of aquatic life by the United States Environmental Agency (USEPA 1987). This criterion does not, however, account for differences in bioavailability of Se species or the fact that Se rapidly accumulates in food sources such as periphyton even at low water concentrations (Conley et al. 2009). The current threshold is under review pending consideration of a new whole-body tissue criterion of 7.91 µg/g based on research done with bluegill sunfish (*Lepomis macrochirus*) (USEPA 2004). This new criterion,

however, may not be protective of more sensitive fish species. More data must be generated to understand how Se accumulates in aquatic food chains. It may be necessary to develop more habitat- or species-specific Se tissue criteria due to significant differences in factors influencing bioaccumulation including lentic versus lotic systems, sedimentation, fish diversity, and the species of Se released into the system (Adams et al. 2000).

Lemly (2002) proposed that 10 and 12 mg/kg Se in the ovary and liver, respectively, could cause reproductive failure in fish. Concentrations of Se in ovary/eggs and liver collected from the main stem of the Mud River in this study often exceeded the levels proposed by Lemly (2002), indicating that the fish from the main stem of the Mud River are likely experiencing reproductive impairment. Fish in the Mud River should continue to be monitored for signs of Se accumulation and toxicity and research into how Se travels through a stream food chain is necessary to understand how Se from MTR/VF effluent can impact an ecosystem. It is important to measure Se concentrations in separate tissue compartments, with a focus on liver and ovaries, because Se preferentially accumulates in these tissues as indicated by data presented here and by previous research (Hamilton 2003, Teh et al. 2004, WVDEP 2010). High concentrations of Se in the ovaries can lead to reproductive failure and extirpation of native fish species (Lemly 2002, Palmer et al. 2010, WVDEP 2010).

Histological analysis of creek chub ovaries did not reveal any abnormalities in

either LFMR or MR7 samples. Gill tissues from LFMR creek chubs showed elevated numbers of aneurysms during the May 2012 sampling season, although the same aneurysms were not present in LFMR creek chub gill samples from June 2013. MR7 creek chub gill samples did not contain any aneurysms. These aneurysms could be due to injury to gill tissue or parasite infestation. It was not possible to determine the cause of the aneurysms with the samples analyzed. LFMR gill tissues contained an expected number of trematode and protozoa parasite infestation. Interestingly, no parasites were observed in MR7 gills. While the lack of observed parasites could be due to sample handling (Snyder 2003) and/or low sample size, it is possible MTR/VF coal mining effluent in MR7 has decreased parasite populations due to a loss of host species for earlier trematode life stages.

Previous research has shown that trematode diversity can decrease in ecosystems heavily polluted with heavy metals and that low trematode diversity can be an indicator of poor environmental health (Poulin 1992, Shea et al. 2012). For example, a decreased number of gravid *Bothriocephalus acheilognathi*, a pseudophyllidean cestode in Belews Lake reservoir, was associated with increased concentrations of Se in the reservoir water (Riggs et al. 1987). Another possible reason for decreased trematode numbers could be the increased conductivity in MR7 water. Invertebrates can be sensitive to increases in conductivity. For example, Pond et al. (2008) showed that MTR/VF coal mining correlated with significant decreases in certain families of macroinvertebrates in West

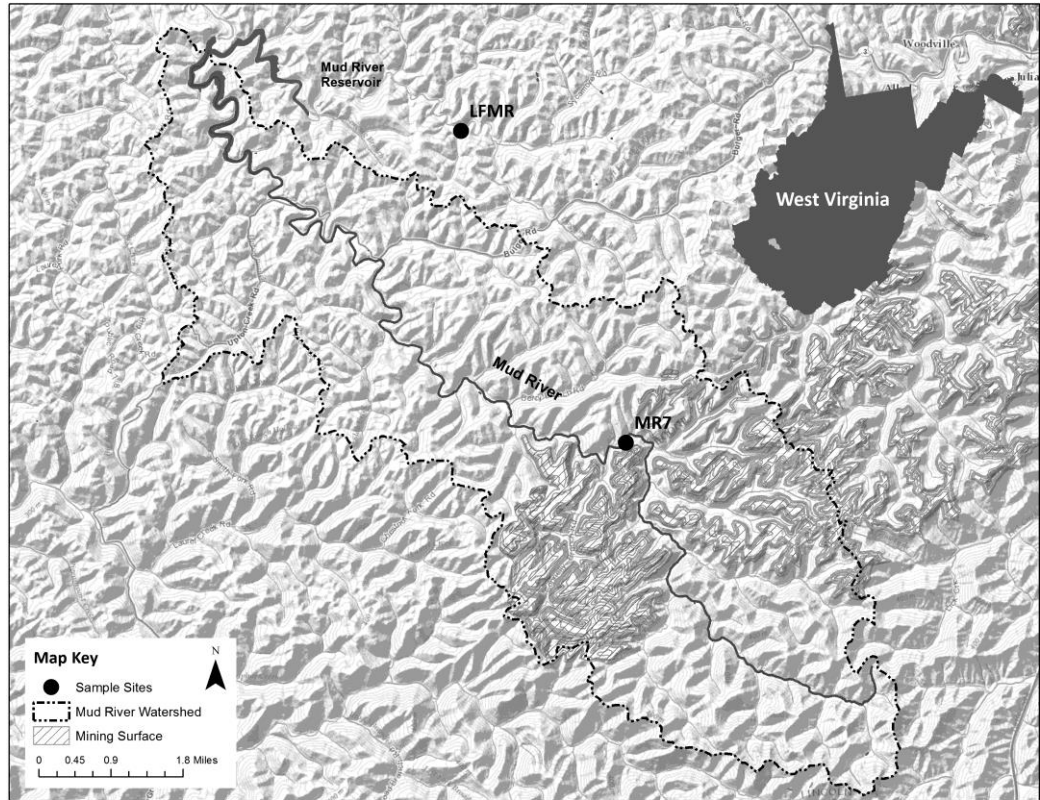
Virginia streams, and Kunz et al. (2013) showed that reconstituted waters representative of alkaline Appalachian mine drainage were toxic to mussels and mayflies, although crustaceans were more tolerant (Kunz et al. 2013).

Fish have previously been shown to store selenium in skeletal muscle tissue as organoselenium compounds such as selenomethionine (Phibbs et al. 2011). The Mud River is a selenate-dominated system (Vesper et al. 2008), where fish are exposed to selenium as they consume lower level food chain organisms that have chemically transformed selenate into selenomethionine. Fish accumulate selenomethionine from environmental sources such as water exposure and diet (Hamilton 2003, Muscatello et al. 2008). Differences in Se speciation for the three tissues analyzed here indicate that Se is metabolized and/or stored differently in each tissue type. Se-methionine composed approximately 22-40% of all tissues analyzed, which is consistent with previous results (Misra et al. 2012). One male creek chub liver sample (MR7 32) contained methyl-Se-cysteine, which was not found river trout livers (Misra et al. 2012). Interestingly, we observed a significant amount of Se-cystine in liver and ovaries but not fillets, where instead there was methyl-Se-cysteine. Selenocysteine is unstable in the presence of oxygen and can be oxidized to Se-cystine (Beld et al. 2007, Misra et al. 2012).

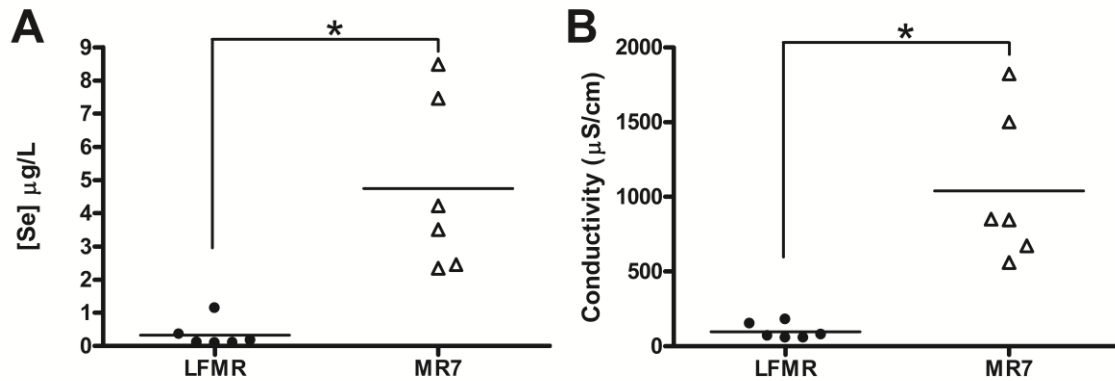
Insect samples were also composed of Se-methionine and Se-cystine, similar to fish liver and ovary samples, but insects also contained a higher fraction of inorganic Se species selenite (~30%) and selenate (10%). Although the selenate found in Mud River

water is considerably less toxic and bioavailable to higher trophic organism, the results presented here suggest that water-borne selenate was taken up by lower trophic organisms such as bacteria or algae, transformed into more readily bioavailable forms of selenium, and transferred through a food chain to higher trophic organisms including insects and fish.

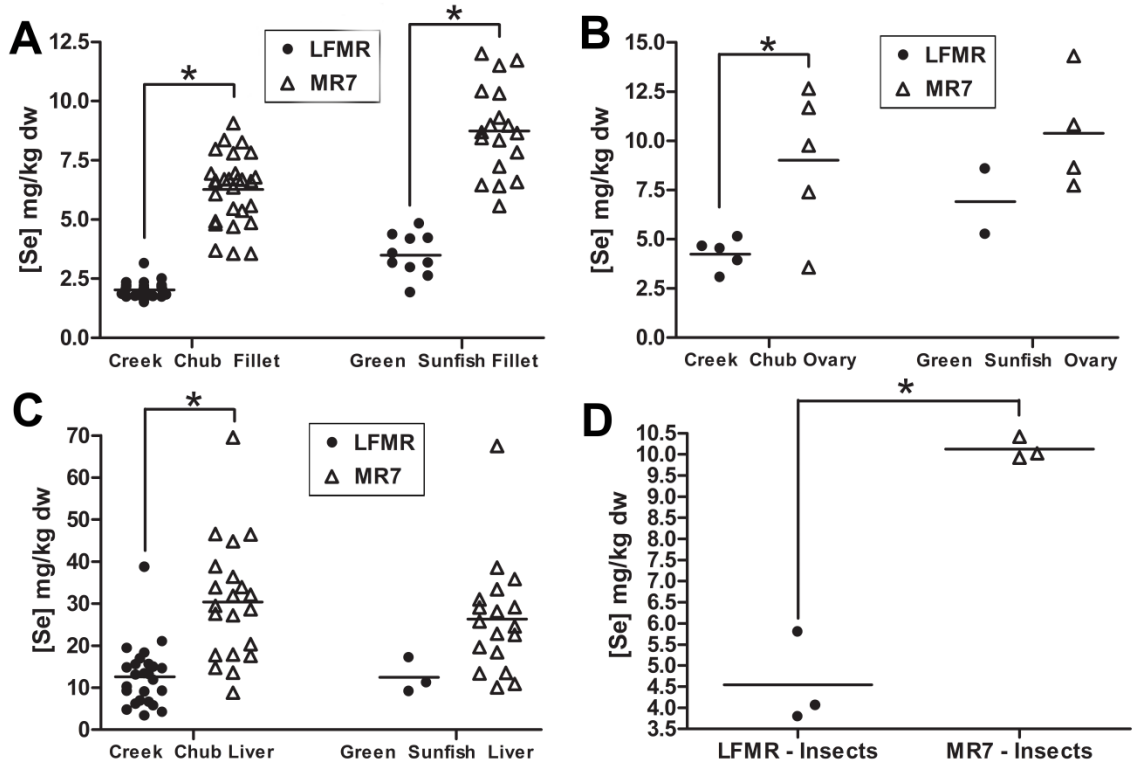
Future research efforts should continue to investigate Se accumulation and speciation in the Mud River fish as well as focus on lower levels of the aquatic food chain including invertebrates and biofilms to understand how different forms of Se move through the food chain. A population survey of the fish species comparing the main stem and left fork of the Mud River would also help determine if mining effluent in the main stem Mud River has negatively impacted local fish populations. Our results suggest that the main stem Mud River wild fish populations are exposed to significant concentrations of biologically available Se that are readily accumulating in various tissue compartments at concentrations that could negatively impact reproduction and overall health.



**Figure 7: Map of Mud River sampling sites LFMR and MR7 labeled in black dots. The region bordered by the dashed line denotes the Mud River watershed. The area covered by hash marks indicates surface mining. The grey insert in the bottom left is the state of West Virginia. Map created by Gretchen Kroeger of Duke University using ArcMap (ArcGIS, esri®, Redlands, CA, USA)**



**Figure 8: A. Concentrations of soluble selenium ( $\mu\text{g/L}$ ) at LFMR and MR7 (N=6 per site) during sampling efforts during spring and early summer of 2011-2013. Asterisk indicates significance ( $p < 0.01$ ). B. Conductivity ( $\mu\text{S/cm}$ ) at LFMR and MR7 (N=6 per site). Asterisk indicates significance ( $p < 0.005$ ). Bars represent mean**

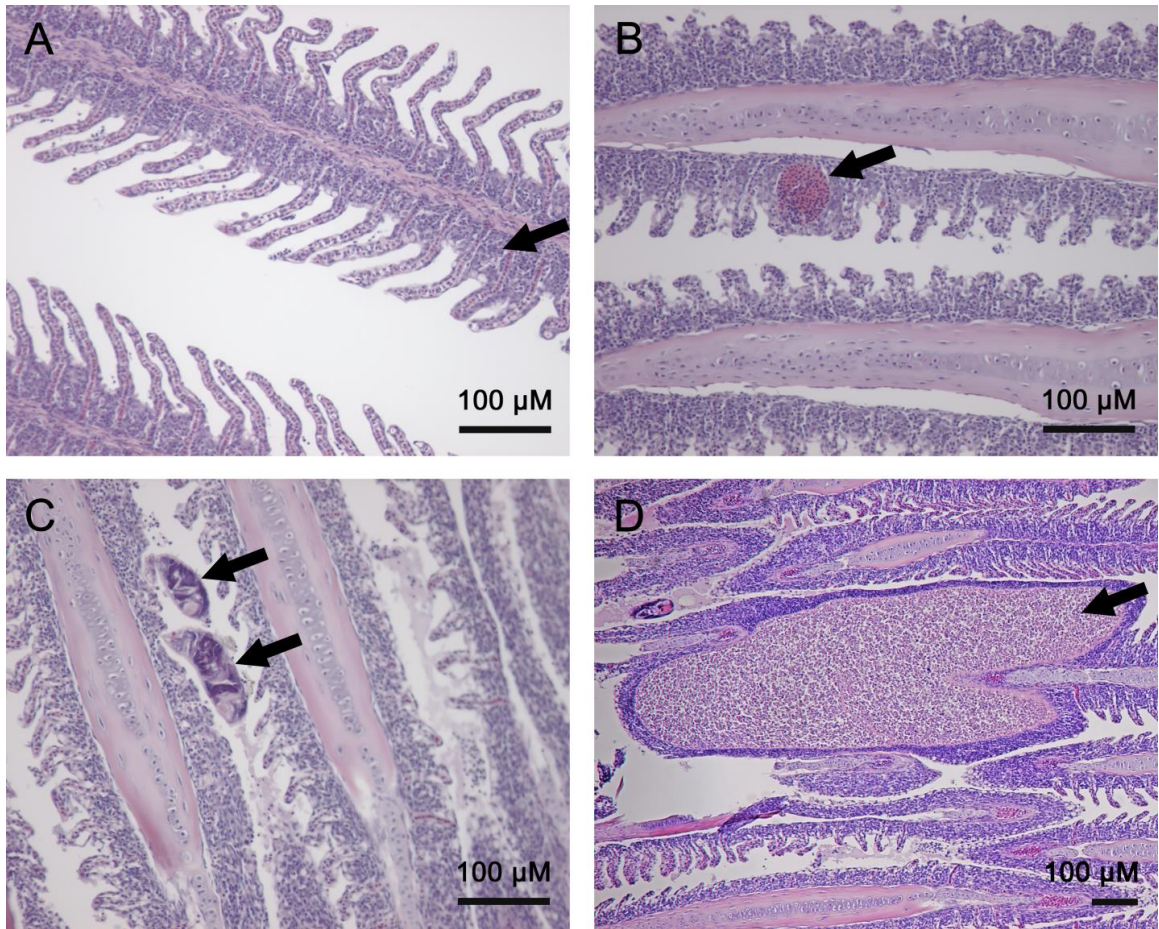


**Figure 9: A.** Se (mg/kg dw) in skinless fillets, as determined by ICP-MS, from fish collected in the main stem of the Mud River (MR7, dark grey) and the Left Fork of the Mud River (LFMR, light grey) Asterisk indicate significance ( $p < 0.0001$ ). Bars represent mean. **B.** Se (mg/kg dw) in liver ( $p < 0.0001$ ). **C.** Se (mg/kg dw) in ovaries ( $p < 0.02$ ). **D.** Se (mg/kg dw) in composite insect samples ( $p < 0.001$ ).

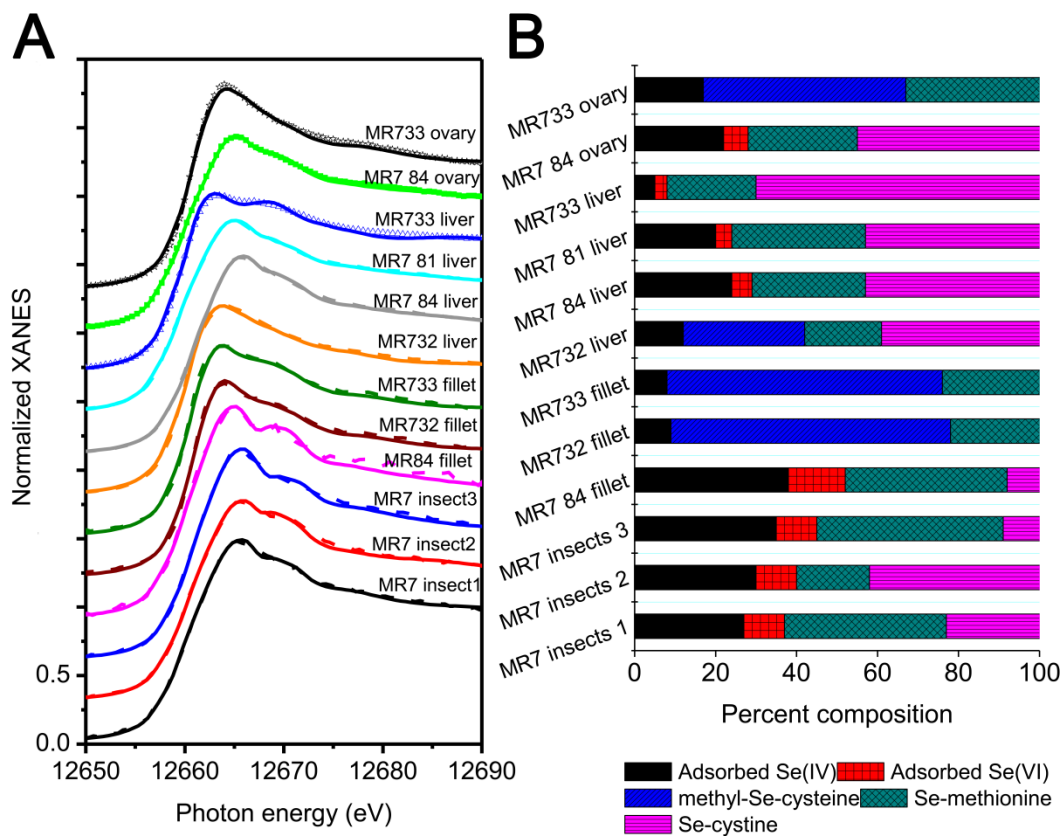




**Figure 10: A green sunfish (*Lepomis cyanellus*) from the main stem of the Mud River at site MR7 exhibiting a significant maxillary deformity consistent with Se exposure**



**Figure 11: A. Normal gill tissue from MR7 creek chub at 20X. Except for minimal increase in interlamellar tissue at right margin of field (arrow), all respiratory lamellae are equal in length and thickness. B. Arrow points to single aneurysm in gill tissue from LFMR creek chub at 20X. Both primary lamellae in this field are thickened and somewhat distorted. C. Arrows point to trematode parasites in gill tissue from LFMR creek chub. D. Arrow pointing to large protozoal infestation in primary lamellae of LFMR creek chub gill at 10X.**



**Figure 12: A. X-ray absorption near edge spectroscopy (XANES) spectra showing the fitted results for MR7 fish and insect tissues B. Relative fraction of selenium species as a percentage of total selenium in MR7 fish tissues and insects**

### **3. Otoliths as a tool for measuring lifetime selenium exposure in fish from the Mud River, West Virginia**

This chapter will be submitted as: Arnold, M.C., Friedrich, L.A., Lindberg, T.T., Halden, N.M., Ross, M., Palace, V., Bernhardt, E., and Di Giulio, R.T. Microchemical analysis of selenium in otoliths of two fish species captured near mountaintop coal removal mining operations in West Virginia.

#### ***3.1 Introduction***

Selenium (Se) is a micronutrient with a narrow range between essentiality and toxicity in oviparous vertebrates such as fish and birds (Eisler 2000, Muscatello et al. 2006, Janz et al. 2010). Symptoms of Se toxicity in fish include reproductive failure, teratogenesis, and increased mortality of early life stages (Lemly 2002, Teh et al. 2004, Muscatello et al. 2009). Although Se is a naturally occurring element, anthropogenic activities such as mining can facilitate leaching of Se into surrounding aquatic ecosystems. Mountaintop removal/valley fill (MTR/VF) coal mining is a type of surface mining practiced throughout the Appalachian mountains that has previously been demonstrated to facilitate the release of selenium from spoil rock into surrounding streams and rivers (Merovich et al. 2007, Lindberg et al. 2011, Arnold et al. 2014).

Current monitoring programs typically use whole body or specific tissue concentrations of Se, in addition to surface water concentrations, in an effort understand the fate of Se in an ecosystem and to estimate potential negative impacts of Se on wild

fish populations (Ferreri et al. 2004, WVDEP 2010). While analyses of tissue or whole-body burdens for Se are valuable for determining recent exposure, species differences in Se bioaccumulation, depuration, and migration can make interpretation of such Se values difficult. Hard tissues such as otoliths, calcified inner ear structures of teleost fish, can provide a long-term exposure history of stream contaminants including Se (Palace et al. 2007, Friedrich et al. 2011). However, the specific relationship between concentrations of elements in otoliths and soft tissues remain to be elucidated.

Otoliths are made up of layers of aragonite continuously deposited in a protein matrix (Degens et al. 1969, Campana et al. 1985) throughout the lifetime of the fish, creating annual growth rings. Because otoliths incorporate trace elements that reflect surface water chemistry and these deposits are metabolically stable, otoliths can act as records of exposure for certain contaminants (Halden et al. 2008, Martin et al. 2013). For example, Friedrich *et al.* (2011) showed increased concentrations of Se in later stage otolith annuli from three coldwater species of fish in Canada, indicating exposure to Se during latter life stages and suggesting that fish captured from sites highly impacted by mining were recent immigrants to these high-Se waters. This insight would not be possible with analysis of soft tissue Se alone, and it provides valuable insight into habitat use by fish that could be impacted by Se exposure (Friedrich et al. 2011).

The concentration of Se in MTR-impacted surface waters in West Virginia can be elevated 3-4 times background levels depending on flow conditions and season, where

high discharges in winter typically dilute Se (Lindberg et al., 2011). High summer temperatures increase evapotranspiration, which when combined with low rainfall can drive up surface water Se concentrations. We hypothesize that this seasonal Se concentration variation could be linked to otolith Se concentrations in fish. Previous work has demonstrated that otolith chemistry often track surface water chemistry (Martin et al. 2013), suggesting that patterns in otolith Se concentration may be a combination of life-history traits and Se concentration in the water column.

In this study, we used otolith microchemical analysis to understand lifetime exposure of wild fish to Se. Fish were collected from the main stem of the Mud River, in West Virginia at a site previously demonstrated to be significantly impacted for several decades by Se released from nearby mountaintop removal coal mining operations (WVDEP 2010, Lindberg et al. 2011, Arnold et al. 2014). We also collected fish from a reference site and a site newly impacted by MTR/VF. Potential relationships between muscle Se concentrations and otolith Se concentrations in green sunfish and creek chubs collected from the sampling sites were explored. Results from this study suggest that a retrospective reconstruction of Se concentrations in muscle can be derived from Se concentrations in otoliths, but only in creek chub (*Semotilus atromaculatus*) and not green sunfish (*Lepomis cyanellus*), underling the importance of species differences for determining partitioning of Se among specific tissues.

## ***3.2 Materials and methods***

### **3.2.1 Sampling sites**

The main stem of the Mud River passes directly through the Hobet 21 surface mine, an MTR/VF coal mining operation that has been discharging mining effluent into a 9 km section of the Mud River for over 40 years (Lindberg et al. 2011). The Mud River splits into two forks referred to here as specific sites on the main stem Mud River (MR7) and Left Fork Mud River (LFMR) (Figure 13). Naming conventions are in keeping with previously published research (Lindberg et al. 2011, Arnold et al. 2014). LFMR and the main stem Mud River are connected via the Mud River Reservoir, allowing fish to potentially migrate between these sites. MR7 is located 9.4 km from the confluence at the Mud River Reservoir and LFMR is located 9.2 km from the confluence. The main stem Mud River receives discharge contaminated with Se from multiple valley fills permitted by the National Pollutant Discharge Elimination System (Lindberg et al. 2011). Both sites show similar hydrology (Lindberg et al. 2011) but LFMR does not receive input from MTR/VF coal mining. The Big Ugly Creek (BU), located near the Big Ugly Wildlife Management Area, was permitted for blasting in May 2008-2009 and has been mined intermittently since.

### **3.2.2 Fish and otolith collection**

Mature green sunfish and creek chubs were collected from the three sites using a backpack electroshocker (HT-2000 Battery Backpack Electro-Fisher, Halltech Aquatic Research Inc., Ontario, Canada) during six sampling trips from March to July, 2011-2013 as previously described in Arnold et al. (2014). Captured fish were euthanized with 3 g/L ethyl 3-aminobenzoate methanesulfonate (MS-222, Sigma-Aldrich Co., LLC) for at least 30 minutes until opercular movement ceased followed by decapitation. Wet weight and standard length were measured on site. Fish were transported back to Duke University and stored at -80°C until dissection. Otoliths were removed from each fish, gently cleaned with double deionized water (ddH<sub>2</sub>O), and stored in microtubes until analysis.

Muscle tissue from green sunfish and creek chubs from LFMR and MR7 was collected and Se concentrations results have been previously published (Arnold et al. 2014). A subset of these samples was used in this study. Selenium tissue concentrations were determined as described in Arnold et al. (2014).

### **3.2.3 LA-ICP-MS otolith analysis**

Creek chub and green sunfish saggital otoliths were embedded in epoxy resin and cut transverse to create a dorso-ventral cross section through the nucleus to expose all annular growth zones. The posterior half of each cut otolith was re-embedded in a Lucite microprobe mount, then hand ground and polished, as previously described (Friedrich et al. 2008). Prior to analysis, samples were washed in an ultrasonic cleaner with double



distilled water and allowed to air dry. Laser ablation inductively coupled plasma mass spectrometry (LA-ICP-MS) analyses were performed using a Thermo Finnigan Element 2 ICP-MS system (Thermo Fisher Scientific, Waltham, MA). Samples were ablated in two sets; the first set was ablated with a NIR (785 nm) femtosecond (65 fs) laser, using a Quantronix Ti-light seed laser (Quantronix Inc., Hamden, CT) with an Integra C amplifier (Contium ®, San Jose, CA). The second set was ablated with a Merchantek LUV 213 Nd:YAG laser (Merckantek Electro-Optics, Carlsbad, CA). Isotope  $^{77}\text{Se}$  was measured rather than the more abundant  $^{80}\text{Se}$ , which has molecular interferences in the plasma gas and otolith matrix (e.g.,  $^{40}\text{Ar}^{40}\text{Ar}$  and  $^{40}\text{Ca}^{40}\text{Ar}$ ). Platinum cones were used to eliminate any interference that nickel cones may produce for  $^{77}\text{Se}$  analyses (e.g.,  $^{61}\text{Ni}^{16}\text{O}$ ).

Laser running conditions were set to optimize sensitivity and resolution of the annular growth zones, and included a 50  $\mu\text{m}$  laser beam travelling at 2  $\mu\text{m}/\text{s}$ . Calcium as 56 wt% CaO was used as an internal standard and the external calibration was done using NIST 610 glass (Pearce et al. 1997). Line scans were run across the otolith surface from core to edge, at a high angle to the growth zones in time-resolved or scanning mode. Analyses of standard reference material (NIST 610 glass) were completed at the beginning and end of each program, bracketing otolith scans. Data were reduced using the “Trace Elements” data reduction scheme of the Iolite software package running on Igor Pro software program (WaveMetrics, Tigard, OR) (Paton et al. 2011).

### **3.2.4 Comparing hydrology to otolith selenium**

To explore the relationship between hydrologic conditions and otolith Se accumulation, we used historic hydrologic data from a U.S. Geological Survey (USGS) gauging station at Mud River Reservoir (station number: 03204350) approximately 8 km downstream of our study sites. Data are reported in terms of reservoir surface water depth (meters) and broadly represents hydrologic conditions over the lifetime of fish collected. Water depth was related to conductivity and previous data from Lindberg et al. (2011) was used to establish the relationship between conductivity and surface water Se concentrations. Late summers in the Mud River are characterized by large decreases in discharge and high temperatures, which prior work in the Mud River demonstrated to be associated with increases in Se concentration in surface waters, especially at base flow (Lindberg et al. 2011).

We attempted to pair this historic hydrologic data with the approximate ages of collected fish on a small subset of MR7 creek chub otoliths in order to explore relationships between otolith Se concentration and background surface water conditions. All fish hatch dates were arbitrarily fixed to April 1st and linearly interpolated from year to year.

### **3.2.5 Statistics**

Selenium concentration values are presented as mean  $\pm$  standard error. Average muscle and yearly otolith Se concentrations were analyzed using a one-way analysis of

variance (ANOVA) with a Holm-Sidak post hoc test. When the data did not meet normality or equal variance assumptions an ANOVA by ranks (Kruskal-Wallis) was used with a Dunn's Method post-hoc test. A linear regression analysis was performed on log transformed muscle and otolith Se concentrations to determine any relationships. GraphPad Prism 6 (GraphPad Software, La Jolla, CA) and SigmaPlot 12.5 (Systat Software Inc., San Jose, CA) were used for data analysis.

### ***3.3 Results***

#### **3.3.1 Otolith selenium concentrations**

Twenty-two creek chubs and 15 green sunfish were collected in total from the three sampling sites. In general, green sunfish from all sites contained background (<1 µg/g) to low (1-4 µg/g) average concentrations of otolith Se whereas creek chub otolith Se values were increased in fish collected from MR7 ( $\geq 5$  µg/g peaks). Otoliths containing 1-4 µg/g Se were predominantly creek chubs (12) from LFMR, MR7, and BU and one LFMR green sunfish (Figure 14). Interestingly, two MR7 creek chub females contained unusually low otolith Se. One of these creek chubs showed an increase in otolith Se as it aged, with the baseline otolith Se concentration increasing from an average of 0.5 µg/g in 2008 to 1.2 µg/g in 2009, with peaks up to 3.8 µg/g. The second MR7 creek chub female had an average otolith Se concentration of 2 µg/g, although it also contained spikes of otolith Se up to 4.2 µg/g.

Nine fish contained otolith Se peaks  $\geq 5 \mu\text{g/g}$ . These fish were exclusively creek chubs, the majority (77.8%, 7/9) of which were collected from MR7. These fish were generally between 1-2 years old, with the exception of two fish ages 4 and 6 years old. One of the 2-year old female creek chubs contained a  $66 \mu\text{g/g}$  Se peak in the otolith in 2012. It is noteworthy that two LFMR female creek chubs contained 5-8  $\mu\text{g/g}$  Se peaks in their otoliths, which may indicate travel to the mined fork of the Mud River.

By examining the earliest otolith growth, the primordial, we can assess whether individual fish experienced early life stage exposure. We found that eight of the nine fish with high otolith Se had greater than  $1 \mu\text{g/g}$  Se in the otolith primordia. Both LFMR fish with high otolith Se also contained Se in the otolith primordia. The only fish with low otolith Se to have primordial Se accumulation was an MR7 female creek chub. The remaining fish did not contain primordial otolith Se concentrations above the baseline levels ( $<1 \mu\text{g/g}$ ).

### **3.3.2 Muscle selenium concentration**

Green sunfish muscle Se concentrations varied between sampling sites, with MR7 green sunfish containing significantly higher concentrations compared to LFMR and BU ( $p < 0.05$ ). MR7 green sunfish fillets had an average of  $8.42 \pm 0.7 \mu\text{g/g dw}$  Se whereas LFMR and BU green sunfish contained  $3.16 \pm 0.4$  and  $3.29 \pm 0.2 \mu\text{g/g dw}$ , respectively. MR7 creek chubs contained an average muscle Se concentration of  $10.6 \pm 2 \mu\text{g/g dw}$ ,

which was significantly higher ( $p < 0.05$ ) than LFMR creek chub muscle ( $1.94 \pm 0.07$  mg/kg dw) and BU creek chubs ( $1.64 \pm 0.2$   $\mu\text{g/g}$  dw).

A significant linear regression was found for the relationship between creek chub muscle Se and the average Se concentrations in the final 10% or 20% of the otoliths ( $R^2 = 0.46$ ,  $p < 0.001$  and  $R^2 = 0.54$ ,  $p = 0.0002$ , respectively, Figure 15A and B). One outlier, a BU creek chub with notably low otolith Se ( $< 0.005$   $\mu\text{g/g}$  for the last 10% average compared to an average value of  $2\mu\text{g/g}$  for other creek chubs) was removed from data set when calculating the linear regression. No significant relationships were found between muscle and the last 10% ( $R^2 = 0.06$ ,  $p = 0.31$ ) or 20% ( $R^2 = 0.03$ ,  $p = 0.47$ ) average otolith Se for green sunfish.

### **3.3.3 The relationship between hydrology data and otolith selenium**

When comparing a subset of MR7 creek chubs with high otolith Se to hydrologic data from the Mud River Reservoir, a relationship was observable between the two data sets (Figure 16A-C). All large ( $\sim 40$   $\mu\text{g/g}$ ) Se peaks in creek chub otoliths correlated with a significant drop in water level at the Mud River Reservoir in the late summers of 2011 and 2012. Summer drops in water level were strongly correlated ( $R^2 = 0.86$ ), with increases in conductivity, and, by correlation ( $R^2 = 0.76$ ), Se. Thus, peaks in Se Otolith concentration during late summer months correspond with peak Se concentrations in surface waters.

### ***3.4 Discussion***

Soft tissue analysis of Se provides a “snapshot” view of Se accumulation because fish can metabolize and depurate Se, indicating that the concentrations found in these tissues only represent what the fish has been exposed to recently, ignoring historical exposure and migration between polluted and unpolluted waters. Otolith analysis is becoming increasingly utilized as a tool to circumvent these problems associated with soft tissue data. Otolith Se analysis is particularly valuable because in addition to providing an estimate for the age of a fish, otoliths incorporate contaminants such as Se as the fish grows, creating a time series of exposure.

The results presented here reveal species differences in otolith accumulation of Se. Green sunfish otolith Se concentrations did not vary with muscle Se concentrations at the mined (MR7 and BU) versus unmined (LFMR) sites. In contrast, regression analysis revealed that creek chub otolith Se concentrations were predictive of muscle Se concentrations, with MR7 creek chubs accumulating Se at higher concentrations in both otolith and muscle compared to LFMR and BU creek chubs. The species differences in otolith Se accumulation meant that the majority of green sunfish from all three sites contained background concentrations of otolith Se (<1 µg/g) whereas creek chubs from LFMR and BU contained predominantly 1-4 µg/g and MR7 creek chubs contained high otolith Se with peaks  $\geq 5$  µg/g.

These data indicate that otoliths from green sunfish would not be good monitors for bioaccumulation of Se, but otoliths could potentially be used to determine lifetime Se accumulation in muscle tissue from creek chubs. Several of the MR7 creek chubs had very high otolith Se concentrations (28-60  $\mu\text{g/g}$ ). The disparity in Se accumulation patterns between the two species may be due to species-specific differences in otolith deposition (Dove et al. 1996, Swearer et al. 2003). It could also suggest very low residence times of green sunfish in the Se enriched sites, although an apparently established colony of green sunfish composed of a range of ages was observed at MR7 (unpublished observation).

When otolith and muscle Se concentrations were compared within species, creek chub otolith Se were more predictive of muscle Se concentrations. The average otolith Se concentration from the final 10% and 20% of the otolith typically increased with increasing muscle Se concentrations for MR7 creek chubs. The lower muscle Se at LFMR was often reflected by lower concentrations of Se in LFMR otoliths. In contrast, green sunfish otolith Se was not related to muscle Se concentrations (Figure 3B). Green sunfish otolith concentrations were very low, on average less than 1  $\mu\text{g/g}$  across all three sites despite the increased muscle Se at MR7.

Selenium is significantly toxic to oviparous vertebrates such as fish and birds because it is readily maternally transferred via yolk proteins such as vitellogenin (Kroll et al. 1991), leading to developmental defects or decreases in reproductive success due to

embryo lethality (reviewed by Lemly, 2002). Previous work has shown that composite aquatic insect samples collected from MR7 showed significantly higher concentrations of Se compared to LFMR (Arnold et al. 2014), and MR7 female creek chubs feeding on these insects can transfer the Se to their progeny. The majority of MR7 creek chubs collected in the Mud River showed elevated concentrations of Se in their otolith primordia, which is likely due to Se exposure via maternal transfer.

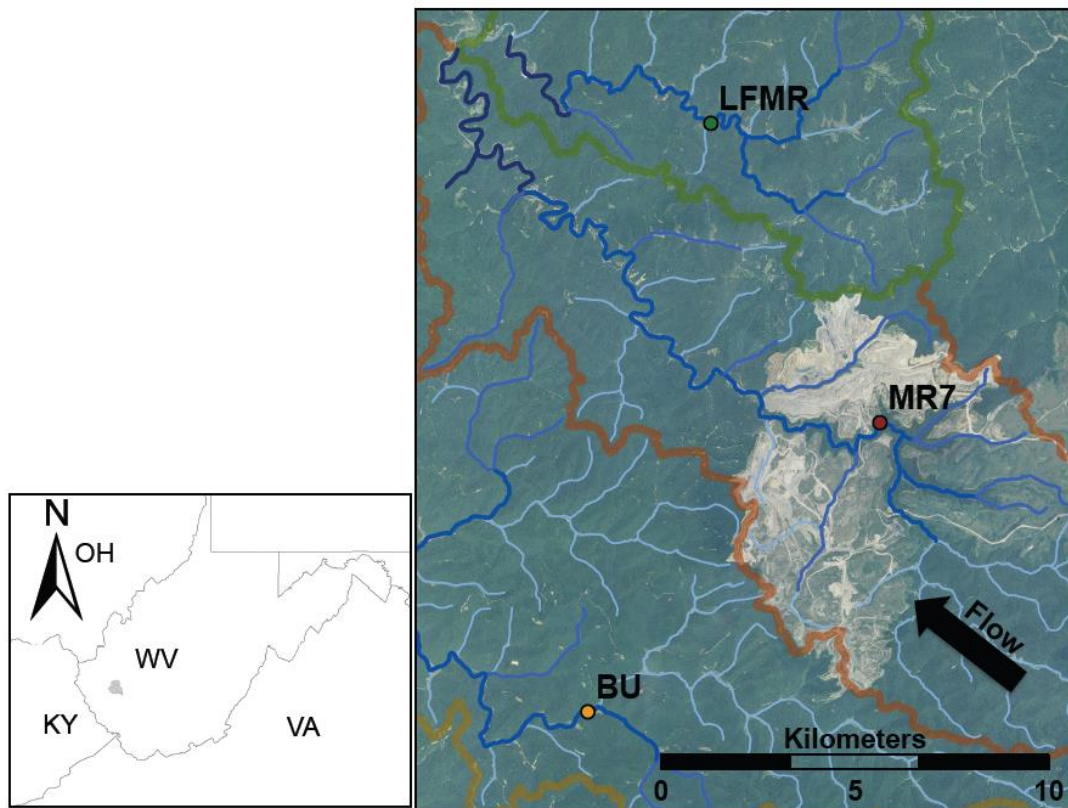
Peaks in otolith Se concentrations in MR7 creek chubs coincided with seasonal lows in discharge as measured at the Mud River Reservoir. These seasonal decreases in stream flow correlate with increases in Se residence time in the surface waters of the Mud River and a 3-4 fold increase in concentration (Lindberg et al. 2011), leading to increases in Se exposures in MR7 biota. Whereas life history and bioaccumulation (Arnold et al. 2014) may explain some of the seasonal peaks in Se, changing ambient surface water chemistry due to fluctuations in water flow from rainfall could play an equally strong role (Martin et al. 2013). Although the data presented here are limited, the results suggest that surface water depth time series data in combination with otolith chemistry can provide a robust interpretation of prior Se exposure, including seasonal patterns.

These data suggest that body burdens of Se in fish can vary considerably over time and that both the timing of sampling and species choice could heavily influence Se assessments. Monitoring programs should take into account species differences in Se accumulation, timing and location of sampling, and types of tissues sampled. For

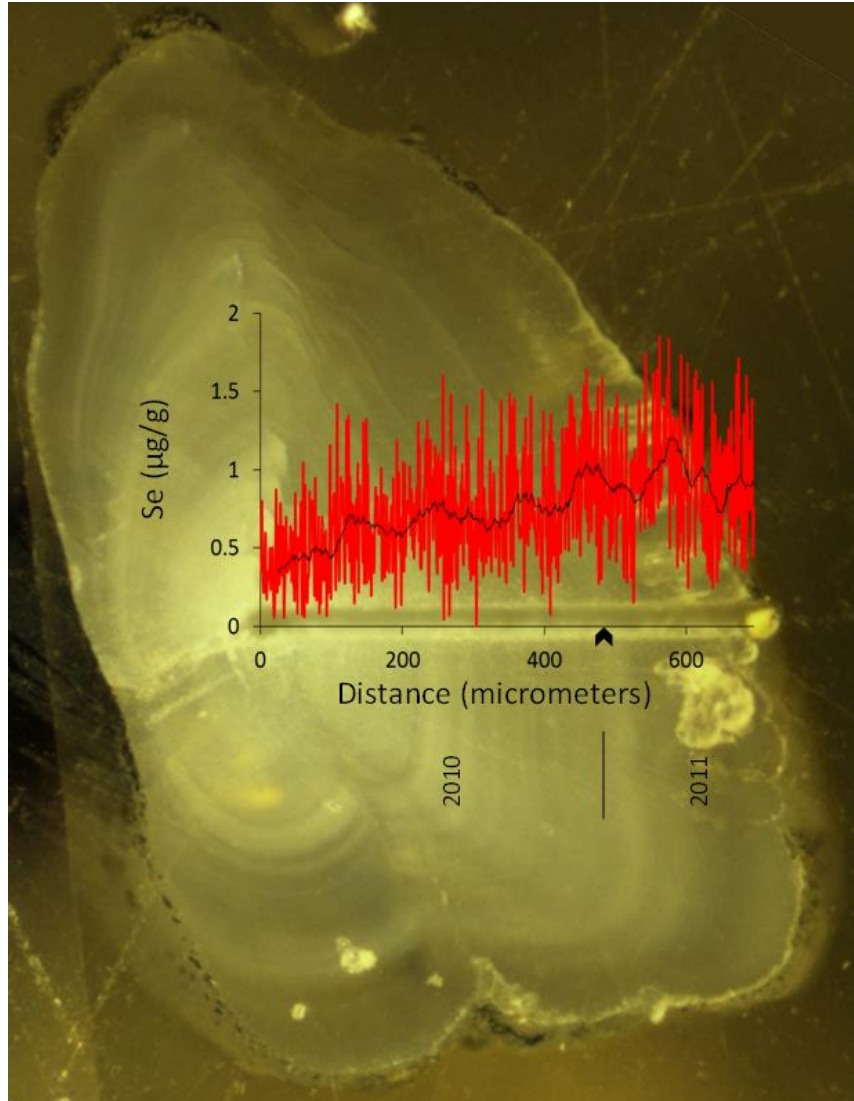


example, fish collected in winter months compared to fish collected from the Mud River in summer may have significantly different tissue Se concentrations due to winter dilution of Se in the system from high flows. Soft tissue analysis of Se reflects more recent exposure whereas otolith analysis provides a more comprehensive picture of Se exposure, although the data presented here indicate that the utility of otoliths as a good record of Se exposure is limited by species-specific differences in accumulation.

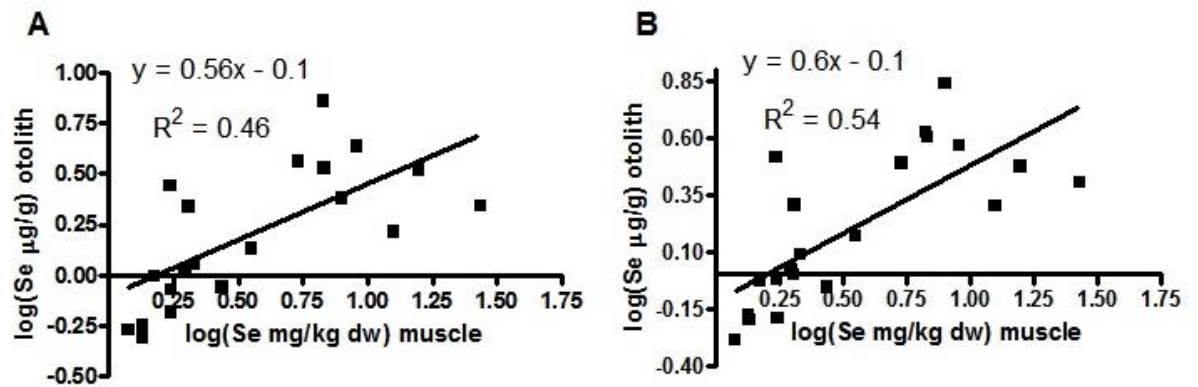
Otolith analysis was a useful tool for creek chubs but not green sunfish. Two of the fish in the “high” otolith Se category that also had otolith primordia Se were from the LFMR site, which has been previously shown to contain minimal amounts of water column Se (Arnold et al. 2014). These data suggest that some fish captured in LFMR may originate from and/or are migrating to seleniferous water such as the Mud River Reservoir, which connects the main stem and the Left Fork. The majority of MR7 creek chubs contained high concentrations of otolith Se, including in the primordia, so it can be assumed that a significant portion of these fish are residing in the Se-impacted Mud River.



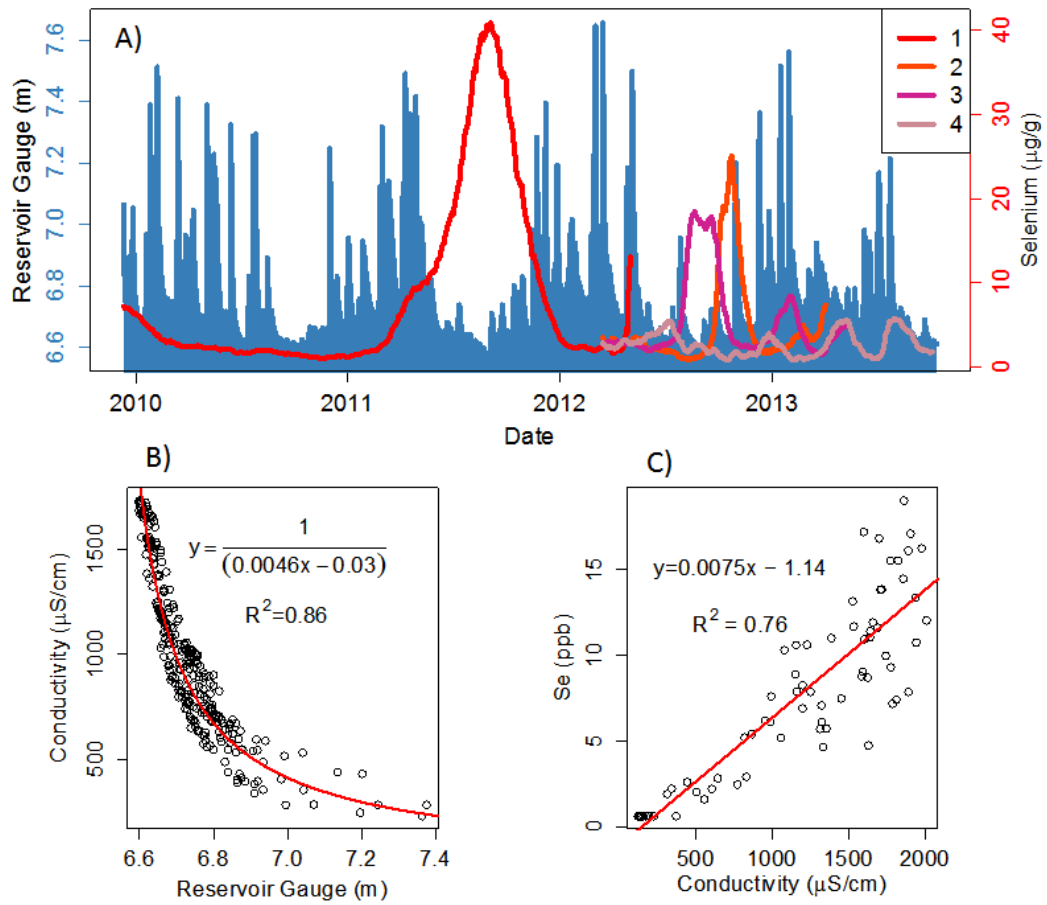
**Figure 13: Map showing sampling sites Left Fork Mud River (LFMR), Big Ugly (BU), and Mud River 7 (MR7) in West Virginia. Yellow, orange, and green lines outline the BU, MR7, and LFMR watersheds, respectively. Black arrow indicates direction of flow.**



**Figure 14: Otolith Se concentrations ( $\mu\text{g/g}$ ) determined using LA-ICP-MS for a creek chub caught in Big Ugly Creek. The x-axis shows the distance (micrometers) across the otolith and the year.**



**Figure 15: A. A. Linear regression between  $\log(\text{Se})$  from the average of the last 10% of the otolith versus  $\log(\text{Se})$  in muscle from creek chubs ( $p < 0.001$ ). B. Linear regression between  $\log(\text{Se})$  from the average of the last 20% of the otolith versus  $\log(\text{Se})$  in muscle from creek chubs ( $p = 0.0002$ ).**



**Figure 16: A. Time series of Se concentration for four different creek chubs (1-4) with Se peaks in late summer for three of the four fish shown. Se peaks (left y-axis, µg/g) correspond to times when reservoir depth (right y-axis, m) were lowest and surface water conductivity and Se were elevated B. Correlation between reservoir water level and conductivity in the Mud River and C. Correlation between conductivity and Se concentrations (ppb, based on data from Lindberg et al., 2011.)**

## **4. Biofilm mediated uptake and trophic transfer of selenium to fathead minnows**

This chapter was submitted as: Arnold, M.C., Lindberg, T.T., Bier, R.L., Bernhardt, E., and Di Giulio, R.T. Biofilm mediated uptake and trophic transfer of selenium to fathead minnows. *Freshwater Science*.

### ***4.1 Introduction***

The fate of a contaminant often depends on the chemistry of the surrounding aquatic environment. Of the contaminants released from geologic processes, selenium is of particular concern because it is a known teratogen that causes significant deformities in oviparous vertebrates (Lemly 1993). Selenium (Se) is a metalloid found in aquatic biofilms growing in Se-rich water (Hockin et al. 2003). Selenium occurs naturally in rocks associated with low-sulfur coal deposits, including those in the Appalachian Mountains of the United States (Vesper et al. 2008) and can be released to aquatic systems during land alteration such as mountaintop removal/valley fill (MTR/VF) coal mining.

During the process of MTR/VF coal mining, the tops of mountains are blasted off to access coal seams, and Se-enriched rocks and coal seams are exposed to weathering, causing the release of significant amounts of Se in the form of selenate ( $\text{SeO}_4^{2-}$ ) into the environment (Palmer et al. 2010, Lindberg et al. 2011). Although Se is a micronutrient essential for the function of several key enzymes including glutathione peroxidases

(Flohe et al. 1973, Rotruck et al. 1973, Arthur et al. 1990), exposure to elevated concentrations beyond those required for enzymatic function can cause severe developmental abnormalities in fish and birds (Ohlendorf et al. 1988, Lemly 1997). Diet is the primary route of Se exposure in vertebrates (Lemly 2002), indicating that biofilms, which make up the base of some aquatic food webs, could be an important source of Se for higher trophic organisms (Figure 17).

Selenate, in anoxic conditions, can be readily transformed into more biologically available forms including selenomethionine by bacteria and algae (reviewed by Maher et al., 2010) . Because Se toxicity often requires uptake via dietary exposure, most previous research on Se bioaccumulation and toxicity has been conducted in lakes or reservoirs (lotic systems) where sediments are regularly anoxic. However, large amounts of Se are also released directly into flowing water (lotic) systems from mountaintop removal/valley fill coal mines (MTR/VF), and stream biofilms may allow for anoxic zones in these otherwise well oxygenated habitats. Microhabitats created by the biofilms may be very important in the biotransformation of inorganic Se.

Aquatic biofilms can serve as a primary route of contaminant exposure via ingestion, yet little documentation exists describing their role in contaminant sequestration and transfer in aquatic ecosystems. In part, our limited knowledge is due to the extraordinary complexity of these ecosystem components. The assemblage of microorganisms, extracellular polymeric substances, water, and macroinvertebrates that comprise biofilms (Wimpenny et al. 2000, Van Hullebusch et al. 2003) readily

accumulate and chemically transform dissolved metals from stream water through biosorption, metal reductive precipitation, and other mechanisms described by van Hullenbusch (2003). The position of biofilms as the basis of an aquatic food chain combined with an ability to accumulate metal contaminants suggests that biofilm chemical composition may be useful in understanding how metals transfer to higher trophic organisms. Determining the role biofilms play in the accumulation, transformation, and bioavailability of stream contaminants is critical to accurately model their fate, transport, and effects on the greater stream ecosystem.

The ability of biofilms to concentrate Se suggests that biofilms may be important in the accumulation and trophic transfer of Se released into a stream via MTR/VF coal mining. For example, algae living in seleniferous water biomagnified 1400-fold the concentration of Se from water, and the Se was subsequently transferred to higher trophic organisms (Fan et al. 2002). Although water column and fish tissue Se data are often available, there are few data available on the role of biofilms in Se bioaccumulation in lotic systems and how readily this Se can be transferred to higher trophic organisms such as fish.

Because there is a strong positive correlation between the metal concentrations within biofilms and the concentration of those metals in the tissues of grazing aquatic invertebrates (Rhea et al. 2006, Farag et al. 2007, Conley et al. 2009), fish that feed on biofilms may also accumulate Se. In this study we collected biofilms from the MTR/VF coal mining-impacted main stem of the Mud River (mined), a lotic system receiving



effluent from the Hobet 21 coal mine in southwest West Virginia. Biofilms were also collected from the Left Fork of the Mud River (unmined), which acted as a reference site because it does not receive MTR/VF coal mining effluent. Biofilms were transported back to the laboratory where they were analyzed for Se content and fed to adult female fathead minnows over the course of approximately two weeks in order to simulate stream minnows feeding on natural biofilms. The objectives of this study were to determine how much Se accumulates in biofilms from a MTR/VF coal mining impacted lotic system and to model the trophic transfer of this Se from biofilm to fish.

## ***4.2 Materials and Methods***

### **4.2.1 Collecting and processing biofilms**

Biofilm samples for the feeding experiment were collected during three sampling trips from both mining impacted and reference sites in May and June, 2012 and 2013 (Figure 18). Mined and unmined sampling sites were located on the Mud River, WV at locations previously described (Lindberg et al. 2011, Arnold et al. 2014). Biofilm samples were collected from both natural (rock) and artificial (acrylic plates) substrates. Biofilm scrapings were stored in metal-free certified 50 mL polypropylene tubes (VWR International, LLC, Radnor, PA, USA) on ice for transport back to the laboratory where they were stored at -80°C until analyses could be completed.

Biofilms used in fish feeding experiments were colonized on acrylic plates measuring 25.5 x 20 cm in floating PVC holder rigs containing five plates per rig that were positioned parallel to the current (Appendix Figure 32). Plates were suspended in a

vertical orientation in order to reduce detrital accumulation (Lane et al. 2003), approximately 4-6 inches below the surface of the water. Biofilm rigs were held by wires at a length that allowed the rigs to move up and down with water flow; two biofilm rigs were placed at each site. Acrylic plates were roughened with p80 grain sandpaper during the summer 2013 collection season to promote greater biofilm growth. Biofilm rigs were placed in the Mud River for approximately 6 weeks, after which they were collected and stored in individual bags containing river water at 4°C until use.

To assess differences in the Se content of separate biofilm components, filamentous algae samples were collected from in-stream rock surfaces at both sites in addition to biofilms grown on acrylic plates. Biofilms collected from artificial substrates had grown on three acrylic plates deployed in the Mud River system in June 2013. Acrylic plate biofilms were collected approximately 3.5 months later by scraping each plate into a metal-free certified 50 mL polypropylene tube (VWR International, LLC, Radnor, PA, USA). Tubes were kept on ice for transport to the lab and stored at 4°C until density separation.

Light mass fractions dominated by filamentous algae were separated from dense fractions containing diatoms using density fractionation (Hamilton et al. 2005). Briefly, biofilm slurries were added to a 70% v/v colloidal silica-water mixture (Ludox® TM-50, Aldrich, USA) in 5 mL aliquots and centrifuged at 1000 rpm for 10 minutes. This initial spin of each aliquot separated sediments from biofilms and resulted in a bright green upper layer and dark brown lower layer. These layers were transferred via pipette into

two separate 50 mL centrifuge tubes containing 70% v/v colloidal silica and re-centrifuged to purify each fraction. Serial transfer and centrifuging was repeated until fractions were visibly separated and centrifuging yielded only one layer. The separated fractions were then repeatedly washed with double deionized water (ddH<sub>2</sub>O), centrifuged, and transferred to a new 50 mL tube of ddH<sub>2</sub>O until no silica pellet was visible.

To visualize the biofilms, samples were imaged using electron microscopy at Chapel Hill Analytical and Nanofabrication Laboratory, Department of Applied Physical Sciences, University of North Carolina, Chapel Hill, NC. Biofilms grown on acrylic plates were fixed in a solution containing 2.5% glutaraldehyde, 2% paraformaldehyde with 0.05% CaCl<sub>2</sub> in 0.1M cacodylate buffer, pH 7.4 for several days. Samples were rinsed overnight in 0.1M cacodylate buffer and then taken through a dehydration series (50%, 70%, 95%) to absolute ethanol.

Scanning electron microscopy (SEM) samples were critical point dried, mounted onto stubs and coated with Au/Pd and imaged on a Hitachi 4800F at 15-20kV accelerating voltage. X-ray microanalysis was performed with an Oxford Inca system. Samples for transmission electron microscopy (TEM) were removed from the substrate and infiltrated in Polybed 812/ ethanol (50:50) for 4 hours, transferred to 100% Polybed 812 overnight and embedded into blocks and cured at 65°C for two days. Ultrathin sections were cut on a RMC 6000 at 90-100nm. Sections were imaged with or without

poststaining (uranyl acetate and lead citrate) on a Jeol 100CX (Jeol, Ltd., Tokyo, Japan) at 80 kV.

#### **4.2.2 Feeding experiments**

Adult female fathead minnows (*Pimephales promelas*) purchased from Aquatic Biosystems (Fort Collins, CO, USA) were housed in aquaria provided with aeration and filtration via hanging filters. Fathead minnows (average standard length of  $4.5 \pm 0.8$  cm) were held in large tanks for several days before they were randomly placed in the 150 L treatment tanks. Halved PVC pipes were provided for shelter in each aquarium. For each experiment, four fish were placed with either a mined or unmined biofilm plate and there were two replicate groups per treatment for a total of four groups of four fish. The experiments were repeated three times with new fish and biofilm plates collected from the field. Feeding experiments were replicated three times.

Biofilm plates were placed in the aquaria with the four-fish groups (2 unmined and 2 mined groups of 4 fish each for a total of 16 fish per experiment) one at a time until five plates per treatment replicate were used. Plates were replaced every 3-4 days until the end of the experiment (14-16 days) after which the fish were allowed to depurate for three days to remove biofilm material from the gut before euthanasia via ethyl 3-aminobenzoate methanesulfonate (MS-222, Sigma-Aldrich Co., LLC) and decapitation. During the depuration period fish were fed Aquatox flake food (Zeigler®, Gardners, PA) containing 1 mg/kg dw Se, as determined by ICP-MS methods described below. Tissues

including skinless fillet, liver, and ovaries were collected from freshly euthanized fish and stored at  $-80^{\circ}\text{C}$  until analysis.

Water quality including temperature, general hardness (dGH), carbonate hardness (DkH), pH, and temperature were monitored daily. Average GH was  $82.8 \pm 3$  dGH and  $81.8 \pm 3$  dGH while KH was  $115.8 \pm 7$  dKH and  $116.3 \pm 7$  dKH for unmined and mined treatment tanks, respectively. Average pH was  $7.8 \pm 0.6$  for both unmined and mined while temperature was  $22.3 \pm 0.08^{\circ}\text{C}$  and  $23.2 \pm 0.1^{\circ}\text{C}$  for unmined and mined, respectively. gH and kH were modified using sodium bicarbonate and Epsom salts in carbon filtered water. Water samples at the beginning and end of the experiment were collected to determine leaching of Se into aquaria water. Water samples were acidified with trace metal grade nitric acid and stored at  $4^{\circ}\text{C}$  until analysis. Scrapings of each biofilm plate were collected before the plates were placed in the aquaria for the 2013 experiments. Two representative biofilm scrapings were taken from two plates for each site during the 2012 experiment. Biofilm scrapings were stored at  $-80^{\circ}\text{C}$  until they were processed for analysis as described below.

#### **4.2.3 ICP-MS analysis of biofilm and fathead minnow samples**

Freeze-dried samples were weighed, and digested at  $75^{\circ}\text{C}$  for at least 6 hours in concentrated nitric acid. Biofilm samples were then decanted and the remaining undigested sediment was freeze-dried and weighed. The weight of undigested sediment was subtracted from the original weight of the biofilm sample to accurately calculate trace element concentrations. Digested samples were diluted 1:10 with ddH<sub>2</sub>O, followed

by a 1:5 dilution with a 2%  $\text{HNO}_3$ /0.5%  $\text{HCl}$  mixture.

Selenium content in fish tissue and biofilm samples was measured using inductively coupled plasma-mass spectrometry (ICP-MS, Agilent 7700X ICP-MS equipped with an Octopole Reaction System) according to the methods described in Arnold et al. (2014). The concentration of Se was determined using a hydrogen gas reaction at a  $\text{H}_2(\text{g})$  flow rate of  $4 \text{ mL min}^{-1}$ . A certified reference material (DORM-2 dogfish protein, National Research Council, Canada) containing known amounts of trace elements including Se was digested in parallel with fish and biofilm samples, and the recovery of the certified Se values were  $86.8 \pm 1 \%$  ( $N=26$ ). Double-deionized water acted as a method blank and was digested in parallel with samples.

#### **4.2.4 Statistics**

Statistical analysis was done using JMP® 10.0.2 (SAS Institute, Inc, Cary, NC) and graphs were made using GraphPad Prism 4 software (GraphPad Software, La Jolla, CA). The concentration of Se in biofilms were compared using Student's t-test ( $\alpha=0.05$ ) and are reported as mean  $\pm$  standard error. Values that were below detection limit (BDL) were assigned a value of zero for analysis. Fathead minnow tissue Se concentrations were averaged in the treatment groups (up to 4 fish per group) and then compared using Student's t-test. Samples containing outlier Se concentrations in any of the fish tissues, as determined by Grubbs' test, were excluded from analysis. One unmined treatment replicate (including four fish) and one fish from another unmined replicate were

excluded. These fish were likely outliers because they were from a different supplier that used unknown feed with unknown concentrations of Se (Foster Lake & Pond Management, Raleigh, NC). This supplier was used due to unexpected mortality that occurred immediately before the onset of the experiment.

### **4.3 Results**

#### **4.3.1 Water and biofilm selenium concentrations**

Analysis of water quality showed that the average soluble Se was significantly higher  $p < 0.05$  ( $6.1 \pm 1.9 \mu\text{g/L}$ ) in mined samples compared to unmined samples ( $0.1 \pm 0.004 \mu\text{g/L}$ ). Conductivity was also significantly higher ( $p < 0.02$ ) at the mined site ( $1270 \pm 290 \mu\text{S}$ ) compared to the unmined site ( $101 \pm 25 \mu\text{S}$ ). The concentration of Se was significantly higher ( $p < 0.05$ ) in the mined biofilms ( $2.7 \pm 0.2 \text{ mg/kg dw}$ ) compared to biofilms from unmined site ( $1.3 \pm 0.1 \text{ mg Se/kg dw}$ ) (Figure 19). Mined biofilms used in the fathead minnow feeding experiments had an average bioconcentration factor (BCF) of  $688 \pm 350$  fold for Se from the Mud River water. Unmined biofilms had an average BCF of  $14505 \pm 2700$  fold.

Electron microscopy revealed that the mined biofilms grown on acrylic plates were dominated by diatoms but also included some filamentous algae (Figure 20). Diatoms observed in mine biofilms included members of the genera *Navicula*, *Achnathadium*, *Eunotia*, *Nitzshia*, *Heliozan*, and *Cocconeis*. Mined biofilms often included a large amount of incorporated sand within the polysaccharide matrix.

When mined filamentous green algae was separated from the biofilm samples, Se was significantly higher ( $p < 0.05$ ) in the fraction not containing filamentous green algae at  $2.3 \pm 1.3$  mg/kg dw compared to  $1.1 \pm 0.6$  mg/kg dw in the algal fraction (Figure 21). Fractions could not be statistically compared for the unmined acrylic samples due to limited sample size, but the trend is similar. Concentrations of Se were higher in mined biofilms used for fractionation compared to biofilms collected for feeding experiments and this increase is likely due to the fact that these biofilms were left to grow on the acrylic plates for approximately three times longer than plates grown for feeding experiments. Rock scrapings of filamentous green algae were collected in parallel with the fractionated biofilms. Although sample size was limited ( $N = 2$ ), the range of Se concentrations in the mined filamentous algae from mined site rocks was slightly lower (BDL-3.3 mg/kg dw) than the concentrations found in filamentous algae from biofilm plates collected after 3.5 months of deployment ( $N = 3$ , 2.9-5.1 mg/kg dw).

#### **4.3.2 Fathead minnow uptake of selenium from biofilms**

After approximately two weeks of feeding on biofilms, adult female fathead minnows fed on mined biofilms accumulated significantly higher concentrations of Se in livers and ovaries (mean concentration  $2.4 \pm 0.1$  and  $3.1 \pm 0.3$  mg/kg dw respectively) compared to unmined biofilm-fed fathead minnows (mean concentration  $1.5 \pm 0.3$  and  $1.4 \pm 0.4$  mg/kg dw respectively,  $p < 0.02$  for both tissues, Figure 22). There was no difference in Se concentrations in skinless fillets between treatments. In general,



aquarium water samples contained Se concentrations below detection limit or very low (0.01-0.04  $\mu\text{g/L}$ ) for all sampling time points in the unmined tanks and initial mined water samples. Final water samples for mined treatment tanks showed minimally elevated concentrations of Se (0.02-0.2  $\mu\text{g/L}$ ).

#### ***4.4 Discussion***

As humans continue to extract and process geologic energy resources, contamination of nearby freshwaters from metals is a growing concern. When aquatic ecosystems are exposed to contaminants from these sources, there may be consequences to aquatic biota through multiple facets. Because aquatic biofilms lie at the base of this foodweb, they can serve as the entry point for inorganic contaminants into the rest of the aquatic foodweb (Kimball et al. 1995, Farag et al. 2007, Ancion et al. 2013). However, research has only more recently begun to thoroughly investigate the role of aquatic biofilms in contaminant transfer and the likelihood that, due to their complex redox characteristics, they may be responsible for tissue-specific partitioning of the contaminants within higher-level organisms.

Mined biofilms accumulated significantly higher concentrations of Se compared to unmined biofilms. Interestingly, BCF values for unmined biofilms were significantly larger ( $14500 \pm 2700$  fold,  $p < 0.01$ ) than mined biofilms ( $690 \pm 350$  fold). Conley et al. (2009) found an average BCF of  $1113 \pm 430$  fold for biofilms spiked with selenite for 7-9 days, which is similar to the values calculated in this study for the mined site. The

unmined water column Se concentrations were significantly lower compared to mined, but the unmined biofilm concentrations were approximately half of the mined biofilm values. The amount of Se accumulated in the unmined biofilms was surprising considering the very low concentrations of dissolved Se in the water column. The values of Se in the fish ovaries reflect the concentrations of Se in the biofilms. There may be a limiting factor in the bioaccumulation of Se in biofilms. It is possible that the biofilms collected from the two sites contain different taxa, leading to differences in uptake and biotransformation of Se.

The data presented here are particularly significant because despite the fact that the majority of Se released from MTRVF mines into rivers is as the less bioavailable form (selenate), biofilms in streams below the Hobet mine complex accumulated and concentrated significant quantities of Se. Although it has previously been shown that Se accumulates at higher concentrations in lentic food chains (reviewed by Simmons and Wallaschläger, 2005), the results presented here and previously (Arnold et al. 2014) indicate that Se can be a concern for trophic transfer in flowing water systems as well.

Electron microscopy revealed that the biofilms contained a large amount of diatoms, which have been previously shown to accumulate Se (Riedel et al. 1991). Fractionation of the biofilms showed that filamentous green algae contained approximately one third of the Se in the mined biofilms grown on artificial substrate. Although sample sizes were too small to permit statistical analysis, the trend was similar for unmined biofilm plate samples. Filamentous algae from rocks at the mined site

contained slightly less Se compared to filamentous algae from the biofilm plates. It is important to understand how Se partitions into the various components of biofilms because consumers may preferentially feed on specific compartments and could be exposed to greater or lesser concentrations of Se depending on how they feed. Future research should look at seasonal patterns of Se accumulation in biofilm fractions because there may be significant differences in how algae and/or diatoms accumulate Se depending on factors such as light availability, temperature or rainfall.

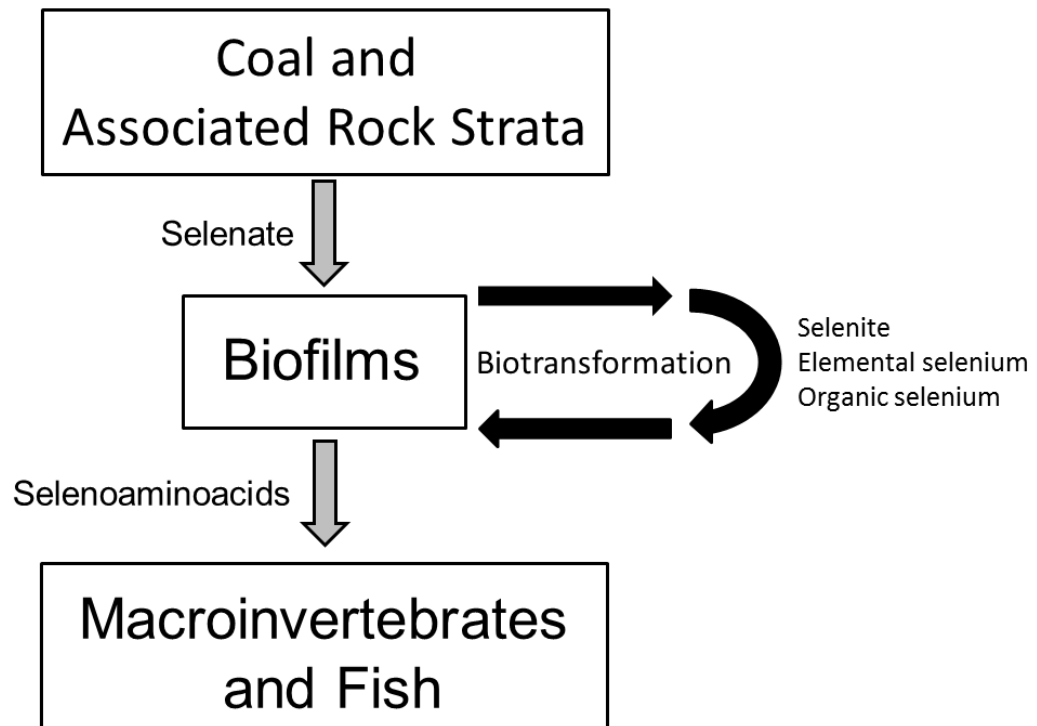
After 14-16 days of feeding on naturally-derived biofilms from a mountaintop removal coal mining-impacted site on the Mud River, adult female fathead minnows accumulated significantly higher concentrations of Se in their ovaries compared to fathead minnows fed on biofilms from the unimpacted site. Although it is possible that differences in the biofilm nutritional content could be different between the plates from the mined compared to the unmined site, it is noteworthy that fathead minnows fed on the mined plates accumulated elevated concentrations of Se in a relatively short period of time. In the laboratory, the fathead minnows bioconcentrated Se from the mined biofilms up to an average of 3.1 mg/kg dw in approximately two weeks. The current proposed standard for whole body Se in wild fish is 8.1 mg/kg dw (USEPA 2014), and long term exposure to dietary Se via biofilms could lead to fish Se body burdens that approach the proposed standard. These data indicate that it is possible to model trophic transfer of inorganic contaminants such as Se from biofilms to fish in the laboratory. The

experimental design described here is especially useful because it uses real-world field samples while maintaining laboratory controlled exposure conditions.

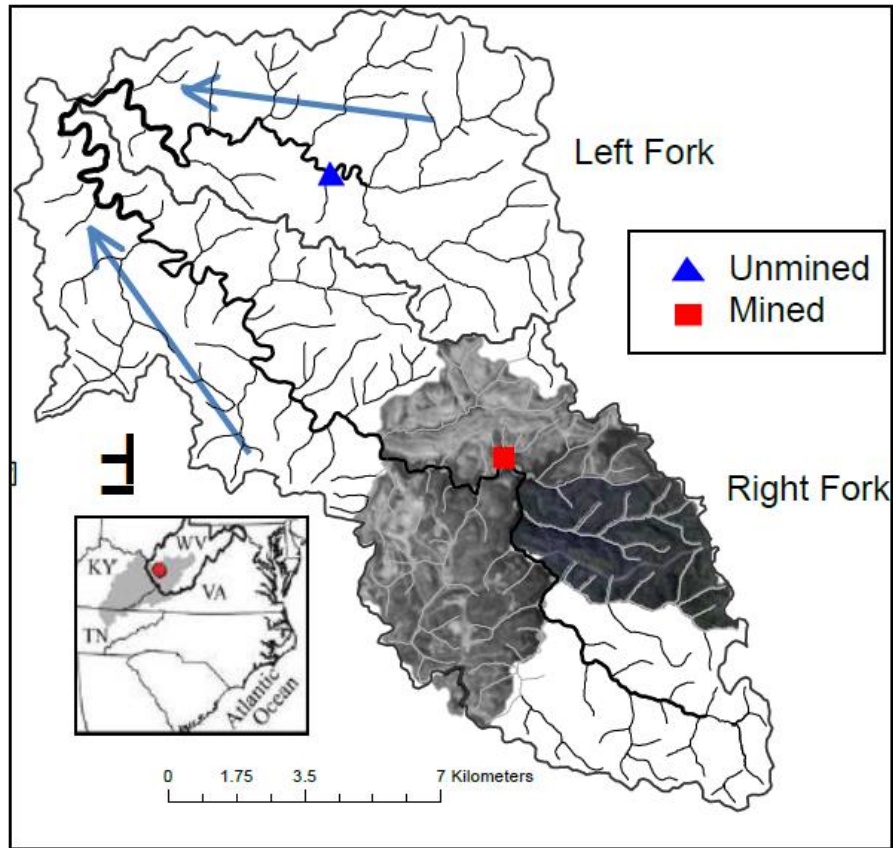
The data shown here indicate that fish can bioaccumulate Se as they feed on the biofilms grown in selenate rich waters. Fish living in the Mud River are at risk from Se toxicity as they feed on natural biofilms and/or on insects that feed on the biofilms. We have previously shown that fish and insects from the same mining-impacted site on the Mud River accumulate elevated concentrations of Se (Arnold et al. 2014). Data from this current study support our hypothesis that this dietary Se exposure could originate in stream biofilms. For example, creek chubs from mined sites contained significantly higher concentrations of selenium in fillet, ovaries, and livers compared to fish from a reference site (Arnold et al. 2014). Creek chubs collected from the Mud River contained biofilm-like materials in their stomachs (unpublished observation), and young creek chubs feed on small macroinvertebrates that were found in the acrylic plate biofilms (Barber et al. 1971, Moshenko et al. 1973). Other common stream fish such as the stoneroller (*Campostoma anomalum*) that feed predominantly on algae may also be at high risk for selenium exposure and toxicity.

Our data suggest that although Se typically accumulates at lower concentrations compared to lentic systems, lotic systems are still at risk for Se toxicity. Moreover, the measurement of waterborne Se concentrations alone is not a good indicator of Se in an aquatic food chain also noted by Hillwalker et al. (2006). Biofilms in complex aquatic ecosystems are important entry points for Se into the aquatic food web. Additional

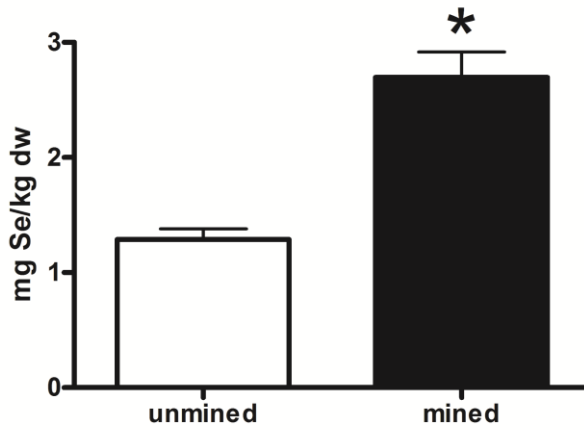
assessments of lotic systems should incorporate biofilms into models of Se fate and transport.



**Figure 17: Movement of selenium through a conceptualized aquatic food chain. Selenium is released into an aquatic habitat from coal and seleniferous rocks as selenate. Biofilms bioaccumulate and biotransform selenate into various selenium species. Organic selenium is accumulated by macroinvertebrates and fish.**

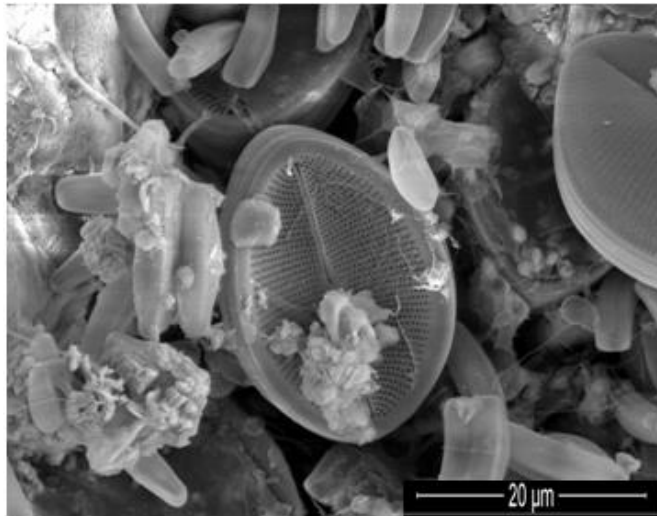


**Figure 18: Mined and unmined sampling sites on Mud River in Boone and Lincoln Counties, West Virginia. Light grey tributary streams run through mined areas, while black tributaries do not. Arrows show flow direction. Inset of US mid-Atlantic states shows Appalachian Coalfield Region as grey shaded area with relative location of study wite in WV in red (not to scale).**

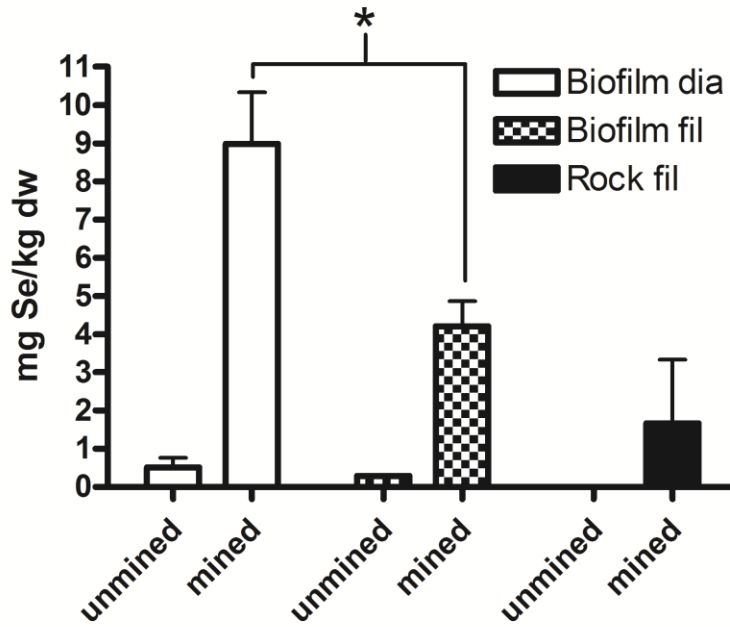


**Figure 19: Concentration of Se (mg/kg dw) in acrylic biofilm scrapings from unmined and mined in the Mud River, WV used to feed fathead minnows in the laboratory (N=22). Bars represent standard errors of the mean. Three replicate experiments are represented in this graph ( $p < 0.0001$ ).**

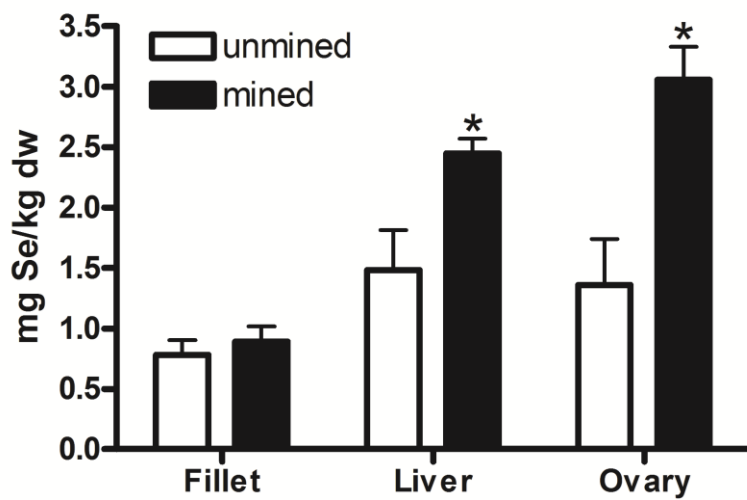




**Figure 20: Image of diatoms (*Cocconeis pediculus*) in biofilms taken from the mined site using scanning electron microscopy. Images were collected by Dr. Wallace Ambrose (Chapel Hill Analytical and Nanofabrication Laboratory, University of North Carolina, Chapel Hill, NC).**



**Figure 21: Mean  $\pm$  standard error of Se (mg/kg dw) in green algae fractions (fil: filamentous algae) versus remaining material (dia: remaining diatom and sediment fraction) in biofilms collected from acrylic plates and rock scrapings at mined and unmined sites in the Mud River, WV (N=3). Asterisk represents significance ( $p < 0.05$ )**



**Figure 22: Mean  $\pm$  standard error of Se (mg/kg dw) in skinless fillet, liver, and ovaries from fathead minnow fed biofilm grown in sites unmined and mined (N=6 except for unmined liver and ovary where N=4). Asterisks represents significant difference ( $p < 0.02$ ).**

## **5. Embryo toxicity of selenium in zebrafish (*Danio rerio*)**

This chapter will be submitted as: Arnold, M.C., Forte, J., and Di Giulio, R.T. The role of oxidative stress in embryo toxicity of selenium compounds in zebrafish (*Danio rerio*)

### **5.1 Introduction**

Selenium (Se) is an essential micronutrient necessary for proper function of important antioxidant enzymes, including glutathione peroxidases (Eisler 2000). However, Se has a narrow margin between essentiality and toxicity, meaning that concentrations above those that are nutritionally beneficial can be significantly harmful (Janz et al. 2010). Oviparous vertebrates including fish, birds, amphibians, and reptiles are sensitive to Se toxicity because Se is readily transferred from mother to embryo through vitellogenin and other egg yolk proteins (Kroll et al. 1991, Unrine et al. 2006, Bergeron et al. 2010). There are several hypothesized mechanisms of Se toxicity, including sulfur substitution, immune system disruption, and oxidative stress (reviewed by Janz et al., 2010). Recently oxidative stress has received more attention as a possible mechanism for the teratogenicity associated with Se exposure.

Some forms of Se, including selenite, are active prooxidants and may combine with glutathione to generate superoxide radicals, indicating that Se toxicity may be a result of oxidative stress (Spallholz 1997). Inorganic forms of Se including selenate or

selenite are the species more commonly found in surface waters contaminated by Se. Inorganic Se can be biotransformed by algae and bacteria into organic forms including selenomethionine, which is considered to be one of the more toxic forms of Se (Maher et al. 2010). There is evidence that selenomethionine, a selenoamino acid, may be transformed by rainbow trout (*Oncorhynchus mykiss*) embryos into methylselenol, which redox cycles with glutathione to produce the superoxide anion radical (Figure 23) (Palace et al. 2004).

The expression of several genes can be induced by exposure to reactive oxygen species (ROS) (reviewed by Di Giulio and Meyer, 2008). For example, ROS have been demonstrated to regulate the expression of glutathione cysteine ligase catalytic subunit (GCLc), glutathione-S-transferase (GST), glutathione peroxidase 1 (GPX1), and manganese superoxide dismutase (MnSOD) (Di Giulio et al. 2008). GCLc is part of a heterodimeric enzyme involved in the synthesis of glutathione (GSH), an important non-enzymatic antioxidant (Franklin et al. 2009). GST pi class 2 (GSTp2) is a cytosolic enzyme important in the phase II detoxification of a variety of xenobiotic chemicals through conjugation with reduced glutathione (Schlenk et al. 2008). GPX1 is an important antioxidant enzyme found in the cytosol and mitochondrial matrix that contains a selenocysteine moiety in its active site and is capable of reducing excess hydrogen peroxide to water and lipid hydroperoxides to their corresponding alcohols (Jones et al. 1981, Hussain et al. 2004). MnSOD is a mitochondrial antioxidant enzyme that

dismutates superoxide to hydrogen peroxide and water (Oberley et al. 1988). An increase in the expression of these genes can indicate a response to elevated cellular ROS production.

It is currently unknown if piscine GCLc, GPX1, MnSOD, or GSTp2 contain antioxidant response elements (ARE), through which a redox-sensitive response in expression can be regulated. However, ARE-like sequences are found in human, rat, and mouse GSTp (Ikeda et al. 2002, Ikeda et al. 2004) and zebrafish GSTp1 contains an ARE-like sequence (Suzuki et al. 2005). Human GCLc can be induced via an ARE sequence (Wild et al. 2000) and human GPx2 and GPx3 contain an ARE (Bierl et al. 2004). Mouse MnSOD also contains an ARE sequence (Jones et al. 1995).

The purpose of this study was to investigate the role of oxidative stress in Se-induced zebrafish (*Danio rerio*) embryo deformities. Zebrafish are highly valuable to toxicological research because of their rapid development, high fecundity, and fully sequenced genome, which provides the opportunity to conduct analysis on changes in gene expression that can provide insight into the mechanism behind Se embryo toxicity. Additionally, previous research has demonstrated that selenomethionine is one of the more toxic forms of Se to newly hatched zebrafish larvae (Niimi et al. 1976). We attempted to induce deformities in zebrafish embryos using aqueous exposures of several Se compounds, including selenate, selenite, and L-selenomethionine (SeMet). N-acetylcysteine (NAC) was used in an attempt to rescue Se-induced deformities. N-

acetylcysteine is an antioxidant shown to increase reduced glutathione (GSH) and scavenge oxidant species (Moldeus et al. 1986). Finally, gene expression analysis was performed on several genes involved in the oxidative stress response.

## ***5.2 Materials and methods***

### **5.2.1 Chemicals**

Sodium selenite and L-selenomethionine were purchased from Acros Organics (Thermo Fisher Scientific, Fair Law, NJ). Sodium selenate was purchased from Alfa Aesar (Ward Hill, MA). N-acetylcysteine was purchased from Sigma-Aldrich, LLC. (St. Louis, MO).

### **5.2.2 Aqueous selenium zebrafish embryo exposures**

Zebrafish adults were placed in breeding chambers overnight and allowed to breed in the morning. Embryos were collected from the breeding chambers and screened for viability and cell stage. Healthy embryos that successfully reached the 8-32 cell stage were selected to use in dosing experiments. Embryos were rinsed with 30% Danieau media before treatment.

Selenium dosing media was made in 30% Danieau media. Zebrafish embryos were treated with control, 1, 50, 100, or 400  $\mu\text{g/L}$  SeMet. In a separate dosing experiment embryos were exposed to control, 30, 45, or 60  $\mu\text{g/L}$  selenate ( $\text{Na}_2\text{SeO}_4$ ) or selenite ( $\text{Na}_2\text{SeO}_3$ ). Three replicate wells were dosed for each treatment and the experiments were

repeated at least twice with the exception of the 30 µg/L treatment. At least three replicate wells were dosed for each treatment and the 1, 50, and 400 µg/L exposures were repeated twice and the 100 µg/L exposures were repeated five times. Zebrafish embryos (12-30) were placed in each well of a 6-well plastic plate with 4 mL dosing media. Dosed plates were stored in an incubator at 28°C.

Embryos were screened for toxicity at 48 hours post fertilization (hpf) using a Nikon SMZ 1500 microscope equipped with a Nikon Sight DS-Fi1 camera and NIS elements F4.00.06 imaging software (Nikon, Melville, NY, USA). Dead embryos were removed from wells. Embryos were counted as “deformed” if they had malformations of the spine (lordosis, kyphosis), pericardial edema, eye malformations, and/or craniofacial deformities.

### **5.2.3 Antioxidant rescue**

Zebrafish embryos were collected and screened as described above. Five to 20 embryos were placed in 5 mL of either 100 µM NAC in 30% Danieau as a pre-treatment or 30% Danieau only as controls in round glass petri dishes. N-acetylcysteine is a sulphhydryl donor and precursor to GSH (Kelly 1998). Treatment with NAC has previously been demonstrated to increase GSH in rabbit hearts and increase expression of GSSG-reductase in rat liver and lung cells, indicating that it can increase GSH regeneration (Deflora et al. 1985, Ceconi et al. 1988). Embryos were placed in an incubator overnight and then rinsed well with 30% Danieau before they were transferred



back into the cleaned glass plates with 5 mL of either 400 µg/L SeMet in 30% Danieau or control media (30% Danieau), creating the following treatments: control, 100 µM NAC, 400 µg/L SeMet, and 100 µM NAC+ 400 µg/L SeMet. Embryos were screened for toxicity as described above at 96 hpf. Each treatment group was replicated three times and the entire dosing experiment was repeated four times.

#### **5.2.4 Quantitative real-time PCR**

Twelve to 30 zebrafish embryos exposed individually to selenate, selenite, or L-selenomethionine in 96-well plates as described above in section 5.2.2 were manually dechorionated using mild suction from a small transfer pipette and stored at -80°C until processing. Embryos were homogenized and RNA was isolated from samples using RNA-Bee/chloroform extraction (Tel-test, Inc, Friendswood, TX) or a Direct-zol RNA MiniPrep kit (Zymo Research, Irvine, CA). cDNA was created using an Omniscript TR kit (Qiagen, Valencia, CA). Quantitative real-time PCR using Sybgreen Lightcycler Master Mix (Applied Biosystems, Foster City, CA) was conducted on an ABI 7300 quantitative real-time PCR machine. A thermal cycle of 10 minutes at 95°C, followed by 35 replicates of 15 seconds at 95°C, 1 minute at 60°C, and finally a dissociation curve. Samples were run in duplicate.

Primers were designed using PrimerQuest software (Integrated DNA Technologies, Inc., Coralville, IA); sequences are shown in Appendix Table 2. Quantitative real-time PCR data were analyzed on the ABI PRISM 7300 Sequence

Detection System, Version 1.1 (Applied Biosystems, Inc., Foster City, CA). Average fold induction of mRNA was determined by comparing the  $C_T$  of the gene to the reference genes  $\beta$ -actin for the selenate/selenite exposures or Eif1b for the SeMet exposures.

### **5.2.5 Statistics**

The proportion of deformed embryos in each treatment was calculated using the number of embryos left alive and deformed in each well. All results are reported as mean  $\pm$  standard error. Statistical analysis was performed using SigmaPlot 12.5 (Systat Software Inc., San Jose, CA) and GraphPad Prism 6 (La Jolla, CA). Selenate, selenite, and SeMet exposure mortality and deformity data (as expressed in proportion dead or deformed) was analyzed using a Kruskal-Wallis one-way analysis of variance (ANOVA) by ranks followed by a multiple comparisons test using Dunn's Method. A two-way ANOVA was performed on the mortality and deformity values for the antioxidant rescue experiments ( $\alpha = 0.05$ ).

A four parameter nonlinear dose response analysis without constraints was used to determine the  $EC_{50}$  for the SeMet exposures. Gene expression  $C_t$  values were also compared using an unpaired t-test. Fold change expression values were calculated by normalizing  $C_t$  values to the control gene (either  $\beta$ -actin or Eif1b) using  $ddC_t$ .  $\beta$ -actin was used as the control gene for the selenate/selenite exposures while Eif1b was used for the SeMet exposures.

## **5.3 Results**

### **5.3.1 Selenium induced deformities in zebrafish embryos**

Mortality was significantly different between treatment groups in the SeMet dosed embryos ( $H=19.384$ ,  $p < 0.001$ ) with 100 and 400  $\mu\text{g/L}$  SeMet exposures resulting in significantly higher mortality compared to controls ( $p < 0.05$ , Figure 24A). Zebrafish embryos exposed to the highest two doses SeMet also exhibited significantly increased incidences of lordosis and craniofacial deformities (Figure 25A-C,  $H = 19.384$ ,  $p < 0.001$ ). For example, at 100 ppb and 400 ppb SeMet,  $56 \pm 36\%$  and  $75 \pm 30\%$  of embryos displayed deformities, respectively, which was significantly higher when compared to a deformity rate of  $0.9 \pm 2.6\%$  in controls ( $p < 0.05$ , Figure 26A). An  $EC_{50}$  value of 84  $\mu\text{g/L}$  was calculated for SeMet for zebrafish embryo deformities (95% confidence interval of 57.33-123.2  $\mu\text{g/L}$ , Figure 27).

No significant differences were found in percent mortality between selenate or selenite treated embryos compared to controls (Figure 24B). Craniofacial and skeletal deformities were occasionally found in zebrafish embryos exposed to aqueous selenate (maximum average  $2.4 \pm 2.4\%$ ) and selenite (maximum average  $2.6 \pm 1.7\%$ , Figure 25D), although the frequencies of these deformities were not significantly different from controls ( $1.2 \pm 0.7\%$ ) at any dose ( $p = 0.73$ , Figure 26B).

### **5.3.2 Antioxidant rescue with N-acetylcysteine**

A statistically significant interaction between SeMet and NAC was observed ( $p = 0.022$ ), meaning that significant differences in the proportion of deformities were observed between embryos exposed to NAC in combination with SeMet versus embryos treated with SeMet alone ( $p < 0.001$ , Figure 28). Pre-treatment with NAC was protective against SeMet induced embryo deformities; embryos pre-treated with NAC showed a frequency of deformity that was indistinguishable from controls while embryos without the pre-treatment exposed to SeMet alone showed an average deformity frequency of almost 50%. No significant difference in deformities was observed between control embryos and NAC only treated embryos. Deformities in the co-exposed NAC + SeMet treatment groups were frequently mild (slight lordosis or craniofacial deformity, small pericardial edema) while the SeMet alone exposed embryos showed deformities ranging from mild to severe (lack of trunk/tail development, significant craniofacial deformity and pericardial edema). No significant differences in mortality were observed between treatment groups ( $p = 0.389$ , Figure 24C).

### **5.3.3 Gene expression**

Gene expression analyses of oxidative stress responsive genes in embryos exposed to 30  $\mu\text{g/L}$  aqueous selenite showed a  $6.6 \pm 1$  fold increase in expression of GSTp2 compared to controls. MnSOD, GPX1, and GCLc expression was not increased in selenite exposed embryos (Figure 29). Embryos exposed to 30  $\mu\text{g/L}$  aqueous selenate did not show changes in expression for any of the genes analyzed. Of the genes tested in

zebrafish embryos exposed to aqueous exposure to SeMet, only MnSOD showed a slight but significant decrease ( $0.67 \pm 0.09$ -fold) in expression in the 50  $\mu\text{g/L}$  treatment group (Figure 30,  $p \leq 0.05$ ).

## **5.4 Discussion**

While we observed no detectable effects of selenite or selenate, Se added as SeMet led to a high frequency and severity of deformities in embryonic zebrafish. Since the detrimental effects of SeMet did not occur with pre-treatment with NAC, we attributed at least some portion of the observed toxicity of this form of Se to oxidative stress. Aqueous exposure to either selenate or selenite did not lead to an increase in craniofacial and skeletal deformities relative to controls. In contrast, aqueous, SeMet exposure did cause a significant number of deformities in zebrafish embryos with a calculated  $\text{EC}_{50}$  concentration of 84  $\mu\text{g/L}$ . A dose-dependent increase in deformities was observed with increasing doses of SeMet. Deformities observed in embryos dosed with 100 or 400  $\mu\text{g/L}$  SeMet were often severe, including a total lack of head development and/or significant truncation of the spine and tail; these embryos did not typically hatch.

Previous evidence exists for the involvement of oxidative stress in Se toxicity. Mallard ducks (*Anas platyrhynchos*) fed SeMet showed a dose-dependent increase in the hepatic ratio of GSSG to GSH and an increase in hydroperoxides that are associated with lipid peroxidation (Heinz et al. 1988, Hoffman et al. 1998). Selenomethionine is oxidized to selenoxide in Japanese medaka embryos, resulting in a depletion of glutathione

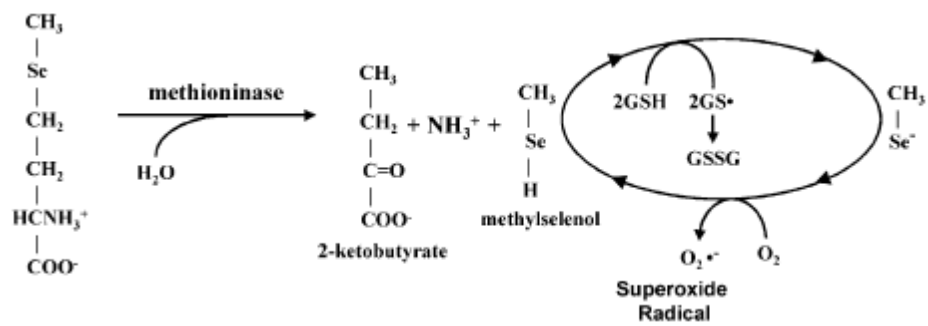
following aqueous exposure (Lavado et al. 2012). Additionally, research shows that rainbow trout (*Oncorhynchus mykiss*) embryos are capable of enzymatically cleaving parentally derived organic Se into metabolites such as methylselenol, which can generate reactive oxygen species, causing oxidative stress (Wang et al. 2002, Palace et al. 2004). Homogenate from rainbow trout embryos was shown to transform SeMet into methylselenol, which can produce superoxide in the presence of GSH (Palace et al. 2004). Rainbow trout exposed to Se develop yolk sac and pericardial edema, and these forms of edema may be caused by oxidative stress (Palace et al. 2004, Bauder et al. 2005).

While only SeMet generated deformities, we found that it was only selenite that led to significant changes in gene expression profiles. Aqueous exposure to selenite caused an approximately 6.6-fold increase in the expression of glutathione-S-transferase pi class 2, a gene involved in the xenobiotic detoxification process. Although it is not currently known whether zebrafish GSTp2 contains an antioxidant response element (ARE), zebrafish GSTp1, which shares significant homology with GSTp2, contains an ARE-like sequence that is regulated by Nrf 2 (Suzuki et al. 2005), indicating that GSTp2 expression may be sensitive to changes in redox status. The expression values of other oxidative stress response genes did not show significant changes compared to controls for aqueous selenate, selenite, or SeMet with the one exception of SeMet exposure eliciting a small decrease in expression of MnSOD for embryos exposed to 50 µg/L SeMet. Limited

previous data exist for changes in MnSOD expression in fish exposed to Se. MnSOD expression in macrophages and rat hepatocytes varied with selenite exposure depending on the tissue type and species tested (Shilo et al. 2004, Shilo et al. 2008).

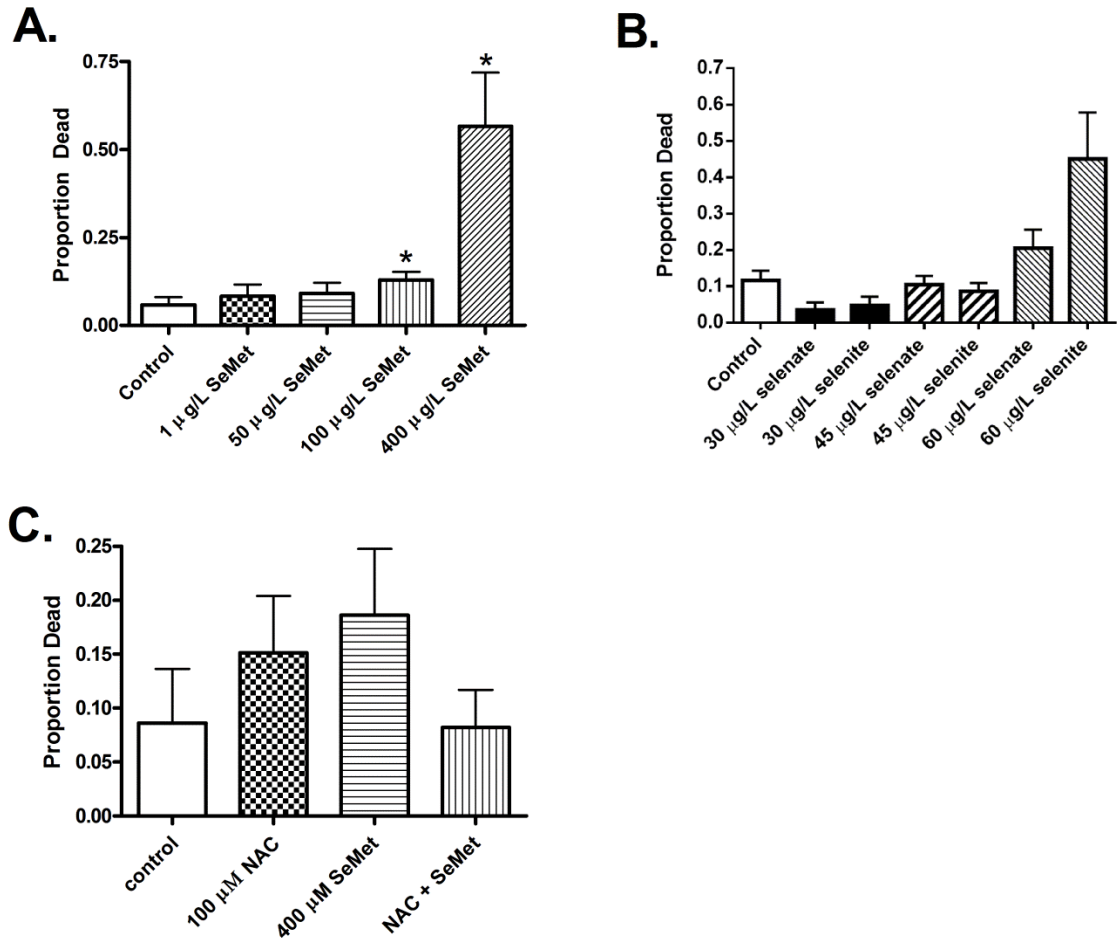
The evidence for the role of oxidative stress in Se-induced embryo toxicity however remains conflicted. Kupsco et al. (2014) found no evidence of lipid peroxidation in Japanese medaka exposed to aqueous selenomethionine and hypersalinity, and they suggest that other mechanisms such as activation of the unfolded protein response may play a role in embryo toxicity. These data in addition to the results presented here suggest that Se induced embryo toxicity may be caused by multiple mechanisms including substitution-based protein malfunction, oxidative stress, unfolded protein response, and possibly immune dysfunction (reviewed by Janz et al., 2010).

Our NAC rescue results suggest, however, do support the hypothesis that oxidative stress plays a role in SeMet-mediated deformities, that could be deleterious to vulnerable fish populations inhabiting Se contaminated ecosystems. There is considerable previous research demonstrating that Se exposure can cause significant developmental defects in fish embryos (Teh et al. 2004, Holm et al. 2005, Muscatello et al. 2006), and oxidative stress may be an important mechanism behind this teratogenicity.

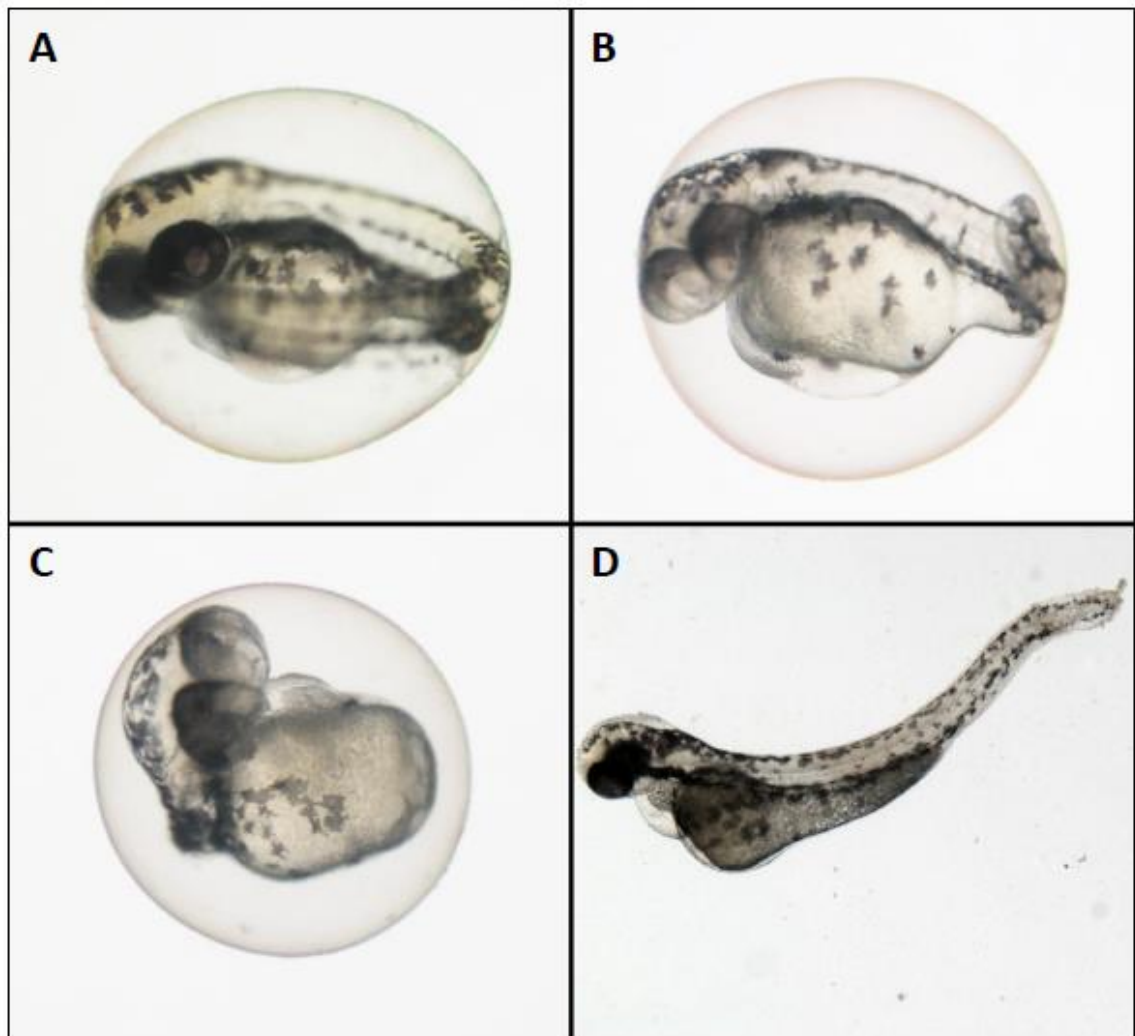


**Figure 23: Proposed mechanism for the production of superoxide radical from selenomethionine by methioninase enzyme activity in rainbow trout embryos (GSH, reduced glutathione, GSSG, oxidized glutathione). Reprinted with permission from Palace et al. (2004).**

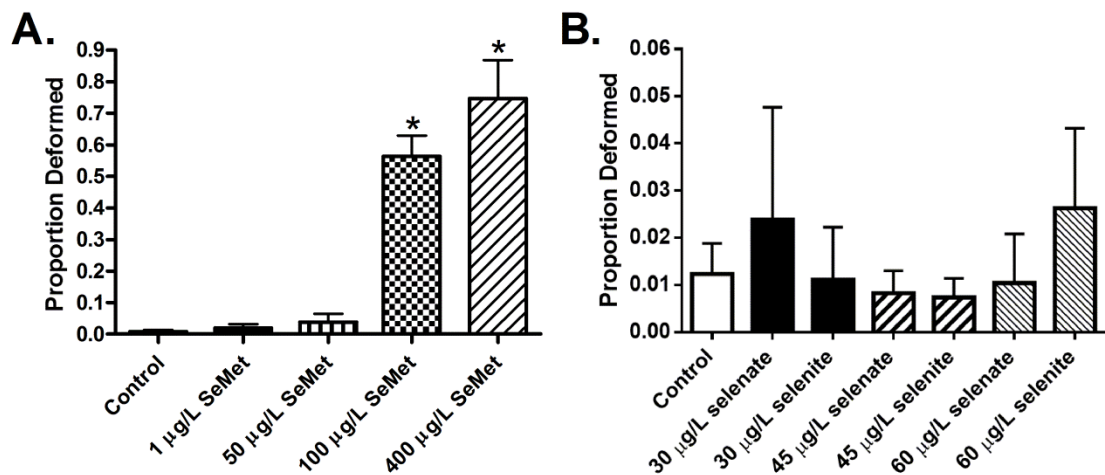




**Figure 24: Proportion dead in embryos treated with A. L-selenomethionine (SeMet), B. selenate or selenite, and C. N-acetylcysteine (NAC) or SeMet combinations. Asterisks represent significant differences from control ( $p < 0.05$ )**



**Figure 25: Zebrafish (*Danio rerio*) embryos A. control (48 hpf) B. 100 µg/L L-selenomethionine (48 hpf) exposed embryo with raniofacial and tail deformities with pericardial edema. C. 100 µg/L L-selenomethionine (48 hpf) exposed embryo with significant deformity. D. 30 µg/L selenite (72 hpf) exposed embryo with lordosis and pericardial edema.**



**Figure 26: Proportion deformed zebrafish embryos with aqueous exposure to either A. L-selenomethionine or B. selenate or selenite. Note differences in Y-axis scales. Bars represent standard error. Asterisks represent significant differences from controls ( $p < 0.05$ ).**

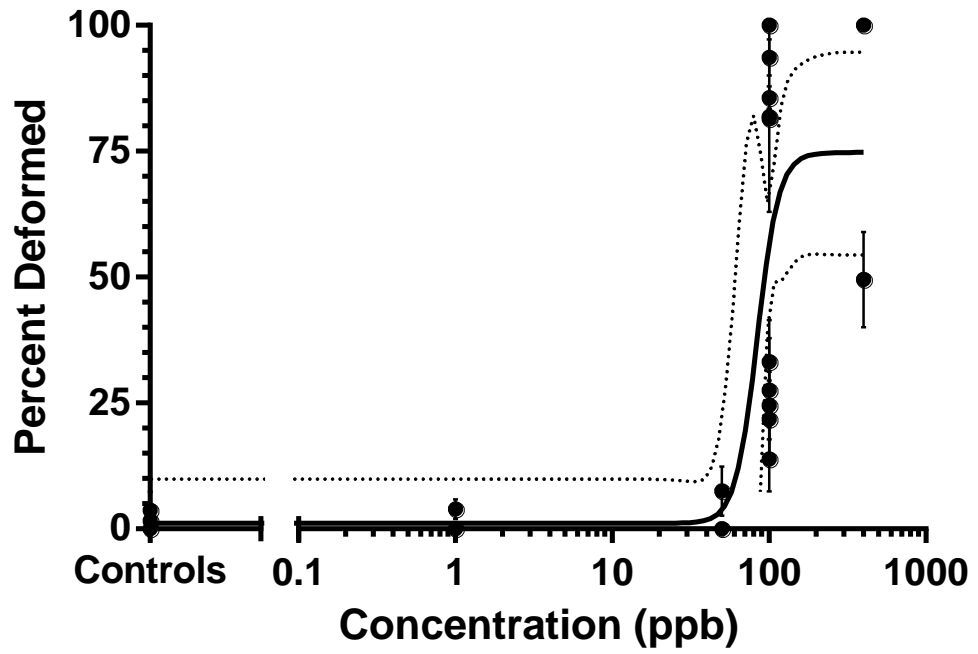
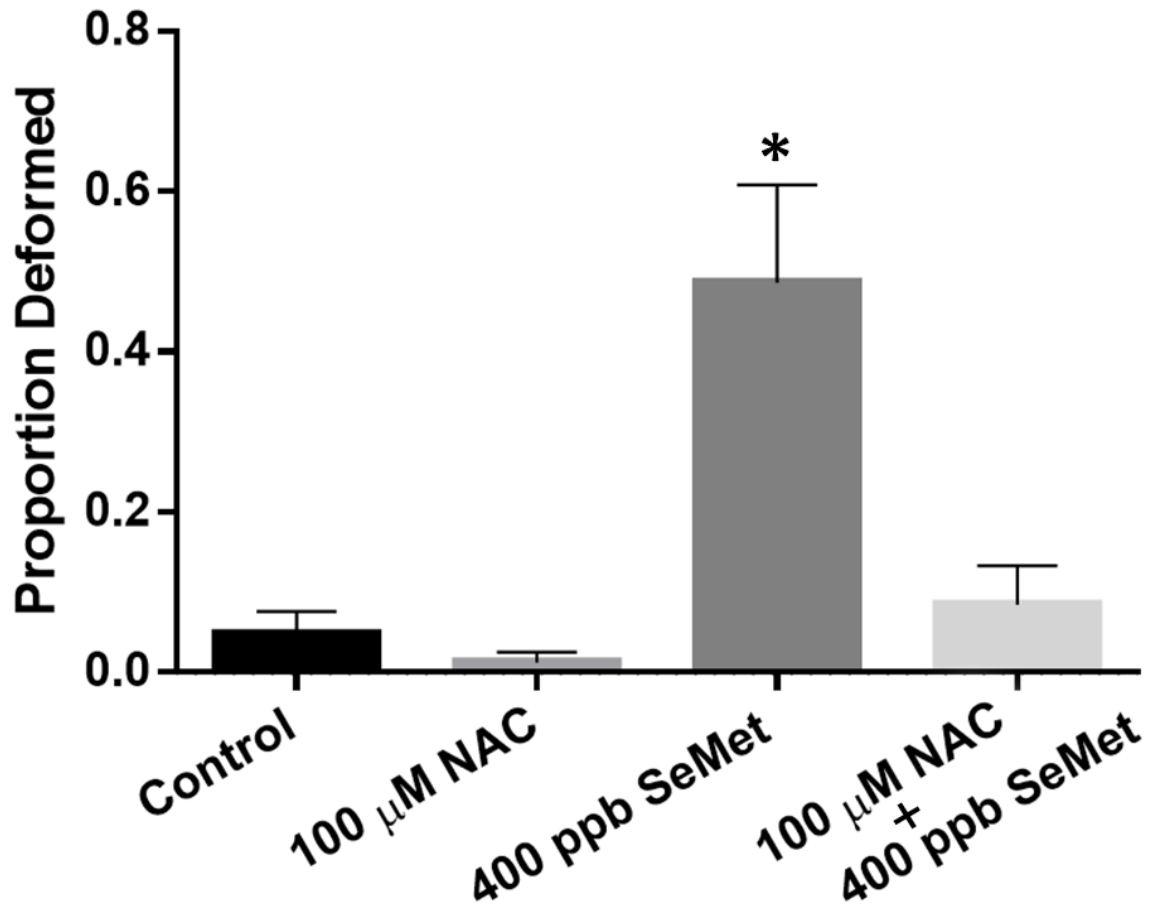
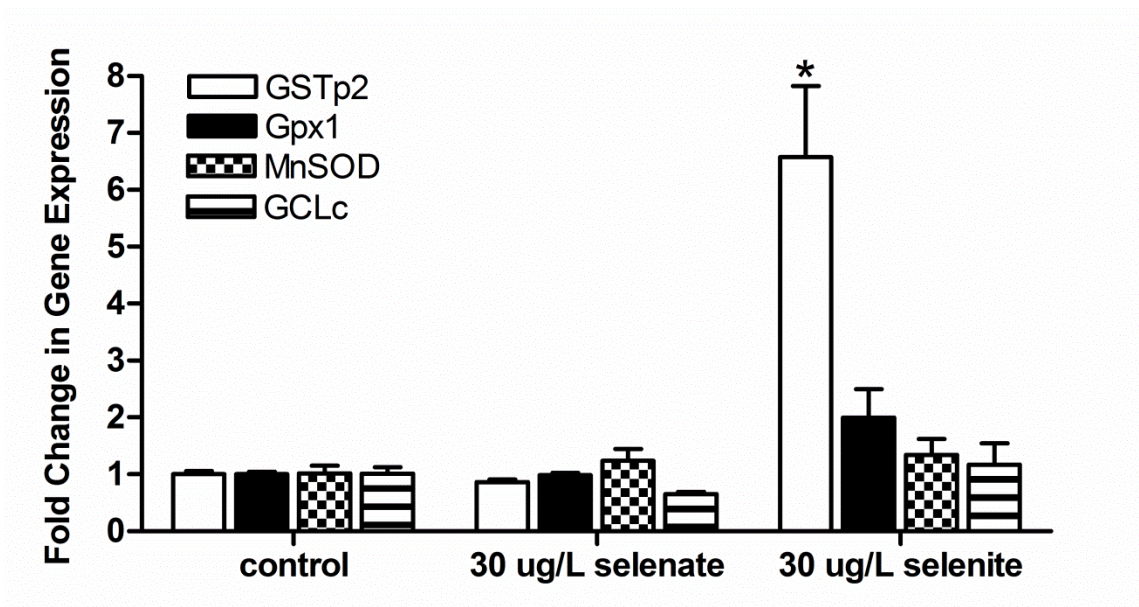


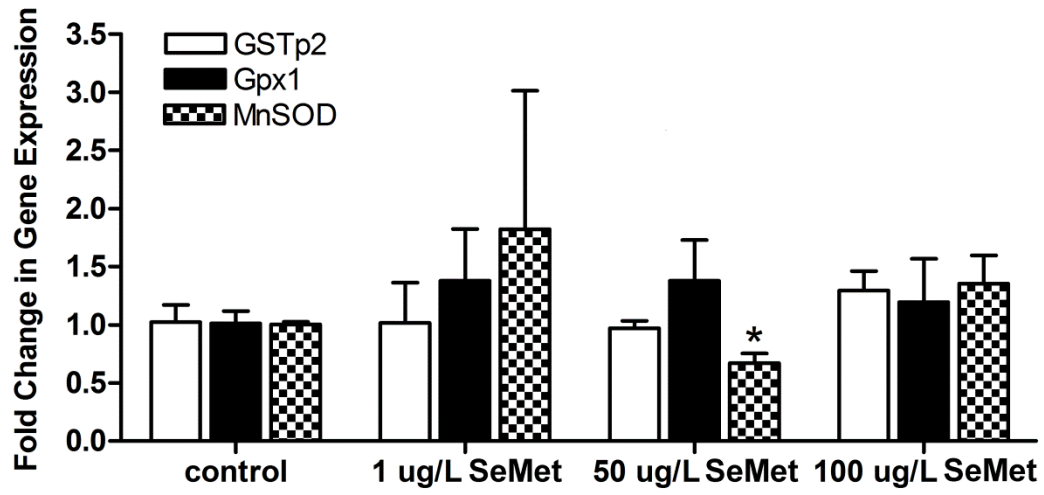
Figure 27: Dose response curve for deformities induced in zebrafish embryos by L-selenomethionine. Dotted lines represent the 95% confidence interval. Calculated EC<sub>50</sub> = 84 µg/L.



**Figure 28: Proportion deformed in control, N-acetylcysteine only (NAC), L-selenomethionine (SeMet), or NAC + SeMet treated zebrafish embryos. Bars represent standard error. Asterisk represents significant difference between SeMet treated embryos and NAC + SeMet treated embryos ( $p < 0.001$ )**



**Figure 29: Fold change in gene expression for embryos treated with control, selenate, or selenite aqueous exposures. Asterisk indicates significance ( $p \leq 0.05$ ) from respective control. Bars represent standard error.**



**Figure 30: Fold change in gene expression for embryos treated with control or L-selenomethionine (SeMet) aqueous exposures. Asterisk indicates significance ( $p \leq 0.05$ ) from respective control. Bars represent standard error.**

## **6. Summary and Future Directions**

### ***6.1 Summary***

In this dissertation I examined the fate and environmental impacts of selenium released from MTR/VF coal mining. Selenium enriched effluent from local MTR/VF coal mining operations has been released into the main stem of the Mud River since the 1970's (Lindberg et al. 2011, Arnold et al. 2014). Selenium is a naturally occurring element that is required for the function of several key enzymes in animals, including glutathione peroxidases and thyronine deiodinases (Flohe et al. 1973, Arthur et al. 1990). At elevated concentrations, however, selenium is toxic. Oviparous vertebrates such as fish and birds are sensitive to selenium, which can cause teratogenesis and reproductive failure (Ohlendorf et al. 1988, Janz et al. 2010).

Four major questions about the fate and toxicity of selenium from mountaintop removal/valley fill coal mining effluent were addressed in this dissertation: (1) how much and in what form of selenium is bioaccumulating in wild Mud River fish; (2) what is the historical exposure of selenium in Mud River fish as described by otolith and muscle concentrations and are there species differences; (3) do biofilms from the Mud River accumulate selenium and can this selenium be transferred to fish via diet; and (4) does oxidative stress play a role in selenium-induced embryo toxicity? To answer these questions I used a combination of field and laboratory research. Field samples including water, insects, biofilms, and fish were collected for selenium concentration and speciation



analysis. Laboratory fish models including zebrafish and fathead minnows were used to model trophic transfer of selenium as well as to investigate the mechanism(s) behind the deformities commonly found in embryos exposed to selenium.

As described in Chapter 2, I collected water, composite aquatic insect samples, and two species of fish from the main stem of the Mud River (mined) and the Left Fork Mud River (unmined reference site) to assess the status of selenium in the ecosystems. Water quality analysis showed significant increases in both conductivity and concentrations of selenium at the mined sampling site compared to the reference site, indicating significant degradation at the mining-impacted site. Insect samples also showed significantly higher concentrations of selenium at the mined site versus the reference site. Both the green sunfish (*Lepomis cyanellus*) and creek chub (*Semotilus atromaculatus*) collected from the mined site contained significantly higher concentrations of selenium in skinless fillet samples compared to reference site fish. Creek chubs from the mined site also had significantly higher selenium in ovary and liver tissues. A similar trend was observed in green sunfish although the differences were not significant possibly due to higher variability and lower sample size. These data are highly valuable for estimating selenium contamination in lotic systems, which are often considered to be less impacted by selenium compared to lentic systems.

Fish were also collected from Big Ugly Creek, which has recently been permitted for surface coal mining (Figure 31 A-C). Conductivity in the surface waters of Big Ugly

Creek was measured as high as 640  $\mu\text{S}/\text{cm}$ , which indicates potential water quality degradation due to surface mining (unpublished data). Both creek chubs and green sunfish collected from Big Ugly contained selenium concentrations similar to LFMR fish (Figure 31). It is noteworthy, however, that Big Ugly green sunfish livers (N=2) contained similar concentrations of selenium compared to MR7 green sunfish livers ( $21.0 \pm 2$  and  $23.1 \pm 16$  mg/kg dw, respectively) and that the one Big Ugly green sunfish ovary analyzed contained a high concentration of selenium (19.5 mg/kg dw) compared to even MR7 green sunfish ( $10.4 \pm 3$  mg/kg dw).

X-ray absorption near edge spectroscopy analysis revealed that insect samples contained Se-methionine and Se-cystine, similar to fish liver and ovary samples, but insects also contained higher fractions of inorganic selenium species (selenite and selenate). Interestingly, histological analysis of mined site creek chub gills showed fewer parasites and aneurysms compared to reference site creek chubs, a result that I hypothesize may be caused by poorer water quality at the mined site, which have previously been demonstrated to cause decreases in parasite populations.

Since soft tissue analysis only indicates relatively recent selenium exposure, I used otoliths from the mined and reference site fish, in addition to fish from a recently mined site (Big Ugly Creek), in an effort to understand long-term selenium exposure and to determine if otolith concentrations relate to muscle concentrations in two fish species exposed to MTR/VF coal mining effluent (Chapter 3). The results showed that otolith

selenium accumulation patterns were different between the two species of fish and that otolith selenium concentrations tended to increase with increasing muscle selenium concentrations. In contrast, no such relationship was found for green sunfish, which typically had very low otolith selenium concentrations even when muscle selenium concentrations were elevated. It is possible that species differences in otolith deposition, selenium metabolism, or length of stay in the selenium enriched environment caused the disparities in otolith selenium accumulation between green sunfish and creek chubs.

Otolith analysis also revealed that mined site creek chubs often contained selenium in the otolith primordia, indicating that these fish are receiving selenium via maternal transfer. The regression data presented in Chapter 3 are valuable because they allow researchers to estimate muscle concentrations throughout the lifetime of the fish, not just the concentration when the fish was captured, which is important when estimating lifetime impacts of selenium exposure.

Next, as described in Chapter 4, I collected biofilms from the main stem and Left Fork Mud River and fed them to fathead minnows (*Pimephales promelas*) in an effort to model the trophic transfer of selenium in a lotic system. Microbially mediated transformations of selenium also play an important role in selenium bioavailability and cycling. Many species of bacteria, fungi, plant, and algae present in biofilm material commonly found in streams can accumulate and metabolize selenium into forms that can increase or decrease bioavailability (Stolz et al. 2006). Higher trophic organisms such as

fish are exposed to selenium predominantly through diet (Hamilton 2003). Organic selenium compounds are known to be significantly more bioavailable to higher organisms than inorganic forms such as selenate or selenite (Schrauzer 2000).

Biofilms from the mined site contained significantly higher concentrations of selenium compared to reference biofilms. Mined biofilms were composed of a variety of diatom species as well as filamentous green algae. When separated into two components, green algae from the mined site contained approximately half of the concentration of selenium compared to the remaining diatom fraction. Fathead minnows fed on the mined biofilms contained significantly more selenium in ovary and liver tissues compared to reference site fed biofilms. Our results showed that selenium can rapidly transfer through a simulated food chain, and indicate that more research is necessary to determine the role of microorganisms and algae in the bioaccumulation of selenium in an aquatic food chain.

Finally, I used aqueous exposures of various chemical species of selenium in zebrafish exposures in an effort to understand the potential role of oxidative stress in selenium-induced embryo toxicity (Chapter 5). Selenate and selenite aqueous exposure did not cause significantly higher incidences of deformities compared to controls. Selenomethionine, however, caused significantly increased numbers of embryo deformities and an  $EC_{50}$  value for deformities was calculated at approximately 84  $\mu\text{g/L}$ . Rescue of these selenomethionine-induced craniofacial and skeletal deformities with the

antioxidant N-acetylcysteine was successful, indicating that oxidative stress may play a role in selenium-induced deformities.

Expression analysis glutathione-S-transferase pi class 2, glutathione peroxidase 1, manganese superoxide dismutase, and glutathione cysteine ligase 1 in general revealed no differences between controls and selenate or selenite exposure with the exception of GSTp2, which was elevated in embryos exposed to 30 µg/L aqueous selenite. Gene expression of GSTp2, MnSOD, GPx1, and GCLc also remained unaltered for embryos exposed to L-selenomethionine. Although success of antioxidant rescue of selenium-induced deformities suggests that oxidative stress may play a role in selenium-induced embryo deformities, further work is necessary to clarify the role of this mechanism. Moreover, other mechanisms have been proposed that also merit study as reviewed by Janz et al., 2010.

## ***6.2 Future Directions***

Although this dissertation attempted to examine the fate and impacts of selenium released from MTR/VF coal mining on aquatic ecosystems, the questions of how selenium speciation changes through a food chain and the ultimate impact of that selenium on fish reproduction and survival were not fully explored. Limitations in resources as well as difficulties in sample preparation meant that only a small fraction of samples could be analyzed for selenium speciation. Although several attempts were made, biofilm samples could not be accurately analyzed using XANES techniques. I

hypothesize that the failure of the XANES technique for the biofilm samples was due to a large fraction of sediment versus organic material, which interfered with the beam line. Fractionation of the samples to remove sediment was not possible due to extremely limited sample size. Future attempts should collect large masses of biofilm materials and fractionate the samples in order to remove contaminating sediments.

Histological analysis of gill tissues from creek chubs at the mined site suggested at a lack of normal parasite infection. These results hint at a possible decrease in parasite populations but the difficulties in collecting samples and the resource-intensive nature of histology hindered further analysis. A more intensive survey of fish gills combined with data on mussel populations, which act as intermediate hosts for many fish parasites, would help provide more insight into the subtle effects of degraded water quality on parasites, which are less visible but important parts of aquatic ecosystems.

In order to address the question of how fish migration and differences in selenium uptake between species I used otolith analysis to determine lifetime exposure in Mud River creek chubs and green sunfish. Otolith LA-ICP-MS analysis is a powerful tool because it can provide a historical record of exposure because selenium is incorporated into the otolith matrix as the fish grows. In the otolith study described in Chapter 3, I compared otolith selenium concentrations to muscle Se concentrations. While the data provided insight into species differences, it would have been more useful from a regulatory standpoint to compare the otolith concentrations to the levels of selenium

found in the ovary. Relationships between ovaries and otoliths could provide valuable information on historical selenium exposure as related to reproductive consequences. This was not possible due to the limited number of female fish with mature ovaries captured at the mined site.

Fish are economically valuable, highly visible organisms that have rightly received significant attention for selenium toxicity studies. However, when we started to collect biofilm samples it quickly became apparent that biofilms may be very important in controlling the fate and bioavailability of selenium in the Mud River. Increased attention should be given to the lowest organisms on the food chain because as our results indicate, they may act as gatekeepers for selenium as higher organisms eat the biofilms. There would likely be significant value in better characterizing the biofilm components (e.g. diatom species, percent sediment, etc.), the partitioning of selenium in these components and how the biofilms differ between mined and unmined sites.

It was surprising to us that the Left Fork Mud River biofilms, collected from a site shown to have little water column selenium, sometimes contained similar concentrations of selenium to the biofilms collected from the mining impacted site. It is possible that biofilms can accumulate even small amounts of selenium, meaning they act as sinks for selenium that could then potentially enter into the food chain. Also, as mentioned previously, determining selenium speciation in biofilms would be particularly valuable because if the biofilms are accumulating inorganic forms of selenium such as selenate,

which is the dominant form of selenium released into the Mud River, and transforming it into the highly bioavailable organic forms such as selenomethionine, this could increase the toxicity of Se-enriched effluent in an aquatic ecosystem. Data from laboratory dosed biofilms suggest that this transformation from inorganic to organic selenium does occur (Conley et al. 2013).

In an attempt to investigate trophic transfer of selenium from biofilms to fish from a more mechanistic standpoint I fed laboratory-spiked biofilms to zebrafish. The biofilms were incubated with selenate and then fed to adult zebrafish females for approximately one week. Embryos were collected, screened for deformities, and tissues were analyzed for selenium uptake. Initial analysis of the tissues, however, revealed little to no uptake of selenium in the adult zebrafish. The failure of this experiment highlights the difficulties in balancing the needs of the experimenter (i.e. a model that has a variety of genetic tools available like the zebrafish) with the model's basic biology, (the fact that zebrafish do not readily eat biofilms because they are surface feeders). The lack of selenium uptake by the adult females likely caused a lack of developmental deformities predicted but not observed in the embryos.

In order to discover if oxidative stress played a role in selenium-induced embryo toxicity, I used aqueous selenium exposures in zebrafish embryos. Aqueous exposures were chosen for ease and speed, because large numbers of embryos could be exposed as opposed to dietary exposures that take a large amount of time, space, adult fish, and



sometimes the adults do not produce enough embryos at the end of the experiment. Unfortunately, the aqueous route of exposure is limited because certain species of selenium do not seem to easily cross the chorion and this route of exposure is less environmentally relevant than dietary exposures. Experiments using selenium-spiked diets fed to adults and examining effects in offspring would better reflect realistic environmental conditions. Some evidence, however, has suggested that increased salinity can increase aqueous selenium toxicity in medaka (*Oryzias latipes*) embryos (Kupsco et al. 2014), indicating that the aqueous route of exposure may be important under certain environmental conditions. The pursuit of research into the mechanism behind selenium toxicity would likely be better in a fish model—such as the medaka—that can tolerate a wide range of environmental conditions that would be favorable to selenium crossing the chorion.

One issue regarding selenium-induced embryo toxicity I found was a lack of consistency in embryo response to selenium. It was quickly apparent that sometimes aqueous selenomethionine exposure caused a large number of deformities while other exposures at the same concentrations induced only mild deformities. Although significant effort was made to ensure consistency in exposure protocols, it could be that slight differences in exposure water conditions or embryonic stages at time of exposure could change the observed toxicity. Interestingly, I observed what appeared to be differences in sensitivity between embryos that came from different groups of adult fish, one of which I

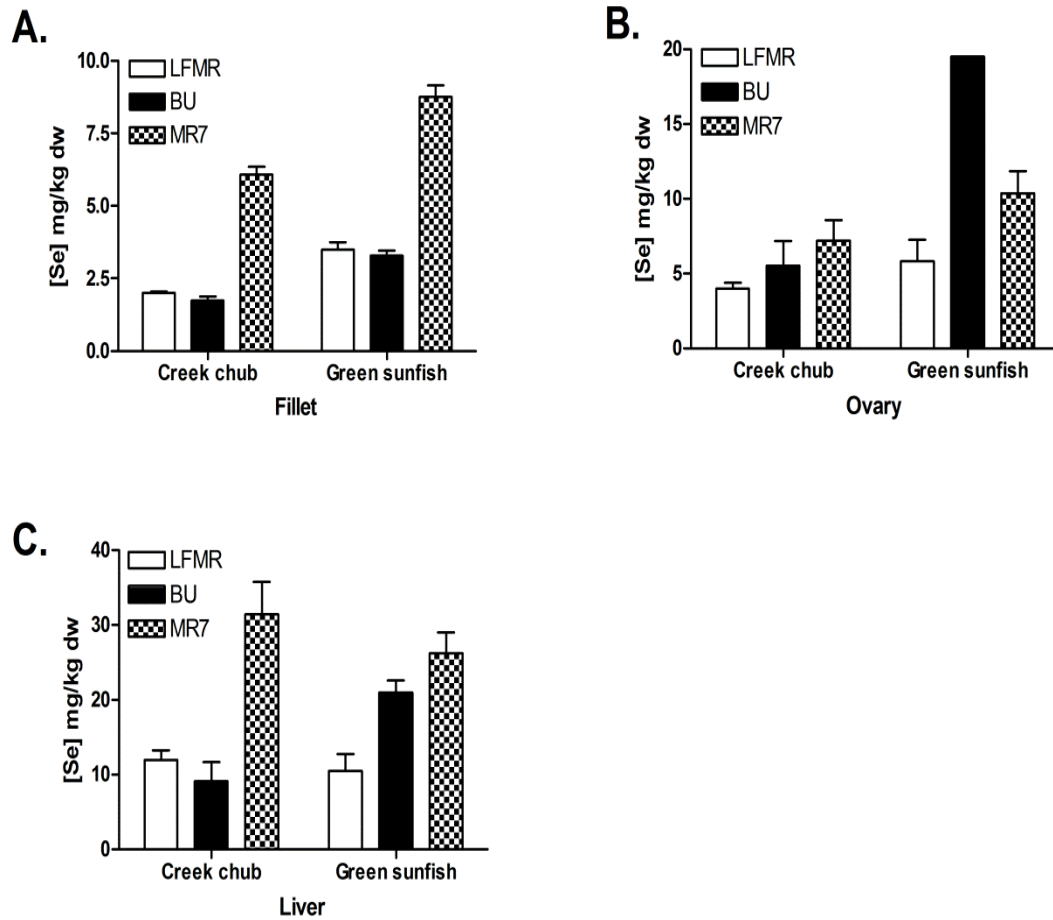
found out later was likely immunocompromised. A more detailed analysis should be conducted on differences in genetic strain sensitivity to selenium and how changes in exposure protocols, including stage of exposure, could influence selenium toxicity.

Gene expression analysis can be a useful tool for determining which mRNAs are up- or down-regulated when an organism is exposed to an environmental contaminant, which suggests possible mechanisms for observable toxicity. In Chapter 5, I attempted to use gene expression analysis targeted at several genes involved in the oxidative stress response. As microarrays decrease in cost and alternative technologies such as serial analysis of gene expression become more widely available, it will become easier to analyze entire genomes for changes in expression. These new technologies will be especially helpful in determining mechanisms behind Se-induced embryo toxicity, which is likely due to a combination of effects including substitution-based protein malfunction, oxidative stress, and possibly immune dysfunction (reviewed by Janz et al., 2010).

In conclusion, this dissertation described the bioaccumulation, fate, and toxicity of selenium, a contaminant of concern released in effluent flowing from MTR/VF coal mining operations. Both laboratory and field based techniques were used to describe species differences in selenium accumulation as well as how selenium would move through a simulated food chain from biofilms to fish. Finally, zebrafish embryos were used in an effort to explore oxidative stress as a mechanism for selenium-induced toxicity.

Over the course of my doctoral research I learned several key facts from these data: 1) choice of tissue, species, and exposure route are very important when designing selenium toxicity studies and argue for at the least tissue specific criteria to protect fish in vulnerable aquatic ecosystems; 2) lotic systems are still capable of accumulating toxic forms of selenium in an aquatic food chain despite significant water and sediment flow; 3) less visible organisms such as algae may be important for selenium cycling in an aquatic ecosystem; and 4) more work must be done to clarify the role of oxidative stress in selenium teratogenicity. The findings described in this work contribute to the limited knowledge base on the environment impacts of selenium in a lotic system and these data are important because they can help guide policymakers who set selenium standards that are protective of aquatic life. Although the methodologies described here represent a broad range of techniques in selenium research, the variety of field and laboratory techniques allowed me to explore a greater range of selenium toxicity research.

These data also helped generate several new questions that should be pursued to help fill the large data gaps concerning selenium toxicity, including: does water quality degradation from mining impact parasite populations in the Mud River? What species of selenium are present in biofilms? What forms of selenium are found in various compartments of biofilms? Is there evidence for oxidative stress in embryos exposed to selenium via maternal exposure? These questions and more represent future work that can be pursued in the selenium field of research.



**Figure 31: Selenium (mg/kg dw) in creek chubs and green sunfish from LFMR, MR7, and Big Ugly. A. Skinless fillet. B. Ovary. C. Liver. Bars represent standard error. Figure adapted from Arnold et al. (2014).**

## Appendix

**Table 1: Linear combination fitting results for Se K-edge XANES spectra of MR7 fish and insect tissues. The data show the proportion (in units of mol%) of the reference spectra that resulted in the best fit to the sample data.†**

Sample	Adsorbed Se(IV)†	Adsorbed Se(VI)†	Methyl-Se-cysteine	Se-methionine	Se-cystine	<i>R-factor</i> ( $\times 10^4$ )*
MR7 insects 1	27 ± 1	10 ± 1		40 ± 2	23 ± 2	1.5
MR7 insects 2	30 ± 6	10 ± 1		18 ± 4	42 ± 4	2.4
MR7 insects 3	35 ± 1	10 ± 1		46 ± 5	9 ± 5	2.8
MR7 84 fillet	38 ± 2	14 ± 1		40 ± 10	8 ± 11	11.4
MR732 fillet	9 ± 1		69 ± 5	22 ± 5		2.5
MR733 fillet	8 ± 1		68 ± 5	24 ± 5		3
MR732 liver	12 ± 1		30 ± 4	19 ± 3	39 ± 2	1.7
MR7 84 liver	24 ± 1	5 ± 1		28 ± 2	43 ± 2	0.8
MR7 81 liver	20 ± 1	4 ± 0		33 ± 2	43 ± 2	0.6
MR733 liver	5 ± 1	3 ± 4		22 ± 3	70 ± 3	3.1
MR7 84 ovary	22 ± 1	6 ± 1		27 ± 1	45 ± 1	0.8
MR733 ovary	17 ± 1		50 ± 4	33 ± 4		2.5

† Mean ± standard deviation. Weighting factors on each fit summed to 100 ± 1 mol% and were normalized to 100%. Although seven Se species were used as end-members in LCF analyses, for all samples the best fits were obtained using the combination of adsorbed selenite [Se(IV)], adsorbed selenate [Se(VI)], methyl-Se-cysteine, Se-methionine, and Se-cystine.

‡ Selenite and selenate adsorbed on poorly crystalline aluminum hydroxide at pH 7.

\* Normalized sum of the squared residuals of the fit ( $R\text{-factor} = \frac{\sum(\text{data}-\text{fit})^2}{\sum \text{data}^2}$ ).



**Figure 32: Biofilm rig used to collect biofilms from the Mud River, West Virginia**

**Table 2: Primers used for zebrafish (*Danio rerio*) quantitative real time polymerase chain reaction assay**

B-actin	Forward	5'-AAG ATC AAG ATC ATT GCT CCC-3'
	Reverse	5'-CCA GAC TCA TCG TAC TCC T-3'
Eif1b	Forward	5'-GCC TTC AAG AAG AAA TTT GCC-3'
	Reverse	5'-CCG TGG ACT TTG AGC TG-3'
GSTp2	Forward	5'TCT GGA CTC TTT CCC GTC TCT CAA-3'
	Reverse	5'-ATT CAC TGT TTG CCG TTG CCG-3'
MnSOD	Forward	5'-CTA GCC CGC TGA CAT TAC ATC-3'
	Reverse	5'-GAG CGG AAG ATT GAG GAT TG-3'
GPX1	Forward	5'-AGA TGT CAT TCC TGC ACA CG-3'
	Reverse	5'-AAG GAG AAG CTT CCT CAG CC-3'
GCLc	Forward	5'-AAG TGG ATG AGG GAG TTT GTT GCC-3'
	Reverse	5'-CTT GTG GAG CAG GTC GTA GTT GAT-3'

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## Biography

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### Publications:

**Arnold, M.C.**, Lindberg, T.T., Bier, R.L., Bernhardt, E., and Di Giulio, R.T. Biofilm mediated uptake and trophic transfer of selenium to fathead minnows. (Submitted) *Freshwater Science*.

Matson, C., Auffan, M., Lindberg, T., **Arnold, M.**, Hsu-Kim, H., Wiesner, M., Di Giulio, R. (in review) Silver toxicity across salinity gradients: The role of dissolved silver chloride species ( $\text{AgCl}_x$ ) in Atlantic killifish (*Fundulus heteroclitus*) and medaka (*Oryzias latipes*) early life-stage toxicity. *Environmental Toxicology and Chemistry*.

**Arnold, M.C.**, Lindberg, T.T., Liu, Y.-T., Porter, K.A., Hsu-Kim, H., Hinton, D.E., Di Giulio, R.T. (2014) Bioaccumulation and speciation of selenium in fish and insects collected from a mountaintop removal coal mining-impacted stream in West Virginia. *Ecotoxicology*, 23: 929-938.

Turner, E.A., Kroeger, G.L., **Arnold, M.C.**, Thornton, B.L.M., Di Giulio, R.T., Meyer, J.N. (2013) Assessing different mechanisms of toxicity in mountaintop removal/valley fill coal mining-affected watershed samples using *Caenorhabditis elegans*. *PLoS One*, 8: e75329. Available online <http://www.plosone.org/article/info%3Adoi%2F10.1371%2Fjournal.pone.0075329>.

Auffan, M. Matson, C. Rose, J. **Arnold M.**, Proux, O., Fayard, B., Chaurand, P., Wiesner, M.R., Bottero, J.-Y., Di Giulio, R. (2013) Salinity-dependent silver nanoparticle uptake and biotransformation in Atlantic Killifish (*Fundulus heteroclitus*) embryos. *Nanotoxicology*, in press: 1-10.

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*Caenorhabditis elegans*. *Archives of Environmental Contamination and Toxicology*. 65: 224-233.

Gelsleichter, J., Szabo, N.J., **Arnold, M.** (2009) Ecoestrogen exposure and effects in the tidal Caloosahatchee River. *Florida Scientist* 72:325-345.

#### Awards and Scholarships

- EPA STAR 2014 Fellowship Grant Finalist (Awarded but grant declined)
- Student Platform Presentation Award, 3<sup>rd</sup> place, Carolinas Society of Environmental Toxicology and Chemistry (CSETAC) Meeting 2013, Raleigh, NC
- Student Poster Award, 2<sup>nd</sup> place, CSETAC 2011, Boone, NC.
- SETAC Student Travel Award, 2010, Portland, OR
- James B. Duke Fellowship, Duke University, 2009-2013
- Chancellor's Scholarship, Duke University, 2009
- Fulbright Scholarship: Malaysia, Universiti Malaysia Terengganu, 2008-2009, Kuala Terengganu, Malaysia.