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In February of 2014, a coal ash spill occurred in the Dan River in Eden, NC. Coal ash is a potential sulfur and heavy metal source and can disrupt aquatic and riparian food webs. Sulfur can stimulate mercury methylation, which bioaccumulates in the food web, threatening human and wildlife health. This study aimed to determine if dominant river and riparian invertebrates assimilated coal ash derived sulfur in relation to distance from the spill using stable isotopes of sulfur and carbon. Sulfur $\delta^{34}S$ analysis showed that approximately 1.5 years after the spill, riparian spiders downstream from the spill were more enriched in ^{34}S than upstream spiders, consistent with incorporation of coal ash derived sulfur. Spider $\delta^{34}S$ also increased with distance from the spill site. $\delta^{34}S$ of the Asian clam, *Corbicula fluminea*, declined downstream of the spill site, a change that was not consistent with coal ash S, and $\delta^{13}C$ suggested that *Corbicula* shifted their feeding mode in relation to location from the spill. Methylmercury analysis for both clams and spiders were not significantly different between upstream and downstream sites, indicating that mercury from the spill was a not significant problem in these components of the Dan River food web.

COAL ASH DERIVED SULFUR AND MERCURY IN THE DAN RIVER

INVERTEBRATE FOOD WEB

by

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Approved by

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To my advisor, lab mates, friends, and family who have patiently supported and encouraged me to pursue my scientific passions.

APPROVAL PAGE

This thesis, written by Kimber Corson, has been approved by the following committee of the Faculty of The Graduate School at The University of North Carolina at Greensboro.

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CHAPTER I

INTRODUCTION

On February 2, 2014, at Duke Energy's Dan River Steam Station (DRSS) near Eden, North Carolina, a storm water pipe running under an unlined coal ash retention pond into the Dan River ruptured, spilling more than 39,000 tons of coal ash and 27 million gallons of contaminated water directly into the Dan River (Zucchino 2014). This was the third largest coal ash spill in United States history. Coal ash is a product of coal fired power plants, produced during the combustion of coal. Coal combustion results in two ash products—fly ash that is mostly released into the atmosphere, and bottom ash, or coal ash, that is mixed with water and pumped into a nearby open retention pond (Duke Energy 2015). Coal ash contains heavy metals such as mercury, arsenic, cadmium, chromium, and lead, as well as sulfur in the form of sulfate (National Research Council 2006). In addition to the risk of spills, coal ash components can leach through the ground water and into the river from unlined coal ash retention ponds, such as the two ponds adjacent to the Dan River built in 1980, with the original pond being constructed in 1956 (EPA 2009).

Sulfur occurs naturally as pyrite (FeS_2) and gypsum ($CaSO_4$) in rocks and sediment, as well as coal. Weathering of rocks releases these sulfur compounds into streams (Kellog et al. 1972). The burning of fossil fuels emits sulfur dioxide, which can dry deposit as sulfate and can react with water to form sulfuric acid, resulting in acid rain, and also can provide fresh water systems with sulfur (Kellog et al. 1972).

Sulfur is an essential element in both terrestrial and aquatic food webs, and is found in all organisms. Sulfur is found in four common amino acids: methionine, cysteine, homocysteine, and taurine; the former two are necessary for protein production in both prokaryotes and eukaryotes (Brosnan and Brosnan 2006). Methionine is the beginning amino acid in the production of all proteins, and cysteine plays a key role in creating disulfide bonds, giving proteins their complex protein-folding pathways and structure (Brosnan and Brosnan 2006).

As freshwater systems are normally depleted in sulfate (Muyzer and Stams 2008), increasing the amount of sulfate in a river (such as by introducing coal ash into the system) may lead to proliferation of communities of sulfate reducing bacteria (hereafter referred to as SRB). Anaerobic SRB living in the sediment of freshwater systems convert SO_4^{2-} to H_2S via their metabolism.

Though inorganic mercury is toxic, methylmercury is the most toxic form of mercury to humans and other organisms, as methylmercury binds to proteins and is lipophilic, easily passing through the cell membrane (Hong et al. 2012). Anaerobic SRB residing in the sediment of aquatic systems have the potential to also convert inorganic mercury into the more toxic form methylmercury if mercury is present in the environment (Compeau and Bartha 1985), thereby routing methylmercury into the food web. As sulfur-containing products are assimilated into the biomass of the SRB, so is methylmercury. Consumption of SRB by aquatic invertebrates allows coal ash derived mercury and sulfur to enter the food web. Methylmercury persists in the environment and bioaccumulates as it moves through the food web, posing a risk for human and environmental health long after any traceable amount of mercury in the water is gone (Mergler et al. 2007). Though mercury may not persist in the water column, mercury has been shown to accumulate in sediments following a coal ash spill in Kingston, Tennessee (Ruhl et al. 2009). A separate analysis of this study measured mercury levels in Dan River sediments (Ku et al. 2017).

Among heavy metals, methylmercury exposure poses a particular risk to human and environmental health because it persists in the environment and bioaccumulates with increasing trophic level. Methylmercury binds to proteins and free amino acids and cannot be removed (Mergler et al. 2007). The main route of human exposure is consumption of contaminated fish, which, as top predators, can be high in methylmercury due to bioaccumulation. The human health effects of prolonged mercury poisoning, also known as Minamata disease, include reduced peripheral vision, ataxia, tremor, numbness in the hands and feet, muscle weakness, and damaged speech and hearing (Mergler et al. 2007). Minamata disease results from eating mercury-containing fish and shellfish (Hong et al. 2012). Exposure of methylmercury to pregnant women is especially harmful, as the mercury tends to accumulate in the fetal brain, causing deformities and nervous system disorders such as cerebral palsy (Hong et al. 2012). The environmental health effects of methylmercury exposure in aquatic systems include mouthpart deformities, decreased limb regeneration in regenerative species, loss of equilibrium in fish, delayed molting in invertebrate larvae, gill abnormalities, heart and circadian rhythm disturbances, abnormal behavior, decreased reproductive success, and mortality, all of which can result in decreased fitness of the organism (Vermeulen et al. 2000).

During combustion, the elements in coal fractionate into several isotopic forms. The light isotopes such as ^{32}S are disproportionately burned off and released in fly ash and gas products while more of the heavy isotopes such as ^{34}S are left in the bottom ash and pumped into retention ponds (Elswick et al. 2007). Therefore, bottom ash has a higher $\delta^{34}S$ signature than fly ash. Determining the sulfur stable isotopic ratio ($^{34}S/^{32}S$) is the sole method of differentiating between natural and anthropogenic sulfur sources (Derda et al. 2006). Therefore, sulfur stable isotope analysis (SIA) can serve as an important environmental tracer to map the migration of coal combustion pollution in the environment. Sulfur isotopic composition is measured in parts per thousand (ppt or $^{o}/_{oo}$) relative to the Canyon Diablo troilite (CDT) standard, as follows.

$$\delta = \left(\frac{^{34}S/^{32}S_{sample} - ^{34}S/^{32}S_{standard}}{^{34}S/^{32}S_{sample}} \right) \times 1000 .$$

$\delta^{34}S$ of invertebrate consumers closely resembles the $\delta^{34}S$ of their diet (Peterson and Fry 1987). Some sulfur fractionation does occur in the microbial food web (Aharon and Fu 1999), but fractionation does not occur in the invertebrate food web or across trophic levels (Hesslein et al. 1991). Since coal ash has a specific $\delta^{34}S$ signature that does not significantly fractionate in the invertebrate food web, sulfur stable isotope analysis of aquatic invertebrates can be used to trace the origin of sulfur in the food web to coal ash (Hesslein et al. 1991). This can be done by comparing $\delta^{34}S$ found in invertebrates downstream of the spill site to $\delta^{34}S$ from the coal ash retention pond after accounting for atmospheric deposition, ambient levels from upstream, and pond leaching ^{34}S. Sulfur isotopes are appropriate for use in environmental pollution studies, as areas unaffected by the influence of industrially produced sulfur have $\delta^{34}S$ values that are near 0‰ or negative (Krouse and Grinenko 1991).

Mercury SIA can be used to trach coal ash derived mercury into the invertebrate food web, but such analysis is not cost or labor effective. There are a few university labs in the country that perform mercury SIA, but they do not accept outside samples. However, there are several university labs that offer sulfur SIA, which may be an appropriate surrogate for tracing coal ash derived mercury, as SRB are the primary methylators of mercury (Compeau and Bartha 1985). This study explores the use of sulfur SIA as a surrogate for coal ash derived mercury.

Carbon and nitrogen stable isotope analyses are also important as indicators of food sources for aquatic invertebrates. $\delta^{13}C$ is used to trace the source of carbon in an invertebrate's diet, using the PeeDee limestone standard, because there is minimal fractionation of carbon isotopes through the food web and carbon in different food sources is often isotopically distinct. $\delta^{15}N$ is used to determine an organism's trophic level within an ecosystem, using nitrogen gas in the atmosphere as the standard. $\delta^{15}N$ typically becomes enriched by 3 to 5 ppt with each trophic level increase (Peterson and Fry 1987). Any major alterations in food web rates of transfer within pathways that have occurred due to the coal ash spill can be identified with the combination of sulfur, carbon, and nitrogen isotope analysis.

Aims and Hypotheses/Predictions

Aim 1: To quantify the relationship between distance from a coal ash spill site in the Dan River and consumer use of a food source containing coal ash, using SIA of sulfur to trace coal ash derived sulfur and SIA of carbon and nitrogen to trace organic matter sources.

1A) I predict that coal ash infiltration forces invertebrates to shift their feeding method to primarily feed from the seston rather than sediment, with the greatest effect just downstream of the spill site, and attenuating with distance.

1B) I also predict that the effect of coal ash on the $\delta^{34}S$ signatures in aquatic invertebrates is greatest just downstream of the spill site, and attenuates with distance. The upstream invertebrates have depleted $\delta^{34}S$, reflective of ambient levels and the downstream invertebrates have elevated $\delta^{34}S$ signatures nearest to the spill site that decrease as distance from the spill site increases.

Aim 2: To quantify the relationship between distance from the spill site and coal-ash derived mercury incorporation in the Dan River invertebrate food web using total mercury (THg) and methylmercury (MeHg) analysis.

2) I predict that the THg and MeHg in aquatic invertebrates decreases with distance from the coal ash spill. As distance from the spill site increases, THg and MeHg levels decrease.

Aim 3: To evaluate the validity of using sulfur SIA as a surrogate for mercury SIA in the Dan River.

3) I hypothesize that sulfur SIA can be used as a surrogate for coal ash-derived mercury in Dan River invertebrates. Invertebrates enriched in coal ash derived ^{34}S are also enriched in coal ash derived mercury.

CHAPTER II

MATERIALS AND METHODS

Sampling Sites

The Dan River is a 344-kilometer-long river that flows through North Carolina and Virginia, runs into the Roanoke River and ultimately empties into the Atlantic Ocean at Albemarle Sound in Plymouth, North Carolina. The project encompasses a 103 river-kilometer portion of the Dan River that extends from Eden, NC, to Milton, NC. This stretch of the Dan River is characterized by a stream bed substrate made up of rocks, sand, silt, and organic matter. The discharge is typically around 1,000 cubic feet per second at the Danville USGS gauge.

Study sites (Fig. 1) along the Dan River were divided into three areas: upstream of the spill site, adjacent to the coal ash pond and slightly upstream of the spill pipe, and downstream of the spill site. The three upstream samples near Eden, NC, were collected to indicate background levels of $\delta^{34}S$ and mercury from before the spill. Samples from the three sites adjacent to the coal ash pond but upstream of the pipe were collected to indicate how much of the $\delta^{34}S$ and mercury might be due to long-term leaching into the river from the unlined pond. The five downstream samples include sites around Danville, VA, Milton, NC, and Draper Landing in Eden, NC. Most sites included a channel and shore location when possible. Sites were accessed via a motorboat or inflatable raft from various entry points.

Sample Collection

Aims 1 and 2

Each site was sampled in July 2015 for a warm season sampling. A second sampling session occurred in fall 2016 in order to observe annual changes in the $\delta^{34}S$ signature as well as to collect seston, which we had not thought to collect in 2015, and additional leaf litter, sediment, and invertebrate samples. Samples of sediment cores, leaf litter, and any available invertebrates were collected, including clams, stoneflies, mayflies, dragonflies, damselflies, beetles, true bugs, and riparian spiders, providing organisms from two trophic levels: primary consumers and predators, although not all consumer groups could be collected from all sites. Multiple samples were collected on the shore by hand and in the channel using a dredge for both carbon and nitrogen stable isotope and mercury analysis, preferably with enough biomass for at least three samples, or as many samples that were possible in the allotted sampling time. Collected invertebrates were placed in Nalgene bottles with filtered stream water. Predators were separated into one or two per bottle to prevent them from consuming each other. The bottles were put on ice in a cooler for transport to the lab, thereby providing several hours for the organisms to void their guts, and were then frozen upon return to the lab. Sediment samples were collected by pushing a plastic corer into the sediment. Three cores were taken and were pooled across depth. The sediment samples were stored in 60 ml falcon tubes and taken back to the lab for processing.

Sample Processing

Aims 1 and 3

Frozen bottles of aquatic invertebrates and terrestrial spiders from the field were thawed and identified to family or genus using a dissecting microscope, and were placed into 1.5 ml microcentrifuge tubes, with a target weight of approximately 3.0 mg and up to 3 replicates of each family or genus ($n=3$ per site) unless insufficient biomass for three samples was collected. Specific n values for comparisons are given in Table 1. The microcentrifuge tubes were placed into a 60°C drying oven for at least three days, whereupon they were crushed using a mortar and pestle, weighed out into tin capsules, and placed into 96-well plates. The samples were sent to the Stable Isotope Laboratory at the University of California at Davis for analysis of sulfur, carbon, and nitrogen stable isotopes. The long-term standard deviations are 0.2 per mil for ^{13}C, 0.3 per mil for ^{15}N, and 0.4 per mil for ^{34}S.

Shore sediment samples were homogenized by crushing the sample with an acid-washed pestle and mortar. Channel sediment was first sieved using a 1 mm acid-washed polypropylene mesh to remove large and coarse particles, and then homogenized by crushing the sample with an acid-washed pestle and mortar. All sediment samples were sent in 15 ml falcon tubes to the Colorado Plateau Stable Isotope Laboratory at Northern Arizona University for analysis of carbon, and nitrogen stable isotopes (the amount of sediment mass needed for sulfur SIA could not fit in the instrument).

Aims 2 and 3

Members of Dr. Martin Tsui's lab completed all mercury sample preparation and analysis. Sediment samples collected from the river were freeze-dried and homogenized with an acid-washed mortar and pestle. Teflon-digested weigh boats were used to weigh out subsamples, and were digested with concentrated trace-metal grade hydrogen peroxide and nitric acid (4:1, v:v) overnight at 80^{o}C. An aliquot of the acid digest was subsequently analyzed for THg using cold atomic fluorescence spectroscopy (CVAFS) (Brooks Rand Model III).

Spider and *Corbicula* samples were freeze-dried and homogenized with acid-washed mortars and pestles. Sterile centrifuge vials were used to weigh out subsamples, and were digested with 4.6M trace-metal grade nitric acid for 12 hours at 60^{o}C. These samples were analyzed for methylmercury with a GC-CVAFS using the Hammerschmidt and Fitzgerald protocol (2005). The remaining acidified samples were oxidized with 5% potassium permanganate (KM_nO_4) and 2.5% potassium persulfate ($K_2S_2O_8$) (1:1, v:v) at ambient temperature. Samples were then neutralized with hydroxylamine (H_3NO). The neutralized solution was then analyzed for THg with CVAFS.

All THg and MeHg analyses were paired with reagent blanks, duplicates, and standard reference materials (i.e., National Research Council Canada DORM-4 fish protein for biological samples and MESS-3 marine sediment for sediment samples).

Statistical Methods

Statistical analysis for all aims were run using R statistical software (version 3.2.3) or SPSS (version 24). For Aim 1, two-way repeated ANOVAs were performed on the *Corbicula fluminea* data to evaluate the influence of site (upstream of the spill site, adjacent to the coal ash retention pond, and downstream of the spill site) and location (shore or channel) on $\delta^{34}S$, $\delta^{13}C$, and $\delta^{15}N$ in the organisms. One-way ANOVAs were performed on the data from spiders collected from the riparian zone to evaluate the influence of site on $\delta^{34}S$, $\delta^{13}C$, and $\delta^{15}N$ in the organisms. Tukey's post hoc tests were performed where possible. Simple linear regressions compared distance (from the Smith River Confluence, upstream from all sample sites) with log transformed $\delta^{34}S$ in spiders, in addition to $\delta^{13}C$ in clams, spiders, seston, sediment, and leaf litter. For Aim 2 simple linear regressions were performed to compare distance to methylmercury in clams and spiders, and distance to THg in sediment to evaluate whether THg or MeHg concentrations change with distance from the spill.

CHAPTER III

RESULTS

Corbicula Dietary Shifts and Coal Ash Assimilation

There was no statistical difference in $\delta^{13}C$ between clams collected from the shore or the channel (p=0.464), but there was a difference between clams collected from upstream and downstream of the spill site (p=0.013) with downstream clams being more depleted in ^{13}C (Fig. 2A, Table 1). A simple linear regression also showed that overall, clams become significantly depleted in ^{13}C with increasing distance (Fig. 3A) with a p-value of 0.0211 and R^2 of 0.427, however, a closer look at solely the downstream sites shows no significant variation of ^{13}C with increasing distance (Fig. 3B) with p=0.975. Analysis for $\delta^{15}N$ of clams (Fig. 2B, Table 1) showed no difference between upstream and downstream clams (p=0.627), or shore and channel clams (p=0.377).

Pure coal ash collected from the Dan River stream station had a $\delta^{34}S$ of 7.32 ppt. Upstream clams from the shore and channel had an average $\delta^{34}S$ of 6.27 and 6.76 respectively, while downstream clams from the shore and channel had an average $\delta^{34}S$ of 5.78 and 5.67 respectively (Fig. 2C, Table 1). Upstream clams had a significantly higher $\delta^{34}S$ value, closer to the pure coal ash value of 7.32 ppt, than downstream clams. There was no significant difference between shore and channel clams (p=0.108) for $\delta^{34}S$, but there was a difference between clams collected upstream and downstream of the spill site (p<0.001). A simple linear regression shows that overall, $\delta^{34}S$ in clams decreases with distance from the spill site with p=0.000339 and R^2=0.739 (Fig. 4).

To visually compare clams to their possible food sources—seston, leaf litter, and sediment, averages of $\delta^{13}C$ were plotted against site (upstream/downstream) (Fig. 5). Individual values were also plotted against distance (Fig. 6) to determine whether $\delta^{13}C$ values of potential food sources could be used to predict clam $\delta^{13}C$ values. Upstream clams were more depleted in ^{13}C than downstream clams (Fig. 5A). Upstream seston was more enriched in ^{13}C than downstream seston (Fig. 5B). Upstream and downstream leaf litter were statistically similar (Fig. 5C). Upstream sediment was more enriched in ^{13}C than downstream sediment (Fig. 5D). These plots suggested that seston was likely the most influential food source. On average upstream clams had a $\delta^{13}C$ value more similar to seston than sediment, and downstream clams had a $\delta^{13}C$ value more similar to sediment than seston. Upstream clams had an average of -25.8 ppt, while upstream seston had an average of -26.8 ppt and upstream sediment had an average of -28.9 ppt. Downstream clams had an average of -27.0 ppt, while downstream sediment had an average of -28.1 ppt and downstream seston had an average of -26.1 ppt.

A mixing model with clams as the consumer and seston and sediment as the food sources was made for $\delta^{13}C$ and $\delta^{15}N$ (Table 2). Leaf litter was not included as a food source, as it is a component of sediment organic matter and is not a direct food source for clams. One sample site's % seston and % sediment values for the two isotopes are negative or over 100%, suggesting some influence of an unmeasured food source.

Spider Coal Ash Assimilation

There was no difference between upstream and downstream spiders $\delta^{13}C$ ($p=0.175$) or leaching and downstream spiders ($p=0.284$) (Fig. 7A, Table 1). There was significance between upstream and leaching site spiders ($p=0.014$). Analysis of $\delta^{15}N$ in spiders (Fig. 7B, Table 1) showed no difference between any of the sampled sites, with upstream-leaching $p=0.330$, upstream-downstream $p=0.603$, and leaching-downstream $p=0.860$.

$\delta^{34}S$ analysis of spiders (Fig. 7C) found no difference between leaching and downstream spiders ($p=0.861$). Spiders collected upstream and downstream from the spill site had different $\delta^{34}S$ ($p=0.012$) with downstream spiders having a higher $\delta^{34}S$ than upstream spiders. Upstream and leaching site spiders were also significantly different ($p=0.026$) with leaching site spiders having a higher $\delta^{34}S$ than upstream spiders. A logarithmic linear regression (Fig. 8) shows a positive relationship of increasing $\delta^{34}S$ and increasing distance with $p=0.0274$ and $R^2=0.0274$.

Individual $\delta^{13}C$ values were plotted against distance (Fig. 9) to determine whether $\delta^{13}C$ values of potential food sources could be used to predict spider $\delta^{13}C$ values. These are not direct food sources for spiders, but are the food sources for aquatic insects that riparian spiders will eat once they've emerged, so two-trophic level shift (and therefore a 2 ppt increase between food source and consumer $\delta^{13}C$) must be considered. Overall it appears that sediment likely influences spider $\delta^{13}C$ the most. Leaching site spiders had the most enriched $\delta^{13}C$ values, as did some downstream spiders. Spiders collected from

Milton, NC, the sample site farthest downstream from the coal ash spill, had $\delta^{13}C$ values similar to upstream sites.

Methylmercury in Corbicula and Spiders

Using simple linear regression, there was no relationship between methylmercury and river distance in clams (Fig. 10), with p=0.991 and R^2=0.000002. There was no relationship between clam shell size and methylmercury (Fig. 11), with p=0.486 and R^2=0.0625.

Analysis of spider methylmercury vs. river distance using simple linear regression (Fig. 12) again yielded no relationship with p=0.774 and R^2=0.0109.

Sulfur Isotopes as a Surrogate for Mercury

There were no significant relationships between *Corbicula* MeHg and distance (Fig. 10) with p=0.991, *Corbicula* MeHg and mean shell length (Fig. 11) with p=0.486, or spider MeHg and distance (Fig. 12) with p=774, showing no effect from the coal ash spill on the amount of MeHg found in downstream clams or spiders. *Corbicula* $\delta^{34}S$ was plotted against MeHg (Fig. 13), and the comparisons showed no relationship with $\delta^{34}S$ and MeHg with p=0.560. Spider $\delta^{34}S$ was also plotted against MeHg (Fig. 14), and also showed no relationship with p=0.686. For *Corbicula* and spiders, $\delta^{34}S$ was not a useful surrogate for coal ash derived MeHg.

CHAPTER IV

DISCUSSION

Evaluation of the Effects of Coal Ash on Corbicula

Carbon stable isotope analysis suggests that upstream clams' diet is different than that of the downstream clams. Analysis showed that there was no difference between $\delta^{13}C$ in clams collected from the shore and the channel, however there was a significant difference between clams collected from upstream and downstream of the spill site (Fig. 2A). Clams can feed by deposit feeding in the sediment, or by filtering seston from the water column (Bullard and Hershey 2013). If clams are solely feeding in the sediment, we should see a similar pattern of $\delta^{13}C$ depletion in sediment upstream and downstream of the spill site. However, we found that the $\delta^{13}C$ pattern for sediment was the opposite than that of the clams (Fig. 5). The upstream sediments were more depleted in ^{13}C than downstream sediments (Fig. 5D), indicating that the diets and feeding modes of upstream and downstream clams were very different. Upstream clams appeared to feed primarily from the seston, and downstream clams appeared to incorporate more of their carbon from a sediment food source. $\delta^{13}C$ increases by about 1ppt with increasing trophic level (DeNiro and Epstein 1978). Upstream clam average $\delta^{13}C$ was enriched by 1 ppt of the seston $\delta^{13}C$. Downstream clam average $\delta^{13}C$ was enriched by about 1 ppt of the downstream sediment $\delta^{13}C$. Considering that sample collection took place more than a year after the coal ash spill, and the inference that by that time the coal ash had settled out of the water column or was transported out of the system, I had expected there to be no difference between upstream and downstream seston. however, that was not the case (Fig. 5B). In fact, seston had the same trend as sediment in regard to ^{13}C—upstream seston was more depleted in ^{13}C than downstream seston. This may be due to the river size and effect of dams rather than the coal ash spill.

The discrepancy between upstream and downstream clam diet was further explored with a *Corbicula* mixing model using two food sources—sediment and seston (Table 2). The mixing model contains a % seston value outside of the mixing space that is greater than 100%, indicating that an additional food source is present, but unaccounted for (Phillips 2012). However, with both δ^{13}C and δ^{15}N I found that upstream clams had higher % seston values than downstream clams, indicating that upstream clams were indeed feeding from the seston more than downstream clams. Leaf litter was not considered as a food source in the mixing models, since leaf litter was collected from the sediment and had δ^{13}C and δ^{15}N too far away from *Corbicula* to be an important diet source. Clams also do not have a mechanism to directly feed on leaf litter. The mixing model is nevertheless valid in ascertaining that an unknown food source has had an effect on clam diet. Clearly there are other factors contributing to the diet of the invertebrates that cannot be identified.

My first hypothesis, 1A, is not supported by the clam data. There was a difference in δ^{34}S between clams collected upstream and downstream, and no difference between channel and shore clams. Pure coal ash collected from the coal ash retention pond in question had a $\delta^{34}S$ value of 7.32 ppt. Since ^{34}S does not fractionate in the invertebrate food web, if downstream clams were assimilating coal ash derived sulfur, I'd have expected downstream clams to have higher $\delta^{34}S$ values, perhaps not as high as 7.32 ppt due to the fractionation that occurs during microbial metabolism, but certainly higher than upstream clams. Clearly I did not see that, indicating that clams downstream from the spill are not assimilating coal ash derived sulfur. This result would normally suggest that downstream clams shifted away from feeding on a sediment food source, but considering the $\delta^{13}C$ data, this cannot be the case. All sediments analyzed for $\delta^{34}S$ had no peaks. Seston was not analyzed for $\delta^{34}S$, as this analysis was not possible due to limited mass.

Evaluation of the Effects of Coal Ash on Spiders

The aquatic food sources for leaching site spiders is isotopically different than the aquatic food sources of upstream spiders, indicating a coal ash spill effect on the riparian spiders' aquatic food source of emergent insects at leaching sites. Using $\delta^{13}C$ SIA in spiders, there was a significant difference between upstream and leaching site spiders. There was no difference between downstream spiders and either upstream or leaching site spiders, but we can see that something was affecting the $\delta^{13}C$ of the diet of leaching site spiders. This disparity between diets would have been influenced by the $\delta^{13}C$ of the both emergent and terrestrial insects that are consumed by the spiders (Collier et al. 2002). We collected leaf litter to account for the terrestrial aspect of the spiders' diet, but there was no difference between upstream and downstream leaf litter (Fig 7C), therefore we must assume that any pattern in the $\delta^{13}C$ of spiders must be attributed to their aquatically sourced diet. Unfortunately, we were unable to collect enough biomass to analyze emergent insects, which would have accounted for the aquatic portion of the spiders' diets.

Hypothesis 1B is supported by sulfur stable isotope analysis in spiders, which shows a difference between upstream spiders and both leaching and downstream spiders. The finding that leaching and downstream spiders had a $\delta^{34}S$ closer to the pure coal ash value of 7.32 ppt indicates that leaching and downstream spiders may be incorporating coal ash derived sulfur, likely derived from eating insects from the river. A mixing model to estimate the dietary proportion of coal ash derived sulfur using $\delta^{34}S$ could not be used because insects and other sulfur source materials were not collected. However, spiders residing close to the river channel in other systems are estimated to obtain approximately half of their diet from aquatic sources and half from terrestrial sources (Collier et al. 2002).

The disparity between the $\delta^{34}S$ findings between spiders and clams may be affected by those organisms' life histories and the distribution of coal ash at the time of sampling. The families of spiders collected for this study—Lycosids, Pisaurids, Araneids, and Tetragnathids—typically have lifespans of up to one year, while *Corbicula* have lifespans up to 7 years, with an average of 2-4 years. Slower growing organisms like clams may not show as much isotopic change as faster growing organisms like spiders (Hesslein et al. 1993), however *Corbicula* also turn over their biomass quickly, on average 73-91 days (Vaughn and Hakenkamp 2001). Subsequently, clams will only reflect stable isotope values relative to the river conditions of the two and a half to three months previous to collection, even though most clams collected had spent their entire lives in the river post-coal ash spill. At the time of sampling, we found that in sediment cores, coal ash was not widely or evenly distributed in the river sediments, but rather, coal ash deposits were patchy. Patches of coal ash were also likely to move with each rain event. *Corbicula* were likely not consistently exposed to coal ash deposits and subsequently did not assimilate coal ash derived ^{34}S.

Sulfur Isotopes as a Surrogate for Mercury

The lack of significance between clams and MeHg, and spiders and MeHg, refuting hypothesis 2, raised concerns that the use of ^{34}S as a surrogate for mercury stable isotopes may not have been the most appropriate method for this study. Both the primary and secondary retention ponds at the DRSS were built in 1980 and weren't officially decommissioned until 2012, just two years before the coal ash spill, but the original basin was constructed in 1956 and gradually increased in size until its division into the two existing retention ponds was necessary (EPA 2009). Given that the site had been leaching coal ash derived products for nearly 60 years, and the fact that no significantly different amounts of THg was found in upstream and downstream river sediments, some of the mercury in the coal ash may have already transported out of the system by the time of the 2014 spill. Although we did find occasional layers of coal ash in sediment cores, considering the speed and discharge of the river, it is possible that the bulk of the coal ash did not settle in the river channel, but was deposited in Kerr Lake. To my knowledge, Kerr Lake has not been sampled for coal ash in the lake bed. The coal ash layers that can be found in the Dan River may be too widely dispersed for methylated mercury to be available to the invertebrate food web, since no variation in MeHg was found in invertebrates on either side of the coal ash spill.

The spider and clam data support the possibility that most of the coal ash derived mercury had already dissipated out of the river, as the data do not show any pattern consistent with enrichment in mercury derived from the spill. Although increasing mercury with distance from the spill site was found (Fig. 13), a separate analysis correcting for percent organic matter found that there was no significant difference between upstream and downstream THg in river sediments (Ku et al. 2017).

Hypothesis 3 is not supported. Both clams and spiders showed significantly different amounts of $\delta^{34}S$ in relation to distance from the spill site, but this is not the case for MeHg. Clams had significantly decreasing $\delta^{34}S$ with increasing distance from the spill site, and spiders had significantly increasing $\delta^{34}S$ with increasing distance from the spill site. Clams did not assimilate coal ash derived sulfur, but spiders did. Neither organism assimilated coal ash derived mercury. The use of sulfur isotopes as a surrogate for mercury did not prove useful in this study because there were no differing amounts of invertebrate methylmercury upstream or downstream of the spill.

General Conclusions

Stable isotopes of carbon and nitrogen are often used as indicators of diet source and trophic level in aquatic food webs, and this study is no different in that regard. The literature involving the use of sulfur SIA, however, is more limited. This study sought to expand the literature involving the use of sulfur SIA in clarifying dietary origin as, unlike carbon, it does not fractionate once it enters the invertebrate food web (Hesslein et al. 1991). The coupling of carbon and sulfur isotopes did provide a clearer picture of dietary influences on clams and spiders in the Dan River and would be a useful tool for future studies.

We found that the coal ash spill may have influenced the dietary pattern of *Corbicula* and the food sources of spiders using $\delta^{13}C$, as downstream *Corbicula* fed more from the sediment than the seston, and leach site spiders have $\delta^{13}C$ values distinct from upstream spiders. *Corbicula* did not assimilate coal ash derived sulfur, but spiders did, as seen in the ^{34}S analyses. Longitudinal linear regressions showed that downstream *Corbicula* and spiders have not assimilated more coal ash derived methylmercury than upstream *Corbicula* and spiders. In conclusion, the 2014 Dan River Steam Station coal ash spill did not significantly impact methylmercury levels in aquatic invertebrates in the sampled portion of the Dan River.

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APPENDIX A

TABLES AND FIGURES

Table 1. Summary of Stable Isotope Analysis ANOVAs for *Corbicula* and Spiders with Post Hoc Tests.

A summary of ANOVAs for SIA of carbon, nitrogen, and sulfur in response to site (upstream or downstream) and location (shore or channel) for clams, or site (upstream, leaching, or downstream) for spiders. Tukey post-hoc tests were performed for the Two-way ANOVAs. Significant p-values have been bolded.

Analysis	F-ratio	n	df	p-value
Corbicula $\delta^{13}C$ Two-way ANOVA				
Site (Upstream/Downstream)	10.157	12	1	**0.013**
Location (Shore/Channel)	0.591	12	1	0.464
Site * Location	0.004	12	1	0.950
Corbicula $\delta^{15}N$ Two-way ANOVA				
Site (Upstream/Downstream)	0.255	12	1	0.627
Location (Shore/Channel)	0.874	12	1	0.377
Site * Location	0.201	12	1	0.666
Corbicula $\delta^{34}S$ Two-way ANOVA				
Site (Upstream/Downstream)	51.337	13	1	**5.279E-05**
Location (Shore/Channel)	3.179	13	1	0.108
Site * Location	7.570	13	1	**0.022**
Spider $\delta^{13}C$ One-way ANOVA				
Upstream-Leaching	6.493	4	2	**0.014**
Upstream-Downstream		4	2	0.175
Leaching-Downstream		4	2	0.284
Spider $\delta^{15}N$ One-way ANOVA				
Upstream-Leaching	1.181	4	2	0.330
Upstream-Downstream		4	2	0.603
Leaching-Downstream		4	2	0.860
Spider $\delta^{34}S$ One-way ANOVA				
Upstream-Leaching	8.222	4	2	**0.026**
Upstream-Downstream		4	2	**0.012**
Leaching-Downstream		4	2	0.6861

Table 2. ***Corbicula*** **Mixing Model.**

A mixing model determining percent seston and percent sediment in *Corbicula fluminea*, out of a diet of seston and sediment, as calculated from $\delta^{13}C$ and $\delta^{15}N$. Distance is from the confluence of the Dan and Smith rivers, our point of reference. Site (upstream or downstream) refers to relation to the spill site.

Site	Distance (km)	% seston by $\delta^{13}C$	% sediment by $\delta^{13}C$	% seston by $\delta^{15}N$	% sediment by $\delta^{15}N$
Upstream	0.5	84.13	15.87	46.64	53.36
Upstream	1.42	74.64	25.36	48.40	51.60
Upstream	2.53	136.59	-36.59	--	--
Downstream	36.71	57.13	42.87	29.01	70.99
Downstream	40.65	2.91	97.09	19.71	80.29

Figure 1. Field Sampling Sites.

A Google map of the selected field sites. There are three upstream sites, three leaching sites, and a total of five downstream sites.

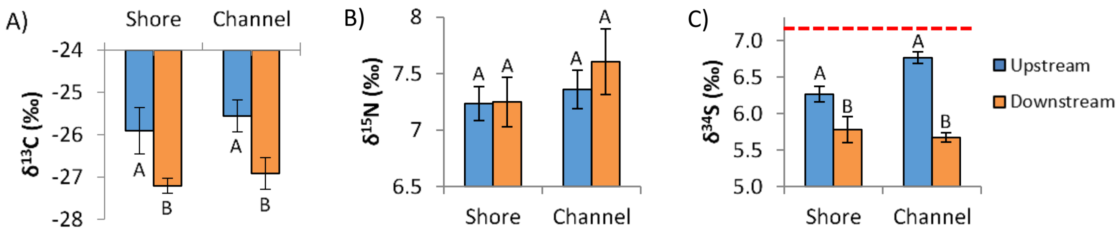

Figure 2. SIA for *Coribcula*.

$\delta^{13}C$, $\delta^{15}N$, and $\delta^{34}S$ in asiatic clams *Corbicula fluminea* collected from the shore and channel both upstream and downstream from the spill site. P-values are from a Two-Factor with Replication ANOVA performed with SPSS. Dissimilar uppercase letters indicate significant difference.

A) Site (Upstream:Downstream) $p=0.013$, Location (Shore:Channel) $p=0.464$, Site*Location $p=0.950$.

B) Site (Upstream:Downstream) $p=0.627$, Location (Shore:Channel) $p=0.377$, Site*Location $p=0.666$.

C) Site (Upstream:Downstream) $p<0.001$. Location (Shore:Channel) $p=0.108$. Site*Location $p=0.022$. Note the red dashed line indicating pure coal ash with a $\delta^{34}S$ value of 7.32 ppt.

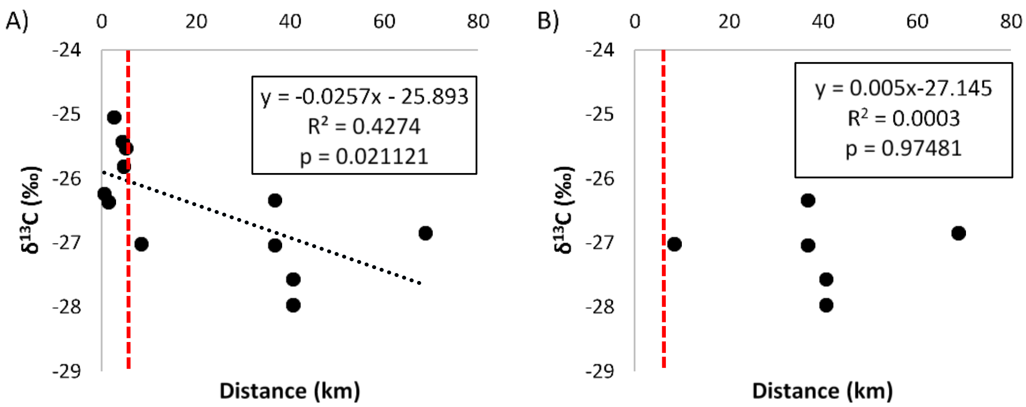

Figure 3. ***Corbicula*** **δ^{13}C vs. Distance.**

Simple linear regression of clam δ^{13}C vs. distance from the Smith River confluence. The spill site is approximately 4 kilometers downstream of the confluence, indicated by red dashed lines. Regression A includes clams collected from all sites, regression B only includes clams collected from downstream sites.

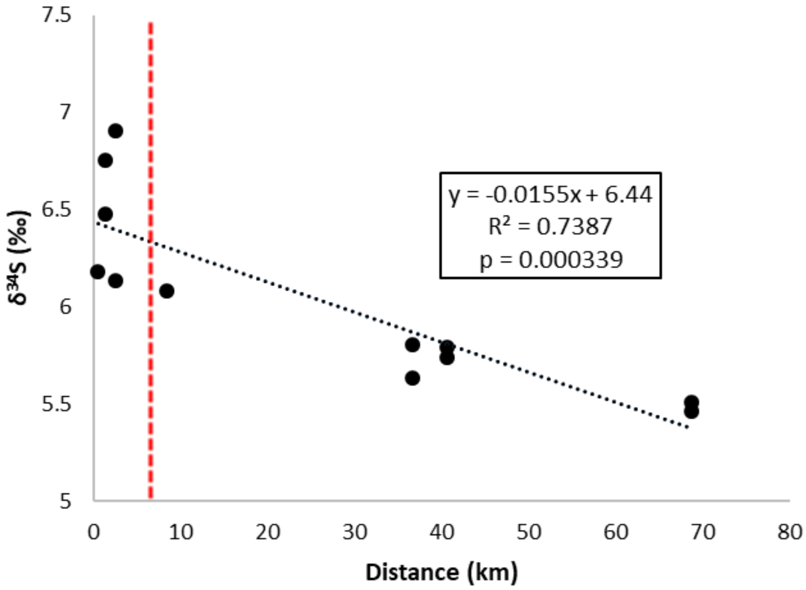

Figure 4. ***Corbicula*** **δ^{34}S vs. Distance**.

Simple linear regression of *Corbicula* δ^{34}S vs. distance from the Smith River confluence.

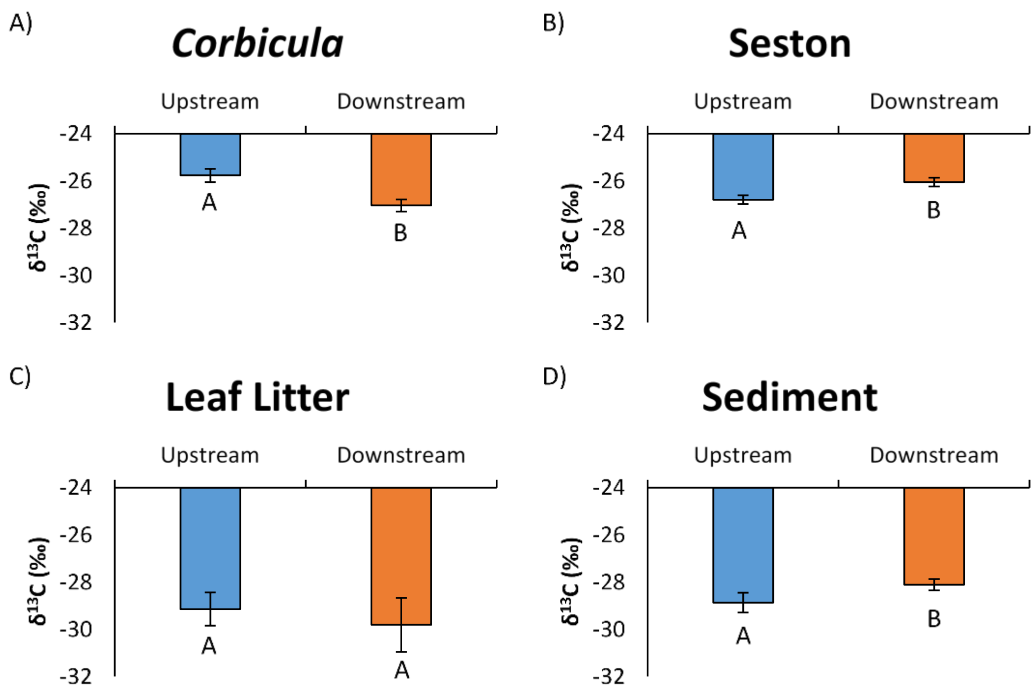

Figure 5. Comparison of $\delta^{13}C$ in *Corbicula* and Dietary Sources.

Possible food sources are seston, sediment, and leaf litter. Dissimilar uppercase letters indicate significant difference.

A) Shore and channel clams have been averaged since there was no difference between them. Upstream:Downstream p=0.00740.

B) $\delta^{13}C$ in seston collected upstream and downstream from the spill. Upstream:Downstream p=0.0369.

C) Comparison of $\delta^{13}C$ in leaf litter collected upstream and downstream of the spill site. Upstream:Downstream p= 0.322.

D) $\delta^{13}C$ in sediment collected upstream and downstream from the spill site. Upstream:Downstream p<0.050.

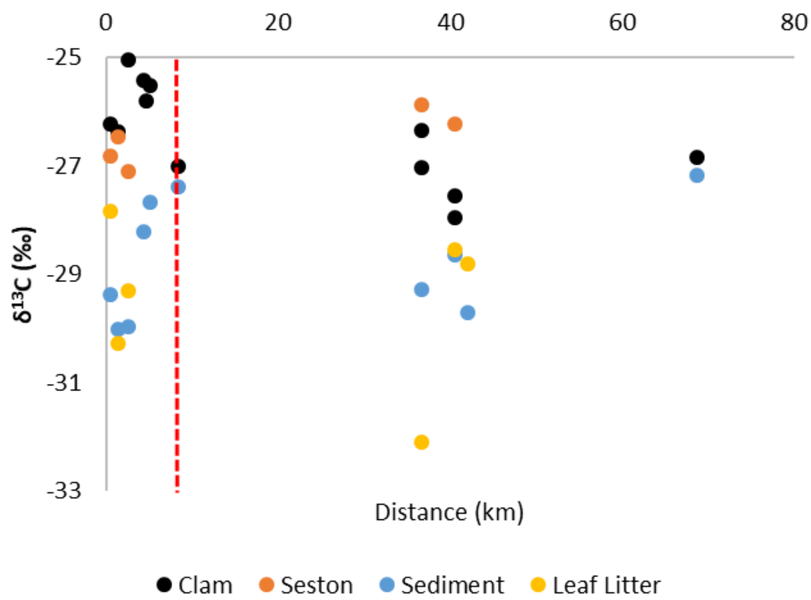

Figure 6. Distance vs. $\delta^{13}C$ in *Corbicula* and Dietary Sources.

A comparison of *Corbicula fluminea* and its possible food sources, seston, sediment, and leaf litter.

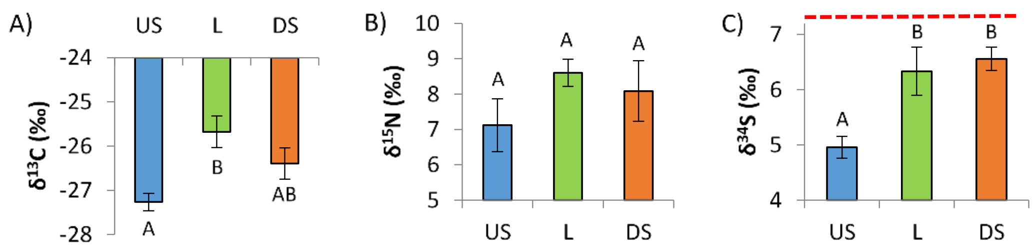

Figure 7. SIA for Spiders.

$\delta^{13}C$, $\delta^{15}N$, and $\delta^{34}S$ in riparian spiders collected from sampling sites upstream of the spill site (US), leaching from the coal ash retention pond (L), and downstream of the spill site (DS). P-values are from One-Factor with Replication ANOVAs performed with SPSS. Dissimilar uppercase letters indicate significant difference.

A) Upstream:Leaching p=0.014, Upstream:Downstream p=0.175, Leaching:Downstream: p=0.284.

B) Upstream:Leaching p=0.330, Upstream:Downstream p=0.603, Leaching:Downstream p=0.860.

C) Upstream:Leaching p=0.026, Upstream:Downstream p=0.012, Leaching:Downstream p=0.861. Note the red dashed line indicating pure coal ash with a $\delta^{34}S$ value of 7.32 ppt.

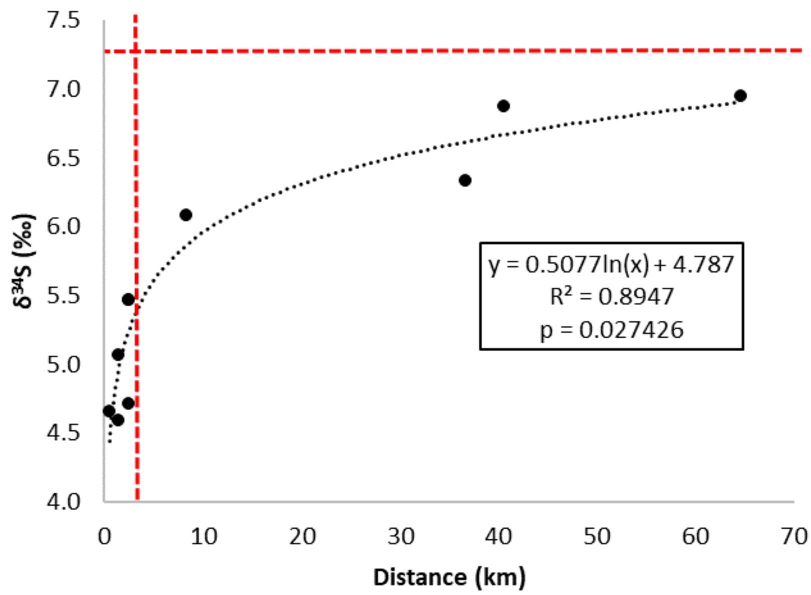

Figure 8. Spider $\delta^{34}S$ vs. Distance.

Simple linear regression of $\delta^{34}S$ in spiders vs. distance from the Smith River confluence. A logarithmic scale was used to reflect the increase in coal ash just at the spill site. The vertical red line indicates the spill site. The horizontal red line indicates the pure coal ash value of 7.32 ppt.

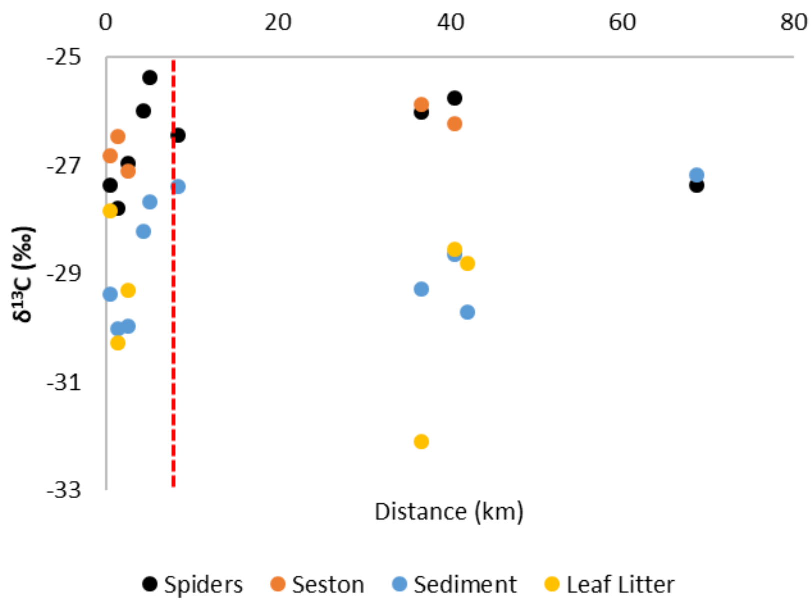

Figure 9. Distance vs δ^{13}C in Spiders and Dietary Sources.

A comparison of spiders and their possible food sources, seston, sediment, and leaf litter.

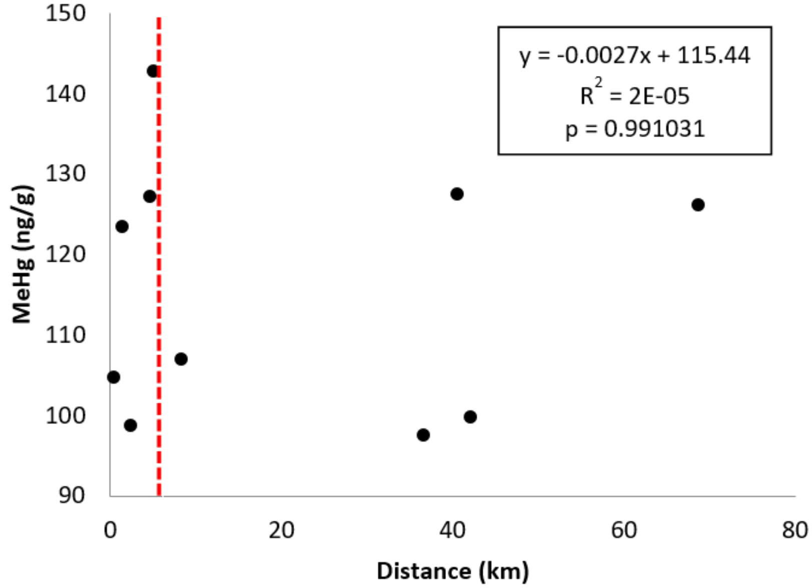

Figure 10. *Corbicula* MeHg vs. Distance.

Comparison of methylmercury (ng/g) and distance in *Corbicula fluminea* collected from upstream, adjacent to, and downstream of the spill site.

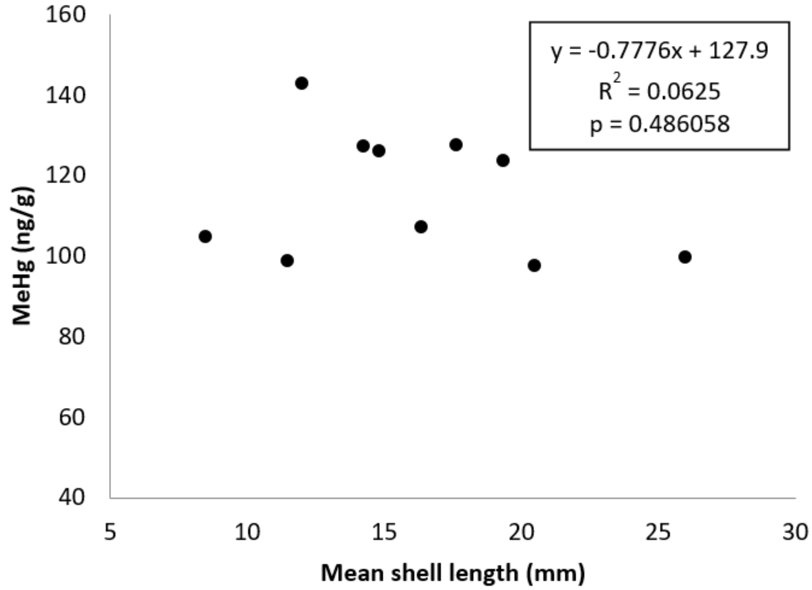

Figure 11. *Corbicula* MeHg vs. Size.

Comparison of methylmercury (ng/g) and average clam shell length in *Corbicula fluminea* collected from upstream, adjacent to, and downstream of the spill site.

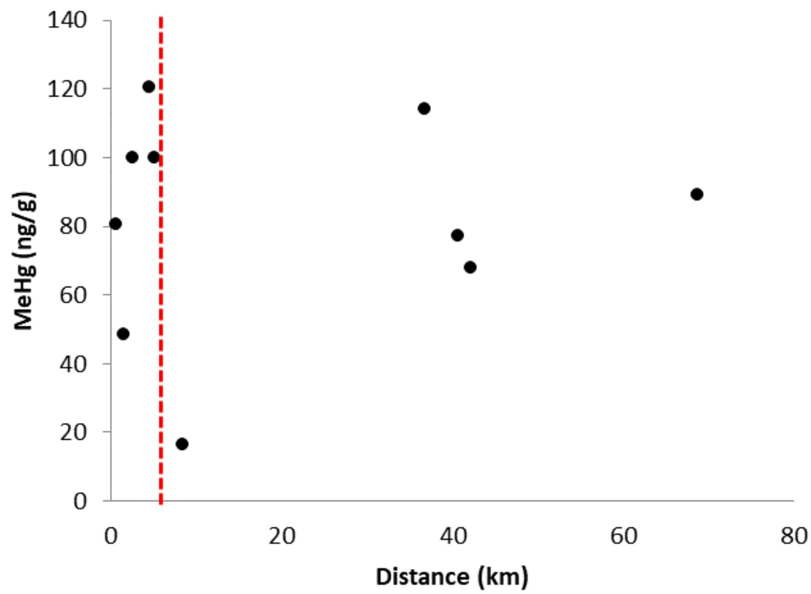

Figure 12. Spider MeHg vs. Distance

Comparison of methylmercury (ng/g) and distance in spiders collected from the riparian zones upstream, adjacent to, and downstream of the spill site.

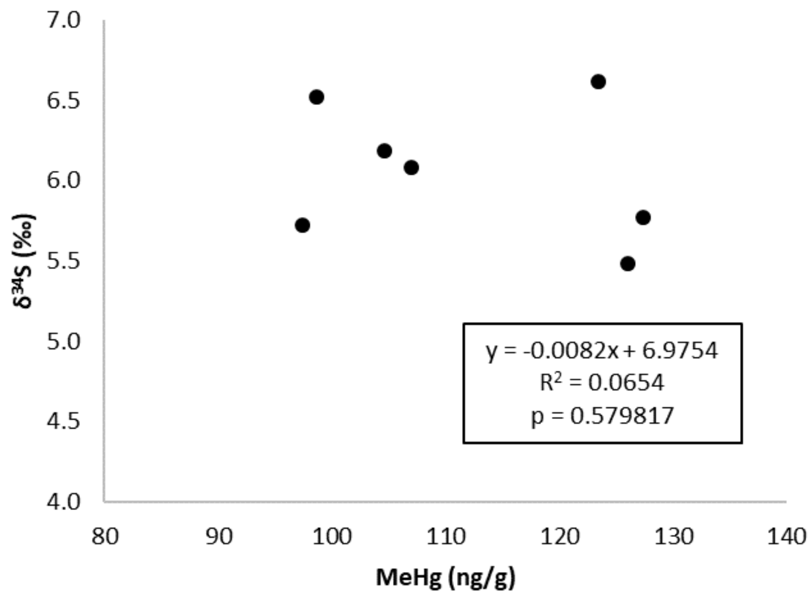

Figure 13. ***Corbicula*** **δ^{34}S vs. MeHg.**

A simple linear regression shows no relationship between δ^{34}S and methylmercury (ng/g) in *Corbicula*.

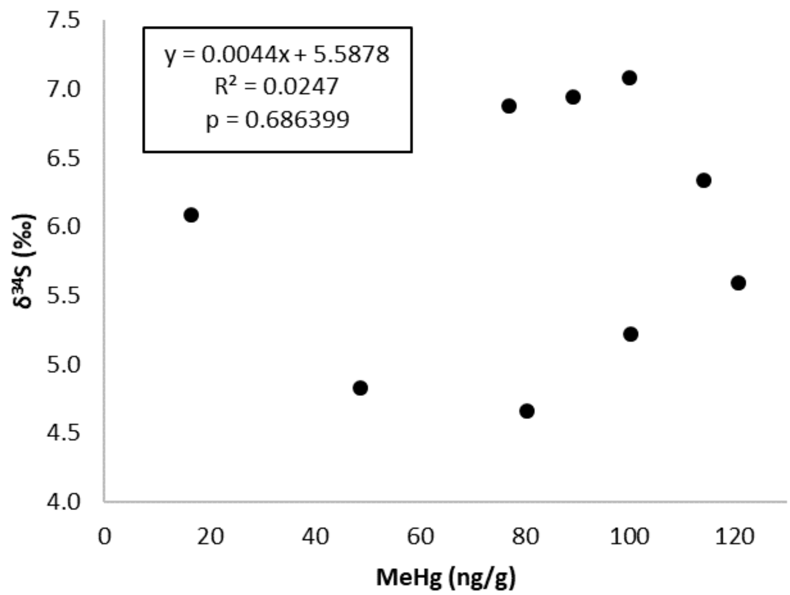

Figure 14. Spider $\delta^{34}S$ vs. MeHg.

A simple linear regression shows no relationship between $\delta^{34}S$ and methylmercury (ng/g) in riparian spiders.