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CAN AMPHIPODS BE USED TO MONITOR MINING-IMPACTED LAKES?

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CAN AMPHIPODS BE USED TO MONITOR MINING-IMPACTED LAKES?

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By

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MASTER'S THESIS

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ABSTRACT

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Metal pollution in aquatic systems is complicated and expensive to manage; establishing a sentinel species for contaminated habitats, rather than measuring abiotic parameters, can provide a more complete perspective of the impacts of pollutants. I conducted a dual field/laboratory study to determine whether an amphipod (*Hyalella azteca*) can serve as a sentinel in a mining-impacted area in Northern Idaho, the Coeur d'Alene (CDA) River Basin. My objectives in the field study were to (1) determine seasonal abundance and metal burden of amphipods in nine chain lakes and a reference lake (Benewah L.) and (2) compare aqueous trace metal levels and limnologic parameters from lake water to patterns in amphipod abundance, size, and metal burden. I predicted that aqueous metal concentrations correlate with amphipod abundance and body burden. I found that amphipod abundance differed across the lakes and over time, but patterns in amphipod abundance and size could not be explained by the factors that I measured. The results confirm that the metals are bioavailable and that uptake is influenced by limnological factors. In the laboratory study, I compared the Zn and Pb tolerances of amphipods from three chain lakes to that of amphipods from the

reference site (Benewah L.) and from a commercial source. I hypothesized that chain lake amphipods would have higher metal tolerance, indicated by higher survival and swimming activity following a 96-hour exposure. Chain lake amphipods exhibited higher Zn tolerance than did the commercial amphipods but were not consistently more tolerant than were reference lake amphipods. The Zn tolerance of all amphipod populations varied seasonally. Differences in Pb tolerance were difficult to detect, likely reflecting issues with Pb solubility. The activity assay was not sensitive enough to detect among-population differences as swimming was strongly affected by all Zn and Pb doses. Taken together, these results confirm that chain lake amphipods are more tolerant of metals than are the commercially-obtained amphipods that are routinely used for toxicity testing. *Hyalella azteca* could become a sentinel for this basin, but only if amphipods from a reference lake serve as the (negative) control population and if the amphipods are monitored year-round.

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INTRODUCTION

Widespread metal pollution in the environment is an inevitable consequence of the "technological metabolism of man" (Wetzel 1975). The need to extract metals from the earth is unlikely to decline in the future, and the appropriate management of the disposal or reclamation of metals will continue to be a complex socioeconomic and environmental problem. As technologies improve, we must also acknowledge the tradeoff between the consequences associated with metal contamination and the quality of human life afforded by the use of metals.

Monitoring the impact of metal contamination and remediation on freshwater ecosystems is critical in regions where legacy contamination exists and where future contamination is likely to occur. Freshwater habitats support diverse communities of organisms and offer ecological services that are economically important (Daily 1997, Costanza et al. 1998). However, because freshwater systems are inherently dynamic and complex, even over very small distances (e.g., within a water column), it is difficult to predict whether metals will be bioavailable (i.e., have the potential to be accumulated by an organism). As an added complication, metals often occur as mixtures with other metals and/or with organic pollutants (Brown 1968), and the mixture composition impacts the bioavailability of each component.

The bioavailability of metals is determined by their chemical properties and soluble fraction (dissolved, particulate) in water, by the unique physical and chemical

factors within a water body, and by the ability of a resident organism to accumulate metals. The biotic ligand model and more recently, the biodynamic model, are tools that allow an evaluation of the influence of environmental factors (i.e., competing cations, inorganic and organic ligands) on the rate of metal-biotic ligand complexation, and thus toxicity to aquatic organisms (Toro et al. 2001, Niyogi and Wood 2003, Luoma et al. 2009). Abiotic factors that influence metal bioavailability include temperature, dissolved oxygen (DO), pH, alkalinity, hardness, and dissolved organic carbon (Bryan 1971, Campbell and Stokes 1985, USEPA 2004, 2007, Besser et al. 2015). Factors that influence an organism's overall potential to accumulate bioavailable metals include variance in morphological resistance to uptake, trophic level, and feeding strategy (e.g., grazing, filter feeding) (for review, Luoma 1983, Hare 1992). Thus, it has been argued for some time (Luoma 1983) that simply monitoring metal concentrations and the abiotic characteristics of freshwaters and their sediments does not adequately capture the impact of metal contamination on an ecosystem. Rather, it is better to monitor a resident sentinel organism.

A sentinel, as defined by Berthet (2013) is "any species providing a warning of dysfunction or an imbalance of the environment…" A sentinel can be a bioindicator species, providing information by its presence, absence, or relative abundance. An effective sentinel is most often an animal that plays a weighted role in the structure or function of the ecosystem. It should meet the following criteria: the animal is primarily constrained to the site of interest, is easy to identify and collect year-round, and its

biology is well-known. The species or population should be widely abundant with broad distribution. The population should exhibit tolerance to chronic exposure to the contaminant of interest (e.g., metals) as documented by dose-effect relationships in laboratory studies (Glickman et al. 1991, Berthet 2013). Ideally, a sentinel species is one in which biomarker patterns (cellular-level responses to the contaminant), dose effect patterns of toxicity, and population-level metrics (abundance, size/frequency distribution) are all known, allowing measurements at one level of organization to inform conclusions at other levels of organization.

Tolerance is the ability of an organism to maintain essential functions such as growth and reproduction during periods of exposure to a pollutant (Amiard-Triquet 2011). It has been identified in several populations of aquatic taxa that are chronically exposed to high levels of Cd, Cu, Pb, and Zn (for review, Johnston 2011). The overall integrity of an impacted ecosystem is influenced by the range of tolerances exhibited by its inhabitants within the context of the environmental factors that influence metal bioavailability. By identifying tolerance in wild populations of animals that are chronically exposed to metals, we can better understand how the ratio of sensitive versus tolerant taxa will change as habitats experience metal contamination.

The amphipod *Hyalella azteca* is a small, freshwater benthic crustacean with potential for use as a sentinel species for metal contamination. It is widely distributed and abundant in North and Central America. Populations of *H. azteca* exist in waters with diverse physical characteristics, such as water chemistry and pH, and diverse

communities (Strong Jr 1972, 1973). *Hyalella azteca* is a well-established model organism for ecotoxicology studies because it has a short maturation period and life cycle, is easy to culture and identify (Lawrence 1981), and can be easily collected (Stephenson and Mackie 1986). It is an ecologically-relevant species as it is an important staple in the diets of fish and other large freshwater consumers (for review, Mebane 2010), and plays an important role in nutrient cycling (Mathias 1971, Strong Jr 1972). *Hyalella azteca* is frequently used in water quality testing and metal toxicity studies, especially with respect to contaminated sediments (Hornig et al. 1988, Borgmann and Munawar 1989, Cubbage et al. 1997, Ingersoll et al. 2005, Besser et al. 2015), because it is sensitive to pollutants (Borgmann et al. 1989), and in particular, to trace metals (Borgmann et al. 1993). However, few studies have explored the metal tolerance of wild *H. azteca* populations collected from contaminated waters (see Clark et al. 2015 for one example with an organic pollutant). Identifying the tolerance of field-collected amphipods to metal stress is an important first step in determining whether these animals can function as a sentinel species for metal-impacted habitats.

The need to identify and monitor sentinels is relevant in populated areas that have been affected by mining; an important component of sentinel development is being able to study the species in a region in which contaminated and unimpacted habitats are in close proximity (e.g., Taylor et al. 2016). A century of mining activity introduced extensive metal contamination into the Coeur d'Alene (CDA) Basin (Figure 1) in northeast Idaho (Sprenke et al. 2000, Balistrieri et al. 2002, NRC 2005, USEPA 2015).

This basin, the principal hydrologic system to the region's mining district (Silver Valley), comprises two drainages. The North Fork and the South Fork meet near Cataldo, ID to comprise the CDA River and extend to Lake Coeur d'Alene.

Inefficient mining practices (pre-1968) delivered approximately 56 million metric tons of metal-laden tailings from the Silver Valley into the South Fork (Long 1998, Balistrieri et al. 2002). Managers of the Basin have improved water quality since 1983 (Hoiland et al. 1994, Clark and Mebane 2014) when the Environmental Protection Agency listed the Bunker Hill Mining and Metallurgical Complex as a Superfund Site under the Comprehensive Environmental Response, Compensation, and Liability Act of 1980. This designation funded extensive terrestrial cleanup within the boundaries of a 21-square mile, primarily urban "Box" (Figure 1). Despite remediation and restoration within the Box, contaminated sediments continue to impact water quality, human health, and ecosystem integrity, as contaminated sediments are regularly redistributed within the CDA River and its floodplain (Balistrieri et al. 2002). As evidence of this, large signs are posted at recreational areas, warning the public of the potential for exposure to metals. Specifically, eleven chain lakes that exist within the floodplain of the river (NRC 2005) are used heavily for recreation. While the CDA River has been extensively studied (Dillon and Mebane 2002, Spears et al. 2006, Lefcort et al. 2010, Mebane et al. 2012) the chain lakes have received comparatively little attention. These lakes are thus the focus of this study.

The physical processes influencing the distribution and concentration of metals moving through the river and into the chain lakes have been reviewed by Balistrieri et al. (2002). Among metals of highest concern in the CDA Basin are Zn and Pb (Hoiland et al. 1994, Balistrieri et al. 2002). Dissolved Zn exists in high concentrations in the river and often exceeds water quality criteria and the Criterion Continuous Concentration (Hoiland et al. 1994). Pb also exists in high concentrations in the river, but is mostly bound to particles and the sediment, rather than in dissolved form in the water column. Of the approximately 250,000 ± 62,000 metric tons of Pb in the lower valley, about 59 percent is in the floodplain, and those sediments can have up to 36,000 ppm Pb (Balistrieri et al. 2002). Sprenke et al. (2000) characterized the sediments in the chain lakes (Table 1), and demonstrated that contamination is concentrated at the sediment surface and subsurface and generally decreases with substrate depth, especially after 1 m. The chain lake sediments include Pb, Zn, Cd, and As at the sediment-water interface. Surface water concentrations reported for four of the chain lakes are listed in Table 2 in Sprenke et al. (2000). According to Chess (2015) and an online source with GPS data from the National Oceanic and Atmospheric Administration (gpsnauticalcharts.com), the lakes are all less than 9 m deep.

Although cleanup and restoration in the CDA Basin has enabled some recolonization of animals in the CDA River, including benthic invertebrates now living in previously uninhabitable areas, high levels of trace metals in the river are biologically available for uptake by aquatic organisms (Farag et al. 1998, Dillon and Mebane 2002).

Given that metals are biologically available in the CDA River, it is reasonable to predict that they may also be available in the chain lakes. Tolerance to trace metal stress has been suggested as one mechanism by which animals are able to survive in the river (Lefcort et al. 2004). If tolerance occurs in river biota, where metal ions are readily bioavailable, it may also occur in chain lake fauna that likely experience periodic fluxes of high concentrations of the same metal ions into the sediment-water interface and water column. With the exception of Sprenke et al. (2000), who measured surface water concentrations of metals in Rose L., Medicine L., Black L., and Anderson L., surface water concentrations and metal content of chain lake invertebrates has not been measured. The extent of metal bioavailability from chain lake sediments is not well understood.

Given the importance of amphipods to the diets of predatory fish, and given that the chain lakes are frequently used for recreation, the presence of *H. azteca* in the lakes warrants investigation of the species' potential utility as a sentinel for the CDA Basin. Additionally, *H. azteca* meets several of the criteria reviewed above for appropriate use as a sentinel. To my knowledge, the tolerance of populations of *H. azteca* living in mining-impacted habitats has not been reported. If metals in the lakes are bioavailable in concentrations that are comparable to published toxicity thresholds for laboratory-cultured amphipods, it is important to determine whether amphipod populations that are currently established in the Basin are more tolerant of trace metal exposure than are populations not chronically exposed to contaminated sediments, as this could have implications for management of polluted watersheds.

HYPOTHESES

My objectives were to (1) test whether exposure to trace metal pollution has contributed to increased tolerance to metals in CDA Basin lentic *H. azteca* amphipod populations, (2) determine seasonal abundance and metal burden of amphipods in nine chain lakes and a reference lake (Benewah L.), and (3) compare aqueous trace metal levels and limnologic parameters from lake water samples to patterns in amphipod abundance, size, and metal burden. The St. Joe River, which feeds Benewah L. at the south end of Lake Coeur d'Alene, is separated from the CDA River drainage by the St. Joe Mountains. The St. Joe River and its associated lakes (e.g., Chatcolet L.) have been used as references for metal-related work in the CDA Basin (Maret et al. 2003, Spears et al. 2006, Shea et al. 2012, Clark and Mebane 2014).

I conducted a laboratory study in which I compared the trace metal tolerance of field-collected amphipods from three chain lakes and compared their tolerances to that of amphipods from the reference site (Benewah L.). I focused on two metals that are present in high levels in the CDA River, Zn and Pb. These metals vary in their degree of regulation in aquatic organisms as one is essential for metabolism (Zn) and the other is nonessential (Pb). They also vary in the physical/chemical properties that make them more or less soluble. I tested the hypothesis that amphipod populations from the chain lakes are more tolerant of Zn and Pb exposure than are those from the reference lake (Benewah L.). My measures of tolerance were the test statistics generated by the survival analysis (compared shapes of survival curves), MST (mean survival time; h), LT_{50}

(median lethal time after exposure) and LC_{50} (lethal concentration for 50 percent of the population; could be calculated only in select cases) values for each population of amphipods exposed to each metal. I hypothesized that chronic metal exposure has contributed to an increase in the tolerance of the amphipods to trace metal toxicity, and that MST, LT_{50}, and LC_{50} values for the chain lake populations are significantly higher than those for the reference population.

While survival is the most commonly studied endpoint in toxicity studies, observations of the behavioral changes in animals exposed to metals and other pollutants can help us understand the sublethal effects of chemicals and the energetic trade-offs that those animals may experience as they cope with the metal stress. This is especially pertinent for animals that live in chronically-polluted habitats. I modelled this test on swimming tests conducted with the amphipod *Gammarus lawrencianus* (Wallace and Estephan 2004), in which amphipods that were previously exposed to Cd-contaminated sediments were less active than were naïve amphipods. The authors suggested that this pattern may be because the animals must expend significantly more energy to move themselves up through the water column. I predicted that amphipods collected from metal-enriched lakes would display more vertical swimming activity following the acute (96 h) metal exposure than would naïve amphipods. This study was designed as a first step towards determining whether these amphipods may be appropriate sentinels for the CDA Basin by testing whether they display tolerance.

Measuring the tolerance of field-collected CDA Basin amphipods to trace metal stress is only appropriate if metals are demonstrated to be bioavailable in the chain lakes. As sediment-metal concentration profiles vary with depth between the lakes (Sprenke et al. 2000), it is reasonable to predict that bioavailability and tolerance to metals will also vary. I complemented the laboratory study with a field survey of the nine chain lakes, in which I characterized the amphipod populations, aqueous trace metal conditions, and a set of limnological variables that influence metal bioavailability. I characterized resident amphipod abundance as catch per unit effort, growth as amphipod length, and bioaccumulation as tissue metal burden. I hypothesized that *H. azteca* abundance and metal burden varies between lakes, and that aqueous tracemetal concentrations, as well as the limnologic features that influence them, vary between lakes and also change from month-to-month. Finally, I predicted that aqueous metal concentrations correlate with amphipod trace metal tissue loads.

METHODS

Hyalella azteca is present in the CDA River (Van de Riet, pers. comm., 2015) and I confirmed its presence in at least seven of the chain lakes prior to the start of this study. The methods described below are divided into two parts: the seasonal (spring, summer, and fall) laboratory study, and the monthly (April – October) field study (Table 2). Amphipods and water samples were collected for each study, but served different roles. Amphipods collected for the seasonal lab study were used for toxicity tests and for

estimates of tissue metal concentrations in each season. Amphipods collected for the monthly field survey were used to calculate Catch Per Unit Effort (CPUE) and to characterize monthly patterns in tissue metal burden. This design allowed me to track short-term (within season) and long-term (across season) patterns in tissue metal burdens. Water samples collected seasonally were used to identify site conditions on the dates that amphipods were collected for the seasonal toxicity tests. Water samples collected monthly were used to track lake characteristics from spring through fall and to compare with amphipod tissue metal concentrations. Detailed protocols for all methods are present in Appendix 1.

For both the laboratory study and the field study, Benewah L., situated where the St. Joe River enters the southern-most portion of Lake Coeur d'Alene, served as a reference lake for the CDA drainage (e.g., Shea et al. 2012) as the St. Joe Mountains separate this drainage from mining activity in the CDA drainage (Figure 2).

All study sites were located on state or federal land with the exception of Black L., in which access was graciously provided by private landowners from the months of June through October. Individuals and families were observed recreating at all lakes throughout the sampling season. Details about sampling sites are in Table 3.

Laboratory study

Water sampling, storage, and analysis

Water samples for trace metal and water quality analyses to inform the laboratory study were collected from Rose L., Medicine L., Anderson L., Thompson L., and Benewah L. The season(s) in which each lake was sampled is presented in Table 2. The initial design included the reference lake (Benewah L.) and three chain lakes (Rose L., Medicine L., and Anderson L.). The sediment profiles (Rember, pers. comm., 2016, Sprenke et al. 2000) and patterns of stratification and mixing tendencies (Chess, pers. comm., 2015) suggested that the greatest variation in Zn and Pb bioavailability would be detectable among Rose L., Medicine L., and Thompson L., with metal availability being lowest in Rose L. (Table 1). However, as sampling visits to Thompson L. in May and June 2016 did not yield enough amphipods, I sampled Anderson L. instead of Thompson L. for the first two seasonal lab studies. In August, an aquatic fungus introduced mortality during acclimation and toxicity tests to the Rose L. amphipods. At the same time, it was discovered that Thompson L. had sufficient amphipod abundance to begin collections for the toxicity tests. Thus, amphipods were collected from Thompson L. in place of those from Rose L. for the fall season test.

Prior to each collection, a recent (<24 hr) weather report was obtained as rain, high flow events, and wind events can alter sediment/water interactions. During each visit (Table 2), water was collected from three sites per lake; sites were separated by a distance of at least 10 m along the shoreline. Time of day and observations of water

conditions, local vegetation, wildlife, and human activity were recorded (Table 3). Water temperature (°C), DO (mg/L), and pH were measured using a YSI model 556 probe that was positioned approximately 16 cm above the sediment surface.

For trace metal and hardness analyses, surface water at the littoral zone was collected into acid-washed (10% nitric acid) plastic bottles (500 mL) at the same depth that the YSI was employed. Each sampling bottle was rinsed three times in lake water, with the sample bottle filled and capped on the fourth fill. Collection bottles were filled completely with little to no head space. Water samples were transported on ice and stored at 4°C until processed (within 72 hours). Water samples from a given lake were acidified with 70% trace metal grade (TMG) nitric acid (5 mL/L) (USEPA 1992) and then pooled (333 mL per site) in an acid-washed glass beaker. The 1 L pooled samples were heated until the sample volume reached 150-200 mL. Samples were concentrated to ensure that all metals would be detected by the instrument. Boiled sample concentrations are compared with unprocessed versions of the same samples in Appendix 3. A 10 mL subsample from each concentrated sample was filtered through a Whatman™ Puradisc 13 mm diameter 0.45 µm PTFE disposable syringe filter, into a 15 mL polyethylene vial, acidified to 2% nitric acid using 70% (TMG) nitric acid and stored at 4°C until analyzed. Metal analysis of water samples was performed by inductively coupled plasma optical emission spectroscopy (ICP-OES) in the EWU Department of Geology using standard methods (USEPA 1982). Seven calibration standards were used to calibrate the ICP-OES. Certified reference materials included High-Purity Standards

Soil Solution B, River Sediment Solution B, and Trace Metals in Drinking Water. Analyses were run in batches of 30-40 samples, generally within 1-2 months of collection. Samples were loaded onto the autosampler randomly. If all samples collected on the same date could not fit into the autosampler to be run on the same day, the remaining samples were run in the next analysis. If an analysis showed QAQC issues, the samples were reanalyzed. The following elements were reported in every analysis: Ca, Mg, Pb, Zn, As, Cd, and Cu.

I analyzed concentrated versions of the lake water samples because certain elements (e.g., Pb) are often present at levels below the level of instrument detection (LOD) in lake water samples (Nezat, pers. comm., 2016). In July, paired sets of original samples (not concentrated) and concentrated samples were analyzed simultaneously to compare detection efficacy. Values were corrected for the dilution factor (to account for water lost) prior to statistical analysis.

Reported values of all samples (concentrated or not) were evaluated with respect to the LOD from that day's run prior to statistical analysis. When the concentration of an element fell below the LOD, a ½ * LOD was reported (Table 4) for that element in statistical analyses (Zhang 2007).

As ICP-OES analysis measures calcium and magnesium content, water hardness was estimated using the following equation, after the values had been corrected for the dilution factor:

$$Total\ Hardness\ \left(\frac{mg}{L} as CaCO_3\right)$$

$$= 2.5\ \times calcium\ conc.\left(\frac{mg}{L} as\ Ca^{2+}\right) + 4.12$$

$$\times magnesium\ conc.\left(\frac{mg}{L} as\ Mg^{2+}\right)$$

At each of the sampling locations per lake, water samples were collected for alkalinity measurements into separate 100 mL plastic bottles and transported to EWU on ice. Samples were stored at 18°C until alkalinity titrations could be performed following standard procedures (Wetzel and Likens 1991). The samples were not pooled prior to alkalinity titrations.

Amphipod collection, acclimation, and test preparation

I conducted a static 96 h water-only Zn toxicity test in the spring (June), summer (August), and fall (October) of 2016, and a Pb test in the spring and summer. Due to the limited daylight availability in late October that limited amphipod collection time, as well as other logistical constraints concerning Pb solubility in the test chambers, a fall Pb toxicity test was not conducted.

Amphipods were collected from one water sampling site per lake; they were collected from the same site each season. Amphipods were collected for seasonal trace metal analysis (N=10/lake) and for the toxicity tests (N = 230/lake) from Rose L., Medicine L., Anderson L., Thompson L., and Benewah L. (Table 2). Amphipods were scooped into metal sieves from the sediment/water interface, from vegetation, or from clumps of algae, and pipetted from the sieves into plastic containers. Amphipods were

stored in lake water and, when air temperatures were above 21°C, on ice, while transported to EWU. Immediately upon arrival to the lab, amphipods to be used in the toxicity tests were transferred with lake water to 2 L aquaria with 1-2 pieces of cheesecloth and a small pinch of finely ground TetraMin® fish food. The aquaria were maintained in an incubator (Precision Scientific Model 815 Refrigerated Incubator) that was set to the collection temperature (on any given sampling day, water temperature did not vary more than 8.5°C across the sampling lakes; this variation reflects time of day, not inherent variation in water temperature across the bodies of water) as recommended by ASTM (2014). Amphipods that were collected only for metal analysis remained in the collection containers; the containers were stored with the caps off in the incubator for up to 72 hours. These amphipods were not fed. Amphipods that were analyzed for metal analysis were measured (total body length) and weighed, then flash-frozen in liquid N_2, and stored at -80°C. Only amphipods that were larger than 2.0 mm were saved for metal analysis.

Amphipods to be used in toxicity tests were acclimated for one week at the temperature that was closest to the average temperature that was measured at the time of collection (spring, 21°C; summer, 23°C; fall, 14°C). Amphipods were maintained in groups of approximately 100 individuals in at least 2,000 mL of water in aquaria that contained several pieces of 3 cm x 3 cm cheesecloth as substrate. Amphipods were fed TetraMin® fish food ad libitum (approximately every other day), and were under a 12L/12D photoperiod (similar to field conditions). After the first 24-36 hours, in which

the amphipods were housed in 100% lake water, the water was changed daily, with a 50% dilution with pre-chilled dechlorinated water until, by day 6, the amphipods were housed in 100% dechlorinated water.

In June and August (spring and summer collections), amphipods collected from Rose L. experienced high mortality during the acclimation period. A fungal infection is the suspected cause of this mortality as observations were made of a fungus on the dead amphipods on a daily basis in the Rose L. aquaria. For the October (fall) collection, amphipods and water samples were collected from Thompson L. instead of Rose L. (Table 2). Aside from Rose L. (June, August; 35%) and Thompson L. (August; 27%), amphipod mortality during the acclimation period in all other lake/season combinations was <1%. Mortality of Thompson L. amphipods during the final collection period may indicate residual contamination of the incubator, air tubing, air stones, or aquaria from the Rose L. amphipods.

To my knowledge there exist no publicly-available, comprehensive trace metal surface water data from the chain lakes. To determine the appropriate doses for the toxicity tests, in May 2016 I tested whether the 96 hour LC_{50} values for Zn and Pb (Table 8, Borgmann et al. 2005) were appropriate for these populations of (presumably metal-tolerant) amphipods by testing 0%, 100%, 200%, 300%, and 400% of the LC_{50}s in hard water (water hardness 100 mg/L $CaCO_3$; 100% = 29.0 mg/L for Zn, 5.4 mg/L for Pb). These preliminary tests were conducted with amphipods that were collected from Rose L., Medicine L., Anderson L., and Benewah L. Given the patterns of mortality in these

tests (data not shown), I selected five doses for Zn tests (0, 14.5, 29, 43.5, and 58 mg/L Zn) and five doses for Pb tests (0, 5.4, 10.8, 16.2, and 21.6 mg/L Pb).

At the start of each season, a 59 g/L stock solution of zinc sulfate and a 1.4 g/L stock solution of lead nitrate were made in Milli-Q (nanopure) water. Toxicity tests were conducted in 0.5 L glass jars, which were acid-washed and rinsed with Milli-Q water prior to each test. Each jar was filled with 500 mL of dechlorinated tap water (test water), which was allowed to come to temperature for 24 hours. Jars were housed in 150 gallon Rubbermaid stock tanks in which dechlorinated water to an approximate depth of 8 cm was maintained at the amphipod acclimation temperature by water chillers (VWR Scientific Model 1167 and Julabo Model F12).

Acute toxicity tests

During the spring and summer seasons, amphipods were collected for the Zn toxicity test and acclimated for one week (dates of collection: Spring Zn, 6/4/2016; Spring Pb, 6/11/2016; Summer Zn, 8/20/2016; Summer Pb, 8/27/2016). Amphipods for the Pb test were then collected, and their one-week acclimation period occurred while the Zn toxicity test was conducted. The Pb toxicity test was conducted one week after the Zn toxicity test. In the fall, only the Zn test was conducted (date of collection: 10/8/2016).

Nearly identical methods were used during the Zn and Pb toxicity tests. Amphipods were moved individually from the acclimation containers into the 500 ml test jars using a Pasteur pipette with the outermost 3 cm removed. Care was taken to reduce the effects of water tension on amphipod mobility while transferring the

animals. Each test jar received 10 amphipods from a given lake and a 3 cm x 3 cm piece of cheesecloth. Immediately after amphipods were transferred to the test jars, the jars were dosed with either zinc sulfate or lead nitrate. There were 3-5 replicate jars, each with ten amphipods, at each lake/dose combination. Observations for mortality were made at 24, 48, 72, and 96 h. Mortality was confirmed as no movement within 30 seconds of disturbance (Reish and Oshida 1986). Dead amphipods were removed from each jar at every mortality check. The June (spring) Zn and Pb tests included cultured *H. azteca* (Chesapeake Cultures) as a fifth population in order to confirm that patterns in survival of the Benewah L. amphipods were consistent with those of individuals that are naïve to metal stress. The commercially-obtained amphipods were acclimated as described above, with gradual changes from the water in which they were shipped. During preliminary tests, I observed a precipitate at the bottom of all Pb-dosed test jars. As I suspected that this influenced Pb availability I renewed jar water daily in an attempt to limit variation resulting from the precipitation of minerals that might include Pb. Jar water renewals took place immediately after each 24 hour mortality count. All water used for the renewals was brought to the same temperature as the test jars 24 hours prior to each renewal. The same cheesecloth was used for a given jar over the entire duration of the test.

Sublethal swimming tests

For each Zn and Pb test, select amphipods that survived the full 96-hour exposure period were tested in a vertical swimming activity immediately after the completion of

the toxicity test. The swimming activity tests were modelled on Wallace and Estephan (2004). Surviving amphipods from select jars (time constraints prevented testing all surviving amphipods) were tested individually in Tic-Tac® boxes (60 mm x 35 mm x 12 mm) that were filled with 20 mL of dechlorinated water that was the same temperature as water from the toxicity test. One individual was added to a single box (N for each treatment depended on the number of survivors). Amphipods acclimated for 1 min in the box prior to the 10 min swimming test. The number of times an amphipod surfaced (swam to the surface of the water, then returned to the bottom of the box) was counted over 10 min. Select amphipods were frozen for tissue metal analysis and stored at -80°C.

Amphipod tissues were digested in 30% TMG hydrogen peroxide, and then reconstituted in 2% TMG nitric acid prior to metal analysis (Alcock 1987, Schoonover 2013, Schoonover et al. 2016). Each reconstituted tissue sample was filtered through a Whatman™ Puradisc 13 mm diameter 0.45 µm PTFE disposable syringe filter, into a 15 mL polyethylene vial. Metal analysis was performed as above.

Statistical analysis

I performed Gehan-Breslow survival analyses to compare amphipod survival across metal doses for a given lake in a given toxicity test (Bland and Altman 1998). The nonparametric Gehan-Breslow is a version of the Kaplan-Meier survival analysis that is appropriate for multiple groups; it was conducted in SigmaPlot (version 11.0).

The data included both “true” events (amphipod deaths confirmed by the presence of a carcass) and censored events (amphipods that disappeared from the

assay, that died from mishandling, or that were sacrificed at 96 hours). The test uses a Chi-square approach to generate a p value with post-hoc comparisons that test whether the survival curves generated from the amphipods in each metal dose group differ. The test also generates two measures of central tendency with 95% confidence intervals (CI), the mean survival time (MST) and, when appropriate, the LT_{50} (the median time to mortality for 50% of the test population for each dose). Comparisons among lakes and/or across toxicity tests can be accomplished by comparing these measures of central tendencies; any pairwise comparisons for which the 95% CI do not overlap are considered to be significantly different ($p < 0.05$).

Where appropriate, i.e., those test populations that experienced at least 50% mortality in a majority of the jars, the LC_{50} values (lethal concentration for 50 percent of the population) were calculated with the SAS PROBIT Procedure in a logistic regression (version 9.4; SAS code in Appendix 2). The probit analysis is used to apply a regression approach to a binary response variable (alive/dead); the regression approach is used to estimate the dose that yields a 50% response rate. Unlike the Gehan-Breslow survival analysis, this analysis did not discriminate between true events and censored events (SAS 1999). The analysis uses the $\log_{10}$ value of the independent variable (metal dose), and identifies those observations that have a dose value of zero as the control group (do not contribute to the regression).

Standard nested ANOVA could not be applied to the amphipod swimming data as dose was not nested within lake population with a balanced design (not all dose*lake

combinations were tested) and the number of replicates per dose*lake combination varied considerably. I compared the log(x+1) transformed swim data (number of surfacings) using restricted maximum likelihood (REML) with the MIXED procedure of SAS (VanLeeuwen et al. 2013). The independent variables, Population (lake ID), Dose (metal concentrations), and Season (spring, summer, fall) were fixed effects, while Jar was a random effect (SAS code in Appendix 2). Not all models included the jar term if the model failed to converge because amphipods from only one jar were tested within a given Population*Dose group. Degrees of freedom were determined by the Kenward-Roger approach (VanLeeuwen et al. 2013). As I could not include the jar term in all analyses, I used one-way ANOVA to test whether amphipod swimming differed across jars for those dose*lake combinations in which multiple jars were tested (SigmaPlot); amphipod swimming activity did not vary significantly across jars (data not shown). When testing whether patterns in swimming activity differed across doses within a toxicity test, the model was *logswim* = *Pop* + *Dose* + *Pop*Dose* + *Jar(Pop*Dose)*. When testing whether seasonality affected the swimming behavior of amphipods that were exposed only to control (metal-free) conditions, the model was *logswim* = *Pop* + *Season* + *Pop*Season*. When asking whether seasonality and toxicity test type ("Metal", but note that data are only from control, or metal-free, conditions) influenced the swimming activity of amphipods in the control conditions, the model was *logswim* = *Metal* + *Pop* + *Metal*Pop* + *Season* + *Metal*Season* + *Season*Pop* + *Metal*Season*Pop*; this analysis did not include the Chesapeake Culture amphipods from the spring Zn

toxicity test as they were not tested in any other season nor in the Pb toxicity test. This analysis was conducted twice, once with all three Zn toxicity tests and once with just the spring and summer tests, in which both metals were employed.

Field study

Once each month (April – October 2016), I collected amphipods and water samples from nine chain lakes and from Benewah L. for the purpose of characterizing limnological variables in the lakes, dissolved trace metal concentrations in lake water, and tissue trace metal concentrations in the amphipods (Table 2).

Amphipod sampling

Amphipods were collected monthly from each lake for the purpose of characterizing the amphipod populations and to determine monthly patterns in tissue metal burden. Amphipods were collected on the same day as water samples; water and amphipod samples were processed 36 hours after collection. Amphipods were collected using a 500 µm dip net. Catch per unit effort (CPUE) was calculated using one net scoop of a standardized volume of water (12 in x 6 in D-frame diameter * 1 meter length). Each scoop was transferred to a Ziploc bag and filled with lake water. All CPUE samples were transported to EWU and stored in the bags (opened) at the mean collection temperature until they could be sorted (approximately 36 hours). To characterize the population in each lake, amphipods collected in each scoop were counted, measured (mm), and weighed (g). If a dead amphipod was found, only the length was measured.

The dead amphipod was still counted as part of the CPUE and mortality was noted. Mortality was uncommon (less than 1%).

Separately, I collected amphipods for tissue metal analysis at each lake. Individual amphipods (10-15/lake) were collected in small plastic containers filled with lake water, and transported in a cooler on ice to EWU. Amphipods were stored at the mean collection temperature in the collection containers until they could be processed (approximately 36 hours) and were not fed. Mortality in these containers was less than 5%. Weight (g) and length (mm) measurements were recorded for each individual before they were pooled together, flash-frozen in liquid N_2, and stored at -80°C. Amphipod tissue digests and metal analysis was performed as above for all Field Study amphipod tissue samples.

Water sampling

Each month, water samples were collected in 1 L acid-washed screw top plastic bottles from each of the lakes. The methodology for these monthly samples was identical to that described above. As above, triplicate samples from each lake were pooled prior to analysis of trace metal content, and samples for alkalinity titrations were not pooled. Water temperature (°C), DO (mg/L) and pH were measured in triplicate in the field using the YSI meter. General observations were also recorded at each site (Table 3). Samples were analyzed for trace metals using methods described above. Samples with element concentrations below the LOD for a given collection month are reported in Table 4.

Statistical analysis

Nearly all of the field data analyses were conducted twice, once with the October data from Killarney L. and once without it. In several variables, including Zn, Cd, and As, the samples collected from Killarney L. in October were several standard deviations away from the global mean, indicating outlier status. However, as I was unable to determine *why* these measurements would have occurred, the data were not eliminated.

I compared CPUE and limnological variables across all lakes sampled each month using a repeated measures ANOVA with post hoc comparisons conducted with the Holm-Sidak method (SigmaPlot). For those variables that were not normally distributed, I used Friedman (nonparametric, repeated measures) ANOVA and conducted Wilcoxon Matched Pairs Test with Bonferroni-corrected alpha error rates as a proxy for a post hoc comparison. The CPUE data were (natural) log-transformed prior to analysis. For most variables, inclusion of the October Killarney data did not substantially alter the results, however, as all metal and the hardness data were affected by the October Killarney data point, all lake limnological data are presented without the October Killarney data point.

I tested whether amphipod length varied across the lakes with the MIXED procedure with restricted maximum likelihood estimation in SAS, with degrees of freedom determined by Kenward Roger (Code in Appendix 2). The model terms were lake ID and collection month and I treated site as the repeated term, nested within the lake*month group. The model was *Length* = *Lake* + *Month* + *Lake*Month* + *Site*(*Lake*Month*). This is not a true repeated measures analysis, as it treats month as a

factor with ordered levels rather than nesting the month factor within lake ID. This was necessary as there was substantial variance among collection sites in a lake within a given month. The Type 3 tests of fixed effects were assessed; post hoc comparisons were not made as the number of potential combinations was prohibitively high.

I used Friedman (repeated measures) ANOVA on ranks to test whether metal concentrations in the pooled amphipod tissue samples varied across lakes and Spearman Rank Order correlations to test whether tissue metal levels were correlated.

I used several methods to test whether CPUE was related to total metal levels or water quality parameters. As the relationships between CPUE and the limnological variables could not be modeled with linear regression, I first tested whether a quantile regression approach was appropriate (Ramsey et al. 2005, Schmidt et al. 2012). Quantile regressions were conducted with and without the October Killarney L. data and were conducted with and without Benewah L. data (as Benewah L. is not a chain lake). Quantile regressions were conducted in SAS, following the standard procedure (SAS 2016). As the quantile regression approach did not result in estimates for slope that were significantly nonzero, these data are not presented in the Results. SAS code and representative SAS output are presented in Appendix 2.

Second, relationships between limnological variables and (ln)CPUE were modeled with Principal Components Analysis in R (version 3.2.3.). The PCA was conducted with and without the October Killarney data. A principal component regression (PCR) tested whether (ln)CPUE was related to loading score on each PCA axis.

Repeated measures ANOVA (or Friedman ANOVA) were conducted to test whether PCA axis loading scores differed significantly across lakes for the first two PCA axes.

I tested whether CPUE was related to dissolved metal levels, water quality parameters, or body burden using Spearman rank order correlations.

RESULTS

Seasonal field conditions

At the time that amphipods were collected for each seasonal toxicity test, a number of water quality metrics were collected (Table 5). Across the seasons, the highest total metal content (in lake water) tended to occur in Medicine L., Thompson L., and Anderson L. Hardness varied considerably, but in most lakes increased over time. Alkalinity, pH and DO were highest in Benewah L. and Anderson L. There appears to be a seasonal trend in pH across all lakes, with more acidic values recorded in the fall.

Toxicity tests - survival

Survival analyses were conducted separately for each lake in each seasonal toxicity test (Zn, Pb); post hoc comparisons following the survival analysis allowed for evaluation of dose effects. Survival analyses also provided estimates and 95% confidence intervals of two measures of central tendency, mean survival time (MST) and median survival time (LT_{50}), which allowed for comparisons across lake and/or season. Finally, LC_{50} values were calculated when appropriate.

Several metrics confirm that toxicity tests were conducted in accordance with standard procedures. First, amphipod survival in control jars (0 mg/L) was ≥ 80% across all Zn tests (means and statistical results in Table 6) and ≥ 70% across all Pb tests (means and statistical results in Table 7). Second, the reference populations (Chesapeake Culture amphipods and Benewah L. amphipods) should exhibit low tolerance to metals, illustrated by lower survival and a higher LT_{50} (Tables 6 and 7) when exposed to metals. The commercially-available amphipods had significantly higher survival in control conditions than in any Zn or Pb treatment. In all three Zn toxicity tests, amphipods from the reference site, Benewah L., had higher survival in the control conditions than in any metal-containing treatment. In contrast, Benewah L. amphipod survival was highest in the control conditions versus all other Pb doses only in the spring Pb toxicity test. Third, metal availability should follow a dose-response pattern. The presence of metal in the jar water and in amphipod tissue and additional water quality metrics from select toxicity tests are presented in Tables 8 and 9. Hardness of the dechlorinated water used in multiple toxicity tests ranged from 105 – 110 mg/L (Table 8). Jar water Zn concentration tended to follow the dose pattern (Table 9); this pattern was less apparent in the Pb samples. Amphipod tissue metal concentrations were determined from pooled individuals from Benewah L. and Anderson L. prior to the spring Zn test; Zn tissue levels were 2.49 and 2.83 mg/g Zn, respectfully. Amphipods exposed to Zn for 96 h had at least 8.86 mg/g Zn (Table 9).

Zinc toxicity tests

As illustrated in Figure 3, amphipods from both reference populations had significantly lower survival in metal-containing treatments than in the control treatment in the spring Zn toxicity test (Tables 6 and 7). While no Chesapeake Cultures amphipods that were exposed to Zn lived to the 96 h mark, up to 20% of the Benewah L. amphipods that were exposed to Zn survived to 96 h. When mean amphipod survival (MST) differed significantly between these two reference populations (14.5 and 43.5 mg/L Zn), survival was higher in the Benewah L. population. Differences in Chesapeake Cultures amphipod survival followed a general dose response pattern, with amphipod survival in the intermediate doses (14.5 and 29 mg/L Zn; light gray lines in Figure 3a) tending to be higher than it was in the two highest Zn doses (43.5 and 58 mg/L Zn; dark gray lines in Figure 3a). The pattern across Zn doses was different for the Benewah L. amphipods; survival in the highest dose (58 mg/L Zn; solid dark gray line in Figure 3b) was significantly lower than that in any other Zn treatment. The effects of seasonality cannot be assessed in the Chesapeake Cultures amphipods as they were used only in the spring toxicity test. As noted previously, Benewah L. amphipod survival in all seasons was significantly decreased in the presence of any Zn dose in comparison to control conditions. However, differences in Benewah L. amphipod survival across the Zn doses became less pronounced in the later seasons (Table 6). This is illustrated with comparisons of the MST and LT_{50} values, which tended to be highest in the fall.

Rose L. amphipod survival was higher in the control conditions than in the two highest Zn doses in both spring and summer toxicity tests (Table 6) and higher than in the lowest metal dose (14.5 mg/L Zn) in the summer test. At two doses (14.5 and 58 mg/L Zn) Rose L. amphipod survival (MST) was significantly higher in the spring than in the summer. This seasonal pattern is supported by the estimates of LC_{50}, which were 36.2 mg/L Zn in the spring and 6.12 mg/L Zn in the summer; these values should be interpreted with caution as they are not accompanied by 95% confidence intervals given the low sample size. Rose L. amphipods were not tested in the fall toxicity test.

Medicine L. amphipod survival was significantly higher in the control than in all metal-containing treatments in all Zn toxicity tests (except 0 vs. 14.5 mg/L Zn in summer; Table 6). The only time that Medicine L. amphipod survival differed significantly among Zn doses was the elevated survival of amphipods exposed to 14.5 mg/L Zn versus those in the two highest doses in the summer toxicity test. The summer toxicity test also was the only test in which an LC_{50} could be calculated; it was 58.9 mg/L Zn (95% CI, 47.7-88.5 mg/L Zn).

Anderson L. amphipod survival was highest in the control group across all Zn tests (Table 6). In the summer and fall tests, amphipod survival was significantly higher in the lowest dose than in the two highest doses; this pattern was not present in the spring assay. Anderson L. amphipods had significantly lower tolerance to Zn in the summer than in the fall, as indicated by LT_{50} values at 29.0, 43.5, and 58.0 mg/L doses

(Table 6) and by LC_{50} values of 9.86 mg/L (95% CI 4.1 – 14.2 mg/L Zn; summer) and 25.3 mg/L Zn (95% CI 18.8 – 31.2 mg/L Zn; fall).

Thompson L. amphipod survival was highest in the control group in the summer and fall tests (Thompson not tested in the spring; Table 6). Dose-specific patterns in amphipod survival were not consistent across season; this may reflect the low number of replicates (jars) in the summer toxicity test. The greatest replication was used in the highest Zn dose (58 mg/L Zn); amphipod survival (MST and LT_{50}) at this Zn dose was higher in the fall than in the summer. In the fall test, the only season in which it could be calculated, the LC_{50} was 24 mg/L Zn (95% CI 13.2 – 32.4 mg/L Zn).

Tolerance to Zn was greater in the chain lake amphipod populations than in the reference population(s) in the spring test but not in the summer and fall tests (Table 6). In the spring, survival at the highest Zn dose (contained the most replicates) was significantly lower in Benewah L. amphipods than in Rose, Medicine, and Anderson L. amphipods. There is no evidence in the spring test that Zn tolerance differed among chain lake populations. All field-collected amphipods exhibited higher Zn tolerance than did the commercially-obtained amphipods in the spring. In the summer toxicity test, Benewah L. amphipods had lower tolerance of Zn than did amphipods from Medicine L. (all doses) and Thompson L. (highest dose only). In contrast, the Zn tolerance exhibited by Benewah L., Rose L., and Anderson L. amphipods was indistinguishable (except for lower survival of Rose L. amphipods at 14.5 mg/L Zn). Unlike in the previous seasons, Medicine L. amphipods in the fall test had similar or lower Zn tolerance than did

Benewah L. amphipods. Anderson L. and Thompson L. amphipods also had similar Zn tolerances to those of Benewah L. amphipods in the fall test; these patterns may reflect the significant increase in the Zn tolerance of Benewah L. amphipods in this test in comparison to earlier seasons. While differences in LC_{50} between Benewah L. and chain lake amphipods could only be calculated for the fall assay and should interpreted with caution as the 95% CI could not be calculated, the estimate available for the Benewah L. data, the point estimate of the LC_{50} for Benewah L. amphipods is lower (8.13 mg/L Zn) than is the 95% CI's of Anderson L. (18.8 – 31.2 mg/L Zn) and Thompson L. (13.2 – 32.4 mg/L Zn) amphipods.

Lead toxicity tests

As illustrated in Figure 4, both reference populations, the commercially-obtained amphipods and those from Benewah L., had decreased survival when exposed to metal. The Chesapeake Culture amphipods were exposed to Pb only during the spring Pb toxicity test, during which survival in the control conditions (100%) was significantly greater than was survival in all metal-containing treatments (Table 7). The dose response pattern for the Chesapeake Culture amphipods (both from survival analysis and MST) suggests a two-step change in survival, with amphipod survival in the two lowest Pb doses (5.4 and 10.8 mg/L Pb) being lower than in the two highest doses (16.2 and 21.6 mg/L Pb). The Chesapeake Cultures amphipods were the only population in either Pb test to have enough mortality that an LC_{50} value could be calculated; the LC_{50} was 15.9 mg/L Pb (95% CI, 12.0 – 28.2 mg/L Pb). There were no consistent differences in

Benewah L. amphipod survival across Pb doses (Table 7); in the summer assay, Benewah L. amphipod survival was significantly higher in control conditions than in the two highest Pb doses. Differences in Pb tolerance between the two populations only were detected at the two highest doses, at which Benewah L. amphipods had higher survival.

No dose-specific or seasonal patterns in survival were detected in amphipods from Rose L., Medicine L., Anderson L. or Thompson L. (Table 7). At the two highest Pb doses in the spring test, the Chesapeake Cultures amphipods had significantly lower MST than did any other amphipods in the test.

Swimming activity

To assess the uniformity of the swimming activity test, I evaluated the swimming activities (number of surfacings during a ten-minute period) of amphipods that were exposed to control (metal-free) conditions, with specific comparisons across metal assay type, season, and lake population (Figure 5). When modeled with just the first two seasons and without the Chesapeake Cultures data, lake population was the only individual factor that was statistically significant (Lake, $p = 0.0015$; Metal, $p = 0.248$; Season, $p = 0.500$); this likely reflects the higher-than-average swimming activity in Rose L. amphipods and lower swimming activity in Thompson L. amphipods. As only those interaction terms that contained metal were significant (Metal*Pop, $p = 0.0001$; Metal*Season, $p = 0.007$; Metal*Season*Pop, $p = 0.0002$; Season*Pop, $p = 0.091$), the effects of season and lake population on swimming activity was different in the two assay types and the swimming results must be interpreted within assay type. Across the

three zinc toxicity tests (Figure 5a), amphipods from Thompson L. and Rose L. tended to swim more than did the amphipods from the other populations, but differences among lake populations varied considerably across season and not in a consistent pattern (Pop, $p < 0.0001$; Season, $p = 0.0065$; Pop*Season $p < 0.0001$). In the spring Zn toxicity test, the Benewah L. amphipods were more likely to swim and had nearly ten-fold higher average swimming activity than did the amphipods from Chesapeake Cultures (Figure 5a; Table 10). In the spring Pb toxicity test, the Chesapeake Cultures amphipods were not tested; the Benewah L. amphipods had comparable swimming activity to those in the spring Zn assay (Table 10).

To assess the sensitivity of the swimming assay, I tested whether swimming activity was altered in amphipods that had experienced 96 hours of metal exposure in comparison to those in control conditions. In all five toxicity tests, dose significantly decreased swimming activity (all tests, $p \leq 0.009$).

Zinc toxicity tests

I assessed the influence of Zn exposure on amphipod swimming activity independently for each season's test (Table 10 and Figure 6). In all three seasons, Benewah L. amphipods that were exposed to any Zn dose had substantially lower swimming activity than in control conditions, particularly at the two highest Zn doses. Rose L. amphipods that were exposed to any Zn dose had significantly lower swimming activity. In the summer, Medicine L. amphipods swam more in control conditions than in (at least) the two highest Zn doses; amphipod swimming was low in all Zn doses in the fall. In general,

Anderson L. amphipods swam more in the spring toxicity test than in later tests. In the spring, Anderson L. amphipods in the lowest Zn dose had comparable swimming activity to those in the control, but the pattern was bimodal, with half of the amphipods having very high swimming (> 40 surfacings) and half having low activity (< 10 surfacings). At the three higher doses in the spring toxicity test, Anderson L. amphipod swimming was substantially lower than in the control; this pattern was not repeated in the summer or in the fall, during which all Zn-exposed amphipods had little to no swimming activity. In both summer and fall toxicity tests, Zn-exposed Thompson L. amphipods exhibited almost no swimming activity.

In only the summer toxicity test did amphipod swimming activity differ by lake population; Medicine L. amphipods had the highest swimming activity in the control conditions and following exposure to 29 mg/L Zn. In the fall toxicity test, both lake population and dose significantly altered amphipod swimming activity. In the control conditions, Thompson L. amphipods had consistently high swimming activity; in contrast, at the highest Zn dose, Benewah L. amphipods had the highest swimming.

While this swimming activity test reliably indicated metal exposure, the predicted differences among lake populations were not as clear. In the spring toxicity test, only some of the Anderson L. amphipods and none of the Rose L. amphipods maintained higher swimming activity following Zn exposure than did the Benewah L. amphipods. In the summer toxicity test, Medicine L. amphipods tended to swim more than Benewah L. amphipods at all Zn doses tested. In the fall toxicity tests, Benewah L.

amphipods had comparable swimming to the chain lake populations at the three lower doses; at the highest dose, three Benewah L. amphipods had higher swimming activity than did amphipods from any other lake.

Lead toxicity tests

I assessed the influence of Pb exposure on amphipod swimming activity independently for each season's test (Table 11). Amphipods that were exposed to all doses were tested in the spring toxicity tests, but only amphipods from the control and the highest dose were tested in the fall. In the spring, swimming was significantly altered by dose ($p = 0.009$) and by the population*dose interaction ($p = 0.005$), but not by population alone ($p = 0.25$). In the summer toxicity test, swimming activity differed by dose ($p < 0.001$) and population ($p = 0.005$), but not the interaction ($p = 0.14$).

Benewah L. amphipod swimming activity was higher in the control and lowest dose tested than in the three highest doses; all amphipods tested at the control and lowest dose swam at least once, whereas some amphipods in the three highest doses did not swim. In the summer toxicity test, Benewah L. amphipod swimming activity was higher in the control than in the highest dose (only dose tested).

Rose L. amphipods were only tested in the spring toxicity test. As was the case with Benewah L. amphipods, swimming activity was higher in the control and lowest dose tested, than at the high Pb doses, and all amphipods from control and lowest dose swam, but some amphipods from the higher doses did not swim.

The patterns in swimming activity were less clear for Medicine L. and Anderson L. amphipods. Dose response patterns were not apparent in the spring toxicity test; in the summer test, Pb-exposed amphipods from these two lakes had lower swimming activity than did those in control conditions.

Thompson L. amphipods were only tested in the summer toxicity test. The overall swimming activity of Thompson L. amphipods was lower than that of amphipods from other lakes, likely driving the significance of the population term. A larger proportion of Thompson L. amphipods swam following exposure to control conditions (9/10) than following the highest Pb dose (11/19).

Monthly field conditions

As noted in the Methods, the Killarney L. October sample was somewhat different from the rest of the samples from Killarney L. (Figure 7) and from all other lakes (Table 12). For example, total Zn in the October Killarney L. sample was nearly ten-fold higher than the next highest data point (October Killarney L., 4,200 µg/L Zn; September Killarney L., 460 µg/L Zn) and 100-fold (or more) higher than nearly half of the samples in the monthly field study. Similar trends were detected for Cd. Although this sample clearly would be considered an outlier for these datasets, where appropriate, the results are presented with and without this sample.

Amphipod populations

Amphipod CPUE varied significantly across the nine chain lakes and the reference lake (RM-ANOVA, $p < 0.001$ with and without October Killarney L. data; Figure 8, Table 12), from zero amphipods at any collection time in Bull Run L. (significantly lower CPUE than versus all lakes except Killarney L.) to a maximum of 211 amphipods collected from one site at Cave L. in October (Cave L. significantly higher than Bull Run L. and Killarney L.). Amphipods were always present at all sites in Rose L. and Benewah L., and the highest CPUE values for both lakes were observed in the fall (137 in September in Rose L. and 126 in October in Benewah L.). Rose L. and Benewah L. amphipod CPUE was significantly higher than in Bull Run L., Killarney L., Harrison L., Medicine L, and Black L. (Rose L. only).

Total amphipod abundance increased steadily over the months (May, 54; June, 318; July, 326; August, 474; September, 939; October, 958 amphipods; Table 12). The temporal pattern of amphipod abundance varied across lakes, but fell into several general patterns. Abundance increased steadily over time in Medicine L. and Cave L. (Figure 9), with much higher abundance in Cave L. (RM-ANOVA post hoc test of Cave L. vs. Medicine L., $p = 0.065$). Amphipod abundance in Thompson Lake and Harrison Slough also increased over time (Figure 10), but this trend began later in the year (August) than was the case with the other lakes. Amphipods were not detected at any site at Killarney L. until July (Figure 11a); when amphipods were present in Killarney L., CPUE was never higher than five. Killarney L., Black L., and Anderson L. (Figure 11) had low amphipod abundance overall; temporal changes fluctuated over the duration of the field season,

with no consistent pattern. In contrast, Rose L. and Benewah L. had very low amphipod abundance in May, and then peaked twice (Figure 12), with no difference in overall abundance (post hoc p = 1.0).

Body lengths were determined for all amphipods collected throughout the field season (N = 3,069 amphipods; Figure 13). For most lakes, amphipod length varied both by collection site and by month. For example, in Benewah L., amphipod average length was smaller at site 2 than at site 3 (data not shown) and longer in September and October than in August (Figure 12b). Therefore, the ANOVA model that assessed variance in amphipod length had to include site as a term, nested within lake*month combination. Amphipod length differed significantly by month (p = 0.0012) and by the interaction between lake ID and month (p = 0.0004), but not by lake (p = 0.282). Although average amphipod length did not vary by lake, the maximum length varied considerably (Figure 13), with the smallest amphipods in Killarney L. and Black L. Amphipod length tended to increase with month (Figures 8, 10-12), particularly after August, likely reflecting increased abundance (and growth) of the juveniles. Lake-specific patterns in amphipod length may reflect different timing of reproductive events, and tended to correspond to changes in amphipod CPUE, as illustrated most clearly in Benewah L. and Rose L. (Figure 12). In Benewah L., Rose L., Anderson L. and Thompson L., amphipod length was higher in the initial collection (May) than in the next several collections, likely reflecting the abundance of overwintering adults and the appearance of juveniles in mid-summer.

Amphipod tissue metal concentrations were measured in pooled samples of amphipods that varied in number from 6 to 29 individuals (Table 12). Amphipod As tissue concentrations (Table 12) did not differ significantly across lake populations ($p = 0.501$); amphipods from Black L. had approximately double the As burden (for three of the five months) than did amphipods from any other lake. The Cd tissue concentrations did not differ significantly across lake populations ($p = 0.075$; Table 12 and Figure 14), likely reflecting the low sample size (N = 2, Harrison L.; N = 3, Killarney L., N = 4, Medicine L., N = 5, all other lakes). Nonetheless, Cd levels clearly were higher in amphipods from Medicine L., Thompson L. and Black L. than in those from Rose L. and Benewah L. The Cu tissue concentrations differed significantly across the lake populations ($p < 0.001$; Table 12, Figure 14), with lower Cu body burden in amphipods from Killarney L. than in Rose L., Medicine L., Black L., Anderson L., Thompson L., and Benewah L. Similarly, amphipods from Harrison Sl. had significantly lower Cu body burden than did amphipods from Rose L., Black L., and Anderson L. While differences in Pb tissue concentrations between all lakes were nonsignificant ($p = 0.17$; Table 12), a similar pattern to that of Cd is apparent (Figure 14), wherein amphipods in Rose L. and Benewah L. had the lowest Pb body burden. Zn amphipod tissue burdens were not significantly different across the lakes ($p = 0.487$; Table 12). Across all lakes and months, As tissue concentrations were positively correlated with Pb and Zn tissue concentrations (Figure 15; $p \leq 0.0002$) and Cd and Pb tissue concentrations were correlated.

Limnological and metal characteristics of the lakes

Water temperature differed significantly across lakes ($p < 0.001$; Table 12 and Figure 16a) and followed the expected seasonal pattern. Anderson L. was warmer than Rose L., Bull Run L., Killarney L., Medicine L., Cave L., and Harrison Sl. Similarly, Thompson L. was warmer than Killarney L. DO levels also differed significantly across lakes ($p < 0.001$; Table 12 and Figure 16b) but did not follow a consistent seasonal pattern across lakes. Bull Run L. had significantly lower DO than did all lakes except Cave L. Similarly, Cave L. had significantly lower DO than Medicine L., Black L., Anderson L., Thompson L., and Benewah L. Finally, Harrison Sl. had lower DO than did Black L.

Water pH varied across the lakes ($p < 0.001$; Table 12 and Figure 17a) with no consistent seasonal trend; pH was significantly lower in Bull Run L. than in Rose L., Medicine L., Black L., Anderson L., Thompson L., Harrison Sl., and Benewah L. Alkalinity differed across lakes ($p = 0.00016$; Table 12 and Figure 17b). As the alkalinity data were analyzed with Friedman ANOVA, only one significant pair could be detected; alkalinity was significantly higher in Killarney L. than in Rose L. ($p = 0.018$; Table 12). Water hardness also varied significantly across the lakes ($p = 0.0007$; Table 12 and Figure 17c); this observation did not change when the October Killarney sample was included in the analysis ($p = 0.00005$). And as was the case with alkalinity, hardness in Killarney L. was significantly higher than in Rose L. ($p = 0.018$).

Total aqueous concentrations of As, Cd, Pb, and Zn varied significantly ($p \leq 0.019$; Table 12) across the lakes. Rose L. and Benewah L. were the only lakes with significantly

lower aqueous metal concentrations with respect to the chain lakes. Benewah L. had significantly lower concentrations of Cd and Pb than did Killarney L. and Medicine L., respectively ($p \leq 0.028$), while Rose L. was significantly lower in As, Cu, Pb, and Zn than were Killarney L., Anderson L., Medicine L., and Thompson L., respectively ($p \leq 0.028$). Several correlations (Spearman-Rank) were apparent between aqueous metals (Figure 15). For example, Zn was significantly correlated with all other metals, and Cd was significantly correlated with Cu, Pb, and Zn ($p \leq 0.0007$).

Relationships between amphipod populations and limnological and metal characteristics

I used a principal components analysis (PCA) to test whether combinations of total aqueous metal (As, Cd, Cu, Pb, and Zn) and water quality metrics (temp., pH, DO, alkalinity, and hardness) could explain variation in monthly amphipod abundance as Catch Per Unit Effort (CPUE). This analysis was run with and without the October Killarney data point; while general trends were similar in the two models, this data point strongly influenced the results and here I only describe the PCA conducted without this data point. The first principal component axis (PCA1), was negatively correlated with all five metals (reinforcing Spearman-Rank correlation results) and explained 38% of the variance in amphipod abundance. All metals reported from the metal analyses increased as CPUE decreased (Figure 18). The second axis explained 18% of the variance in amphipod abundance; on Axis 2, alkalinity and hardness were positively correlated with variance in CPUE and temperature, pH, and DO were negatively related to amphipod

abundance. It is important to note that together, the first two principal component axes only explain 56% of the variation in amphipod abundance. Multiple linear regression with the PCA scores from ten axes, lake ID and collection date as predictor variables explained 71% of the variation in lnCPUE (data not shown); when PCA scores were removed stepwise, the model with just lake ID and collection date explained 73% of the variation in amphipod abundance. Principal components regressions, in which lnCPUE is the response variable and the PCA scores for each axis are tested individually as the predictor variables failed to explain more than 16% of the variance in amphipod CPUE (data not shown). Finally, I used Kruskal-Wallis ANOVA to test whether the axis scores differed across lake (Figures 19a and 19b). Axis 1 scores (the five metals) differed significantly across lakes ($p < 0.001$), with significantly higher scores (indicating lower metal levels) in Rose L. than in Killarney L., Harrison Sl., Anderson L., and Medicine L. The Axis 1 scores for Killarney L. also were significantly lower than those of Benewah L. and Black L (and Rose L.). Axis 2 scores differed across lakes ($p < 0.001$), with Bull Run L. having significantly higher scores (indicating higher alkalinity and hardness and lower temperature, pH and DO) than Medicine L., Thompson L., Anderson L., and Black L.

Given the non-normality of the data, relationships between tissue and water metal were assessed only with Spearman-rank correlations. Amphipod tissue metal burden was generally not correlated with aqueous metal concentrations, with the exception of Pb burden, which was positively correlated with all aqueous metals except for Cu (Figure 15; Spearman-Rank, $p \leq 0.001$).

DISCUSSION

This study was designed to be the first step in determining whether *H. azteca* could serve as a sentinel species for the CDA Basin as I confirmed that it exists across a gradient of trace metal pollution. A species can be a sentinel if it exhibits tolerance to the pollutant that can be documented by dose response patterns in laboratory studies. I tested whether amphipods from chain lakes in the CDA drainage exhibited tolerance to total aqueous Zn and Pb. I also characterized the amphipod populations across the (presumably) metal-impacted chain lakes and an unimpacted reference lake and tested whether patterns in amphipod abundance, size and metal body burden could be explained by limnological factors and aqueous metal concentrations.

I found that amphipods from the chain lakes exhibited consistently higher Zn tolerance than did the commercial amphipods; this pattern was not as clear when chain lake amphipods were compared to the reference lake amphipods. The Zn tolerance of all amphipods varied seasonally, and not in the same manner for all amphipod populations. Differences in Pb tolerance between field-caught and commercially-obtained amphipods were apparent only at the highest Pb doses, likely reflecting issues with Pb solubility in the toxicity tests. Our activity assay was not sensitive enough for detection of among-population differences as swimming activity was strongly affected by all Zn and Pb doses. Amphipod populations differed considerably across the chain lakes and over time, but patterns in amphipod abundance and/or size could not be explained solely by the limnological or metal factors that I measured. The presence of

metals in amphipod tissues confirms that the metals are bioavailable in all lakes tested and that, as expected, spatial and temporal differences in limnological factors influence amphipod metal body burden.

Taken together, the laboratory and field results confirm that chain lake amphipods are more tolerant of metals than are the commercially-obtained amphipods that are routinely used for toxicity testing and for setting water quality criteria. If I had only used laboratory-cultured amphipods as the "naïve amphipod control," then I could conclude that chain lake amphipods have developed tolerance to chronic metal exposure and are suitable for use as a sentinel species for the CDA Basin. However, Benewah L. amphipods were included in this study to serve as a region-specific, field-sourced "naïve amphipod control," partly because *H. azteca* exist as a multi-species complex and because other laboratory-cultured organisms tend to be more susceptible to exogenous stress than do natural populations (Calisi and Bentley 2009, Melvin and Houlahan 2012). Benewah L. amphipods were less tolerant of Zn (but not of Pb) than were chain lake amphipods in spring and summer, but not in fall, highlighting the strong effect of seasonality on stress tolerance (Helmuth et al. 2010) and the importance of conducting this type of comparative study in multiple seasons. These results suggest that *H. azteca* could be a sentinel for this basin, but only if amphipods from Benewah L. or other unimpacted lakes in the region are used as the (negative) control population against which chain lake populations are calibrated year-round.

Seasonal toxicity tests

Toxicity testing for metals must be interpreted within the context of water hardness; our tests were performed in hard water (105-110 mg/L), at pH 7.4-8.2. Reported LC_{50} values for laboratory-cultured *H. azteca* in 96 h Zn water only toxicity tests range from 436 µg/L in hard water (100 mg/L) at pH 7.8-8.2 (Eisenhauer et al. 1999), to 1,500 µg/L in very hard water (280-300 mg/L) at pH 7.0-7.5 (Schubauer-Berigan et al. 1993). I was not able to estimate LC_{50}s for the Chesapeake Cultures or Benewah populations for either metal because survival at all metal doses was very low, indicating that the true LC_{50} was lower than my lowest dose of 14.5 mg/L Zn. The doses that I used in this experiment are considerably higher than those used in other water-only toxicity tests (Borgmann et al. 1993, Schubauer-Berigan et al. 1993, Eisenhauer et al. 1999) because I needed to employ doses that would be stressful for the chain lake populations. Unfortunately, this came at the cost of being able to compare the survival patterns of my two naïve populations (Chesapeake Cultures and Benewah L. populations) with published reports beyond the generalization that the naïve populations, particularly Chesapeake Cultures amphipods, were susceptible to the Zn and Pb doses.

The Zn LC_{50} values that I calculated for amphipods from Medicine L. and Anderson L. are orders of magnitude higher than the LC_{50} values for commercially-cultured amphipods, which confirms the prediction that chain lake amphipods are more robust to Zn than are the amphipods that are traditionally used as standards. The single estimate for Zn LC_{50} for Benewah L. amphipods from the fall toxicity test must be

interpreted cautiously as it is not accompanied by a confidence interval, but it, too, is orders of magnitude higher than that of the commercial standards, supporting my conclusion that the only appropriate control for amphipods from the Basin is field-caught amphipods. Patterns in MSTs for the lab-cultured amphipods, Benewah L. amphipods and chain lake amphipods support these conclusions, but at a greater sensitivity as the data are available for nearly all doses and populations. Patterns in swimming activity confirm that Zn treatment decreases swimming activity, but that population-level differences can only be detected if the Zn doses are substantially lower and if tests are conducted in multiple seasons. Thus, this swimming activity test may not be as appropriate for detecting among-population differences as are tests of metal-avoidance behavior in snails from Cave L. (Lefcort et al. 2004).

Reported LC_{50} values for 96 h Pb water only tests in very hard water (280-300 mg/L) at pH 7.0-7.5 were > 5,400 µg/L (Schubauer-Berigan et al. 1993), which is the lowest dose used in my toxicity tests. Borgmann et al. (2005) reported an LC_{50} of 147 µg/L at a hardness comparable to ours (124 mg/L; pH 8.3) for a one-week toxicity test. The only Pb LC_{50} that I could generate was an estimate of 16,000 µg/L for Chesapeake Cultures amphipods. This number is unreasonably high and likely reflects logistical difficulties. Similar to the current results, Besser et al. (2005) was not able to obtain a reliable LC_{50} value for *H. azteca* in Pb tests (hardness, 126 mg/L) because survival did not differ among the test concentrations as the Pb precipitated from the test water (they reported < 6 % of nominal Pb stayed in solution). I also observed Pb precipitate forming

at the bottom of test jars immediately after delivery. To compensate for this precipitation, I changed the test jar water daily. As is evident from the overall decreased swimming activity of amphipods in the Pb tests in comparison to the Zn tests and the absence of dose response patterns in the Pb tests, this daily water change was disruptive to the amphipods and did not alleviate the precipitation issue. Therefore, differences between the tolerances of Chesapeake Cultures amphipods and the chain lake populations cannot be evaluated with LC_{50}s. The MST at the highest Pb dose was higher in all of the chain lake populations than in the lab-cultured population, but not higher than that of Benewah L. amphipods. As in the Zn toxicity tests, this pattern reinforces the importance of comparing multiple populations of field-caught amphipods.

These findings are consistent with those of Pieterek and Pietrock (2012) who reported that laboratory-cultured *H. azteca* were more sensitive to acute Se exposure than were field-collected amphipods (collected in the summer). These findings are also consistent with those of Clark et al. (2015 and references within), who reported seasonal patterns in the sensitivity of field-collected amphipods to an insecticide and substantially increased pesticide tolerance in chronically-exposed (field) amphipods compared to commercially-obtained amphipods. He recommends that regulatory decision-making processes about insecticides incorporate data collected from "in situ communities" (p 2260 in Clark et al. 2015) in addition to that collected from the standard tests with commercially-available amphipods. I recommend that this logic be extended to habitats impacted by trace metal pollution. However, my results differ from

those of Clark et al (2015), and are more closely aligned with those of Pieterek and Pietrock (2012). Field-collected naïve controls in Clark et al. (2015) had similar tolerance to the commercially-available amphipods, whereas Benewah L. amphipods did not exhibit tolerance comparable to the laboratory-cultured amphipods; my field-collected reference population exhibited tolerance that was intermediate between the laboratory-cultured and chain lake amphipods that were tested, and field-collected amphipods in Pieterek and Pietrock (2012) were also more robust than the commercially-obtained amphipods. Thus, in building on the recommendations of the above-mentioned studies, I suggest adding local amphipod populations that display natural tolerance to monitoring efforts where metals are the pollutant of interest. Finally, recent sequence analysis has shown that *H*. *azteca* is a multi-species complex, both within the standard cultured resources and in the wild; as different clades exhibit variable metal tolerance, the substantial differences in metal tolerance between the Chesapeake Cultures amphipods and field-collected amphipods may reflect, in part, genetic differences (Leung et al. 2016).

Monthly field study

Surface water metal concentrations were elevated with respect to the reference lake and to Rose L. which would be expected given (1) the hydraulic connectivity of the Coeur d'Alene River and the chain lakes, and (2) the sediment profiles of the chain lakes (Sprenke et al. 2000). These results should be interpreted with caution as the concentrations that I used were from concentrated samples (see Appendix 3) and are

generally lower than the actual concentrations. Most of the chain lakes except Rose L. have elevated concentrations of As, Cd, Pb, and Zn at or near the sediment-water interface; for example, Killarney L. and Medicine L. had significantly higher surface water concentrations of Cd and Pb than did Benewah L. The sediment profiles for these two lakes show that sediment Cd concentrations within the uppermost 25 cm are ≥ 50 mg/kg, and Pb concentrations within the uppermost 50 cm are ≥ 15 mg/kg. Compared to surface water concentrations that I have reported here, Sprenke et al. (2000) reported higher Cd and Zn concentrations, and comparable As and Pb concentrations for Rose L., Medicine L., Black L., and Anderson L. This comparison suggests that surface water Cd and Zn concentrations in the chain lakes could be in decline or that the concentrations that I measured were an underestimate (see Appendix 3).

The bioavailability of elevated concentrations of total aqueous metals to aquatic organisms should be considered within the context of limnologic variables, as these variables influence the degree to which an organism will accumulate and tolerate metals. Essential and non-essential metals are clearly bioavailable in all of the chain lakes as evidenced by the patterns of metal body burden in the amphipods. However, the patterns in body burden reflect metal-specific patterns of regulation or lack thereof (e.g., Rainbow and White 1989) and are not consistently linked to any measured limnological factor, which is not entirely unexpected (for review, Luoma 1983, Luoma and Rainbow 2005, Rainbow and Luoma 2011).

This bioaccumulation may be partly explained by low water hardness that was observed in all but one of the lakes (Killarney L.). Hardness (as $CaCO_3$) is the total concentration of the divalent cations in a solution, and generally is governed by the concentrations of Ca^{2+} and Mg^{2+}. These essential cations compete with dissolved trace metal cations for ligand binding sites on the gill surface. Thus, higher hardness should limit the bioaccumulation of metals via respiratory structures even when aqueous metal concentrations are high. Some of the lowest tissue metal concentrations (particularly when assessed with reference to the aqueous metal levels) were in amphipods collected from Killarney L., likely reflecting, in part, the effect of high water hardness (particularly the high Ca) in Killarney L. water.

The effects of pH must also be considered. pH influences the ability of metals to dissociate in water (low pH) or to bind to surfaces (high pH). Metal cations that are bound (to organic matter for example) are less likely to be absorbed by respiratory structures. However, they can still be accumulated via dietary intake. pH did vary among the chain lakes and likely influenced metal availability in the chain lakes. For example, pH in Anderson L. decreased as amphipod body burden increased from spring to fall. However, the effect of pH on the bioavailability of metals in Anderson L. could be additive or synergistic with those of alkalinity (the pH buffering capacity) and hardness; both factors increased substantially from spring to summer. Together, these three variables likely decreased the bioavailability of metals to the amphipods. And, Anderson L. amphipod acute Zn tolerance decreased from spring to fall. It is possible that

amphipods were experiencing lower metal levels (even though aqueous concentrations were elevated) and that they were less efficient at tolerating those metals when they were exposed to acute Zn under laboratory conditions.

Dissolved organic carbon (DOC) is another limnologic parameter that influences bioavailability because free metal ions tend to bind to DOC (Stephenson and Mackie 1988, Boeckman and Bidwell 2006 and references within), however, given logistical constraints, we could not measure DOC. It is likely that DOC levels contributed to differences in metal accumulation among the populations.

I also did not investigate the bioavailability of metals through dietary uptake, but it is important to recognize that exposure via this route likely influenced amphipod tissue metal concentrations in addition to total aqueous metals, as it is a key component in bioaccumulation prediction models (Luoma 1983, Luoma and Rainbow 2005). When dissolved metal ions are not available for uptake, as is likely the case under high hardness and high DOC conditions, dietary uptake may be the greatest contributor of bioavailable metals to *H*. *azteca*. Dietary uptake as a route of metal accumulation in CDA Basin amphipods should be investigated in the future.

Despite variation in Zn concentrations among the lakes, Zn body burdens did not vary significantly. This is reasonable given that Zn is an essential metal and is physiologically regulated; several aquatic macroinvertebrates, including arthropods, use metal-binding proteins such as metallothioneins to transport essential metals through cells (Hare 1992, Amiard-Triquet et al. 2013). Crustaceans tend to store and detoxify

metals in a number of digestive and excretory organs (Brown 1982) or in intracellular compartments such as granules, where metals can be isolated in high concentrations in order to reduce the toxic effects of accumulation, or be stored indefinitely (Icely and Nott 1980, Hare 1992). Amphipods (*Gammarus* sp.) form granules in the caeca of the intestine (Icely and Nott 1980 and references within). Once formed, granules can be excreted when the animal molts or stored indefinitely. It is not clear if, or how non-essential metals (e.g., As, Cd, Pb) are regulated. Amphipods may be less efficient at regulating these metals and this may explain the higher observed concentrations of Cd and Pb in amphipods collected from the chain lakes than in amphipods that were collected from Benewah L.

I consistently found the highest abundance of amphipods in Rose L. and Benewah L., although Cave L. had the highest single-site abundance (211/m^2 in October). The maximum length of amphipods was indistinguishable among Rose L., Cave L., Anderson L., and only slightly lower in Medicine L., Thompson L., Harrison Sl., and Benewah L. The median lengths of amphipods from all field populations are smaller than the average size at reproductive maturity (Nelson and Brunson 1995), although the largest 1/3 of amphipods collected across lakes (identified as outliers in the boxplot in Figure 13) are within reported ranges for adult *H. azteca*. The small average size of the amphipods I collected could be interpreted as evidence that either the amphipods reach reproductive maturity at smaller lengths when exposed to metals and/or that most of the amphipods I collected were immature. The total aqueous metal concentrations that

I measured, when evaluated within the context of laboratory toxicity tests, would lead to the prediction that amphipods should be the most abundant and largest at Benewah L., intermediate in abundance and size at Rose L., and in low abundance (if not completely absent) and very small in the chain lakes. While the CPUE patterns generally support these predictions, the pattern is not strong and the predicted maximum length pattern is not present. This mismatch between prediction and result may reflect an increasingly-recognized disconnect between laboratory toxicity tests and field distribution patterns (Clements et al. 2013) and the protective effects of multiple metals when experience simultaneously (Mebane et al. 2012, Clements et al. 2013). Amphipod absence in Bull Run is most likely due to the consistently low pH and DO (with possible synergistic effects with the intermediate aqueous metal concentrations); although Sprenke et al. (2000) characterizes this water body as a lake, it is mostly emergent vegetation, with high levels of plant decomposition. Finally, the amphipod populations in Killarney L. and Black L. were low in abundance and small in size; this might be related to the As tissue metal burden, which was high in amphipods from both of these lakes, independent of the aqueous As concentrations in the lakes. Although a formal fish survey has not yet been released for the chain lakes (but apparently is due in May 2016), recreational fishing is abundant in Killarney L. and fish are reported by landowners to be historically abundant in Black L.; it is possible that the absence of large amphipods in these two lakes also reflects fish predation. Vegetation was sparse in Black L. and intermediate in Killarney L. in comparison to Anderson L. All three of these

lakes had low amphipod abundance, but only in Anderson L. (among these three) were large amphipods found, possibly indicating the beneficial aspects (evading predators, substrate, dietary usage) of submerged vegetation in Anderson L.

The amphipod population in Rose L. behaved more like the population in Benewah L. than those of the other chain lakes. For instance, amphipod abundance and size for Benewah L. and Rose L. changed in a similar pattern over the year, with what appeared to be two boom/bust cycles, perhaps indicating multiple reproductive events in these lakes (juvenile growth to reproductive maturity could be as rapid as 27 days; Nelson and Brunson 1995). Metal concentrations for Rose L. also sometimes tracked in a temporal pattern similar to that in Benewah L. (e.g., Cu), and metal concentrations in Rose L. were also always low relative to concentrations in the other lakes (often indistinguishable from concentrations in Benewah L.). This is important because the management history of Rose L. is unique among the chain lakes; Rose L. was historically dammed when metal pollution traveling from upstream was at its peak (pers. comm. Rember, 2015). This is reflected in the sediment profile for the lake, which shows lower metal levels compared to metal levels in the other chain lakes (Sprenke et al. 2000). However, Rose L. amphipods had greater tolerance to metals stress than did Benewah L. amphipods in the spring toxicity test.

Conclusions

As discussed above, the CDA Basin provides an unusual opportunity to study how multi-metal pollution impacts aquatic habitats. Because the CDA River is rigorously monitored

but the chain lakes in its floodplain have been, until recently, virtually ignored by state and federal agencies (likely due to extremely limited budgets and budget priorities focusing on human health), we do not understand how and when the metals move across the sediment/water interface nor how metals bioaccumulate in plants or animals. This system of chain lakes that have variable contact with a river that at times transports high levels of metals could provide the opportunity to understand how geography and geochemistry alter the long-term consequences of metal contamination if this system received the same attention as and thus could be compared to the nearby Clark Fork Basin in MT (Hornberger et al. 1997). Much of the work exploring mining contamination has focused on riverine systems and aquatic insects (as in the Clark Fork Basin), with limited applicability to lentic habitats.

Given that the CDA Basin habitats are heavily used for recreation, it is important to develop tools that will help us evaluate when metals will be bioavailable in the fish and waterfowl that are consumed by humans. As *H. azteca* frequently interact with the sediment and eat decomposing plant matter and are consumed by fish and waterfowl, these amphipods could be a route for metal transport up the food chain. As I have documented, amphipods in the chain lakes had higher levels of nonessential metals (Pb and Cd) than did the amphipods from Benewah L., illustrating that the metals are bioavailable. These results suggest that monitoring metal content of *H. azteca* from the chain lakes would be an appropriate indicator of metal bioavailability.

Whether *H. azteca* could be developed into a sentinel species for the Basin is still an open question. I was not able to draw direct connections between the lab toxicity results and the field data (a problem that has been reported before), nor was I able to explain patterns in amphipod CPUE and size with the limnological and metal data. My results suggest that the processes occurring in each lake are independent and strongly influenced by season, even though these lakes are in the floodplain of the same river and, in some cases, are even hydraulically connected (Medicine L. and Cave L.). My results, particularly the PCA, also suggest that there are variables that I did not measure that strongly influence amphipod populations. The variables that are most likely to be informative include metal content in the possible amphipod food sources and concentrations of dissolved organic carbon. The PCA axis 1 and 2 scores illustrate that these lakes are not homogeneous with respect to metals and limnology, suggesting that successful management of this basin should include a sentinel species that is present in each lake and monitored with equal rigor in each lake. The lack of strong correlations between amphipod population metrics and the data that are ultimately of greatest interest (metal bioavailability) does not rule out the potential utility of the amphipods as sentinels for the Basin. However, future investigations of the potential of *H. azteca* (or really any other organisms) as a sentinel for the Basin must involve monitoring amphipods, limnology, and metals from each lake at monthly intervals (at minimum) and must be done within the structure of comparing Benewah L. as the "negative

control", Rose L. as an intermediate point, and the remaining chain lakes on a spectrum rather than under the assumption that any one chain lake is representative of the rest.

LITERATURE

Alcock, N. W. 1987. A hydrogen-peroxide digestion system for tissue trace-metal analysis. Biological Trace Element Research **13**:363-370.

Amiard-Triquet, C. 2011. Pollution tolerance: from fundamental biological mechanisms to ecological consequences. Pages 1-23 *in* C. Amiard-Triquet, P. S. Rainbow, and M. Romeo, editors. Tolerance to Environmental Contaminants. CRC Press, Boca Raton, FL.

Amiard-Triquet, C., C. Cossu-Leguille, and C. Mouneyrac. 2013. Biomarkers of defense, tolerance, and ecological consequences. Pages 45-74 *in* C. Amiard-Triquet, J.-C. Amiard, and P. S. Rainbow, editors. Ecological Biomarkers: Indicators of Ecotoxicological Effects. CRC Press, Boca Raton, FL.

ASTM. 2014. E729-96(2014) Standard guide for conducting acute toxicity tests on test materials with fishes, macroinvertebrates, and amphibians. ASTM International, West Conshohocken, PA.

Balistrieri, L. S., S. E. Box, A. A. Bookstrom, R. L. Hooper, and J. B. Mahoney. 2002. Impacts of historical mining in the Coeur d'Alene River Basin. Pages 1-34 *in* L. S. Balistrieri and L. L. Stillings, editors. Pathways of metal transfer from mineralized sources to bioreceptors. US Geological Survey Bulletin 2191. U.S. Geological Survey.

BEIPC. Basin Environmental Improvement Project Commission. http://www.basincommission.com/about.asp.

Berthet, B. 2013. Sentinel Species. Pages 155-185 *in* C. Amiard-Triquet, J.-C. Amiard, and P. S. Rainbow, editors. Ecological biomarkers: Indicators of Ecotoxicological Effects. CRC Press, Boca Raton, FL.

Besser, J. M., W. G. Brumbaugh, E. L. Brunson, and C. G. Ingersoll. 2005. Acute and chronic toxicity of lead in water and diet to the amphipod *Hyalella azteca*. Environmental Toxicology and Chemistry **27**:1807-1815.

Besser, J. M., W. G. Brumbaugh, and C. G. Ingersoll. 2015. Characterizing toxicity of metal-contaminated sediments from mining. Applied Geochemistry **57**:73-84.

Bland, J. M., and D. G. Altman. 1998. Survival probabilities (the Kaplan-Meier method). BMJ **317**:1572-1580.

Boeckman, C. J., and J. R. Bidwell. 2006. The effects of temperature, suspended solids, and organic carbon on copper toxicity to two aquatic invertebrates. Water, Air, and Soil Pollution **171**:185-202.

Borgmann, U., Y. Couillard, P. Doyle, and D. G. Dixon. 2005. Toxicity of sixty-three metals and metalloids to *Hyalella azteca* at two levels of water hardness. Environmental Toxicology and Chemistry **24**:641-652.

Borgmann, U., and M. Munawar. 1989. A new standardized sediment bioassay protocol using the amphipod *Hyalella azteca* (Saussure). Hydrobiologia **188**:425-431.

Borgmann, U., W. P. Norwood, and C. Clark. 1993. Accumulation, regulation and toxicity of copper, zinc, lead and mercury in *Hyalella azteca*. Hydrobiolgia **259**:79-89.

Borgmann, U., K. M. Ralph, and W. P. Norwood. 1989. Toxicity test procedures for *Hyalella azteca*, and chronic toxicity of cadmium and pentachlorophenol to *H. azteca*, *Gammarus fasciatus*, and *Daphnia magna*. Archives of Environmental Contamination and Toxicology **18**:756-764.

Brown, B. E. 1982. The form and function of metal-containing 'granules' in invertebrate tissues. Biological Reviews **57**:621-667.

Brown, V. M. 1968. The calculation of the acute toxicity of mixtures of poisons to Rainbow trout. Water Research **2**:723-733.

Bryan, G. W. 1971. The effects of heavy metals (other than mercury) on marine and estuarine organisms. Proceedings of the Royal Society of London B. **177**:389-410.

Calisi, R. M., and G. E. Bentley. 2009. Lab and field experiments: Are they the same animal? Hormones and Behavior **56**:1-10.

Campbell, P., and P. Stokes. 1985. Acidification and toxicity of metals to aquatic biota. Canadian Journal of Fisheries and Aquatic Sciences **42**:2034-2049.

Chess, D. 2015. Personal Communication. Coeur d'Alene Tribe Lake Management Dept.

Clark, G. M., and C. A. Mebane. 2014. Sources, transport, and trends for selected trace metals and nutrients in the Coeur d'Alene and Spokane River Basins, northern Idaho, 1990-2013. U.S. Geological Survey Scientific Investigations Report 2014-5204, Prepared by the U.S. Geological Survey in cooperation with the U.S. Environmental Protection Agency, Reston, Virginia.

Clark, S. L., R. S. Ogle, A. Gantner, L. W. Hall, G. Mitchell, J. Giddings, M. McCoole, M. Dobbs, K. Henry, and T. Valenti. 2015. Comparative sensitivity of field and laboratory populations of *Hyalella azteca* to the pyrethroid insecticides bifenthrin and cypermethrin. Environmental Toxicology and Chemistry **34**:2250-2262.

Clements, W. H., P. Cadmus, and S. F. Brinkman. 2013. Responses of aquatic insects to Cu, and Zn in stream microcosms: Understanding differences between single species tests and field responses. Environmental Science & Technology **47**:7506-7513.

Costanza, R., R. d'Arge, R. De Groot, S. Farber, M. Grasso, B. Hannon, K. Limburg, S. Naeem, R. V. O'neill, and J. Paruelo. 1998. The value of the world's ecosystem services and natural capital. Ecological Economics **1**:3-15.

Cubbage, J., D. Batts, and S. Breidenbach. 1997. Creation and analysis of freshwater sediment quality values in Washington State. 97-323a, Washington State Department of Ecology, Olympia, WA.

Daily, G. 1997. Nature's services: Societal dependence on natural ecosystems. Island Press.

Dillon, F. S., and C. A. Mebane. 2002. Development of site-specific water quality criteria for the South Fork Coeur d'Alene River, Idaho. 1, WindWard Environmental, Seattle, WA.

Eisenhauer, J., K. Brown Sullivan, and M. J. Lydy. 1999. Response of genotypes of *Hyalella azteca* to zinc toxicity. Bulletin of Environmental Contamination and Toxicology **63**:125-132.

Farag, A., D. Woodward, J. Goldstein, W. Brumbaugh, and J. Meyer. 1998. Concentrations of metals associated with mining waste in sediments, biofilm, benthic macroinvertebrates, and fish from the Coeur d'Alene River Basin, Idaho. Archives of Environmental Contamination and Toxicology. **34**:119-127.

Glickman, L. T., A. Fairbrother, A. M. Guarino, H. L. Bergman, and W. B. Buck. 1991. Animals as sentinels of environmental health hazards. 1, National Resource Council, Washington, D.C.

Hare, L. 1992. Aquatic insects and trace metals: Bioavailability, bioaccumulation, and toxicity. Critical Reviews in Toxicology **22**:327-369.

Helmuth, B., B. R. Broitman, L. Yamane, S. E. Gilman, K. Mach, K. A. S. Mislan, and M. W. Denny. 2010. Organismal climatology: analyzing environmental variability at scales relevant to physiological stress. Journal of Experimental Biology **213**:995-1003.

Hoiland, W. K., F. W. Rabe, and R. C. Biggam. 1994. Recovery of macroinvertebrate communities from metal pollution in the South Fork and mainstem of the Coeur d'Alene River, Idaho. Water Environment Research:84-88.

Hornberger, M. I., J. H. Lambing, S. N. Luoma, and E. V. Axtmann. 1997. Spatial and temporal trends of trace metals in surface water, bed sediment, and biota of the Upper Clark Fork Basin, Montana, 1985-95. 97-669, U.S. Geological Survey, Menlo Park, CA.

Hornig, E. C., D. A. Terpening, and W. M. Bogue. 1988. Coeur d'Alene Basin EPA water quality monitoring (1972-1986). EPA 910/9-88-216, USEPA, Seattle,WA.

Icely, J. D., and J. A. Nott. 1980. Accumulation of copper within the "Hepatopancreatic" caeca of *Corophium volutator* (Crustacea: Amphipoda). Marine Bioogy **57**:193-199.

Ingersoll, C. G., N. Wang, J. M. R. Hayward, J. R. Jones, S. B. Jones, and S. D. Ireland. 2005. A field assessment of long-term laboratory sediment toxicity tests with the amphipod *Hyalella azteca*. Environmental Toxicology and Chemistry **24**:2853-2870.

Johnston, E. L. 2011. Tolerance to contaminants.*in* C. Amiard-Triquet, P. S. Rainbow, and M. Romeo, editors. Tolerance to Environmental Contaminants. CRC Press, Boca Raton, FL.

Lawrence, S. G. e. 1981. Manual for the culture of selected freshwater invertebrates. Canadian Special Publication of Fisheries and Aquatic Sciences **54**:169.

Lefcort, H., D. P. Abbott, D. A. Cleary, E. Howell, N. C. Keller, and M. M. Smith. 2004. Aquatic snails from mining sites have evolved to detect and avoid heavy metals. Archives of Environmental Contamination and Toxicology. **46**:478-484.

Lefcort, H., J. Vancura, and E. L. Lider. 2010. 75 years after mining ends stream insect diversity is still affected by heavy metals. Ecotoxicology **19**:1416-1425.

Leung, J., J. D. S. Witt, W. Norwood, and D. G. Dixon. 2016. Implications of Cu and Ni toxicity in two members of the *Hyalella azteca* cryptic species complex: Mortality, growth, and bioaccumulation parameters. Environmental Toxicology and Chemistry **35**:2817-2826.

Long, K. R. 1998. Production and disposal of mill tailings in the Coeur d'Alene mining region, Shoshone County, Idaho; preliminary estimates. 98-595, U.S. Geological Survey.

Luoma, S. N. 1983. Bioavailability of trace metals to aquatic organisms - A review. The Science of the Total Environment **28**:1-22.

Luoma, S. N., D. J. Cain, and P. S. Rainbow. 2009. Calibrating biomonitors to ecological disturbance: a new technique for explaining metal effects in natural waters. Integrated Environmental Assessment and Management **6**:199-209.

Luoma, S. N., and P. S. Rainbow. 2005. Why is metal bioaccumulation so variable? Biodynamics as a unifying concept. Environmental Science & Technology **39**:1921-1931.

Maret, T. R., D. J. Cain, D. E. MacCoy, and T. M. Short. 2003. Response of benthic invertebrate assemblages to metal exposure and bioaccumulation associated with hard-rock mining in northwestern streams, USA. Journal of North American Benthological Society. **22**:598-620.

Mathias, J. A. 1971. Energy flow and secondary production of the amphipods *Hyalella azteca* and *Crangonyx richmondensis occidentalis* in Marion Lake, British Columbia. Journal of the Fisheries Board of Canada **28**:711-726.

Mebane, C. A. 2010. Cadmium risks to freshwater life: Derivation and validation of low-effect criteria values using laboratory and field studies (version 1.2). Scientific Investigations Report 2006-5245, U.S. Geological Survey, Reston, Virginia.

Mebane, C. A., F. S. Dillon, and D. P. Hennessy. 2012. Acute toxicity of cadmium, lead, zinc, and their mixtures to stream-resident fish and invertebrates. Environmental Toxicology and Chemistry **31**:1334-1348.

Melvin, S. D., and J. E. Houlahan. 2012. Tadpole mortality varies across experimental venues: do laboratory populations predict responses in nature? Oecologia **169**:861-868.

Nelson, M. K., and E. L. Brunson. 1995. Postembryonic growth and development of *Hyalella azteca* in laboratory cultures and contaminated sediments. Chemosphere **31**:3129-3140.

Niyogi, S., and C. M. Wood. 2003. Effects of chronic waterborne and dietary metal exposures on gill metal-binding: implications for the biotic ligand model. Human and Ecological Risk Assessment **9**:813-846.

NRC. 2005. Superfund and mining megasites: Lessons from the Coeur d'Alene River Basin. 1, National Resource Council, Washington, DC.

Pieterek, T., and M. Pietrock. 2012. Comparative selenium toxicity to laboratory-reared and field-collected *Hyalella azteca* (Amphipoda, Hyalellidae). Water Air Soil Pollution **223**:4245-4252.

Rainbow, P. S., and S. N. Luoma. 2011. Biodynamic parameters of the accumulation of toxic metals, detoxification, and the aquisition of metal tolerance. Page 25 *in* C. Amiard-Triquet, P. S. Rainbow, and M. Romeo, editors. Tolerance to Environmental Contaminants. CRC Press, Boca Raton, FL.

Rainbow, P. S., and S. L. White. 1989. Comparative strategies of heavy metal accumulation by crustaceans: zinc, copper and cadmium in a decapod, an amphipod and a barnacle. Hydrobiologia **174**:245-262.

Ramsey, P. W., M. C. Rillig, K. P. Feris, J. N. Moore, and J. E. Gannon. 2005. Mine waste contamination limits soil respiration rates: a case study using quantile regression. Soil Biology & Biochemistry **37**:1177-1183.

Reish, D. L., and P. S. Oshida. 1986. Part 10: Short-term static bioassays. 9251025177, Food & Agriculture Organization of the United Nations, Rome, Italy.

SAS. 1999. The PROBIT Procedure. SAS OnlineDoc. SAS Institute Inc., Cary, NC.

SAS. 2016. Chapter 97: The QUANTREG Procedure. SAS/STAT 14.2 User's Guide. SAS Institute Inc, Cary, NC.

Schmidt, T. S., W. H. Clements, and B. S. Cade. 2012. Estimating risks to aquatic life using quantile regression. Society for Freshwater Science **31**:709-723.

Schoonover, C. M. 2013. Effects of heavy metals on the physical activity and survival of freshwater clams. Master's Thesis. Eastern Washington University.

Schoonover, C. M., J. Wieker, R. Pope, C. Brown, E. Cooper, J. DeWitt, S. Gunselman, C. Jensen, W. Stevens, and J. Yri. 2016. Development of functional trait biomarkers for trace metal exposure in freshwater clams (*Musculium* spp.). Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology **200**:21-34.

Schubauer-Berigan, M. K., J. R. Dierkes, P. D. Monson, and G. T. Ankley. 1993. pH-Dependent toxicity of Cd, Cu, Ni, Pb and Zn to *Ceriodaphnia dubia*, *Pimephales promelas*, *Hyalella azteca* and *Lumbriculus variegatus*. Environmental Toxicology and Chemistry **12**:1261-1266.

Shea, J., G. J. Kersten, C. M. Puccia, A. T. Stanton, S. N. Stiso, E. S. Helseson, and E. J. Back. 2012. The use of parasites as indicators of ecosystem health as compared to insects in freshwater lakes of the Inland Northwest. Ecological Indicators **13**:184-188.

Spears, B. L., K. A. Healy, R. E. Brazzle, and J. A. Hansen. 2006. Annual report 2005 biological resource monitoring Coeur d'Alene Basin Operable Unit 3. 1, USFWS, Spokane, Wa.

Sprenke, K., W. Rember, S. Bender, M. Hoffmann, F. Rabbi, and V. Chamberlain. 2000. Toxic metal contamination in the lateral lakes of the Coeur d'Alene River valley, Idaho. Environmental Geology **39**:575-586.

Stephenson, M., and G. Mackie. 1988. Multivariate analysis of correlations between environmental parameters and cadmium concentrations in Hyalella azteca (Crustacea: Amphipoda) from central Ontario lakes. Canadian Journal of Fisheries and Aquatic Sciences **45**:1705-1710.

Stephenson, M., and G. L. Mackie. 1986. Lake acidification as a limiting factor in the distribution of the freshwater amphipod *Hyalella azteca*. Canadian Journal of Fish and Aquatic Sciences. **43**:288-292.

Strong Jr, D. R. 1972. Life history variation among populations of an amphipod (*Hyalella azteca*). Ecology **53**:1103-1111.

Strong Jr, D. R. 1973. Amphipod amplexus, the significance of ecotypic variation. Ecology **54**:1383-1388.

Taylor, L. N., L. Novak, M. Rendas, P. M. C. Antunes, and R. P. Scroggins. 2016. Validation of a new standardized test method for the freshwater amphipod *Hyalella azteca*: Determining the chronic effects of silver in sediment. Environmental Toxicology and Chemistry **9999**:1-9.

Toro, D. M. D., H. E. Allen, H. L. Bergman, J. S. Meyer, P. R. Paquin, and R. C. Santore. 2001. Biotic ligand model of the acute toxicity of metals. 1. Technical Basis. Environmental Toxicology and Chemistry **20**:2383-2396.

USEPA. 1982. Method 200.7 Determination of metals and trace elements in water and wastes by inductively coupled plasma-atomic emission spectrometry. U.S. Environmental Protection Agency, Cincinnati, Ohio.

USEPA. 1992. Method 3005A Acid digestion of waters for total recoverable or dissolved metals for analysis by FLAA or ICP spectroscopy. Page 5. U.S. Environmental Protection Agency.

USEPA. 2004. National recommended water quality criteria. U.S. Environmental Protection Agency, Washington DC.

USEPA. 2007. Aquatic life ambient freshwater quality criteria - copper, 2007 revision. EPA-822-R-07-001, U.S. Environmental Protection Agency, Washington DC.

USEPA. 2015. Bunker Hill Mining & Metallurgical. EPA Region 10 Idaho. USEPA.

VanLeeuwen, D. M., Z. You, and B. Leinauer. 2013. Analyzing partially nested designs with irregular nesting: A cautionary case study. Agronomy Journal **105**:1298-1306.

Wallace, W. G., and A. Estephan. 2004. Differential susceptibility of horizontal and vertical swimming activity to cadmium exposure in a gammaridean amphipod (*Gammarus lawrencianus*). Aquatic Toxicology **69**:289-297.

Wetzel, R. G. 1975. Limnology: Philadelphia. WB Saunders Company **3**.

Wetzel, R. G., and G. Likens. 1991. Limnological Analysis. 2 edition. Springer-Verlag, New York.

Zhang, C. 2007. Fundamentals of Environmental Sampling and Analysis. John Wiley & Sons.

TABLES

Table 1. Maximum sediment concentrations with depth in chain lakes. Bolded lakes provided amphipods for seasonal lab toxicity tests.

Lake	Sediment [Pb] mg/kg*	Sediment [Zn] mg/kg*	Depth below sediment surface (cm)*	Lake Mixing or Turnover†	Amphipods collected for toxicity tests
Rose	3,585	3,296	10		spring, summer
Bull Run	30,160	28,406	30		
Killarney	37,400	34,150	50		
Medicine	19,495	11,784	70	mixing	spring, summer, fall
Cave	9,352	5,766	10	mixing	
Black	11,520	5,720	40		
Anderson	8,130	6,310	10		spring, summer, fall
Thompson	17,435	11,250	50	turnover	summer, fall
Harrison Sl.	N/A	N/A	N/A		
Benewah	N/A	N/A	N/A	turnover	spring, summer, fall

Abbreviations: N/A, not available

*Sediment metal concentration profiles were obtained from Rember (presentation Spokane, WA 2/24/2016; used with permission).

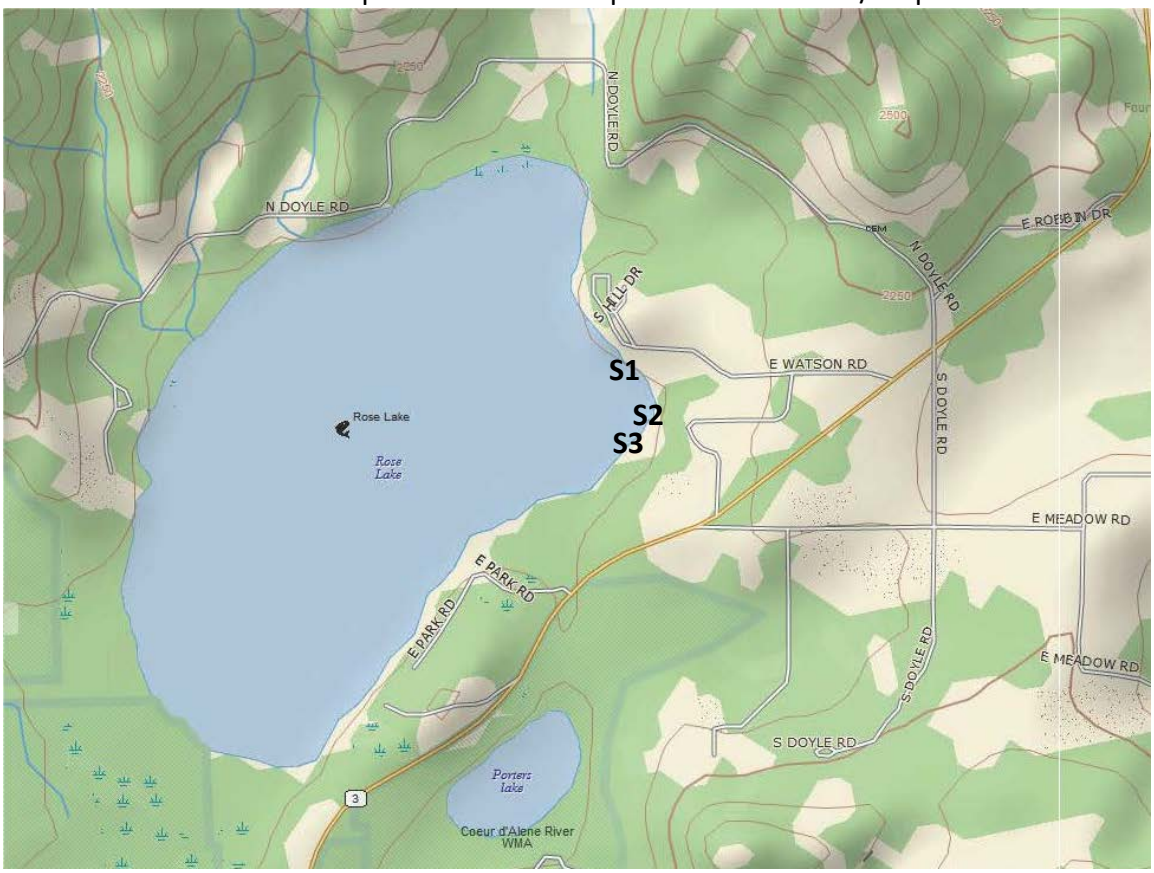
†Information regarding mixing/turnover tendencies was obtained from Chess, personal communication in 2016.

Table 2. Timing of Lab and Field studies. Toxicity and swimming tests that began in late August (summer tests) continued into the first week of September. The fall Zn test was conducted in late October.

Collection month/Activity	Apr 2016	May 2016	Jun 2016	Jul 2016	Aug 2016	Sept 2016	Oct 2016
Seasonal collection (lab), Pb, Zn toxicity tests, swimming activity			*		¥		§
Monthly collection (field)	†	†	‡	‡	‡	‡	‡

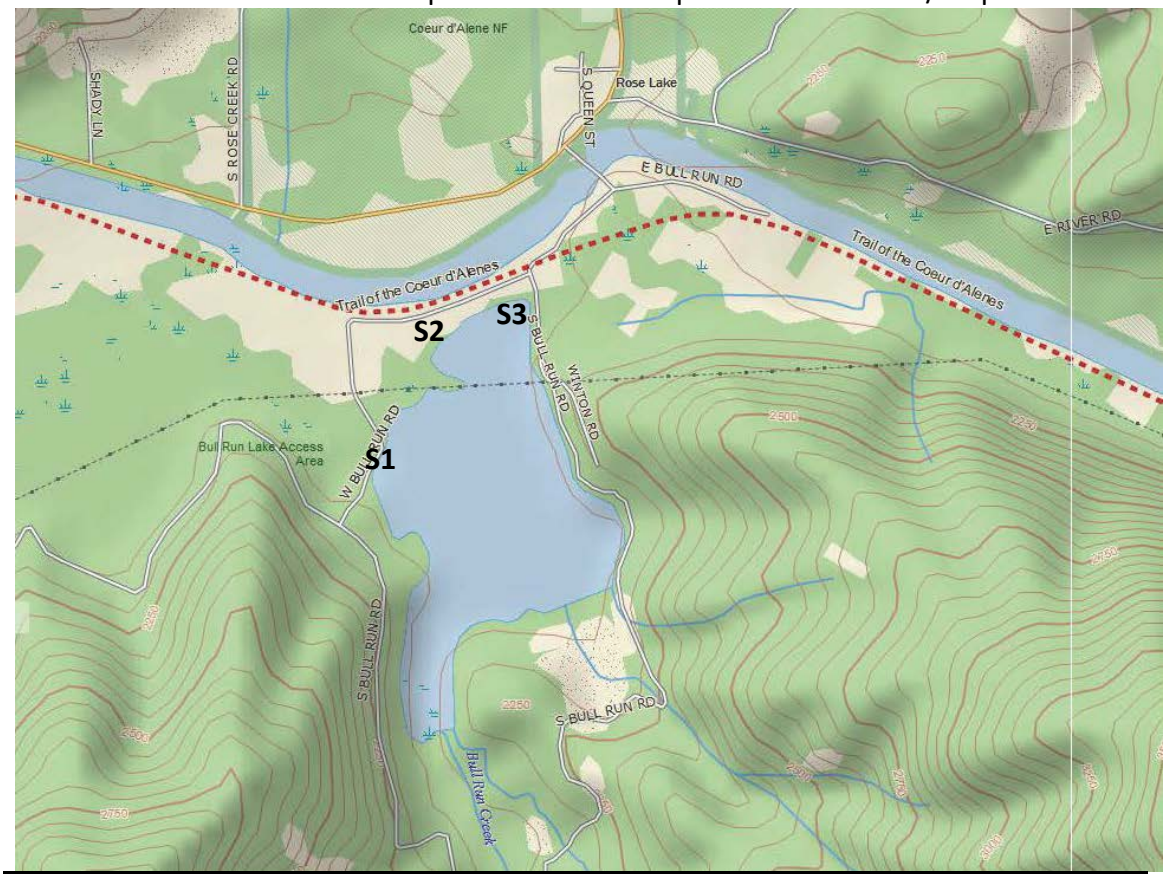
*****Sampled lakes: Rose L., Medicine L., Anderson L., Benewah L.
¥Sampled lakes: Rose L., Medicine L., Anderson L., Thompson L., Benewah L.
§Sampled lakes: Medicine L., Anderson L., Thompson L., Benewah L.
†Sampled lakes: all chain lakes except Black L.; Benewah L.
‡Sampled lakes: all chain lakes and Benewah L.

Table 3a. Topographic maps of Rose L. with geographic coordinates of sampling sites and field observations. Map obtained from explore.delorme.com/Map.



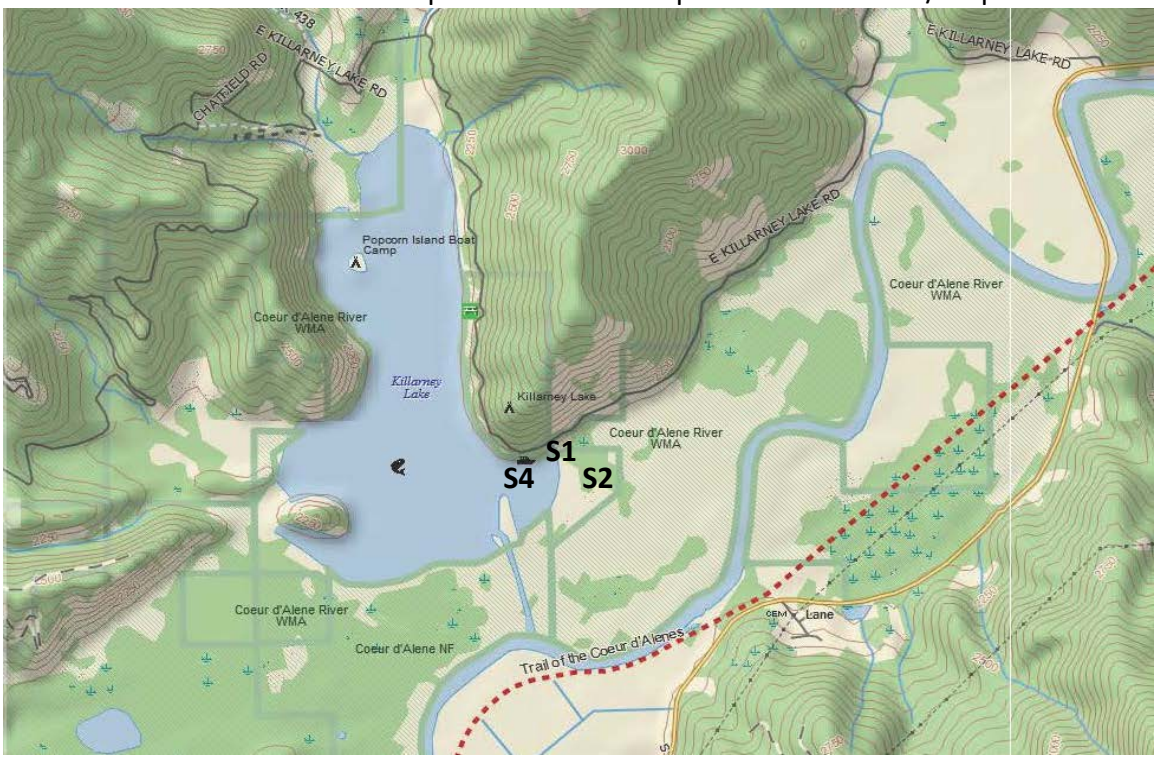
	Site 1 (S1)	Site 2 (S2)	Site 3 (S3)
Rose Lake collection site locations	Lat: 47.553178 Long: -116.458666	Lat: 47.553120 Long: -116.458739	Lat: 47.554638 Long: -116.456500
Access: Idaho Department of Fish & Game (IDFG)			
Observations	Substrate: gravel, mud, organic matter, decomposing vegetation Vegetation: generally dense at sites 1 and 2 especially in summer months; lily pads, wapato arrowhead, marsh cinquefoil, cattails, algae, waterweed, reed canary grass abundant Invertebrates: diving beetle larvae, dragonfly larvae, damselfly larvae, fingernail clams, midges, misc. beetles, snails, leeches, spiders Recreation: typically 2-5 groups per visit (boating, angling, and/or camping) Other: catfish, other small fish present at all sites		

Table 3b. Topographic maps of Bull Run L. with geographic coordinates of sampling sites and field observations. Map obtained from explore.delorme.com/Map.



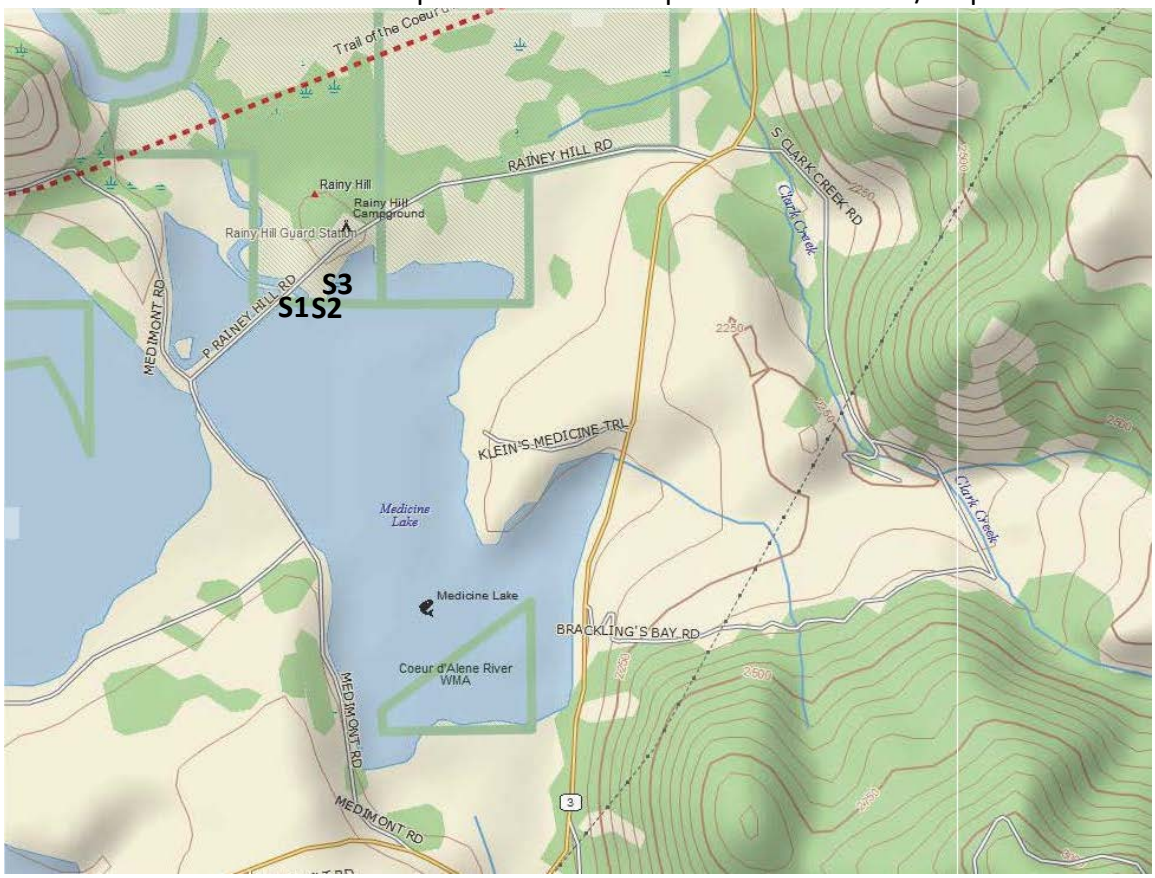
	Site 1 (S1)	Site 2 (S2)	Site 3 (S3)
Bull Run Lake collection site locations	Lat: 47.529344 Long: -116.480632	Lat: 47.533193 Long: -116.479799	Lat: 47.533741 Long: -116.474430
Access: IDFG			
Observations	Substrate: Very fine sediment/mud, often thick, sediment is orange/brown, sometimes red; high decomposition/organic matter/detritus Vegetation: Many lily pads, milfoil, pondweed, some cattails, algae Invertebrates: Spiders, water mites, diving beetle larvae, backswimmers, midges, misc. beetles, cladocerans, leeches, snails, water boatmen, flatworms, fingernail clams, dragonfly larvae, damselfly larvae, **amphipods never observed in this lake** Recreation: 1-2 groups of anglers per visit; one commented that Bull Run is known locally for having many large pike Other: Water frequently has red/orange tint; very turbid; had oily sheen on surface in July and August		

Table 3c. Topographic maps of Killarney L. with geographic coordinates of sampling sites and field observations. Map obtained from explore.delorme.com/Map.



	Site 1 (S1)	Site 2 (S2)	Site 4 (S4)*
Killarney Lake collection site locations	Lat: 47.516642 Long: -116.553020	Lat: 47.51475 Long: -116.549734	Lat: 47.515058 Long: -116.556222
Special Note: Four sites were initially established. Site 3 was dropped in the spring as three collection sites deemed sufficient			
Access: IDFG			
Observations	Substrate: (S1, S2) mud, organic material, decomposing vegetation; high organic matter (black) in fall (S4) gravel, disturbance frequent (boat launch) Vegetation: (S1, S2) generally dense at sites 1 and 3 especially in summer months; lily pads, cattails, algae, waterweed, coontail, milfoil, reed canary grass abundant (S4) narrow-leaved bur-reed, some algae Invertebrates: small beetles, snails, caddisfly larvae, midges, caddisfly larvae, spiders, water striders, water mites, copepods Recreation: typically 3-7 groups per visit (boating, angling, and/or camping), children observed swimming/playing in water at the boat launch Other: (S1, S2) Water frequently turbid		

Table 3d. Topographic maps of Medicine L. with geographic coordinates of sampling sites and field observations. Map obtained from explore.delorme.com/Map.

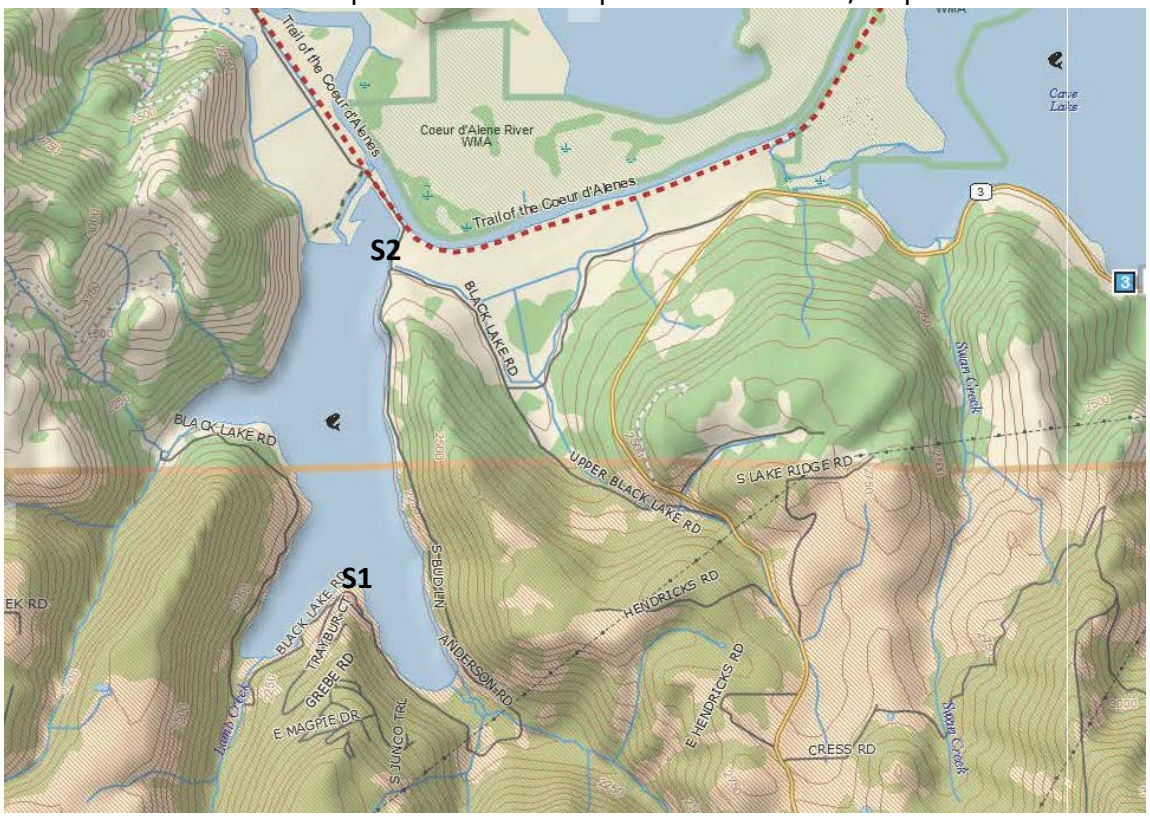


	Site 1 (S1)	Site 2 (S2)	Site 3 (S3)
Medicine Lake collection site locations	Lat: 47.473169 Long: -116.589124	Lat: 47.473086 Long: -116.587728	Lat: 47.473604 Long: -116.587712
Access: IDFG			
Observations	Substrate: Very fine sediment, light brown in spring, red/orange in summer, especially at S1 Vegetation: (S1) many cattails, reed canary grass, horsetails, algae, milfoil, (S2, S3) many horsetails, algae, reed canary grass, some milfoil Invertebrates: Water boatmen, spiders, small beetles, fingernail clams, water mites, damselfly larvae, dragonfly larvae, cladocerans, flat worms, snails, backswimmers, midges, sludge worms, misc. beetles Recreation: 1-5 groups per visit (boating, kayaking, waterskiing, angling, and/or camping) Other: Catfish and pike observed		

Table 3e. Topographic maps of Cave L. with geographic coordinates of sampling sites and field observations. Map obtained from explore.delorme.com/Map.

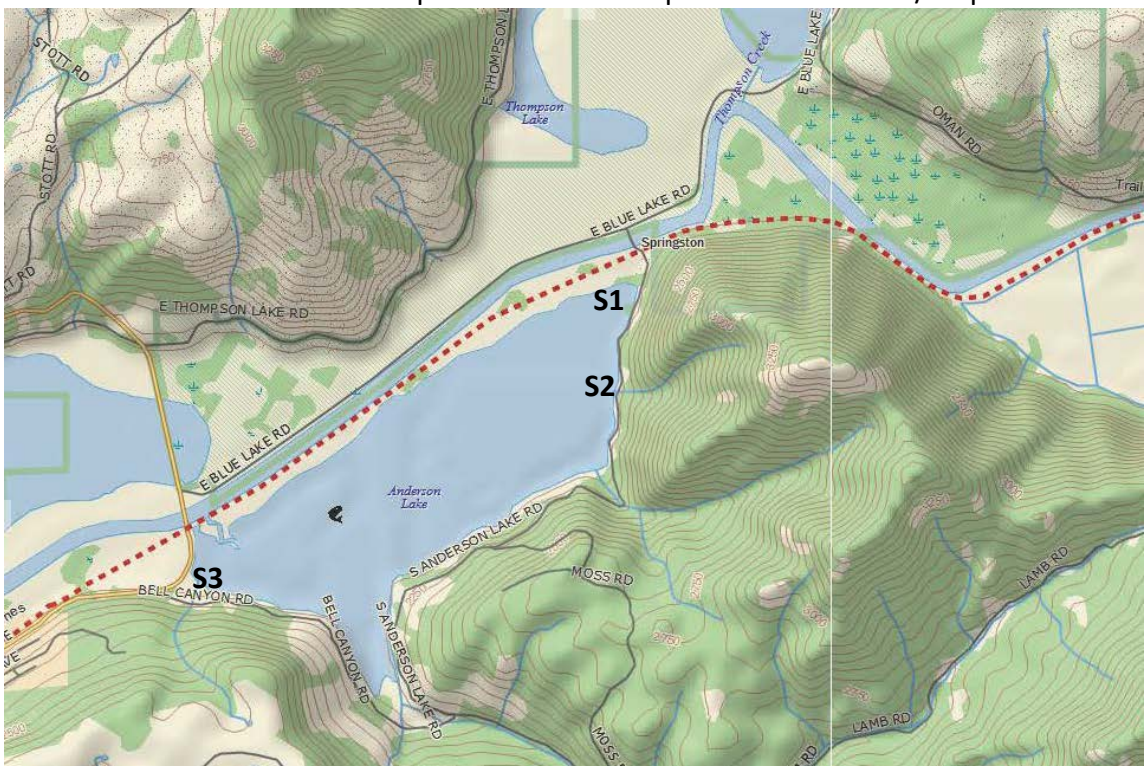
	Site 1 (S1)	Site 2 (S2)	Site 3 (S3)
Cave Lake collection site location	Lat: 47.474415 Long: -116.608152	Lat: 47.469316 Long: -116.591600	Lat: 47.456360 Long: -116.604778
Access: IDFG			
Observations	Substrate: High organic content, decomposing plant matter in late summer/fall, (S1, S3) rocky, coarse sediment, (S2) sediment very fine, light brown Vegetation: Algae, many lily pads, reed canary grass, (S2) many horsetails (S3) waterweed Invertebrates: Midges, flatworms, water mites, backswimmers, leeches, water boatmen, dragonfly larvae, many snails, damselfly larvae, sludge worms, misc. beetles, fingernail clams, cladocerans, spiders Recreation: 1-3 groups per visit (boating, angling, kayaking) Other: (S2) water had orange tint in October		

Table 3f. Topographic maps of Black L. with geographic coordinates of sampling sites and field observations. Map obtained from explore.delorme.com/Map.



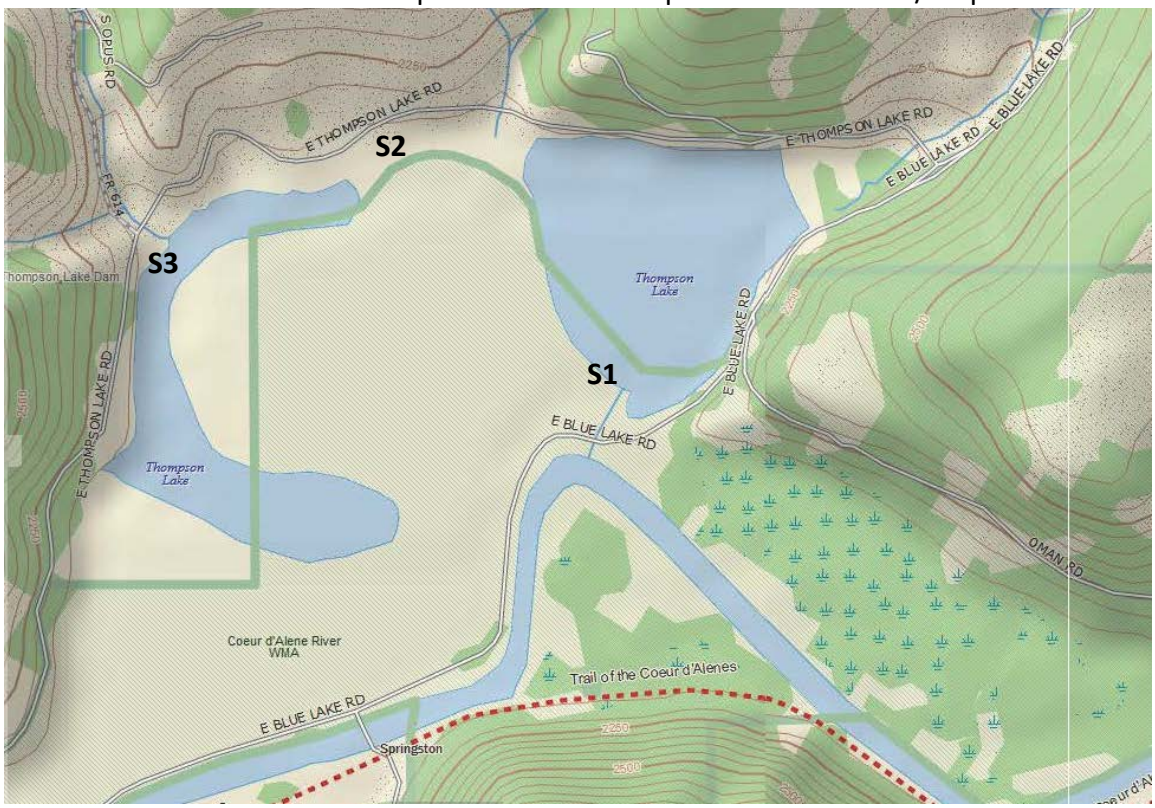
	Site 1 (S1)	Site 2 (S2)
Black Lake collection site location	Lat: 47.441439 Long: -116.660279	Lat: 47.457256 Long: -116.658151
Access: Private land		
Observations	Substrate: Very rocky, course substrate/sediment Vegetation: Algae abundant in late summer, (S1) large patch of woody debris (tree branches, bushes), (S2) reed canary grass Invertebrates: Water boatmen, midges, snails, water mites, leeches, fingernail clams, misc. beetles, sludge worms, caddisfly larvae, damselfly larvae, dragonfly larvae, flatworms Recreation: Many homes have lakefront access, we frequently observed groups boating and angling during summer months Other: Homeowners commented that many anglers were having an abnormally unsuccessful season, also mentioned that the lake experiences an annual algae bloom in the fall	

Table 3g. Topographic maps of Anderson L. with geographic coordinates of sampling sites and field observations. Map obtained from explore.delorme.com/Map.



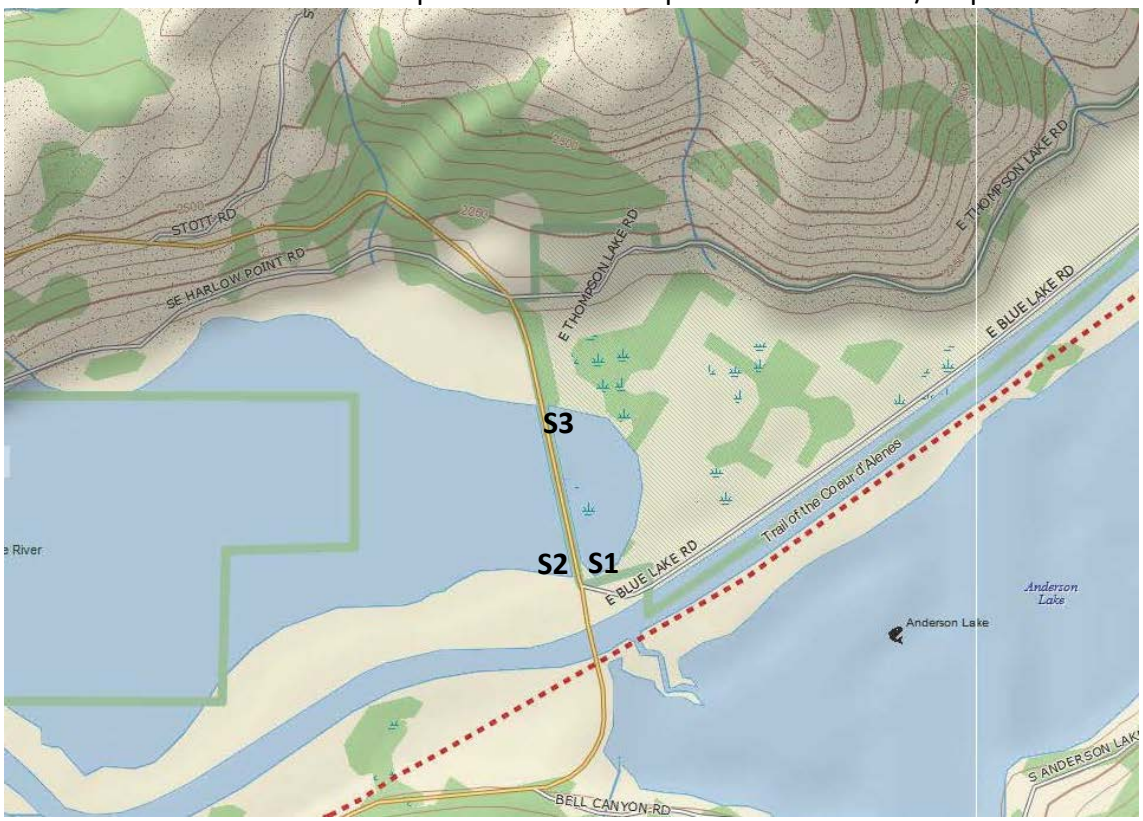
	Site 1 (S1)	Site 2 (S2)	Site 3 (S3)
Anderson Lake collection site location	Lat: 47.476778 Long: -116.732791	Lat: 47.471866 Long: -116.734221	Lat: 47.464235 Long: -116.764710
Access: IDFG			
Observations	Substrate: (S1) sediment very fine, coarse and fine particulate organic matter, deep mud, (S2, S3) substrate mostly rocky Vegetation: Reed canary grass, (S1) lily pads, wapato arrowhead, narrow-leaved bur-reed, (S2, S3) filamentous and green algae abundant, especially in summer/fall, milfoil and coontail abundant Invertebrates: Midges, sludge worms, many snails, leeches, misc. beetles, misc. fly larvae, damselfly larvae, flies, fingernail clams, backswimmers, water boatmen, flatworms, water striders, mayfly larvae, cladocerans, dragonfly larvae, (S1) many large spiders Recreation: 1-2 groups per visit (angling, boating, waterfowl hunting) Other: Large frog, small snake observed; many bluegill fish observed, especially at S2		

Table 3h. Topographic maps of Thompson L. with geographic coordinates of sampling sites and field observations. Map obtained from explore.delorme.com/Map.



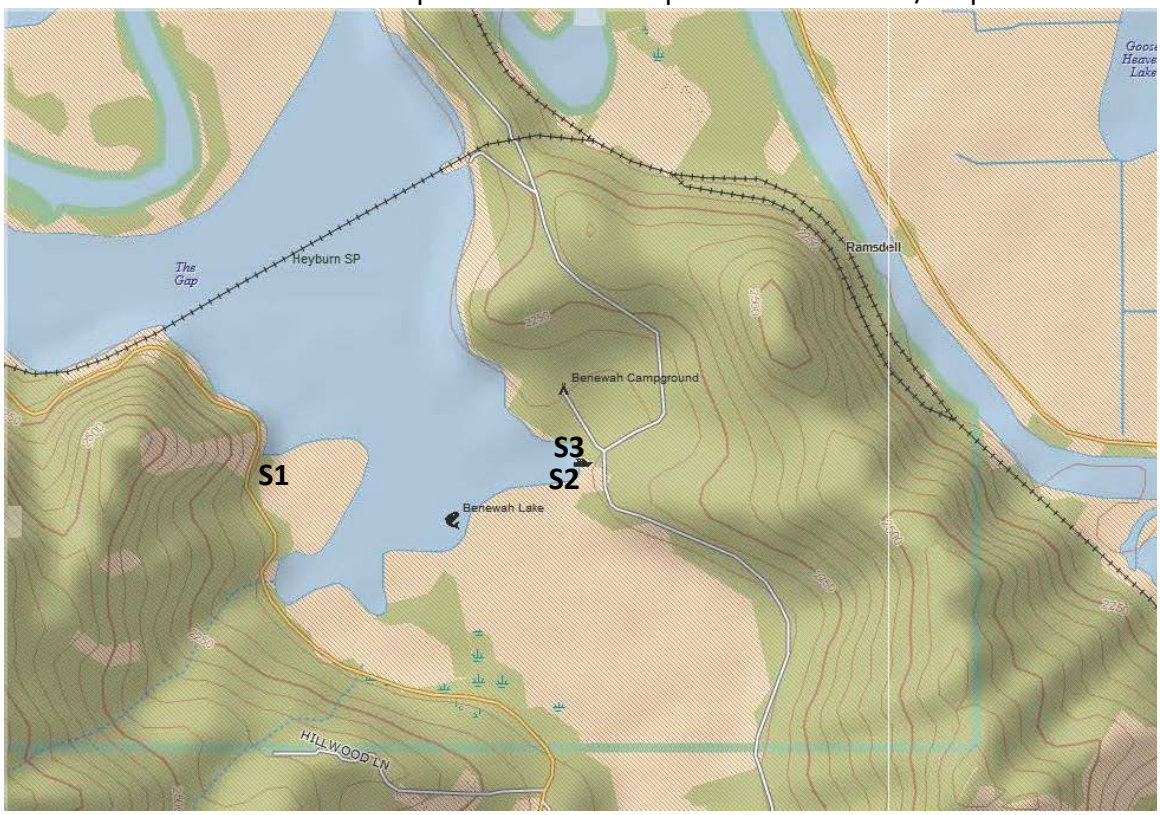
	Site 1 (S1)	Site 2 (S2)	Site 3 (S3)
Thompson Lake collection site location	Lat: 47.487436 Long: -116.724507	Lat: 47.493698 Long: -116.732514	Lat: 47.491021 Long: -116.740909
Access: IDFG			
Observations	Substrate: Very fine sediment, red/orange tint in early spring and late summer/fall, some large rocks Vegetation: Algae, lily pads, reed canary grass, wapato arrowhead, waterweed, milfoil, pondweed, (S1) Many horsetails Invertebrates: Large bryozoan colonies, fingernail clams, water boatmen, spiders, snails, caddisfly larvae, diving beetles, water mites, dragonfly larvae, isopods, midges, damselfly larvae, flatworms, leeches, cladocerans, sludge worms, misc. beetles Recreation: 2-5 groups per visit (angling, boating, hunting, and/or camping, water skiing) Other: Catfish observed; large flocks of waterfowl frequently observed, water light orange color in August		

Table 3i. Topographic maps of Harrison Sl. with geographic coordinates of sampling sites and field observations. Map obtained from explore.delorme.com/Map.



	Site 1 (S1)	Site 2 (S2)	Site 3 (S3)
Harrison Slough collection site location	Lat: 47.467025 Long: -116.765911	Lat: 47.467019 Long: -116.766432	Lat: 47.468672 Long: -116.766512
Access: IDFG			
Observations	Substrate: Rocky Vegetation: Lily pads, green algae, milfoil or coontail (S1, S3) Filamentous algae abundant late spring through fall Invertebrates: Copepods, flatworms, fly larvae, isopods, snails, water boatmen, sludge worms, damselfly larvae, water mites, dragonfly larvae, leeches, fingernail clams, backswimmers, misc. beetles Recreation: 0-1 groups per visit (angling) Other: Water had high organic content in October, also observed black particulate matter and decaying plants, sheen on water surface; tadpoles observed		

Table 3j. Topographic maps of Benewah L. with geographic coordinates of sampling sites and field observations. Map obtained from explore.delorme.com/Map.



	Site 1 (S1)	Site 2 (S2)	Site 3 (S3)
Benewah Lake collection site location	Lat: 47.347249 Long: -116.697821	Lat: 47.347143 Long: -116.686268	Lat: 47.347926 Long: -116.686199
Access: Idaho Parks and Recreation			
Observations	Substrate: (S1) medium to coarse (S2, S3) fine sediment/mud, fine particulate matter; decaying plant matter abundant in late summer/fall Vegetation: Cattails, reed canary grass, milfoil, duck weed, filamentous and green algae, elodea, narrow-leaved bur-reed Invertebrates: Water mites, sludge worms, mayfly larvae, isopods, flatworms, cladocerans, dragonfly larvae, leeches, water boatmen, damselfly larvae, midges, fingernail clams, spiders, beetles, water striders, caddisfly larvae Recreation: 0-4 groups per visit (waterfowl hunting, angling, boating) Other: Large groups of waterfowl frequently observed; bullfrogs abundant; catfish observed		

Table 4. Elements in the concentrated water samples that fell below the limit of detection (LOD) during ICP-OES analysis for samples collected in the lab and field studies.

Collection month/Lake	Apr[c]	May[c]	Jun[a]	Jul[a]	Aug[b]	Sep[bc]	Oct[de]
Rose	As	As, Cu	Cu	Cu	Cu	Cu	Cu
Bull Run		Cu	Cu	Cu	Cu		Cu
Killarney		Cu	Cu	Cu	Cu	Cu	
Medicine			Cu		Cu		Cu
Cave		Cu	Cu		Cu	Cu	Cu
Black	NT	NT	Cu	Cu	Cu	Cu	
Anderson			Cu	Cu	Cu		
Thompson			Cu	Cu	Cu		Cu
Harrison Sl.		Cu	Cu	Cu	Cu	Cu	Cu
Benewah		Cu	Cd, Cu	Cu	Cd, Cu	Cd, Cu	Cu

[a]Samples analyzed 7/28/2016 (Cu had poor recovery for all check standard concentrations on this date, and had a higher LOD with respect to all other analysis dates except 9/23/2016, which may explain why Cu was below the LOD); [b]Samples analyzed 9/23/2016 (Cu had poor recovery for all check standard concentrations on this date), [c]Samples analyzed 10/14/2016, [d]Samples analyzed 1/27/2017, [e]Samples analyzed 2/10/2017. April and May collections were originally analyzed on 6/20/2016. However, there were problems with the quality of analysis, so these samples were reanalyzed on 10/14/2016. NT, not tested.

Table 5a. Seasonal water quality on day of Zn toxicity test collection.

Season (Collection Dates)	Lake	Mean Temp (°C)	Mean DO (mg/L)	Mean pH	Alkalinity ($CaCO_3$, mg/L)	Hardness (mg/L)	As (mg/L)	Cd (mg/L)	Cu (mg/L)	Pb (mg/L)	Zn (mg/L)
Spring (6/4)	Benewah	24	9	8	26.2	15.6	0.0004	0.0000	0.0007	0.0005	0.0054
	Rose	19	7	8	12.8	9.8	0.0004	0.0000	0.0007	0.0033	0.0239
	Medicine	22	9	8	12.9	10.2	0.0021	0.0001	0.0009	0.0172	0.0217
	Anderson	24	13	9	14.9	16.2	0.0034	0.0002	0.0010	0.0105	0.0228
Summer (8/20, 8/27)	Benewah	26	9	7	27.8	22.3	0.0010	0.0000	0.0009	0.0038	0.0301
	Rose	21	7	7	13.8	11.9	0.0006	0.0000	0.0010	0.0061	0.0378
	Medicine	21	8	7	13.8	13.4	0.0035	0.0001	0.0006	0.0225	0.0470
	Anderson	26	11	8	22.0	20.0	0.0035	0.0001	0.0011	0.0107	0.0682
	Thompson	22	7	7	19.2	21.6	0.0018	0.0001	0.0011	0.0225	0.0760
Fall (10/8)	Benewah	16	11	7	32.4	24.6	0.0006	0.0001	0.0015	0.0029	0.0267
	Medicine	11	6	7	15.4	16.3	0.0075	0.0005	0.0005	0.0718	0.0783
	Anderson	14	8	8	52.5	45.5	0.0052	0.0005	0.0023	0.0468	0.0725
	Thompson	13	9	7	19.5	20.3	0.0020	0.0001	0.0006	0.0159	0.0439

Table 5b. Seasonal water quality on day of Pb toxicity test collection.

Season (Collection Dates)	Lake	Mean Temp (°C)	Mean DO (mg/L)	Mean pH	Alkalinity ($CaCO_3$, mg/L)	Hardness (mg/L)	As (mg/L)	Cd (mg/L)	Cu (mg/L)	Pb (mg/L)	Zn (mg/L)
Spring (6/11)	Benewah	20	9	8	22.7	14.9	0.0004	0.0000	0.0008	0.0016	0.0079
	Rose	19	7	8	8.1	3.1	0.0001	0.0000	0.0000	0.0019	0.0069
	Medicine	18	8	7	13.0	3.0	0.0009	0.0000	0.0000	0.0065	0.0100
	Anderson	19	10	9	22.2	16.4	0.0049	0.0007	0.0021	0.0272	0.0696
Summer (8/27)	Benewah	22	9	8	27.8	21.4	0.0004	0.0000	0.0010	0.0066	0.0550
	Medicine	19	8	7	14.0	17.2	0.0048	0.0001	0.0004	0.0282	0.0601
	Anderson	22	9	8	15.8	19.5	0.0036	0.0000	0.0009	0.0091	0.0348
	Thompson	22	7	7	19.2	21.6	0.0018	0.0001	0.0011	0.0225	0.0760

Table 6a. Survival in spring 96 h Zn toxicity test

Season (Start dates)	Population source (Gehan-Breslow statistic; p value)	Pairwise Comparisons (Holm-Sidak) (mg/L)	Dose (mg/L)	No. Jars†	% Survived 96 h (SD)	Mean survival time (h); (95% CI)	LT_{50} (h; median); (95% CI)
Spring (06/13)	Chesapeake Cultures (118.9; p < 0.001)	0 > all doses tested	0.0	4	100 (0)	96*	ND
		14.5 > 43.5	14.5	4	0 (0)	39.0 (34, 44)	48 (44, 52)
		29.0 > 43.5, 58.0	29.0	4	0 (0)	42.0 (37, 47)	48 (43, 53)
		-	43.5	4	0 (0)	27.0 (25, 30)	24 (21, 27)
		58.0 > 43.5	58.0	4	0 (0)	33.0 (29, 37)	24 (18, 30)
	Benewah Lake (98.8; p < 0.001)	0 > all doses tested	0.0	4	90 (8)	93.5 (90, 97)	ND
		14.5 > 58.0	14.5	5	100 (10)	62.4 (56, 69)	72 (66, 78)
		29.0 > 58.0	29.0	3	100 (10)	54.4 (46, 63)	48 (34, 62)
		43.5 > 58.0	43.5	4	100 (13)	53.4 (45, 62)	48 (40, 57)
		-	58.0	4	0 (5)	34.2 (28, 40)	24 (19, 29)
	Rose Lake (19.2; p < 0.001)	0 > 43.5, 58.0	0.0	2	100 (7)	93.7 (88, 100)	ND
		-	14.5	1	50 (ND)	79.2 (63, 96)	ND
		-	29.0	1	70 (ND)	86.4 (72, 101)	ND
		-	43.5	1	30 (ND)	50.4 (28, 73)	24 (12, 36)
		-	58.0	2	50 (4.2)	76.8 (64, 89)	96 (ND)
	Medicine Lake (12.3; p < 0.001)	0 > 58.0	0.0	2	100 (0)	96*	ND
		-	14.5	—	—	—	—
		-	29.0	—	—	—	—
		-	43.5	—	—	—	—
		-	58.0	1	50 (ND)	79.2 (61, 98)	96 (ND)
	Anderson Lake (16.9; p < 0.001)	0 > all doses tested	0.0	3	100 (0)	96*	ND
		-	14.5	3	80 (15)	91.2 (86, 96)	ND
		-	29.0	3	70 (26)	84.0 (75, 93)	ND
		-	43.5	3	70 (6)	87.2 (80, 94)	ND
		-	58.0	4	60 (10)	80.4 (74, 87)	ND

Table 6b. Survival in summer 96 h Zn toxicity test.

Season (Start dates)	Population source (Gehan-Breslow statistic; p value)	Pairwise Comparisons (Holm-Sidak) (mg/L)	Dose (mg/L)	No. Jars†	% Survived 96 h (SD)	Mean survival time (h); (95% CI)	LT_{50} (h; median); (95% CI)
Summer (08/29, 09/05)	Benewah Lake (142.1; p < 0.001)	0 > all doses tested	0.0	7	100 (0)	96*	ND
		14.5 > 29.0, 43.5, 58.0	14.5	5	100 (13)	59.5 (53, 66)	48 (40, 56)
		-	29.0	6	20 (23)	45.2 (38, 53)	24 (19, 29)
		-	43.5	6	10 (13)	40.8 (34, 48)	24 (19, 29)
		-	58.0	7	10 (17)	42.3 (36, 49)	24 (20, 28)
	Rose Lake (53.3; p < 0.001)	0 > 14.5, 43.5, 58.0	0.0	3	10 (6)	93.6	(ND)
		-	14.5	2	5 (7)	36 (28, 44)	24 (15, 33)
		-	29.0	—	—	—	—
		-	43.5	1	0 (ND)	36 (26, 47)	24 (12, 36)
		-	58.0	3	10 (10)	48 (37, 59)	24 (17, 32)
	Medicine Lake (51.1; p < 0.001)	0 > 29.0, 43.5, 58.0	0.0	5	100 (0)	96*	ND
		14.5 > 43.5, 58.0	14.5	5	90 (12)	94.6 (93, 96)	ND
		-	29.0	3	80 (12)	95.2 (94, 97)	ND
		-	43.5	5	60 (18)	88.8 (84, 94)	ND
		-	58.0	5	50 (16)	83.5 (77, 90)	96 (ND)
	Anderson Lake (155.8; p < 0.001)	0 > all doses tested	0.0	9	100 (3)	95.5 (ND)	ND
		14.5 > 43.5, 58.0	14.5	5	40 (17)	64.0 (55, 72)	72 (44, 100)
		-	29.0	5	20 (15)	50.4 (42, 59)	24 (14, 35)
		-	43.5	6	10 (12)	50.0 (42, 58)	24 (15, 33)
		-	58.0	7	0 (5)	40.9 (35, 47)	24 (18, 30)
	Thompson Lake (50.8; p < 0.001)	0 > 14.5, 29.0, 58.0	0.0	3	90 (12)	96.0 (96, 96)	ND
		-	14.5	1	20 (ND)	55.2 (35, 75)	48 (12, 84)
		-	29.0	1	60 (ND)	74.4 (53, 95)	ND
		43.5 > 14.5, 58.0	43.5	1	70 (ND)	91.2 (80, 102)	ND
		-	58.0	3	10 (7)	47.2 (36, 58)	24 (17, 32)

Table 6c. Survival in fall 96 h Zn toxicity test.

Season (Test start dates)	Population source (Gehan-Breslow test statistic; p value)	Pairwise Comparisons (Holm-Sidak) (mg/L)	Dose (mg/L)	No. Jars†	% Survived 96 h (SD)	Mean survival time (h); (95% CI)	LT_{50} (h; median); (95% CI)
Fall (10/17)	Benewah Lake (51.3; p < 0.001)	0 > all doses tested	0.0	6	100 (7)	96.0 (ND)	ND
		-	14.5	6	50 (7)	78.8 (73, 85)	96 (ND)
		-	29.0	6	40 (7)	79.2 (73, 86)	96 (83, 109)
		-	43.5	6	40 (7)	71.2 (63, 79)	96 (ND)
		-	58.0	6	40 (7)	67.6 (60, 76)	72 (41, 76)
	Medicine Lake (54.2; p < 0.001)	0 > all doses tested	0.0	4	100 (9)	96*	ND
		-	14.5	3	20 (10)	64.0 (55, 73)	72 (59, 85)
		-	29.0	4	20 (9)	61.2 (52, 70)	48 (39, 57)
		-	43.5	4	30 (9)	67.2 (58, 77)	72 (42, 102)
		-	58.0	4	30 (9)	58.8 (49, 69)	48 (30, 66)
	Anderson Lake (93.1; p < 0.001)	0 > all doses tested	0.0	6	100 (7)	94.8 (ND)	ND
		14.5 > 43.5, 58.0	14.5	6	70 (7)	90.0 (86, 94)	ND
		-	29.0	6	40 (7)	75.2 (68, 82)	96 (76, 116)
		-	43.5	6	40 (7)	71.2 (64, 79)	72 (46, 98)
		-	58.0	6	20 (7)	58.4 (51, 66)	48 (39, 58)
	Thompson Lake (58.9; p < 0.001)	0 > all doses tested	0.0	6	90 (7)	94.8 (93, 97)	ND
		14.5 > 29.0, 58.0	14.5	5	60 (8)	89.3 (84, 94)	ND
		-	29.0	5	40 (8)	78.7 (72, 85)	96 (82, 111)
		-	43.5	5	50 (8)	75.4 (68, 83)	96 (ND)
		-	58.0	6	3 (7)	72.0 (65, 79)	72 (57, 87)

All assays performed in 2016.

†All jars had 10 amphipods; *No mortality; — Not tested; ND: Could not be determined

Table 7a. Survival in spring 96 h Pb toxicity tests.

Season (Start dates)	Population source (Gehan-Breslow statistic; p value)	Pairwise Comparisons (mg/L)	Dose (mg/L)	No. Jars†	% Survived 96 h (SD)	Mean survival time (h); (95% CI)	LT_{50} (h; median); (95% CI)
Spring (06/20)	Chesapeake Cultures (69.7; p < 0.001)	0 > all doses tested	0.0	5	100 (0)	96*	ND
		5.4 > 16.2, 21.6	5.4	5	70 (23)	92.6 (90, 95)	ND
		10.8 > 16.2, 21.6	10.8	5	78 (15)	91.7 (88, 96)	ND
		-	16.2	3	50 (10)	79.2 (70, 88)	96 (ND)
		-	21.6	3	27 (25)	72.8 (66, 79)	72 (65, 79)
	Benewah Lake (14.6; p = 0.006)	0 > all doses tested	0.0	5	100 (0)	96*	ND
		-	5.4	5	78 (23)	92.2 (88, 96)	ND
		-	10.8	4	85 (13)	94.2 (91, 97)	ND
		-	16.2	5	84 (9)	93.1 (91, 95)	ND
		-	21.6	3	73 (21)	86.4 (80, 93)	ND
	Rose Lake (2.5; P = 0.469)	-	0.0	3	83 (15)	91.2 (87, 96)	ND
		-	5.4	—	—	—	—
		-	10.8	2	90 (14)	91.2 (81, 101)	ND
		-	16.2	4	95 (6)	94.2 (89, 99)	ND
		-	21.6	4	85 (6)	90.5 (85, 96)	ND
	Medicine Lake (8.5; p = 0.074)	-	0.0	3	100 (0)	96*	ND
		-	5.4	3	93 (12)	93.6 (ND)	ND
		-	10.8	3	100 (0)	96*	ND
		-	16.2	3	93 (6)	94.4 (91, 97)	ND
		-	21.6	3	87 (15)	88.8 (81, 97)	ND
	Anderson Lake (10.6; p = 0.032)	-	0.0	6	100 (0)	96*	ND
		-	5.4	5	100 (0)	96*	ND
		-	10.8	6	90 (13)	96.0 (96, 96)	ND
		-	16.2	5	96 (5)	95.5 (94, 97)	ND
		-	21.6	5	96 (5)	95.0 (93, 97)	ND

Table 7b. Survival in summer 96 h Pb toxicity tests.

Season (Start dates)	Population source (Gehan-Breslow statistic; p value)	Pairwise Comparisons (mg/L)	Dose (mg/L)	No. Jars†	% Survived 96 h (SD)	Mean survival time (h); (95% CI)	LT_{50} (h; median); (95% CI)
Summer (09/05)	Benewah (20.9; p < 0.001)	0 > 16.2, 21.6	0.0	7	90 (9)	95.0 (93, 97)	ND
		-	5.4	6	77 (5)	92.4 (89, 96)	ND
		-	10.8	7	84 (8)	93.3 (91, 96)	ND
		-	16.2	5	78 (19)	92.2 (90, 95)	ND
		-	21.6	5	68 (19)	88.8 (84, 93)	ND
	Medicine (3.8; p = 0.438)	-	0.0	7	83 (18)	94.6 (93, 97)	ND
		-	5.4	5	94 (5)	96.0 (96, 96)	ND
		-	10.8	4	93 (10)	96.0 (96, 96)	ND
		-	16.2	6	90 (9)	93.2 (90, 96)	ND
		-	21.6	7	86 (8)	94.3 (93, 96)	ND
	Anderson (10.2; p = 0.037)	-	0.0	7	93 (13)	93.6 (91, 96)	ND
		-	5.4	3	100 (0)	96*	ND
		-	10.8	4	93 (10)	93.0 (88, 98)	ND
		-	16.2	4	88 (15)	93.6 (89, 99)	ND
		-	21.6	5	78 (16)	88.6 (84, 94)	ND
	Thompson (3.7; p = 0.455)	-	0.0	3	97 (6)	96.0 (ND)	ND
		-	5.4	3	90 (0)	92.8 (87, 99)	ND
		-	10.8	3	83 (12)	96.0 (96, 96)	ND
		-	16.2	3	93 (12)	95.2 (93, 97)	ND
		-	21.6	3	80 (26)	94.3 (90, 99)	ND

All assays performed in 2016.

†All jars had 10 amphipods; *No mortality; — Not tested; ND: Could not be determined

Table 8. Jar water quality at the start of the Zn and Pb toxicity tests.

Toxicity Test; Measurement Date	Sample	DO (% saturation)	Temp. (°C)	pH	Hardness (mg/L)
Spring Zn; 6/13/2016	Test Jar	82	21	8	NT
	Test Jar	74	21	8	NT
	Test Jar	68	21	8	NT
	Test Jar	62	21	8	NT
	Test Jar	66	21	8	NT
	Test Jar	66	21	8	NT
	Test Jar	61	21	8	NT
	Test Jar	63	21	8	NT
	Test Jar	62	21	8	NT
Spring Pb; 6/20/2016	Test Jar	81	21	8	NT
	Test Jar	86	21	8	NT
	Test Jar	77	21	8	NT
	Dechlorinated Water Tap	NT	NT	NT	110
Summer Zn; 8/29/2016	Test Jar	80	19	8	NT
	Test Jar	80	19	8	NT
	Test Jar	80	20	8	NT
	Dechlorinated Water Tap	NT	NT	NT	105
Summer Pb; 9/9/2016	Test Jar	58	19	8	NT
	Test Jar	63	19	8	NT
	Test Jar	58	20	8	NT
Fall Zn; 10/18/2016	Test Jar	80	17	7	NT
	Test Jar	78	18	8	NT
	Test Jar	77	18	8	NT
	Test Jar	72	18	8	NT
	Test Jar	77	18	8	NT
	Dechlorinated Water Tap	NT	NT	NT	109

Table 9. Jar water metals at the end of the Zn and Pb toxicity tests.

Toxicity Test; Sample Date	Zn or Pb Dose (mg/L)	Zn or Pb (mg/L)	Hardness (as $CaCO_3$; mg/L)	Amphipod Source Lake	Amphipod Tissue Zn (mg/g)[§]
Spring Zn; 6/21/2016	0	0.005	125	Benewah	2.94
	14.5	2.350	130	Anderson	8.86
	29	> LOQ	122	Anderson	15.74
	43.5	> LOQ	N/A	Anderson	20.09
	58	3.93*	103	Anderson	41.03
Spring Pb; 6/24/16	0	0.0022	125	NT	NT
	5.4	NT	NT	NT	NT
	10.8	0.76	108	NT	NT
	16.2	NT	NT	NT	NT
	21.6	> LOQ	120	NT	NT
Summer Zn; 9/3/2016	0	0.054	93	NT	NT
	14.5	3.60*	98	NT	NT
	29	6.30*	95	NT	NT
	43.5	13.70*	99	NT	NT
	58	10.88*	98	NT	NT

Abbreviations: >LOQ, sample metal greater than the limit of detection for the instrument; NT, not tested; *Sample run as 1:10 dilution of original. [§]Amphipods collected from Benewah L. and Anderson L. for the spring Zn test had tissue levels of 2.493 and 2.833 mg/g Zn, respectfully.

Table 10a. Swimming activity post-96 h Zn spring toxicity test.

Season (Test dates)	Population source (One Way ANOVA)	Dose (mg/L)	No. Individuals	No. individuals did not swim	Mean number of swims; (SD)
Spring (06/13/2016)	Benewah Lake	0.0	10	0	21 (17)
		14.5	3	1	9 (14)
		29.0	3	2	3 (5)
Dose = 0.0008		43.5	2	0	1 (0)
		58.0	2	0	4 (4)
Pop = 0.4165	Rose Lake	0.0	19	1	26 (17)
		14.5	5	3	0 (0)
Pop*Dose = 0.4418		29.0	—	—	—
		43.5	2	1	1 (1)
		58.0	7	5	0 (1)
	Medicine Lake	0.0	—	—	—
		14.5	—	—	—
		29.0	—	—	—
		43.5	—	—	—
		58.0	—	—	—
	Anderson Lake	0.0	20	0	20 (14)
		14.5	9	4	16 (23)
		29.0	8	1	2 (2)
		43.5	17	10	1 (1)
		58.0	13	5	2 (3)
	Chesapeake Cultures	0.0	21	6	3 (9)
		14.5	—	—	—
		29.0	—	—	—
		43.5	—	—	—
		58.0	—	—	—

Table 10b. Swimming activity post-96 h Zn summer toxicity test.

Season (Test dates)	Population source (One Way ANOVA)	Dose (mg/L)	No. Individuals	No. individuals did not swim	Mean number of swims; (SD)
Summer (08/29/2016, 09/05/2016)	Benewah Lake	0.0	30	0	15 (8)
		14.5	6	3	3 (7)
		29.0	12	8	1 (2)
		43.5	6	4	1 (2)
Dose < 0.0001		58.0	8	6	0 (1)
	Rose Lake	0.0	—	—	—
Pop < 0.0001		14.5	—	—	—
		29.0	—	—	—
Pop*Dose = 0.1823		43.5	—	—	—
		58.0	—	—	—
	Medicine Lake	0.0	30	0	25 (10)
		14.5	—	—	—
		29.0	25	10	25 (6)
		43.5	20	7	3 (6)
		58.0	13	7	2 (3)
	Anderson Lake	0.0	31	5	6 (6)
		14.5	14	13	0 (1)
		29.0	7	7	0 (0)
		43.5	5	4	1 (1)
		58.0	3	3	0 (0)
	Thompson Lake	0.0	28	0	15 (14)
		14.5	2	2	0 (0)
		29.0	6	6	0 (0)
		43.5	7	7	0 (0)
		58.0	4	2	1 (1)

Table 10c. Swimming activity post-96 h Zn fall toxicity test.

Season (Test dates)	Population source (One Way ANOVA)	Dose (mg/L)	No. Individuals	No. individuals did not swim	Mean number of swims; (SD)
Fall (10/17/2016)	Benewah Lake	0.0	37	3	17 (12)
		14.5	5	4	1 (2)
		29.0	2	1	2 (3)
Dose < 0.0001		43.5	2	2	0 (0)
		58.0	21	9	3 (4)
Pop = 0.1617	Medicine Lake	0.0	10	2	7 (2)
		14.5	3	2	0 (1)
Pop*Dose = 0.0166		29.0	3	3	0 (0)
		43.5	6	3	1 (1)
		58.0	4	1	2 (1)
	Anderson Lake	0.0	10	0	14 (10)
		14.5	7	6	0 (0)
		29.0	4	4	0 (0)
		43.5	4	3	1 (1)
		58.0	1	1	0 (0)
	Thompson Lake	0.0	9	0	43 (15)
		14.5	7	5	0 (1)
		29.0	6	4	1 (2)
		43.5	3	2	1 (2)
		58.0	4	2	1 (1)

Table 11a. Swimming activity post-96 h Pb spring toxicity test.

Season (Test dates)	Population source (One Way ANOVA)	Dose (mg/L)	No. Individuals	No. individuals did not swim	Mean number of swims; (SD)
Spring (06/20/2016)	Benewah Lake	0.0	10	0	17 (9)
		5.4	5	0	18 (11)
		10.8	8	4	9 (17)
Dose = 0.009		16.2	8	1	6 (8)
		21.6	6	4	12 (22)
Pop = 0.2509	Rose Lake	0.0	6	0	25 (11)
		5.4	—	—	—
Pop*Dose = 0.0046		10.8	6	0	23 (12)
		16.2	8	3	3 (3)
		21.6	17	2	11 (10)
	Medicine Lake	0.0	10	3	10 (12)
		5.4	—	—	—
		10.8	10	0	20 (11)
		16.2	18	3	14 (13)
		21.6	14	3	10 (11)
	Anderson Lake	0.0	11	2	17 (16)
		5.4	8	1	12 (14)
		10.8	8	2	13 (19)
		16.2	9	0	8 (7)
		21.6	22	2	13 (13)
	Chesapeake Cultures	0.0	—	—	—
		5.4	—	—	—
		10.8	—	—	—
		16.2	—	—	—
		21.6	—	—	—

Table 11b. Swimming activity post-96 h Pb summer toxicity test.

Season (Test dates)	Population source (One Way ANOVA)	Dose (mg/L)	No. Individuals	No. individuals did not swim	Mean number of swims; (SD)
Summer (09/05/2016)	Benewah	0.0	28	3	13 (12)
		5.4	—	—	—
Dose < 0.0001		10.8	—	—	—
		16.2	—	—	—
Pop = 0.0048		21.6	11	4	4 (6)
	Medicine	0.0	19	1	19 (14)
Pop*Dose = 0.1361		5.4	—	—	—
		10.8	—	—	—
		16.2	—	—	—
		21.6	16	7	6 (8)
	Anderson	0.0	20	1	22 (8)
		5.4	—	—	—
		10.8	—	—	—
		16.2	—	—	—
		21.6	17	3	4 (5)
	Thompson	0.0	10	1	6 (6)
		5.4	—	—	—
		10.8	—	—	—
		16.2	—	—	—
		21.6	19	8	4 (4)

— Not tested

Table 12a. Monthly amphipod abundance and lake limnology.

Lake	Month	Amphipod Mean CPUE (Per m^2; SD)	Mean Temp. (°C; SD)	Mean DO (mg/L; SD)	Mean pH (SD)	Mean Alkalinity (as mg/L $CaCO_3$; SD)	Water Hardness (as mg/L $CaCO_3$)
Rose	May	12 (12)	18.0 (0.7)	8.4 (0.3)	8 (0.2)	10.0 (2.5)	12.3
	Jun	32 (27)	18.0 (0.3)	8.0 (0.2)	8 (0.3)	8.7 (0.2)	10.8
	Jul	41 (18)	20.6 (0.2)	6.0 (1.1)	7 (0.1)	10.8 (1.1)	11.7
	Aug	24 (12)	20.9 (0.2)	5.3 (1.7)	8 (0.2)	13.5 (1.6)	12.9
	Sep	53 (73)	16.6 (0.6)	8.2 (0.4)	7 (0.2)	14.3 (2.0)	12.3
	Oct	38 (6)	10.4 (0.2)	6.1 (1.8)	7 (0.2)	14.9 (1.7)	15.4
Bull Run	May	0 (0)	19.2 (1.0)	3.4 (0.8)	8 (0.3)	15.4 (9.2)	9.3
	Jun	0 (0)	16.8 (0.8)	1.5 (0.7)	7 (0.3)	12.7 (13.1)	15.9
	Jul	0 (0)	19.6 (0.9)	2.1 (0.5)	6 (0.2)	13.6 (16.1)	17.8
	Aug	0 (0)	18.5 (0.3)	0.5 (0.3)	6 (0.1)	15.1 (11.4)	14.5
	Sep	0 (0)	14.5 (0.8)	2.7 (1.3)	6 (0.1)	13.0 (5.4)	16.7
	Oct	0 (0)	9.2 (0.2)	3.0 (0.9)	7 (0.1)	9.8 (8.6)	13.0
Killarney	May	0 (0)	19.8 (0.7)	7.1 (1.5)	8 (0.3)	30.9 (18.2)	91.8
	Jun	0 (0)	17.6 (1.2)	7.2 (1.1)	7 (0.4)	46.7 (38.0)	78.1
	Jul	1 (2)	19.9 (1.7)	6.4 (2.7)	7 (0.3)	61.1 (46.6)	65.8
	Aug	0 (1)	19.4 (2.4)	4.6 (1.9)	7 (0.4)	60.3 (44.8)	57.2
	Sep	3 (3)	14.8 (1.7)	6.8 (2.5)	7 (0.3)	49.0 (37.9)	46.7
	Oct	1 (0)	10.6 (0.9)	6.1 (3.6)	7 (0.5)	25.3 (8.9)	129.1

Lake	Month	Amphipod Mean CPUE (Per m^2; SD)	Mean Temp. (°C; SD)	Mean DO (mg/L; SD)	Mean pH (SD)	Mean Alkalinity (as mg/L $CaCO_3$; SD)	Water Hardness (as mg/L $CaCO_3$)
Medicine	May	0 (1)	19.2 (0.5)	9.1 (0.9)	8 (0.2)	10.7 (1.6)	15.5
	Jun	2 (2)	17.5 (1.8)	6.3 (4.1)	8 (0.5)	12.4 (2.1)	13.9
	Jul	4 (2)	20.7 (0.5)	8.3 (1.7)	7 (0.4)	13.1 (0.7)	17.5
	Aug	6 (6)	22.6 (0.1)	8.8 (1.2)	8 (0.1)	16.2 (0.6)	17.0
	Sep	6 (2)	15.4 (0.3)	6.9 (1.6)	7 (0.1)	13.8 (2.8)	20.9
	Oct	12 (8)	10.5 (0.1)	7.9 (1.6)	7 (0.4)	28.4 (24.4)	32.6
Cave	May	1 (1)	21.3 (0.7)	8.6 (1.4)	8 (1.2)	7.7 (1.6)	14.6
	Jun	6 (4)	19.7 (1.1)	6.0 (1.9)	7 (0.5)	13.0 (0.4)	13.7
	Jul	17 (17)	21.7 (0.5)	6.8 (1.3)	7 (0.2)	13.1 (3.9)	15.1
	Aug	36 (38)	22.5 (1.6)	6.1 (2.9)	7 (0.2)	15.9 (0.9)	15.9
	Sep	56 (22)	15.7 (1.5)	7.5 (3.8)	7 (0.3)	16.4 (1.2)	15.2
	Oct	112 (103)	11.4 (1.7)	8.7 (6.3)	7 (0.0)	18.3 (10.2)	32.4
Black	May	—	—	—	—	—	—
	Jun	10 (14)	20.0 (0.3)	9.4 (0.1)	8 (0.0)	20.4 (0.6)	21.4
	Jul	3 (3)	21.7 (0.1)	9.8 (0.4)	8 (0.1)	21.7 (0.5)	22.1
	Aug	5 (6)	23.1 (0.0)	10.3 (1.1)	8 (0.3)	25.3 (1.0)	22.5
	Sep	9 (6)	17.2 (0.2)	10.4 (0.4)	7 (0.1)	23.7 (2.2)	21.8
	Oct	15 (ND)	12.3 (ND)	9.2 (ND)	7 (ND)	13.9 (19.6)	22.8

Lake	Month	Amphipod Mean CPUE (Per m^2; SD)	Mean Temp. (°C; SD)	Mean DO (mg/L; SD)	Mean pH (SD)	Mean Alkalinity (as mg/L $CaCO_3$; SD)	Water Hardness (as mg/L $CaCO_3$)
Anderson	May	1 (1)	23.6 (1.9)	9.2 (1.0)	8 (0.3)	21.6 (7.4)	21.3
	Jun	10 (11)	22.4 (0.9)	12.2 (0.3)	9 (0.3)	23.0 (2.0)	19.4
	Jul	11 (9)	23.4 (0.8)	10.9 (2.1)	7 (1.2)	21.1 (1.2)	11.6
	Aug	64 (84)	27.3 (0.7)	9.2 (0.9)	8 (0.5)	22.1 (1.8)	21.3
	Sep	51 (67)	15.3 (1.3)	7.1 (1.1)	6 (0.5)	23.8 (9.6)	29.1
	Oct	13 (14)	12.1 (1.0)	8.5 (3.2)	7 (0.2)	31.9 (6.6)	28.9
Thompson	May	1 (1)	21.8 (1.7)	9.3 (0.4)	8 (0.1)	13.6 (0.6)	15.3
	Jun	11 (12)	21.6 (0.2)	9.7 (1.0)	8 (0.0)	14.2 (2.2)	17.3
	Jul	4 (2)	22.4 (0.9)	8.8 (1.1)	8 (0.1)	15.4 (1.7)	20.4
	Aug	5 (1)	25.0 (1.2)	6.9 (1.0)	8 (0.1)	19.8 (1.1)	25.6
	Sep	100 (24)	16.4 (0.2)	7.1 (1.1)	7 (0.1)	20.0 (3.7)	26.4
	Oct	38 (13)	12.3 (1.3)	9.7 (2.1)	7 (0.1)	22.9 (6.1)	20.1
Harrison Sl.	May	2 (3)	22.5 (2.0)	7.0 (1.8)	8 (0.1)	18.3 (0.7)	21.2
	Jun	1 (2)	20.7 (4.1)	33.0 (41.4)	8 (0.7)	17.7 (4.8)	20.6
	Jul	4 (6)	22.0 (1.2)	6.6 (2.3)	8 (0.1)	20.9 (5.8)	24.5
	Aug	1 (1)	23.2 (3.0)	8.0 (2.1)	7 (0.2)	74.6 (75.7)	40.1
	Sep	22 (30)	14.0 (1.5)	3.7 (3.5)	6 (0.1)	24.5 (8.2)	23.2
	Oct	30 (11)	11.2 (0.4)	10.1 (2.1)	8 (0.3)	23.5 (8.6)	26.7

Lake	Month	Amphipod Mean CPUE (Per m^2; SD)	Mean Temp. (°C; SD)	Mean DO (mg/L; SD)	Mean pH (SD)	Mean Alkalinity (as mg/L $CaCO_3$; SD)	Water Hardness (as mg/L $CaCO_3$)
Benewah	May	2 (1)	22.9 (1.1)	7.5 (1.3)	7 (0.2)	19.0 (3.9)	18.9
	Jun	37 (36)	21.9 (0.6)	9.6 (1.3)	8 (0.7)	22.5 (7.9)	16.9
	Jul	35 (19)	22.8 (0.5)	8.4 (0.9)	7 (0.1)	24.6 (1.4)	22.2
	Aug	19 (26)	25.8 (1.7)	10.1 (0.9)	8 (0.4)	29.5 (7.1)	21.3
	Sep	20 (15)	15.7 (0.3)	9.0 (2.4)	7 (0.3)	27.6 (5.4)	25.8
	Oct	71 (50)	10.1 (2.1)	11.1 (0.9)	7 (0.1)	34.1 (9.1)	24.9

ND: Could not be determined

Table 12b. Metals from water samples (W) and amphipod tissues (T) collected monthly from the chain lakes.

Lake	Month	AsW (µg/L)	AsT (mg/g)	CdW (µg/L)	CdT (mg/g)	CuW (µg/L)	CuT (mg/g)	PbW (µg/L)	PbT (mg/g)	ZnW (µg/L)	ZnT (mg/g)
Rose	May	0.3	0.02	0.03	0.03	0.6	0.52	2.1	0.07	8.0	1.85
	Jun	0.6	0.06	0.04	0.01	0.1	1.44	8.8	0.09	31.6	6.90
	Jul	0.4	0.02	0.01	0.01	0.1	0.97	6.3	0.10	23.2	4.21
	Aug	0.7	0.04	0.03	0.01	0.7	1.91	2.5	0.21	20.3	3.83
	Sep	0.6	0.05	0.07	0.01	1.1	1.09	9.2	0.12	47.7	3.53
	Oct	0.7	0.04	0.05	0.02	0.6	1.90	4.2	0.22	24.0	4.13
Bull Run	May	1.1	—	0.3	—	0.5	—	9.3	—	36.2	—
	Jun	2.0	—	0.2	—	0.1	—	21.9	—	29.6	—
	Jul	2.2	—	0.4	—	0.1	—	40.7	—	59.8	—
	Aug	1.6	—	0.3	—	0.9	—	40.0	—	84.5	—
	Sep	3.2	—	0.9	—	1.1	—	74.5	—	174.5	—
	Oct	0.5	—	0.2	—	0.4	—	7.9	—	72.1	—
Killarney	May	4.7	—	0.7	—	0.5	—	28.5	—	212.7	—
	Jun	4.2	—	0.2	—	0.1	—	30.0	—	39.0	—
	Jul	7.5	—	0.5	—	0.1	—	72.6	—	72.9	—
	Aug	9.8	0.05	0.5	0.01	1.4	0.56	38.5	0.33	102.5	2.83
	Sep	12.3	0.33	3.4	0.03	1.3	0.07	199.4	1.91	462.3	4.92
	Oct	1.9	0.02	39.6	0.00	2.3	0.19	28.1	0.15	4196.7	2.13
Medicine	May	3.7	—	0.2	—	1.6	—	26.4	—	38.0	—
	Jun	2.9	—	0.2	—	0.1	—	24.8	—	67.5	—
	Jul	12.8	—	0.9	—	3.2	—	170.3	—	118.7	—
	Aug	5.4	0.09	0.5	0.12	0.6	1.45	85.9	1.67	74.3	4.83
	Sep	10.0	0.05	0.6	0.11	2.9	1.83	137.2	4.78	102.5	5.15
	Oct	7.5	0.06	0.5	0.06	1.6	0.76	55.8	0.66	125.5	3.22

Lake	Month	AsW (µg/L)	AsT (mg/g)	CdW (µg/L)	CdT (mg/g)	CuW (µg/L)	CuT (mg/g)	PbW (µg/L)	PbT (mg/g)	ZnW (µg/L)	ZnT (mg/g)
Cave	May	1.0	—	0.1	—	0.5	—	11.7	—	17.2	—
	Jun	1.6	0.17	0.1	0.01	0.1	0.98	17.0	2.25	40.1	18.15
	Jul	2.3	0.02	0.6	0.16	2.1	0.74	169.5	0.26	56.6	2.19
	Aug	1.8	0.04	0.1	0.02	0.7	0.91	26.6	0.10	28.7	3.70
	Sep	1.3	0.04	0.05	0.03	1.0	1.05	13.9	0.20	63.6	4.62
	Oct	0.9	0.04	0.2	0.11	0.6	0.85	10.7	0.59	39.4	2.64
Black	May	—	—	—	—	—	—	—	—	—	—
	Jun	1.3	0.20	0.1	0.08	0.1	0.88	12.9	0.32	45.8	13.40
	Jul	1.5	0.15	0.03	0.05	0.2	2.05	9.3	0.23	28.0	6.93
	Aug	1.5	0.05	0.04	0.05	0.6	1.92	7.2	0.16	24.4	4.07
	Sep	2.1	0.11	0.1	0.03	1.3	1.99	17.8	0.21	72.0	9.17
	Oct	1.7	0.03	0.2	0.02	0.3	1.38	26.9	0.35	49.2	3.46
Anderson	May	3.5	—	0.3	—	1.3	—	23.4	—	47.0	—
	Jun	4.7	0.05	0.1	0.09	0.2	1.48	13.0	0.31	39.4	7.43
	Jul	3.4	0.08	0.1	0.02	0.1	1.84	12.8	0.29	32.6	4.27
	Aug	8.0	0.04	1.4	0.02	2.1	1.28	86.9	0.34	138.8	4.62
	Sep	6.3	0.06	3.2	0.01	7.6	1.63	126.4	0.11	366.4	4.90
	Oct	5.2	0.02	0.5	0.01	2.3	1.63	46.8	0.07	72.5	2.71
Thompson	May	1.9	—	0.1	—	1.6	—	22.6	—	22.7	—
	Jun	2.0	0.06	0.1	0.12	0.1	1.63	15.4	0.31	50.7	5.90
	Jul	3.3	0.04	0.4	0.07	0.1	1.29	64.9	0.38	87.0	3.05
	Aug	4.8	0.04	2.9	0.05	4.7	1.45	209.3	0.44	360.6	4.45
	Sep	3.5	0.08	0.4	0.06	1.4	1.41	61.4	0.60	128.0	6.48
	Oct	1.6	0.03	0.1	0.02	1.2	0.97	13.3	0.23	32.7	3.41

Lake	Month	AsW (µg/L)	AsT (mg/g)	CdW (µg/L)	CdT (mg/g)	CuW (µg/L)	CuT (mg/g)	PbW (µg/L)	PbT (mg/g)	ZnW (µg/L)	ZnT (mg/g)
Harrison	May	5.1	—	0.1	—	0.1	—	22.4	—	39.3	—
Sl.	Jun	13.8	—	0.9	—	0.2	—	107.5	—	93.3	—
	Jul	6.3	—	0.3	—	0.7	—	23.0	—	60.4	—
	Aug	3.4	—	0.2	—	0.9	—	19.1	—	60.7	—
	Sep	4.8	0.03	0.3	0.01	0.6	0.44	14.7	0.05	66.4	2.92
	Oct	5.1	0.02	0.1	0.00	0.1	0.40	22.4	0.29	39.3	4.16
Benewah	May	0.4	—	0.04	—	0.5	—	1.3	—	3.9	—
	Jun	0.5	0.03	0.01	0.00	0.1	1.49	0.9	0.08	14.2	3.31
	Jul	0.6	0.04	0.02	0.00	0.1	0.97	6.3	0.02	21.4	4.39
	Aug	0.7	0.04	0.01	0.00	0.7	1.24	3.8	0.04	29.4	2.95
	Sep	1.6	0.02	0.01	0.00	1.1	1.34	9.4	0.05	76.9	3.47

FIGURE LEGENDS

Figure 1. Map of the Coeur d'Alene Basin and "the box" (BEIPC). The CDA River is classified into three reaches, the upper, middle, and lower. The upper reach drains the South Fork from its headwaters near the Idaho-Montana border to Wallace. The South Fork meets the North Fork in the middle reach, which includes the Box, and stretches from Wallace to Cataldo. Finally, the lower reach extends the CDA River from Cataldo to Lake Coeur d'Alene.

Figure 2. Map of the Coeur d'Alene River Basin and chain lakes where amphipod and water samples were collected between March and November 2016. Arrows indicate direction of water flow. I have confirmed amphipod presence in the following lakes: Rose L., Killarney L., Medicine L., Cave L., Thompson L., Anderson L., Harrison Slough, and Benewah L.

Figure 3. Spring Zn 96 h toxicity test survival curves for the reference populations, (a) Chesapeake Cultures and (b) Benewah L.

Figure 4. Spring Pb 96 h toxicity test survival curves for the reference populations, (a) Chesapeake Cultures and (b) Benewah L.

Figure 5. Swimming activity (number of surfacings in 10 minutes) of control treatment amphipods that survived each Zn or Pb toxicity test.

Figure 6. Swimming activity (number of surfacings in 10 minutes) of amphipods that survived each dose in the (a) spring, (b) summer, and (c) fall 96 h Zn toxicity tests.

Figure 7. Total aqueous metal (mg/L) in Killarney L. sampled monthly from May-October 2016. Only one sample was collected each month.

Figure 8. Amphipod abundance as CPUE in each lake from May-October 2016. This figure includes October Killarney data.

Figure 9. Amphipod abundance as CPUE and length (mm) in (a) Cave L. and (b) Medicine L. from May-October 2016.

Figure 10. Amphipod abundance as CPUE and length (mm) in (a) Thompson L. and (b) Harrison Sl. from May-October 2016.

Figure 11. Amphipod abundance as CPUE and length (mm) in (a) Killarney L., (b) Black L., and (c) Anderson L. from May-October 2016.

Figure 12. Amphipod abundance as CPUE and length (mm) in (a) Rose L. and (b) Benewah L. from May-October 2016.

Figure 13. Amphipod lengths (mm) in all lakes. All amphipods collected (N = 3,069) were measured to the nearest tenth of a millimeter. Data are presented as box plots; the line is the median, the box is 25-75%, whiskers are 10-90%, and outliers are dots. See Table 12 for sample size per lake.

Figure 14. Amphipod tissue Cd, Cu, and Pb concentrations (µg/g) from all lakes. Data represent one pooled sample of amphipods (N = 6-29) per month per lake (max = June-October, see Table 12 for individual lake information). Data are presented as box plots, with the line as the median and the box as 25-75%.

Figure 15. Scatterplot of amphipod tissue (tis, µg/g) and total aqueous metal (wat, mg/L) concentrations for all lakes, determined using a Spearman Rank Order correlation. Axis labels to the left of rows indicate the Y axis, axis labels underneath columns indicate the X axis.

Figure 16. Water temperature (°C) and DO (mg/L) for all lakes, collected from May-October 2016. Data are presented as box plots; the line is the median, the box is 25-75%, whiskers are 10-90%, and outliers are dots.

Figure 17. Water (a) pH, (b) alkalinity (mg/L $CaCO_3$), and (c) hardness (mg/L $CaCO_3$) for all lakes, collected May-October 2016. Data are presented as box plots; the line is the median, the box is 25-75%, whiskers are 10-90%, and outliers are dots.

Figure 18. Principal component analysis (PCA) score biplot explaining amphipod abundance by limnologic variables and total aqueous metals. Numbers represent the lake/month samples, vectors represent the explanatory variables entered into the PCA. Axis 1 (Comp. 1) represents aqueous metal concentrations, which are negatively related to the axis. Axis 2 (Comp. 2) represents limnological variables; alkalinity and hardness are positively loaded, temperature, dissolved 0_2, and pH are negatively loaded. PCA was conducted without the October Killarney data point.

Figure 19. Principal component analysis (PCA) axis scores, (a) axis 1 and (b) axis 2 plotted by lake. Scores are presented as boxplots, with the line indicating median and the box indicating 25-75%. PCA was conducted without the October Killarney data point.

FIGURES

Figure 1

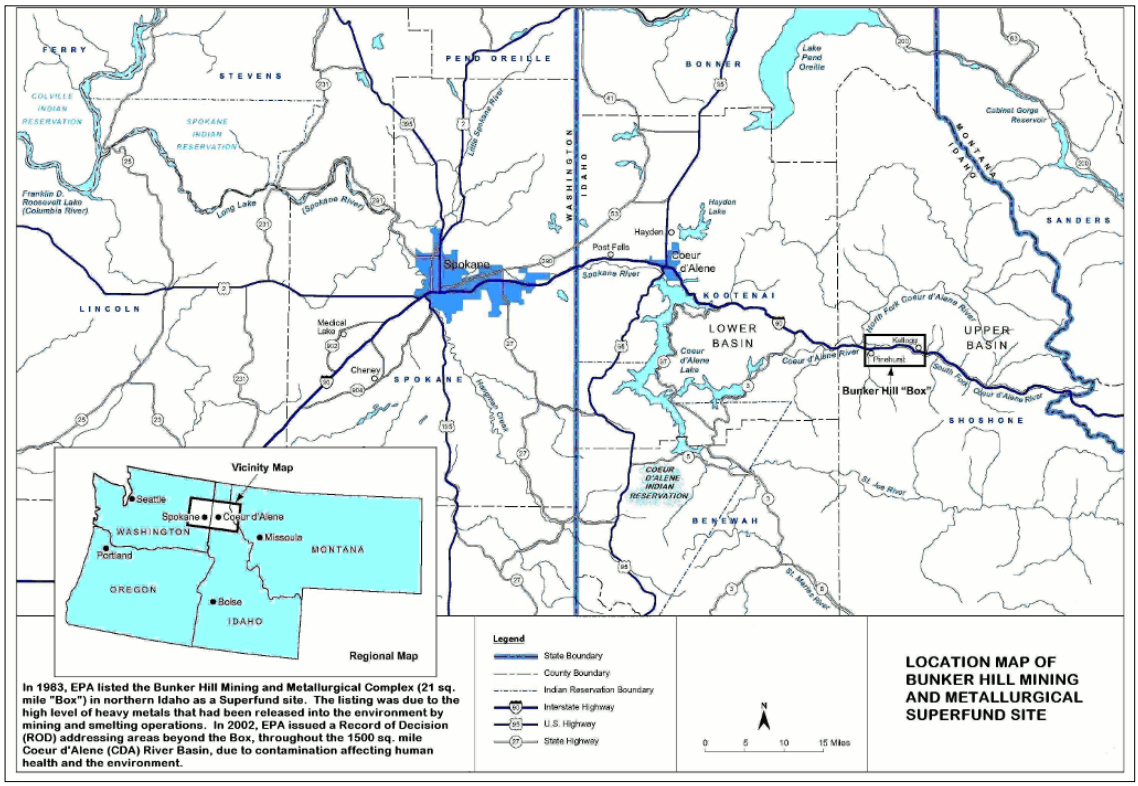


Figure 2


Rose L.
Rose Lake
Bull Run L.
Killarney L.
Thompson L.
CDA R.
Medimont
Harrison Sl.
Anderson L.
Medicine L.
Harrison
Cave L.
Black L.
Worley
Conkling Park
Heyburn State Park
Benewah L.
Plummer
Parkline
St. Joe R.
Harrison Slough
Lake Coeur d'Alene

Figure 3

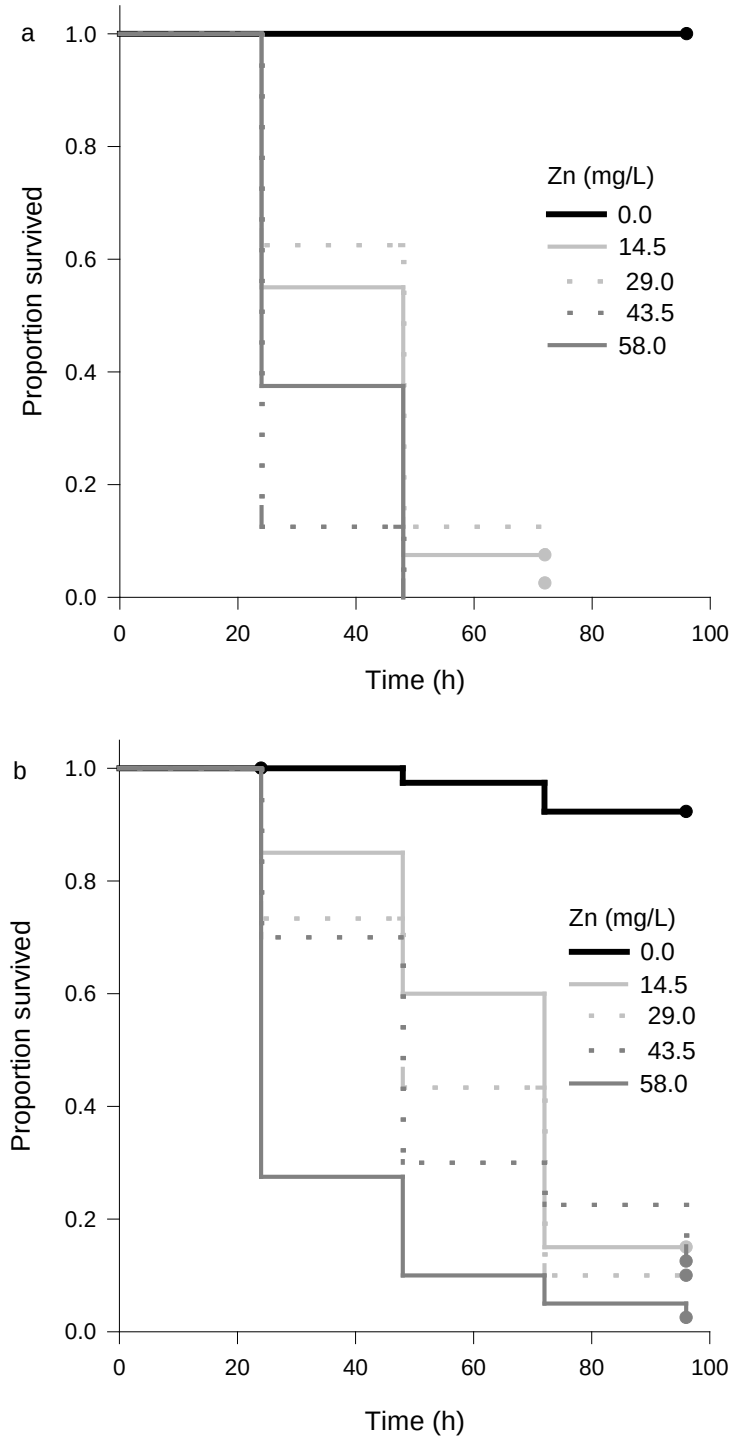

a
b
Proportion survived
Time (h)
Zn (mg/L)
0.0
14.5
29.0
43.5
58.0
0.0
0.2
0.4
0.6
0.8
1.0
0
20
40
60
80
100

Figure 4

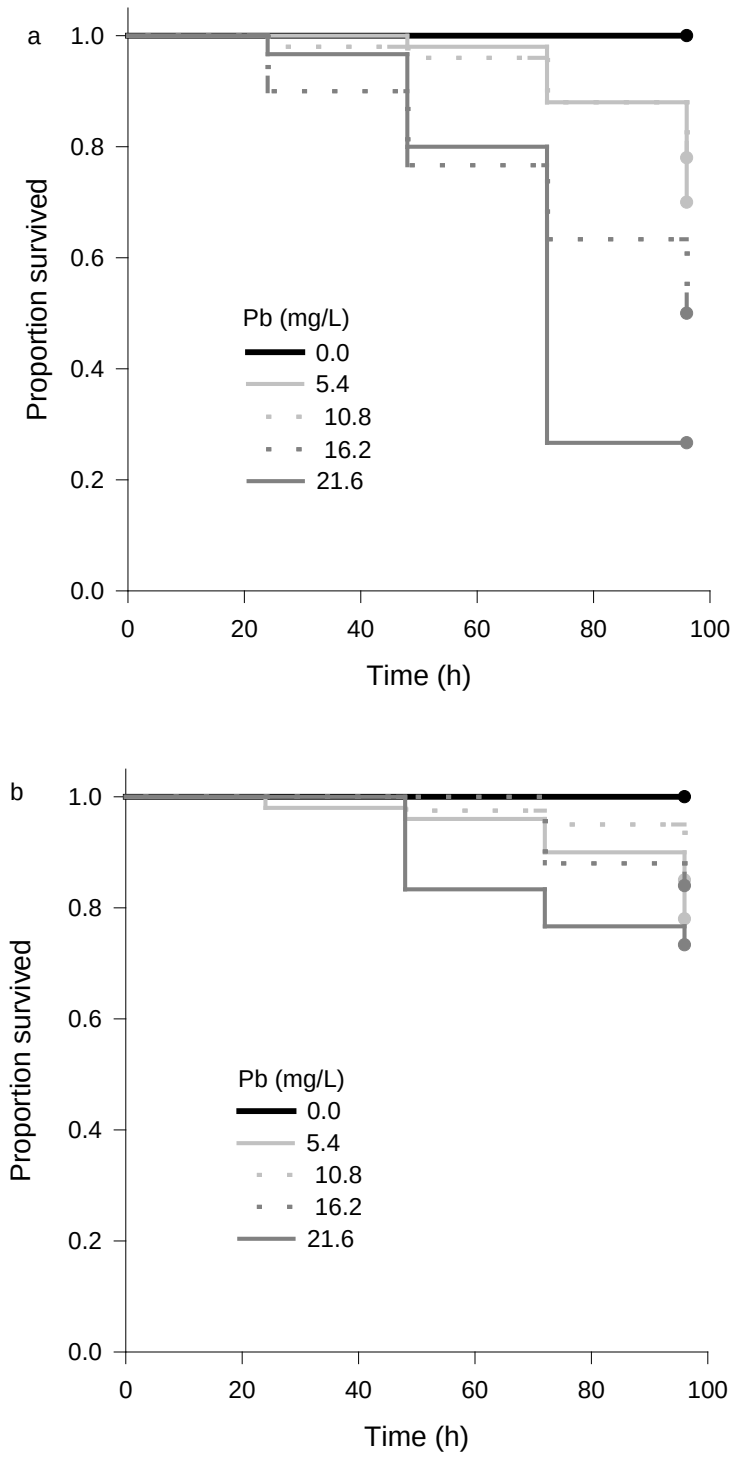
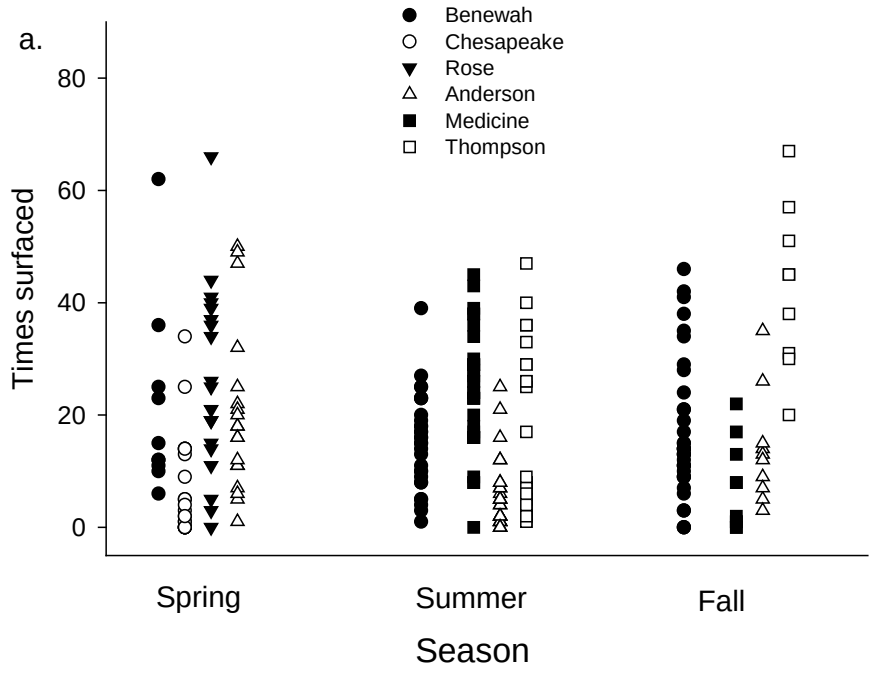

a
1.0
0.8
0.6
0.4
0.2
0.0
Proportion survived
Pb (mg/L)
0.0
5.4
10.8
16.2
21.6
0
20
40
60
80
100
Time (h)
b
1.0
0.8
0.6
0.4
0.2
0.0
Proportion survived
Pb (mg/L)
0.0
5.4
10.8
16.2
21.6
0
20
40
60
80
100
Time (h)

Figure 5


a.
Benewah
Chesapeake
Rose
Anderson
Medicine
Thompson
Times surfaced
0
20
40
60
80
Spring
Summer
Fall
Season

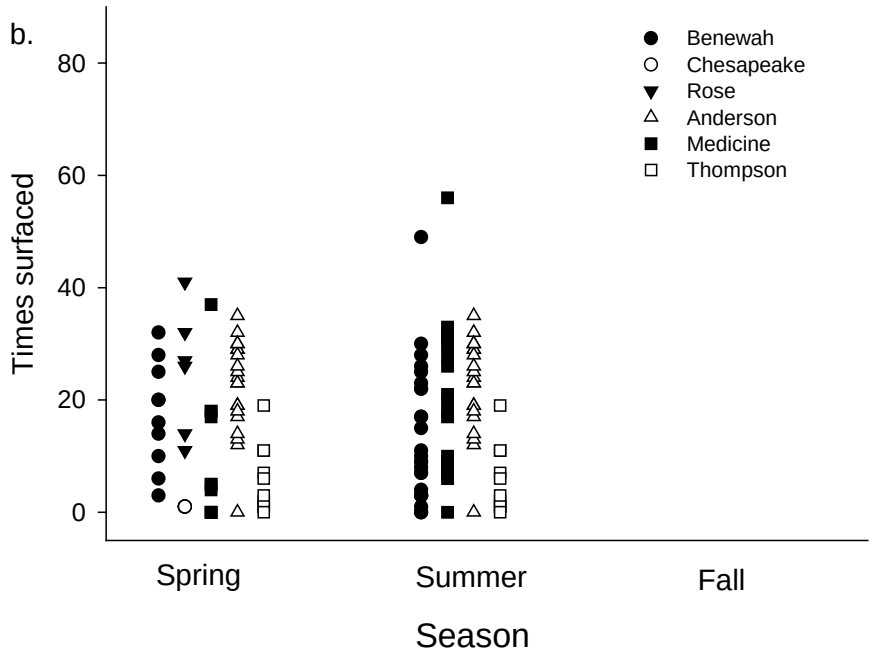

b.
Benewah
Chesapeake
Rose
Anderson
Medicine
Thompson
Times surfaced
0
20
40
60
80
Spring
Summer
Fall
Season

Figure 6

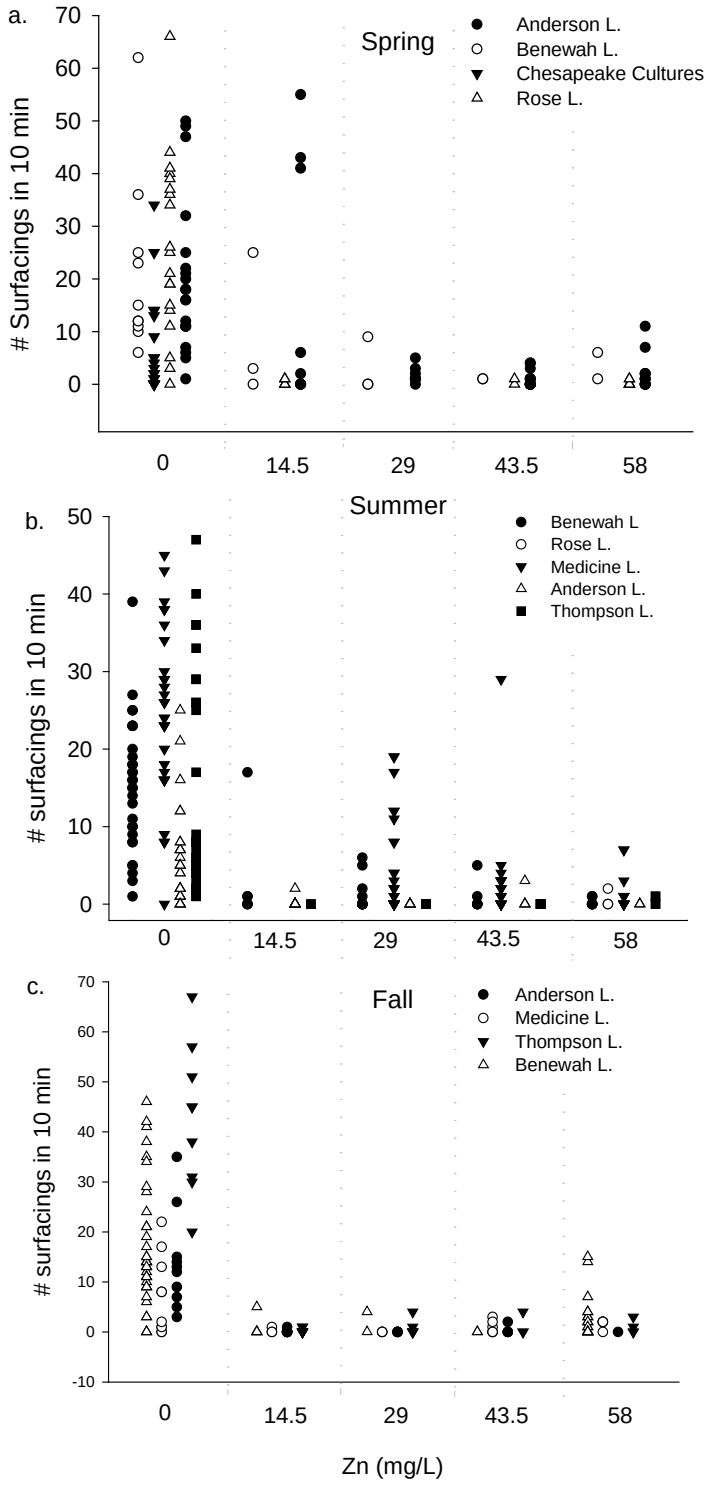

a.
Spring
Anderson L.
Benewah L.
Chesapeake Cultures
Rose L.
Surfacings in 10 min
70
60
50
40
30
20
10
0
0
14.5
29
43.5
58
b.
Summer
Benewah L
Rose L.
Medicine L.
Anderson L.
Thompson L.
surfacings in 10 min
50
40
30
20
10
0
0
14.5
29
43.5
58
c.
Fall
Anderson L.
Medicine L.
Thompson L.
Benewah L.
surfacings in 10 min
70
60
50
40
30
20
10
0
-10
0
14.5
29
43.5
58
Zn (mg/L)

Figure 7

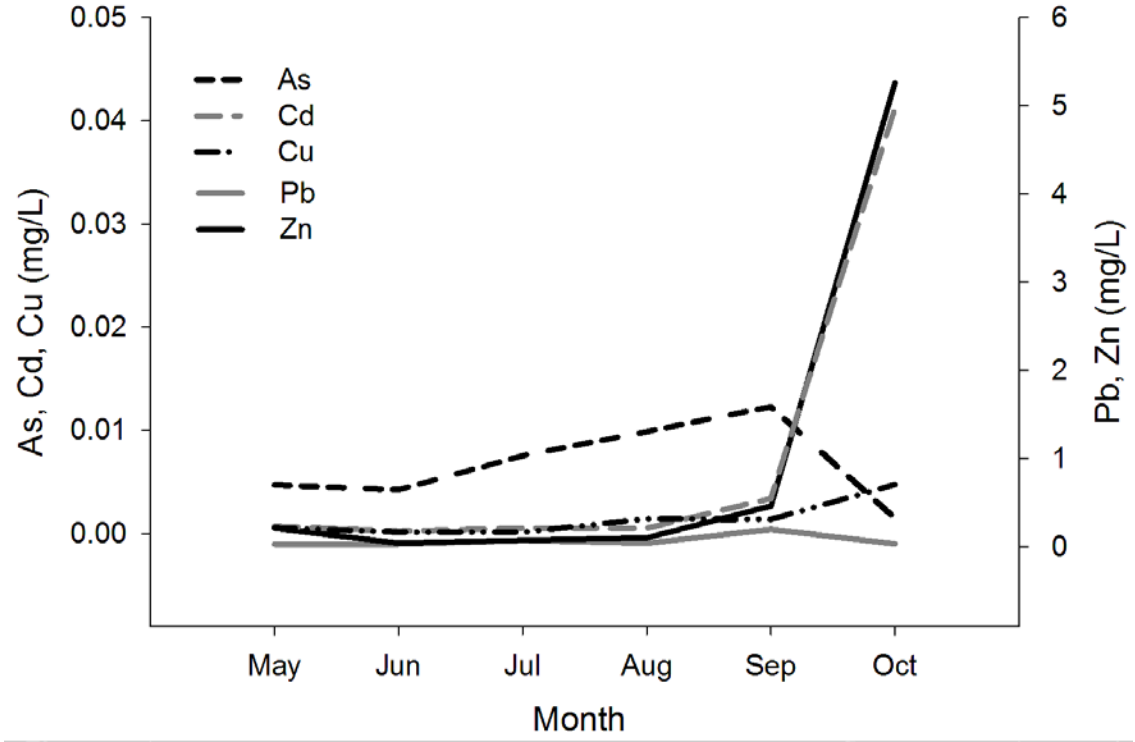

0.05
0.04
0.03
0.02
0.01
0.00
As, Cd, Cu (mg/L)
6
5
4
3
2
1
0
Pb, Zn (mg/L)
As
Cd
Cu
Pb
Zn
May
Jun
Jul
Aug
Sep
Oct
Month

Figure 8

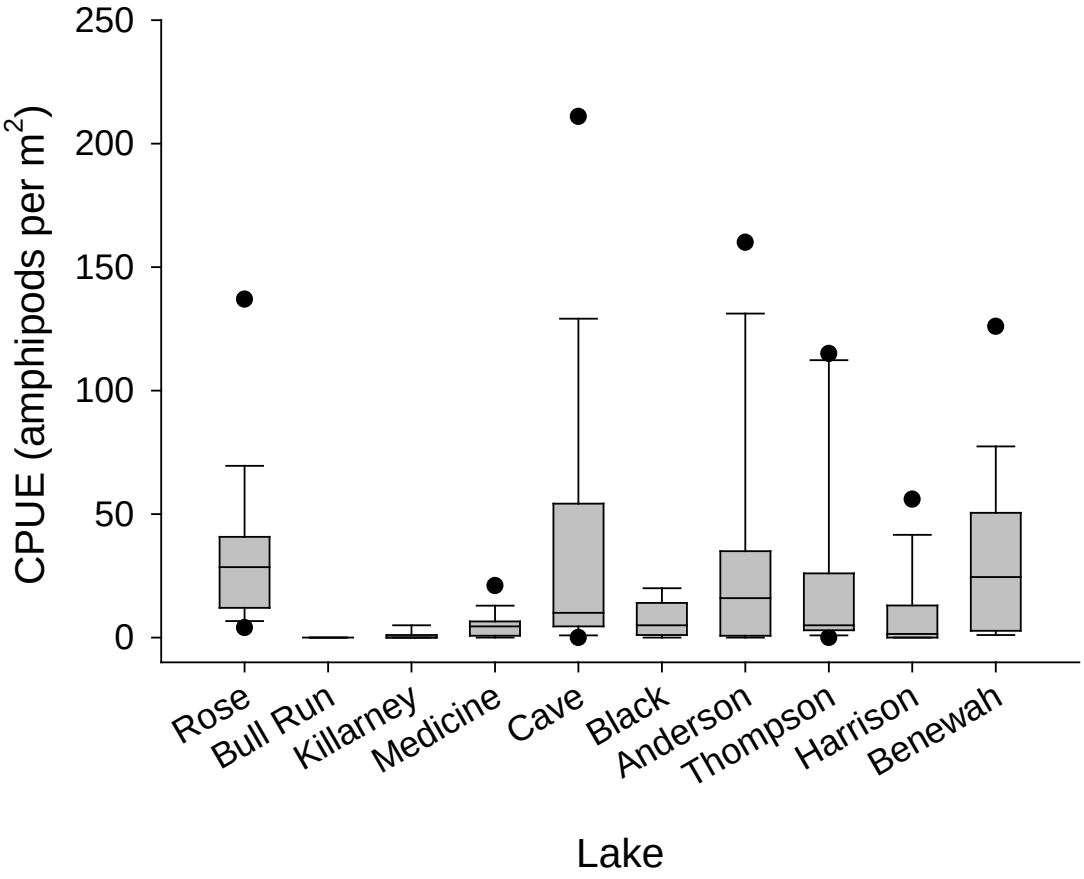

250
200
150
100
50
0
CPUE (amphipods per m2)
Rose
Bull Run
Killarney
Medicine
Cave
Black
Anderson
Thompson
Harrison
Benewah
Lake

Figure 9

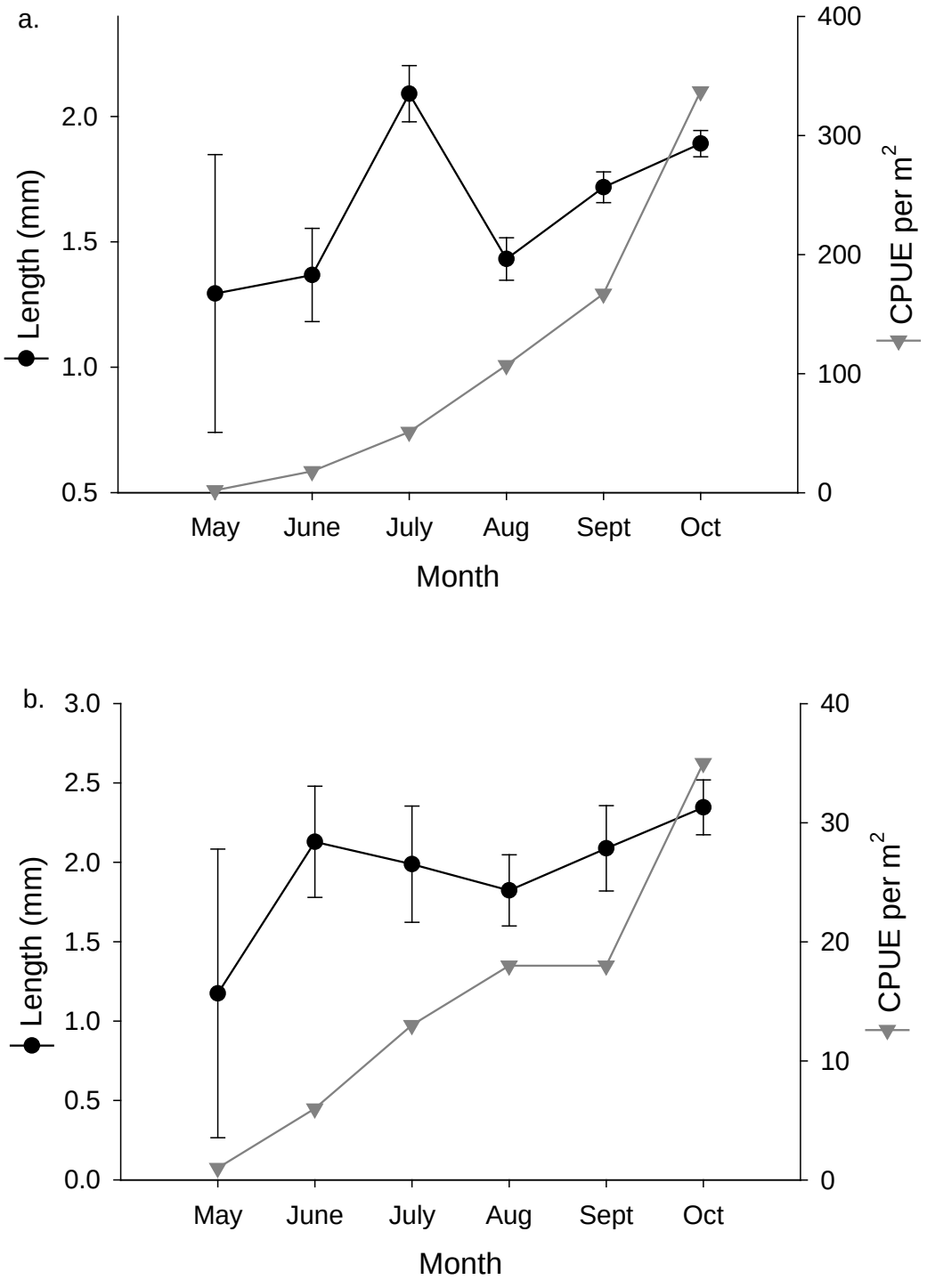
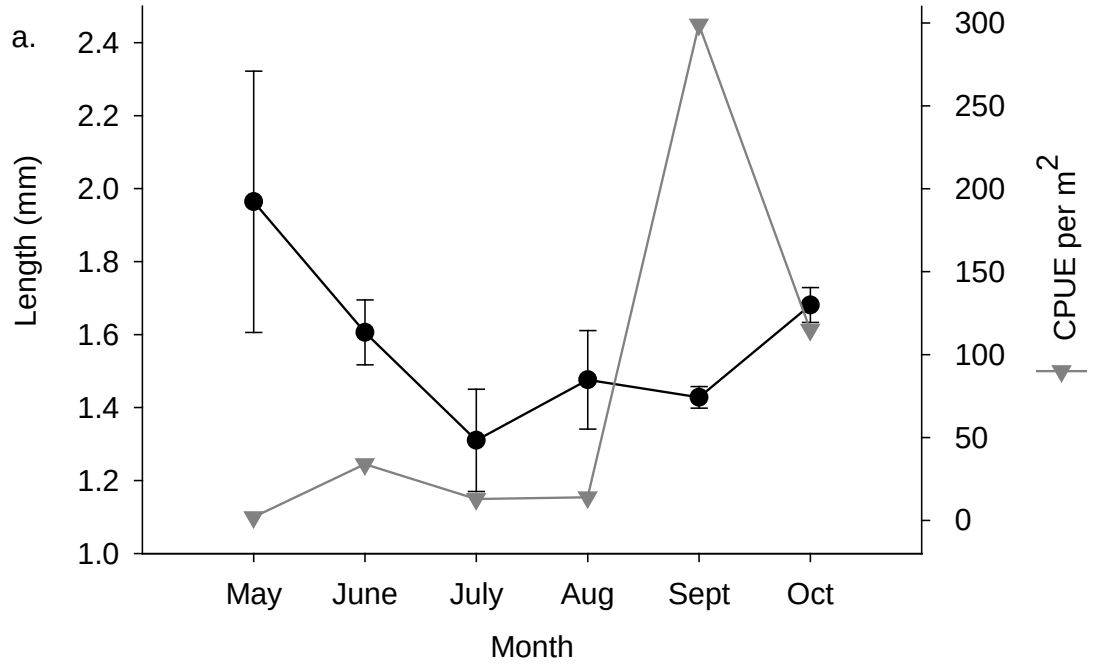

a.
Length (mm)
CPUE per m2
0.5
1.0
1.5
2.0
0
100
200
300
400
May
June
July
Aug
Sept
Oct
Month
b.
Length (mm)
CPUE per m2
0.0
0.5
1.0
1.5
2.0
2.5
3.0
0
10
20
30
40
May
June
July
Aug
Sept
Oct
Month

Figure 10


a.
Length (mm)
2.4
2.2
2.0
1.8
1.6
1.4
1.2
1.0
300
250
200
150
100
50
0
CPUE per m2
May
June
July
Aug
Sept
Oct
Month

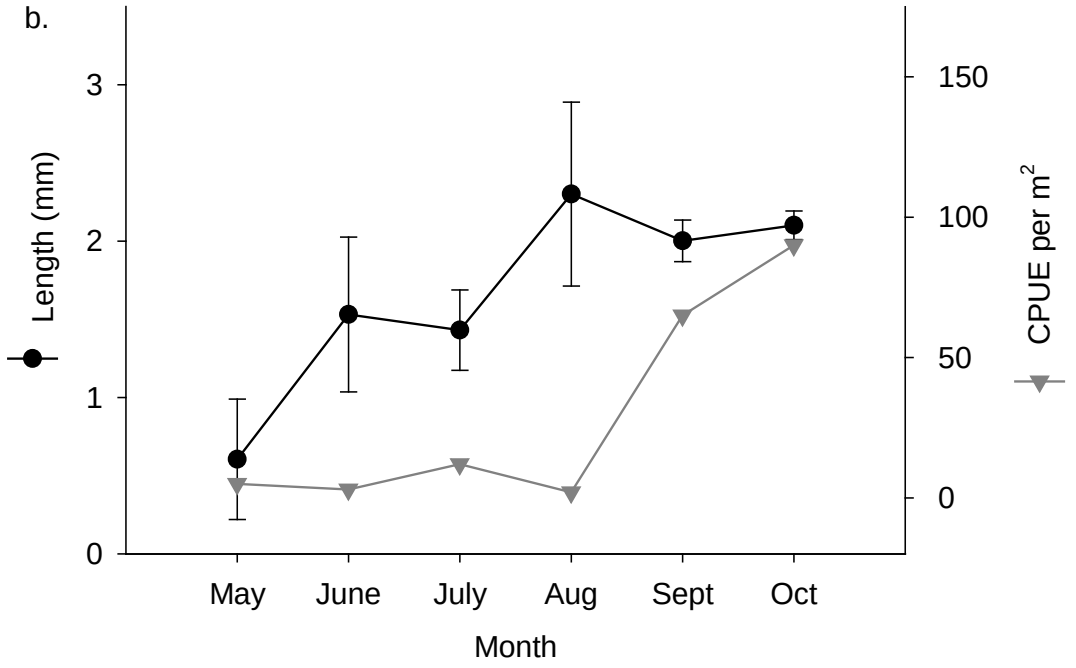

b.
Length (mm)
3
2
1
0
150
100
50
0
CPUE per m2
May
June
July
Aug
Sept
Oct
Month

Figure 11

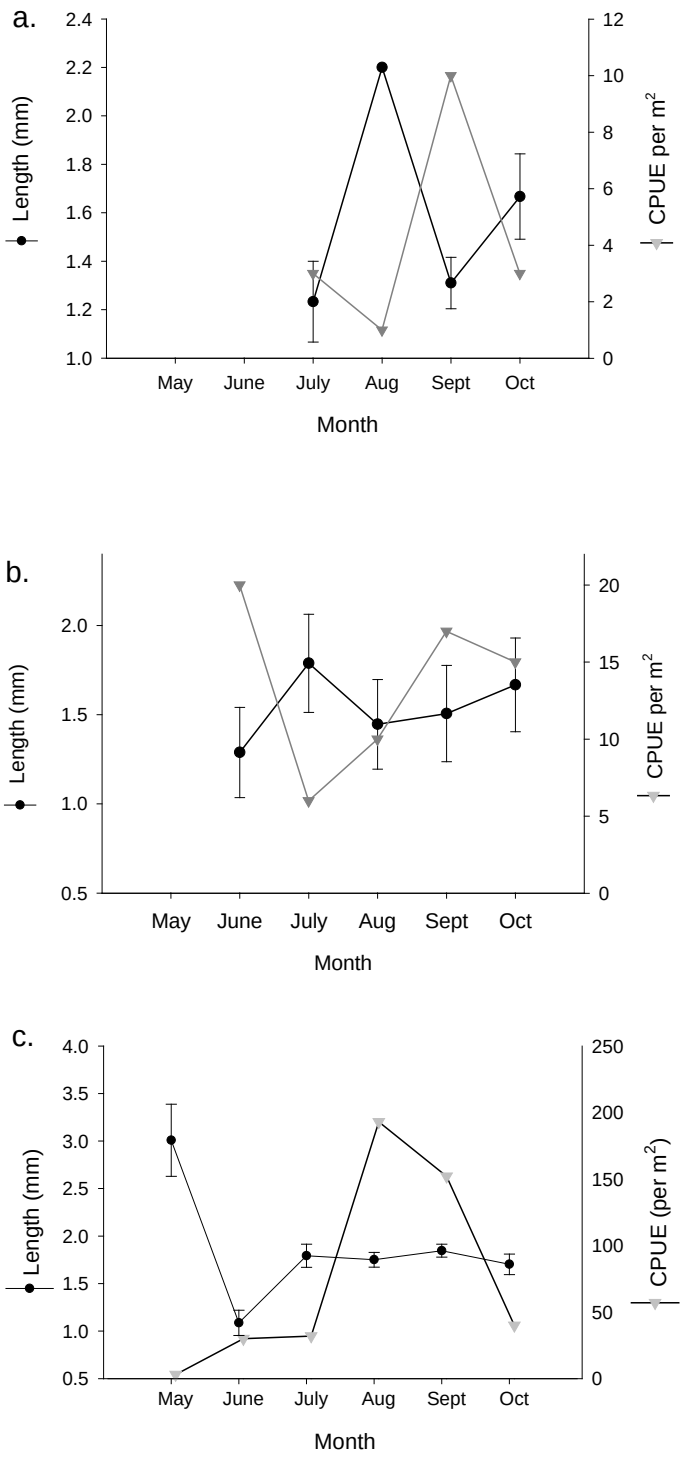
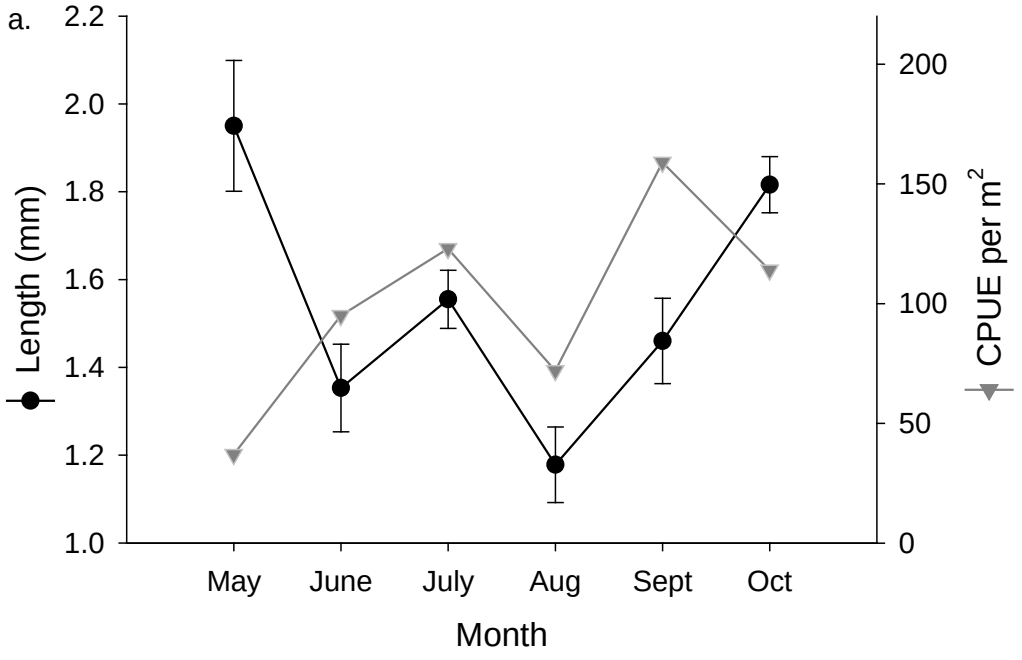

a.
Length (mm)
CPUE per m2
2.4
2.2
2.0
1.8
1.6
1.4
1.2
1.0
12
10
8
6
4
2
0
May
June
July
Aug
Sept
Oct
Month
b.
Length (mm)
CPUE per m2
2.0
1.5
1.0
0.5
20
15
10
5
0
May
June
July
Aug
Sept
Oct
Month
c.
Length (mm)
CPUE (per m2)
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
250
200
150
100
50
0
May
June
July
Aug
Sept
Oct
Month

Figure 12


a.
2.2
2.0
1.8
1.6
1.4
1.2
1.0
Length (mm)
200
150
100
50
0
CPUE per m2
May
June
July
Aug
Sept
Oct
Month

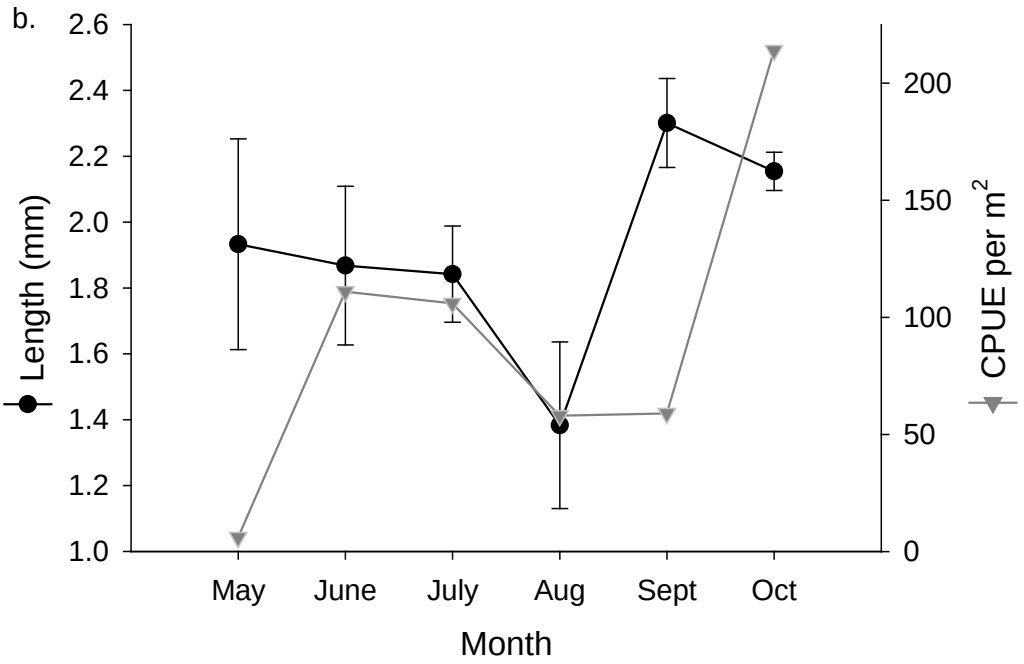

b.
2.6
2.4
2.2
2.0
1.8
1.6
1.4
1.2
1.0
Length (mm)
200
150
100
50
0
CPUE per m2
May
June
July
Aug
Sept
Oct
Month

Figure 13

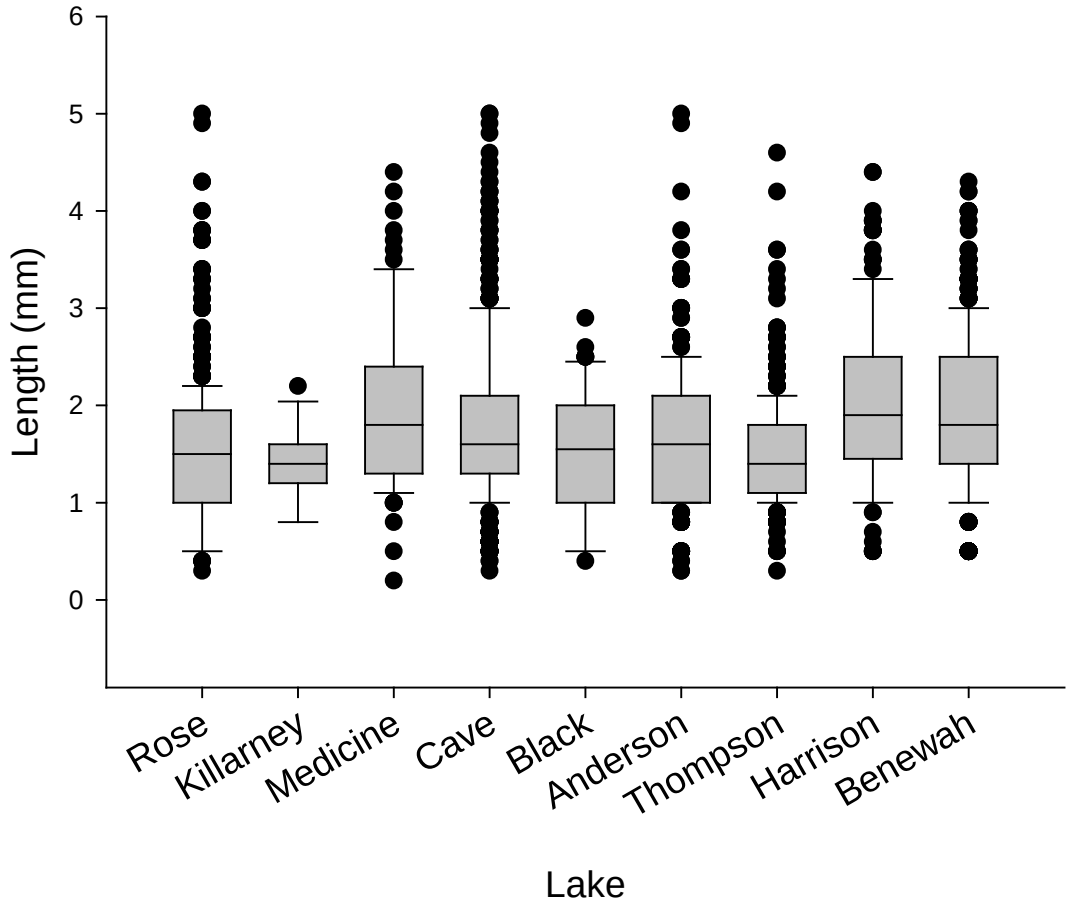

Length (mm)
6
5
4
3
2
1
0
Rose
Killarney
Medicine
Cave
Black
Anderson
Thompson
Harrison
Benewah
Lake

Figure 14

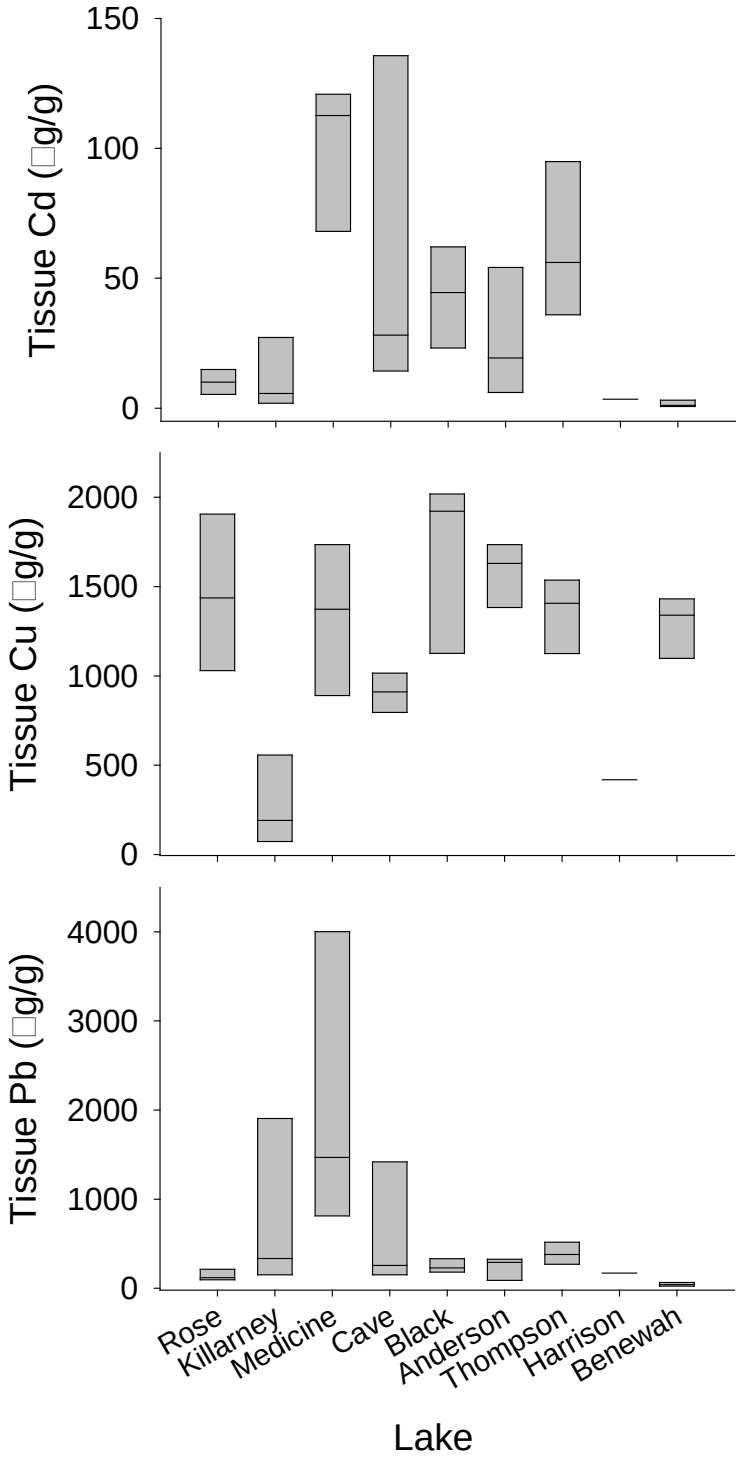

Tissue Cd (□g/g)
150
100
50
0
Tissue Cu (□g/g)
2000
1500
1000
500
0
Tissue Pb (□g/g)
4000
3000
2000
1000
0
Rose
Killarney
Medicine
Cave
Black
Anderson
Thompson
Harrison
Benewah
Lake

Figure 15

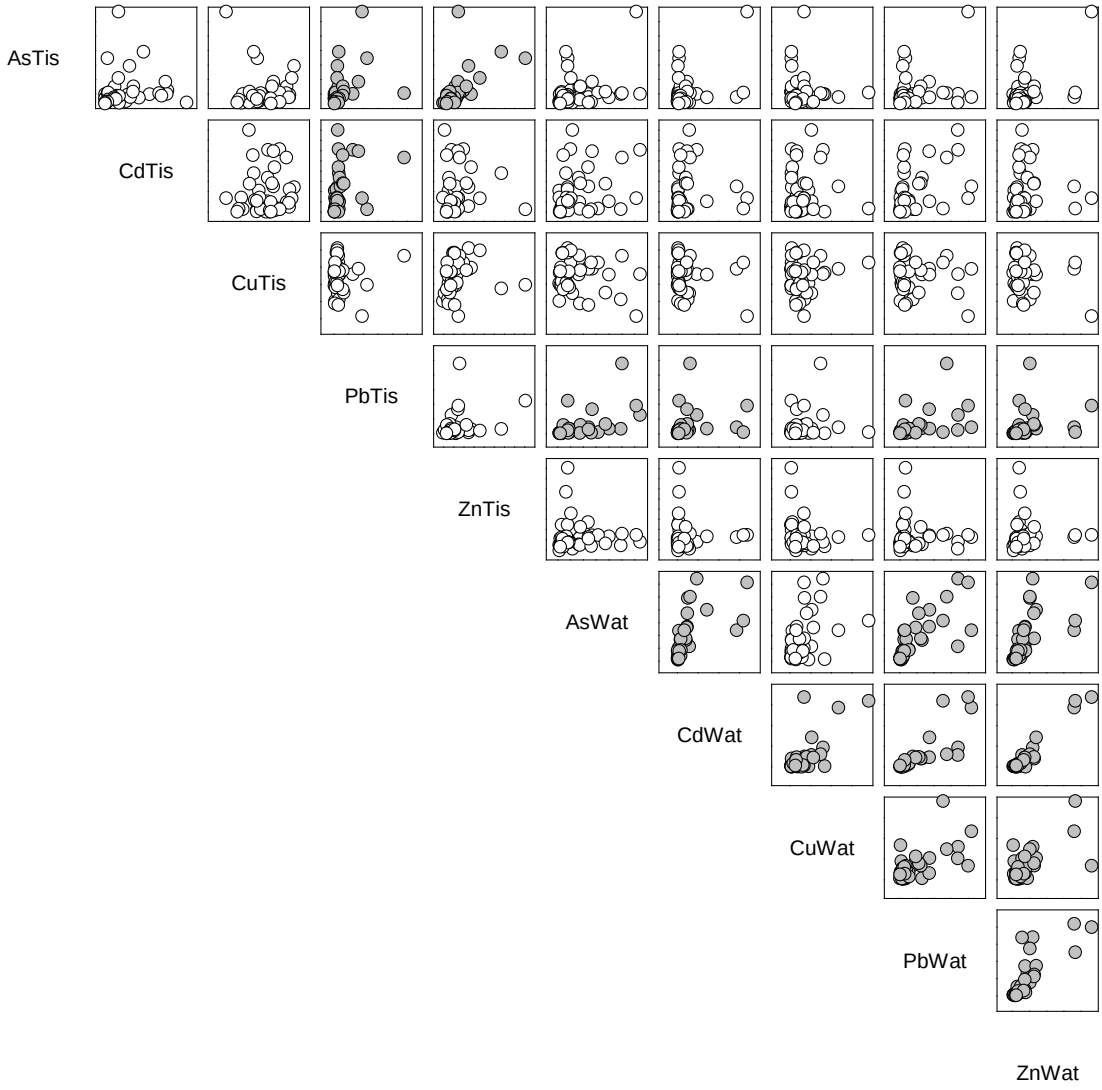
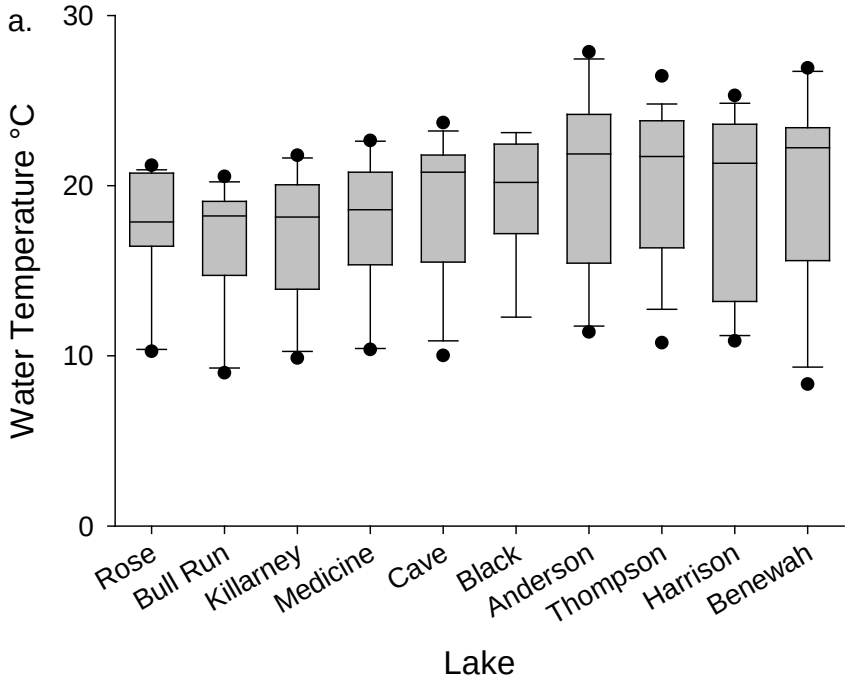

AsTis
CdTis
CuTis
PbTis
ZnTis
AsWat
CdWat
CuWat
PbWat
ZnWat

Figure 16


a.
Water Temperature °C
30
20
10
0
Rose
Bull Run
Killarney
Medicine
Cave
Black
Anderson
Thompson
Harrison
Benewah
Lake

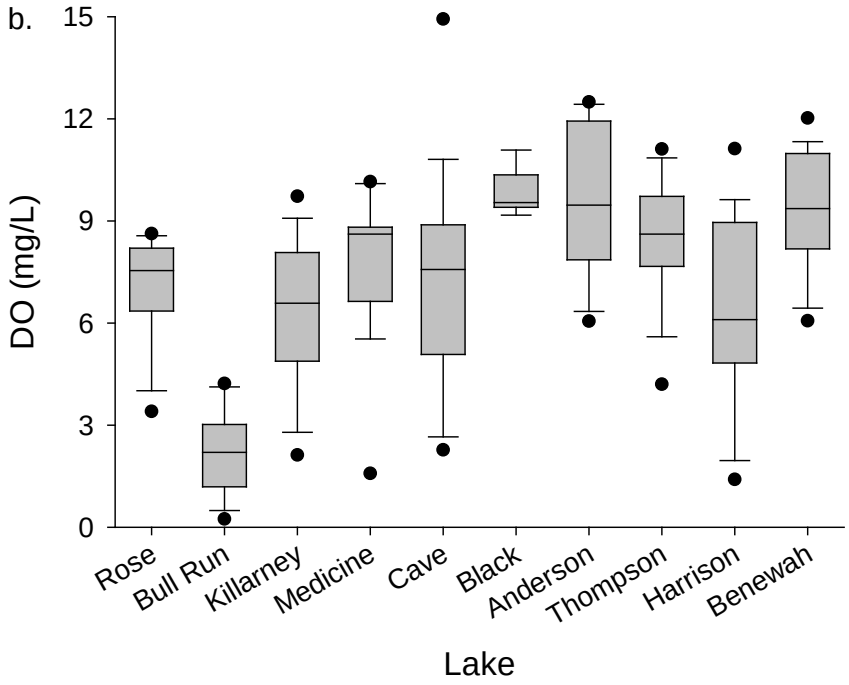

b.
DO (mg/L)
15
12
9
6
3
0
Rose
Bull Run
Killarney
Medicine
Cave
Black
Anderson
Thompson
Harrison
Benewah
Lake

Figure 17

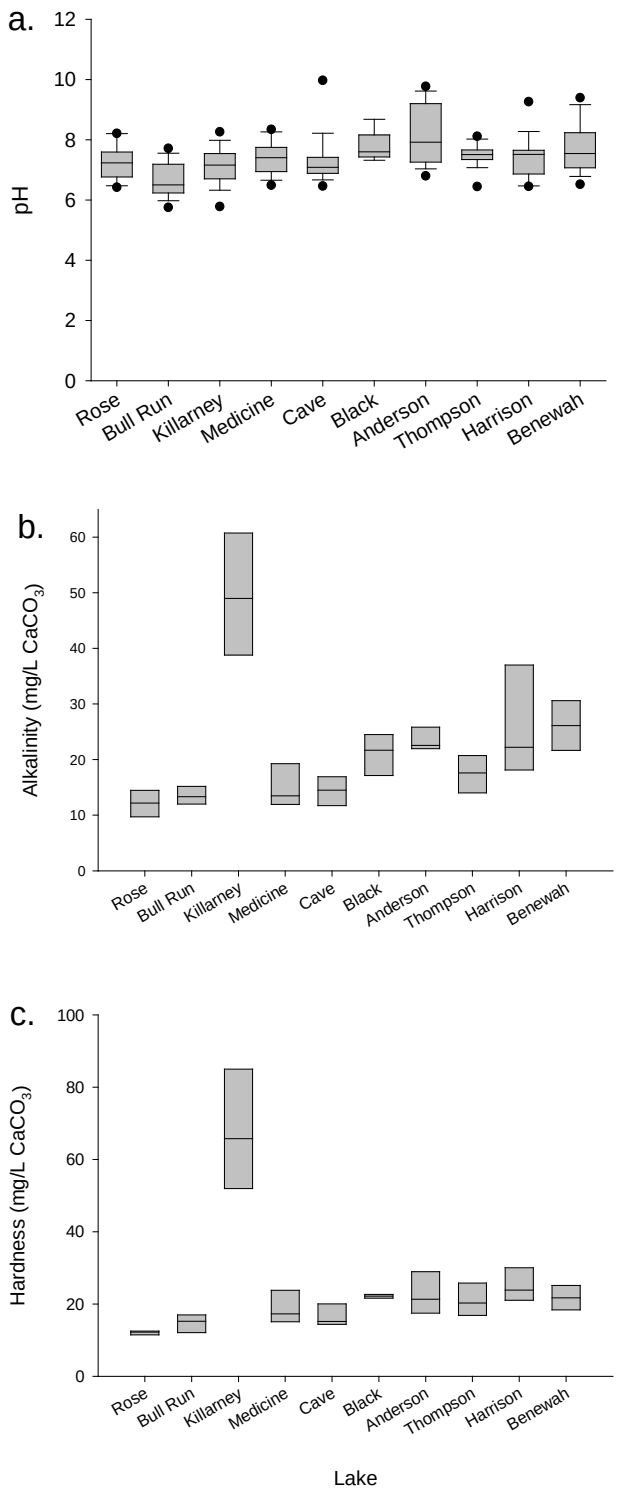

a.
pH
0
2
4
6
8
10
12
Rose
Bull Run
Killarney
Medicine
Cave
Black
Anderson
Thompson
Harrison
Benewah
b.
Alkalinity (mg/L CaCO3)
0
10
20
30
40
50
60
c.
Hardness (mg/L CaCO3)
0
20
40
60
80
100
Lake

Figure 18

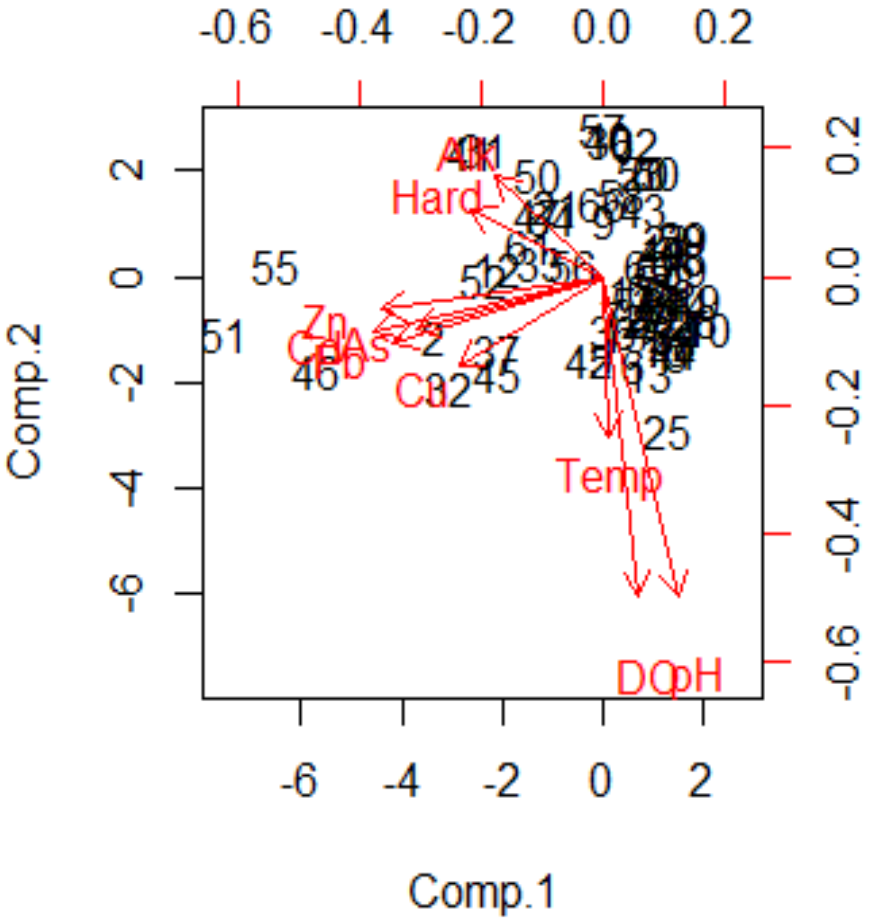
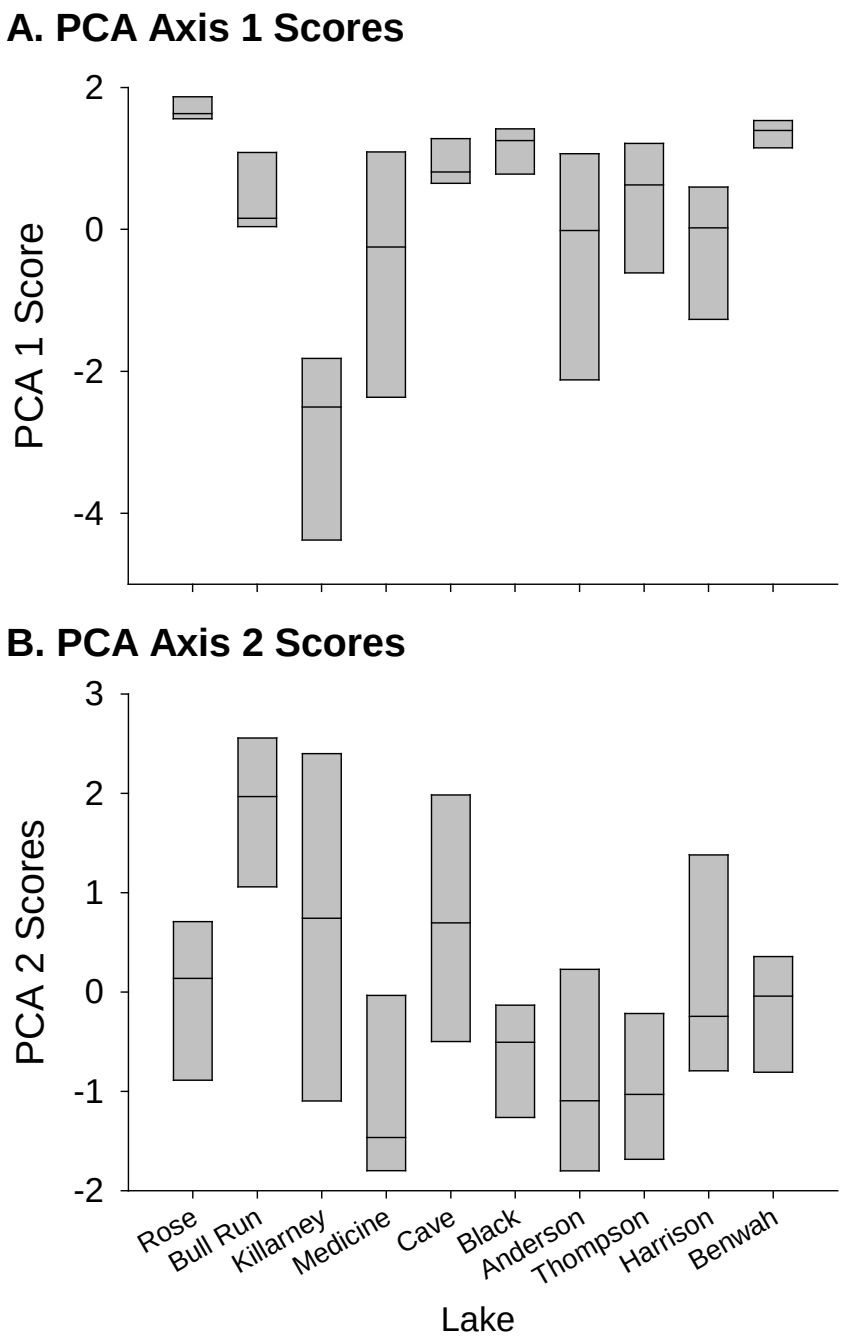


Figure 19


A. PCA Axis 1 Scores
PCA 1 Score
2
0
-2
-4
B. PCA Axis 2 Scores
PCA 2 Scores
3
2
1
0
-1
-2
Rose
Bull Run
Killarney
Medicine
Cave
Black
Anderson
Thompson
Harrison
Benwah
Lake

APPENDIX 1

Protocol 1 – Making 2% and 10% Nitric Acid Solutions

Background

Nitric acid is used frequently to wash glassware and sample bottles, and in solutions to prepare water and tissue samples for metal analysis.

Important considerations

1. **Whenever you prepare a new solution, especially when you are diluting concentrated (70%) nitric acid, be sure to pour the water into the container first, followed by the acid. Never pour water onto acid as this could cause an explosion.**
2. Nitric acid is frequently used in this lab in concentrations of 2%, 10%, and 70%.
3. When you make a solution that will be used with samples that will be analyzed for trace metal content, be sure to use Milli-Q (ultrapure) water. You can get this from Dr. Nezat in the geochemistry lab.
4. When you make any solution that will not be used for preparing samples for trace metal analysis (generally only 10% solutions), you can use the deionized water (white tap).
5. When you make a solution that will be used to prepare samples for trace metal analysis, be sure to use **trace metal grade** nitric acid.
6. When you make any solution that will not be used for preparing samples for trace metal analysis, you can use generic nitric acid, which is generally cheaper.

Part 1. Make 6 L of a 10% nitric acid solution for washing sample bottles and glassware

Background

It is important to acid wash all sample bottles and glassware that will be used to collect or process water and tissue samples that will be analyzed for trace metals. Bottles and glassware that have been washed in Liquinox and deionized water may still have residual trace metals. This residue could contaminate samples and confound analyses if it is not removed. A 10% nitric acid solution should be sufficient to clean glassware.

Objective

To make a 6 L solution of a roughly 10% nitric acid for washing sample bottles and glassware.

Personnel

Two people are required. One person should make the solution while the other person assists as needed.

Supplies

Large (2 gal) plastic Nalgene carboy with spigot

70% nitric acid stock*

*1 L bottle, manufactured by Fluka Analytical, distributed by Sigma-Aldrich (Catalog No. 438073-2.5L), stored under hood in secondary container or obtained from the stock room.

1000 mL Graduated cylinder

Black Sharpie

Label tape

Corrosive acid stickers

Kimwipes

Plastic serving tray*

*These are generally stored on top of the refrigerator.

Safety materials

MSDS 70% nitric acid

Heavy duty rubber gloves appropriate for use when handling concentrated nitric acid

Safety goggles

Preparation

Mark the fluid level lines for each step of the dilution process:

1. Using label tape and a black Sharpie, label the Nalgene carboy, "10 % nitric acid."
2. Place a Corrosive acid sticker near the label.
3. Using a graduated cylinder to measure, fill the Nalgene carboy with 5 L of deionized water.
4. Using a black Sharpie, mark the water level, making an approximately 2 in line and write, "5 L".
5. Measure an additional 1 L of deionized water and pour into the carboy.
6. Using the Sharpie, mark the water level, making an approximately 2 in line and write, "6 L".
7. Cap the carboy then swirl the water to rinse. Discard the water.
8. Fill the carboy to the 5 L line with deionized water.
9. Place the carboy on the serving tray in the fume hood. Place the cap on the bench next to the fume hood.
10. Set the fume hood fan to *high*.

Procedure

1. Put on the heavy duty rubber gloves and safety goggles.
2. Retrieve the bottle of stock 70% nitric acid (this does not need to be trace metal grade – which is more expensive) from the storage area under the fume hood or from the stock room. If you are retrieving the bottle from the stock room, be sure to use a secondary container to transport it and do not transport the acid in between classes when the hallways are crowded.
3. Place the bottle in the fume hood. The carboy should be on the left, the bottle of acid on the right.
4. Unscrew the cap on the bottle of nitric acid. Wait a few seconds to let pressure escape the bottle.
5. Grasp the bottle with your right hand and use your left hand to support the bottle.
6. Move the opening of the bottle to the opening of the carboy.
7. Move your left hand to stabilize the bottom of the bottle as you slowly tip the bottle to pour. Have a helper positioned at the fume hood with you to assist you if needed, and to watch you pour in case you need guidance.
8. Pour the acid in approximately 100-200 mL increments. After each increment, set the bottle down. Have a helper feel the back side and around the bottom of the carboy (far left side away from the bottle of nitric acid). If the carboy is only slightly warm, you may continue poring the next 100-200 mL increment. If the carboy is very warm or hot to the touch, wait to proceed until the heat has dissipated. This may take several minutes. Do not continue pouring if the carboy is very warm or hot as you could cause an explosion.
9. Continue pouring the acid in 100-200 mL increments until the fluid level has reached the 6 L line that you marked in *Preparation*.
10. Set the bottle of nitric acid down and cap tightly.
11. Cap the carboy.
12. Return the bottle of nitric acid either to the storage area under the fume hood or to the stock room.
13. Carry the carboy and the tray over to the sink. Throughout the duration of the project, store the carboy in this location for ease of use.
14. If the spigot leaks, place a paper towel underneath it.

Part 2. Make 80 mL of a 2% trace metal grade nitric acid solution

Background

Completing the amphipod digests as per Protocol 9 requires the use of 2% trace metal grade nitric acid to dilute the amphipod samples for trace metal analysis. This low concentration of nitric acid keeps metal ions in solution and prevents them from sticking to the walls of the 15 mL tubes (what our samples are held in) and also prevents them from clumping up and clogging the Inductively Coupled Plasma Optical Emission Sensor (ICP-OES) that we use to analyze samples for trace metal content. It is important that we use this

low concentration because higher concentrations of nitric acid (>5%) could harm the ICP-OES.

Objective

To make 80 mL of a dilute (2%) solution of trace metal grade nitric acid from a concentrated (70%) stock solution.

Personnel

This procedure only requires one person.

Supplies

2 – 100 mL screw-top glass bottles

10% nitric acid for acid-washing glassware*

*See Part 1.

2000 mL beaker (glass or plastic)

Generic plastic wrap

Label tape

Black Sharpie

Fine tip black Sharpie

Paper towels

Kimwipes

Small Corrosive Acid Label*

*These sticker labels are generally found in the drawer labelled "chem. stickers." Let Dr. Matos know before you run out of stickers so that we can get more from EH&S.

1 L Milli-Q (ultrapure) water*

*This can be obtained from Dr. Nezat in an acid-washed 1 L Nalgene bottle.

70% trace metal grade nitric acid stock*

*1 L bottle, manufactured by Fluka Analytical, distributed by Sigma-Aldrich (Catalog No. 843850), stored under hood in secondary container

Electric pipetteman and 50 mL pipette

P1000 pipette and tips

P200 pipette and tips

2 tip waste containers

Styrofoam bottle holder*

*This is a rectangular piece of Styrofoam that has been carved to hold 100 mL bottles, generally stored in the drawer labelled "plastic disposable tubes"

Safety materials

Latex or vinyl gloves

Lab coat

Safety goggles (optional)

Heavy duty rubber gloves appropriate for use when handling concentrated nitric acid

Preparation Part A. Acid washing glassware

1. To prevent contamination of the 2% trace metal grade nitric acid from the container that the solution will be held in, you must wash the container with 10% nitric acid.
2. Using label tape and a Sharpie, label the 2000 mL beaker "10% nitric acid."
3. Remove the cap from the 100 mL glass bottles.
4. Place the 100 mL glass bottles and their caps inside of the 2000 mL beaker.
5. Put on gloves, lab coat, and safety goggles (if you choose to wear them).
6. Loosen the cap on the carboy that is holding the 10% nitric acid solution.
7. Hold the beaker under the carboy spigot.
8. Open the spigot.
9. Fill the beaker until the bottles and caps are completely submerged.
10. Close the spigot.
11. Set the beaker on a lab bench.
12. Cover the beaker with plastic wrap (this prevents nitric acid fumes from corroding equipment in the lab).
13. Tighten the cap on the carboy.
14. Set a timer for 1 hour (you can leave glassware to be acid-washed for longer than 1 hour). Leave the beaker undisturbed.
15. After at least 1 hour has passed, put on gloves, lab coat and safety goggles.
16. Remove the cap on the carboy that is holding the 10% nitric acid solution.
17. Carefully pour the acid back into the carboy. Use your hand to guard the glass bottles and caps from falling out.
18. Replace the carboy cap.
19. Remove the bottles and caps from the beaker.
20. Screw the caps onto the glass bottles.

21. Using paper towels, wipe excess nitric acid off of the outside of the bottles.
22. Unscrew the bottles.
23. Use Kimwipes to wipe out residual nitric acid. Make sure that the bottles and caps are completely dry before proceeding.

Preparation Part B. Making an aliquot of concentrated (70%) nitric acid

1. Set the fume hood fan to the *high* setting and turn on the fume hood light.
2. Using label tape and a fine tip Sharpie, label 1 100 mL glass bottle "70% trace metal grade nitric acid made on [date] by [your initials]."
3. Place a Corrosive Acid sticker on the bottle.
4. Place the 100 mL glass bottle that you just labelled in the Styrofoam bottle holder. Place these two items in the fume hood.
5. Remove the cap and place it on a Kimwipe so that the inside of the cap is not contaminated by dust.
6. Put on heavy duty rubber gloves, lab coat, and safety goggles.
7. Retrieve the bottle of 70% trace metal grade nitric acid from the storage area under the fume hood and place it in the fume hood next to the glass bottle. Loosen the cap to release any pressure. Wait about 30 seconds.
8. Remove the cap. Slowly pour the acid into the 100 mL glass bottle. Hold the bottle in your right hand to pour, and stabilize the bottle while pouring with your left hand at the bottom of the base of the bottle. Never put your left hand directly under the opening of the bottle as acid could pour out onto your hand. Fill the bottle to the 80 mL mark (approximate).
9. Set the stock bottle down and replace the cap. Make sure that it is tight.
10. Cap the 100 mL glass bottle. Make sure that it is tight.
11. Look over both bottles to check for any nitric acid that may have dripped down the sides. If you find some, wipe it with paper towels.
12. Look over your gloves to check for drops of nitric acid. If you find some, wipe the acid off with paper towels.
13. Return the stock bottle to its secondary container under the fume hood. Close and latch the door.
14. Remove the heavy duty gloves, set them aside.

Preparation Part C. Preparing your container and workspace

1. Label the 2nd 100 mL glass bottle "2% trace metal grade nitric acid made on [date] by [your initials]."
2. Place a Corrosive Acid sticker on the bottle.

3. Place this bottle in the Styrofoam holder with the 100 mL bottle of concentrated nitric acid (there are two places for bottles carved into the Styrofoam holder, so both bottles should fit securely).

4. Place the two tip waste containers in the fume hood. With label tape and a black Sharpie, label one container "tip waste - HNO_3." Label the other container, "tip waste - water."

Procedure

1. Place the bottle of Milli-Q water in the fume hood.
2. Put on latex or vinyl gloves.
3. Remove the cap from the bottle of Milli-Q and from the bottle that you will make the 2% nitric acid solution in. Set the caps on a Kim wipe so that the inside of the caps will not be contaminated by dust from the air.
4. Using the electric pipetteman and a 50 mL pipette, draw up 77.0 mL of Milli-Q water and dispense it into the 100 mL bottle. Dispose of the pipette (autoclave waste bin) and set aside the electric pipetteman.
5. Using the P1000, draw up 0.71 mL Milli-Q water and dispense it into the 100 mL bottle.
6. Dispose of the P1000 tip into the "tip waste – water" container and set the P1000 aside.
7. Replace the cap on the bottle of Milli-Q water.
8. Put on heavy duty rubber gloves.
9. Unscrew the cap from the 100 mL bottle of concentrated (70%) nitric acid. Set it on a Kimwipes so that it is not contaminated by dust.
10. Using the P1000, draw up 2.0 mL of 70% nitric acid and slowly dispense it into the 100 mL bottle holding the Milli-Q water. Wait a few moments for any heat to dissipate. Rinse the pipette tip in the dilute solution by first drawing liquid up, then moving the liquid up and down in the tip a couple of times. Dispose of the pipette tip in the "tip waste – HNO_3" container and set the P1000 aside.
11. Using the P200, draw up 0.29 mL of 70% nitric acid and slowly dispense it into the 100 mL bottle holding the Milli-Q water. Wait a few moments for any heat to dissipate.
12. Dispose of the pipette tip in the "tip waste – HNO_3" container and set the P200 aside.
13. Cap both bottles; make sure they are on tight.
14. Look over both bottles and the heavy duty gloves for any drops of nitric acid. If you find some, use paper towels to wipe it up.
15. Place the bottle of 70% nitric acid under the fume hood in a secondary container. Close and latch the door.
16. Empty the "tip waste – HNO_3" containers into the garbage.

Protocol 2 – YSI 556 Calibration and Use

Background

The YSI (in Dr. McNeely's lab) is a handheld instrument that is used to collect water quality information. The instrument can measure temperature (°C), pH, conductivity (µS/cm or µS/c, cm), and dissolved O_2 (mg/L or percent saturation) in the field. A copy of the YSI Owner's Manual is located in the JMatos Dropbox folder for additional reference.

Objective

To calibrate the YSI pH and dissolved O_2 (DO) probes and to take water quality measurements from the handheld instrument in the field.

Personnel

One person can calibrate the probes. Two people should collect the water quality information in the field; one person can use the handheld instrument while the other person records the measurements in a notebook.

Supplies and Equipment

YSI 556

4, 7, and 10 pH buffer solutions and waste containers*

*These are stored in a box in Dr. McNeely's lab. This box also contains the waste bottles for these buffer solutions.

3 – 50 mL beakers

Kimwipes

Rite-in-the-Rain field notebook

Pencil

Safety Materials

N/A

Preparation

Calibrating the pH probe.

1. This probe should be calibrated once per month if field measurements take place over a period of several months. This calibration can be performed in the lab any time prior to a field day. They YSI can be turned off without needing to be re-calibrated.
2. Press the On/off key to turn on the instrument. When you open the case that is used to house the YSI, the probe and sensors will be sitting in the transport/calibration cup. This cup should always have about 1-2 in of water in it during storage to prevent the probes from drying out.
3. Select *Run* to let the instrument equilibrate. Let the probe sit in the transport/calibration cup undisturbed for about 5 minutes.
4. Select *Escape*. The Main Menu will appear.
5. Use the direction arrows to scroll and highlight *Calibrate*. Select *Calibrate*. The Calibrate Menu will appear.
6. Use the direction arrows to scroll and highlight *pH*. Select *pH*. The pH Calibration Menu will appear.
7. Use the direction arrows to scroll and highlight *3 point*. Select *3 point*.

8. Remove the transport/calibration cup from the probe, then rinse the probe in deionized water and blot it dry with a Kimwipe.
9. Perform a 3 point calibration.
10. Begin with the pH 7 buffer solution.
11. Pour a small amount of buffer solution into a 50 mL beaker.
12. Insert the YSI probe so that the sensors are completely submersed in the solution.
13. Using the keypad, enter 7.00 at the prompt.
14. Press *Enter*. The pH Calibration screen will appear.
15. Allow the probe to remain in the solution for at least one minute. Wait for the probe to equilibrate. When the pH reading is stable for at least 30 seconds, press *Enter*.
16. The screen will indicate that the calibration has been accepted and prompt you to press *Enter* again to continue.
17. Press *Enter*. The pH Calibration Menu will appear.
18. Remove the probe from the buffer solution.
19. Rinse the probe with deionized water and blot it dry with a Kimwipe.
20. Repeat steps 11-19 two more times using the pH 4 buffer followed by the pH 10 buffer.
21. When you are finished, discard the used buffers into the appropriate waste containers.
22. Turn off the YSI.

Calibrating the DO probe.

1. This probe should be calibrated every field day before the first measurements are taken. ***DO NOT turn the YSI off after you have calibrated the instrument for the day. If this happens, you will need to calibrate it again, which requires at least one hour.**
2. Calibrating the DO probe in % saturation automatically calibrates the probe for mg/L.
3. From the Main Menu, use the direction arrows to scroll and highlight *Calibrate*. The Calibration Menu will appear. ***Important*** Check the battery level display when you turn on the instrument, prior to calibration. If the battery level is not at least half full, consider changing the batteries (4 Size C batteries).
4. Use the direction arrows to scroll and highlight *DO %*. Press *Enter*. The DO Barometric Pressure Screen is displayed.
5. Put approximately 3 mm (1/8") of water in the bottom of the transport/calibration cup.
6. Place the sensors into the transport/calibration cup. Make sure that the sensors are NOT immersed in water. If they are, pour out some water.
7. Engage only 1 or 2 threads of the transport/calibration cup to ensure the DO sensor is vented to the atmosphere.
8. Select *Enter*. The DO % saturation calibrations screen will be displayed. Allow approximately one hour for the air in the transport/calibration cup to become water saturated and for the temperature to equilibrate before proceeding.

9. Observe the reading under DO %. When the reading shows no significant change for approximately 30 seconds, press *Enter*. The screen will indicate that the calibration has been accepted and will prompt you to press *Enter* again to continue.
10. Press *Escape* to return to the Calibration Menu.
11. Press *Escape* to return to the Main Menu.
12. Select *Run*.
13. **DO NOT turn off the YSI until you have collected all of your measurements for the day.**

Procedure

Collecting water quality measurements in the field.

1. At each site, collect water samples (Protocol 3) at the same time and from the same location as water quality measurements are recorded from the YSI.
2. One person should stand at the shore to record. Be sure to record observations about the surrounding area, including what types of plants you observe in and out of the water, as well as how many people are recreating and the types of recreation that are happening.
3. The other person will immerse the probe into the water, taking care to not let the probe touch the sediment or be damage by hitting it on any rocks. When possible, stay on the shore and out of the water while immersing the probe so that you do not disturb the sediment.
4. Make sure that the YSI screen says *Run* at the top. All of the parameters should be displayed in real time.
5. Wait for 1-2 minutes while the probe equilibrates. I generally hold the probe with my right hand and the instrument in my left. Swirl the probe in the water. When no significant changes are observed in the values on the screen, read the values aloud to the recorder.
6. The recorder will record these values in a *Rite-in-the-Rain* field notebook using a pencil. The recorder will note the date and time of the recording.
7. Keep the YSI turned on when moving between lakes/sites. The probe should be stored in the transport/calibration cup.
8. When you are done collecting measurements from all lakes/sites, press *On/off*.
9. Be sure that a small amount of water is in the transport/calibration cup. Screw the cup onto the probe tightly and store in the YSI case in the lab until the next field day.

Protocol 3 – Collecting Water Samples for Metal Analysis and Alkalinity

Background

Water samples are collected at every site at every lake, on every trip to the Coeur d'Alene Basin. Sample bottle preparation varies depending on the type of analysis that will be performed on the samples. This protocol will cover the preparation of bottles and sampling techniques for the collection of water samples that will be used for metal analysis and alkalinity titrations. In conjunction with water sample collection, water temperature, pH, and dissolved O_2 should also be measured. See Protocol 2 for information on the collection of water quality parameters using a YSI.

Part 1. Sample bottle preparation and sample collection

Objective

To prepare the sample bottles that will be used to collect water samples for trace metal analysis, and to collect water samples in the field. Acid-washing the bottles removes any trace metals from the inside of the bottles, preventing contamination of the field-collected water samples. See Protocol 1 for details on making the 10% nitric acid solution. Proper sampling technique ensures that the sample bottles have been adequately rinsed with sample water before the actual sample is taken.

Personnel

One person is required to acid wash bottles and collect water samples. However, both activities go much faster with two people working together.

Supplies

Liquinox

13 or 30 - 500 mL or 1000 mL plastic Nalgene sample bottles*

*There are several sample bottles of these sizes in the JMatos lab (in the basement room), but you will also need to borrow some from the stock room. It is better to use 500 mL bottles, but if you do not have enough, 1000 mL bottles work.

Label tape

Black Sharpie

10% nitric acid*

*This is generally kept in a large carboy near the sink.

Generic plastic wrap

2000 mL beaker

3-4 serving trays*

*These are generally kept on top of the refrigerator.

12 or 29 – 100 or 125 mL plastic Nalgene sample bottles*

*These are stored in the basement room in the drawer labeled, "sample bottles."

4 or 10 Ziploc bags

Large Rubbermaid storage tote (no lid needed)

40 frozen ice packs*

*These are stored in the freezer in the lab.

Small Coleman cooler

Safety Materials

Disposable gloves
Lab coat
Safety goggles
Waders
Rubber gloves

Preparation

Acid-washing sample bottles for the collection of water samples that will be sent for trace metal analysis.

1. Wash all of the bottles that you will need in Liquinox and deionized water. The number of sample bottles that you will need depends on the purpose of your collection day. If you are collecting water samples on a Catch Per Unit Effort (CPUE) day, you will be visiting all of the lakes and will need 30 sample bottles (10 lakes, 3 sites per lake - except for Black Lake which only has 2 sites, and one field blank). If you are collecting water samples on an amphipod assay collection day, you will need 13 sample bottles (4 lakes, 3 sites per lake, 1 field blank).
2. Line 3-4 trays with paper towels and place them on the lab bench near the door.
3. Using label tape and a black Sharpie, label each tray, "10% nitric acid."
4. Put on disposable gloves, lab coat, and safety goggles.
5. Fill each bottle to the rim with 10% nitric acid and cover the opening with plastic wrap.
6. Place the bottles on the paper towel-lined trays and leave undisturbed for at least one hour.
7. Place the caps of the bottles into the 2000 mL beaker.
8. Fill the beaker with 10% nitric acid and cover the beaker with generic plastic wrap.
9. Place the beaker on the paper towel-lined trays and leave undisturbed for at least one hour.
10. Slowly pour the acid in the bottles back into the large carboy. Return the bottles to the paper towel-lined trays.
11. Slowly pour the acid in the 2000 mL beaker back into the large carboy, using your left hand to guard the caps from falling into the carboy.
12. Wipe the outside of the caps off with paper towels.
13. Cap the bottles.
14. Remove the nitric acid labels from the sample bottles.
15. Using label tape and a black Sharpie, label all of the bottles with the following, "[Lake, site number, collection date]." As stated above, each lake has 3 sites with the exception of Black Lake, which only has 2 sites. It is helpful to label Benewah sample bottles with blue or green label tape and the chain lakes with any other color of label tape. It is also helpful to label the caps of the bottles with the first letter of the lake and the sample number so that they are easier to find when you are in the field (rather than digging through 30 bottles at each site). Label the bottle that will be used as the field blank, "Field blank, [collection date]."

16. The bottles are now ready to collect sample water for trace metal analysis.

Preparing the field blank.

1. Fill the sample bottle labeled, "Field blank, [collection date]" half full with Milli-Q (ultrapure) water.
2. Shake the bottle vigorously for 30 seconds.
3. Discard the water.
4. Repeat steps 1-3 two more times to completely rinse the bottle.
5. Completely fill the bottle with Milli-Q (ultrapure) water and cap tightly.
6. Bring this bottle with you into the field and treat it the same as all of the other samples that will be processed for trace metal analysis.

Washing sample bottles for the collection of water samples that will be used for alkalinity titrations.

1. Wash all of the bottles that you will need in Liquinox and deionized water. The number of sample bottles that you will need depends on the purpose of your collection day. If you are collecting water samples on a CPUE day, you will be visiting all of the lakes and will need 29 sample bottles (10 lakes, 3 sites per lake - except for Black Lake which only has 2 sites). If you are collecting water samples on an amphipod assay collection day, you will need 12 sample bottles (4 lakes, 3 sites per lake).
2. Using label tape and a black Sharpie, label all of the bottles with the following, "[Lake, site number, collection date] alkalinity." As stated above, each lake has 3 sites with the exception of Black Lake, which only has 2 sites. It is helpful to label Benewah sample bottles with blue or green label tape and the chain lakes with any other color of label tape. It is also helpful to label 1 Ziploc bag for each lake (you can write straight on the bag using a Sharpie) and store all of the bottles for that lake in the Ziploc bag. This will make the sample bottles easier to find when you are in the field (rather than digging through 30 bottles at each site).
3. The bottles are now ready to collect sample water for alkalinity titrations.

<u>Procedure</u>

Before leaving the lab on the field day:

1. Load all of the sample bottles into the large Rubbermaid storage tote. The sample bottles will remain in this tote throughout the field day.
2. Fill the Coleman cooler with the frozen ice packs.

Once you are in the field:

1. At each site, fill one of each type of sample bottle – 1 for metal analysis, 1 for alkalinity.
2. It is important to fill the sample bottles before you take the CPUE scoop or before you begin looking for amphipods so that you avoid collecting water that has been stirred up. At the same time that water samples are collected, YSI readings should also be collected, and in approximately the same location.
3. Wear rubber gloves when working in the chain lakes as the sediments are contaminated with high concentrations of trace metals.
4. To collect a water sample, stand approximately 2 feet deep at the littoral zone. Take care not to disturb the sediment. If possible, stand on a large

boulder or on the shore and reach out to take the sample (without falling in). Fill the bottle with surface water, cap the bottle and shake it vigorously. Repeat this three times.

5. Submerge the bottle as deep as you can reach down without submerging your gloves.
6. Cap the bottle.
7. Repeat steps 4-6 once to collect two water samples total from each site.
8. As you visit each lake/site, transfer the ice packs from the Coleman cooler to the tote where the bottle are stored to keep them cool. This is particularly important during the summer months. On CPUE field days, the tote is filled up with the CPUE sample bags (see Protocol 6) so the bottles will need to be stored in the vehicle elsewhere. Regardless of how they are stored, keep them upright to prevent leaking and keep ice packs on them whenever possible.
9. When you return to the lab, store all of the samples in the refrigerator if there is room or in the incubator. Note the storage conditions/temperature.

Part 2. Preservation of samples for trace metal analysis using EPA Method 3005A

Objective

Upon returning to the lab, the water samples that will be used for metal analysis must be preserved with trace metal grade nitric acid. The acid keeps the metal ions in solution and prevents them from adhering to the sides of the sample bottles. Part 2 is only for samples that will be processed for trace metal analysis, not for alkalinity.

Personnel

Only one person is needed.

Supplies

100 mL bottle of 70% trace metal grade nitric acid stock*

*Stored under the fume hood in a secondary container

Styrofoam bottle holder*

*This is a rectangular piece of Styrofoam that has been carved to hold 100 mL bottles, generally stored in the drawer labelled “plastic disposable tubes.”

P1000 and tips

Waste container to use while working in the fume hood

Safety materials

Heavy duty rubber gloves appropriate for use when handling concentrated nitric acid

Preparation

Preservation of water samples should take place as soon as possible after returning from the field day. It is usually the case that this does not happen until at least 24 hours after return. Note the length of time that passes between the collection date and the preservation date.

1. From each sample bottle, pour out enough sample water (into the appropriate hazardous waste container) so that you can safely pipette the nitric acid into the bottle without it overflowing. This ends up being around 50 mL per bottle if the water level was up to the rim.
2. Turn the fume hood fan to *high*.
3. Put on the heavy duty rubber gloves.

4. Place the Styrofoam bottle holder in the fume hood and retrieve the 100 mL bottle of 70% trace metal grade nitric acid from the storage area below the fume hood. Close and latch the storage area door.

Procedure

1. You should still have the heavy duty rubber gloves on.
2. Remove the caps from the sample bottles. Place the sample bottles in the fume hood and place the caps on Kimwipes on the bench next to the fume hood so that they are not contaminated by dust in the air.
3. For 1000 mL sample bottles: Using the P1000, pipette 5.0 mL of 70% trace metal grade nitric acid into each 1000 mL bottle.
4. For 500 mL sample bottles: Using the P1000, pipette 2.5 mL of 70% trace metal grade nitric acid into each 500 mL bottle.
5. Take care not to touch the pipette to the side of any of the bottles or the sample water to avoid cross contamination.
6. When you are done preserving the samples and are ready to discard the tip, rinse the tip by drawing up some of the sample water and dispensing it out a couple of times. Then discard the tip in the waste container.
7. Cap the bottle of 70% nitric acid and return it to the storage area under the fume hood. Close and latch the door.
8. Cap the bottles tightly. Invert each bottle five times to disperse the acid.
9. Store the bottles in the refrigerator if there is room. If there is no room, store them in the incubator. Record storage conditions and temperature.

Protocol 4 – Processing Water Samples for Metal Analysis

Background

Surface water samples (Coeur d'Alene Basin, ID) were collected in acid-washed 500 mL or 1000 mL Nalgene bottles (see Protocol 3 for details). Samples were acidified as follows according to EPA 3005a:

a) To a 500 mL sample bottle, 2.5 mL 70% trace metal grade nitric acid was added.
b) To a 1000 mL sample bottle, 5.0 mL 70% trace metal grade nitric acid was added.

Samples were stored in the cold incubator in the lab at ~ 18.0°C until they could be processed.

This protocol describes the procedure for processing a single water sample (one lake, from two or three bottles (i.e. one lake, two or three sites). It is possible, and more efficient to process two or more samples at a time.

Objective

To bring large-volume of lake water samples to a volume such that the concentration of metal ions, lead (Pb) and zinc (Zn) is high enough to be detected during ICP-OES analysis. This requires heating the samples in a fume hood until they reach the desired volume. You will end up with two samples to analyze in the ICP (meaning two 15 mL tubes) per pooled water sample. One 15 mL tube will contain the non-boiled version of the sample, and the other tube will contain the condensed/boiled down version of the same sample.

Personnel

This activity does not require more than one person to perform, provided the person can be in the lab for up to 8 hours without interruption.

Materials, supplies, and equipment

Water samples
Labeling tape
Fine tip black Sharpie
Kimwipes
500 mL acid-washed graduated cylinder
50 mL acid-washed graduated cylinder
50 mL graduated cylinder (not acid-washed)
1000 mL acid-washed beaker
50 mL acid-washed beaker
Fume hood
Plastic serving tray*
*These are stored on top of the refrigerator in the lab
Paper towels
Hot plate
2 15 mL centrifuge tubes
Tube rack

Scale
BD 10 ml syringe (Luer-Lok Tip)
Whatman 13 mm disposable filter (45 µm pore size)*
*Filters are Puradisc 13/0.45 PTFE and come in packs of 100, Leur-Lok; Manufactured and distributed by GE Healthcare Life Sciences (Catalog No. 6784-1304)
70% trace metal grade nitric acid stock*
*1 L bottle, manufactured by Fluka Analytical, distributed by Sigma-Aldrich (Catalog No. 843850), stored under hood in secondary container
P1000 pipette
Disposable pipette tips
Pipette tip waste for tips contaminated with nitric acid
Acid-washed 100 ml screw top glass bottle and lid
Styrofoam bottle holder*
*This is a rectangular piece of Styrofoam that has been carved to hold 100 mL bottles, generally stored in the drawer labelled "plastic disposable tubes"
Safety materials
Disposable gloves
Thermal gloves appropriate for handling hot glassware
Thick rubber safety gloves appropriate for handling concentrated (70%) nitric acid
Preparation Part A. Make an aliquot of concentrated (70%) nitric acid
Important Part A should only be completed by people who have received the necessary training from Dr. Matos, and only under her supervision.
In Part A. we make an aliquot of the concentrated nitric acid for two reasons. First, concentrated nitic acid is extremely corrosive; it is less likely that you will spill a small bottle that is stabilized by a Styrofoam holder than a large bottle, and if the bottle does spill, a smaller volume will likely do less damage to your tissues. Second, it is good practice to use an aliquot of any stock solution so that if contamination occurs (e.g., dirty pipette), the stock is not contaminated, preventing potential headaches.

1. Set the fume hood fan to the *high* setting and turn on the fume hood light.
2. Using label tape and a fine tip Sharpie, label the 100 mL glass bottle "70% trace metal grade nitric acid made on [date] by [your initials]."
3. Place a Corrosive Acid sticker on the bottle.
4. Place the 100 mL glass bottle that you just labelled in the Styrofoam bottle holder. Place these two items in the fume hood.
5. Remove the cap and place it on a Kimwipe so that the inside of the cap is not contaminated by dust.
6. Put on heavy duty rubber gloves, lab coat, and safety goggles.
7. Retrieve the bottle of 70% trace metal grade nitric acid from the storage area under the fume hood and place it in the fume hood next to the glass bottle. Loosen the cap to release any pressure. Wait about 30 seconds.
8. Remove the cap. Slowly pour the acid into the 100 mL glass bottle. Hold the bottle in your right hand to pour, and stabilize the bottle while pouring with your left hand at the bottom of the base of the bottle. Never put your left hand directly under the opening of the bottle as acid could pour out onto your hand. Fill the bottle to approximately the 80 mL mark.

9. Set the stock bottle down and replace the cap. Make sure that it is tight.
10. Cap the 100 mL glass bottle. Make sure that it is tight.
11. Look over both bottles to check for any nitric acid that may have dripped down the sides. If you find some, wipe it with paper towels, then dispose of them in the appropriate hazardous waste container.
12. Look over your gloves to check for drops of nitric acid. If you find some, wipe the acid off with paper towels, then dispose of the paper towels in the appropriate hazardous waste container.
13. Return the stock bottle to its secondary container under the fume hood. Also place the glass bottle holding the aliquot that you just made into the secondary container under the fume hood. Close and latch the door.
14. Remove the heavy duty gloves, set them aside.

<u>Procedure Part A. Processing the water sample (not boiled down) for metal analysis</u>

1. Set the fume hood fan to *low*.
2. Label a 15 mL tube with the lake name, sample date, and "Not Boiled."
3. Weigh the tube and record the weight. Always weight the tube with its cap. It is best to place the capped tube on the scale, upside-down.
4. Place the 15 mL tube in a tube rack and set it on the lab bench.
5. Secure the Whatman filter to the Luer-Lok syringe. Using label tape and a black fine tip Sharpie, label the syringe with the lake name and sample date. Set the syringe/filter next to the 15 mL tube.
6. Using label tape, label the 50 mL acid-washed beaker with the lake name and sample date.
7. Obtain all of the sample bottles with the remaining sample water for the lake that you are processing. Shake each bottle.
8. Pour 10 mL of sample from each bottle (two or three bottles, i.e. two or three sites for the lake) into the beaker for a total volume of 20 or 30 mL, depending on how many sites the lake had. Swirl the beaker gently to mix the sample.
9. Walk the beaker over to the fume hood and set it on the hot plate. Set the hot plate to 400°C.
10. Let the beaker warm for approximately 2-5 minutes. Warming the beaker and sample water will make the sample easier to filter.
11. When the beaker has warmed, turn off the hot plate.
12. Walk the beaker over to the lab bench and set it down on a tray covered with a paper towel (never set a warm beaker on a cold surface as it could crack/break).
13. Swirl the beaker gently.
14. Remove the cap from the 15 mL tube and set it down on a Kimwipe so that the inside is not contaminated by dust in the air.
15. Remove the plunger from the syringe and set it on a Kimwipe.
16. Hold the syringe over the open 15 mL tube. Slowly pour about 6 mL of sample into the syringe.
17. Set the beaker down.
18. Return the plunger to the syringe and press it down to filter the sample into the tube. Be careful not to put too much pressure on the tube by resting the

syringe/filter on the rim of the tube as the connection between the filter and the syringe can break or the filter can slip off of the rim of the tube and become lodged in the tube.

19. Repeat step 17 once to filter a total of approximately 12 mL of sample into the tube.
20. Save syringe/filter, it will be used later to filter the condensed/boiled down sample.
21. Cap the 15 mL tube.
22. Weigh the tube, parafilm the cap, and then refrigerate the tube.

Procedure Part B. Boiling down the sample to a condensed form for metal analysis

To condense the sample:

1. Using the labeling tape and fine-tip black Sharpie, label the 1000 mL beaker with the following information: lake name and collection date.
2. Wear disposable gloves.
3. Pool the three samples. To do this, first shake each sample bottle, then measure 333 mL of sample water from each site/bottle using an acid-washed graduated cylinder. Pour the sample water into the 1000 mL beaker.
4. Set the bottles and remaining sample water aside.
5. Place the beaker containing the pooled lake water sample on the hot plate in the fume hood. Set the temperature to 450°C. Adjust the temperature periodically as needed such that the sample never comes to a full boil. Occasionally swirl the beaker to prevent particulates (e.g. small pieces of algae/organic matter) from sticking to the beaker walls.
6. Heat the sample until the volume reaches approximately 200 mL.
7. Turn off the hot plate. Place a thermal glove in the fume hood and transfer the beaker to the thermal glove (never set a hot beaker on a cold surface as it could crack or break). Allow the sample to cool for about 2-5 minutes on the thermal glove. See *Other considerations* below.
8. While the sample is cooling, label the 15 mL tube with the following information: lake name and collection date.
9. Weigh the 15 mL tube, record the label and weight, then place the tube in a rack on the lab bench where you will be filtering.
10. Remove the cap from the 15 mL tube and place it on a Kimwipe so that the inside not contaminated by dust in the air.
11. Using the same syringe/filter that you used in *Procedure Part A*, remove the plunger from the syringe and set it on a Kimwipe. Hold the syringe over the 15 mL tube. Swirl the beaker/sample gently a few times, then carefully pour about 5 mL into the syringe. Filter the 5 mL into the 15 mL tube.
12. Repeat step 11 once. Cap the 15 mL tube.
13. Weigh the 15 mL tube.
14. Pour the remaining sample into a 50 mL graduated cylinder. Try to get every drop out of the beaker, and into the graduated cylinder. Record the volume.
15. To obtain the final condensed sample volume, add the volume of the sample that is in the 15 mL tube (sample weight – tube weight; weight (g) is

approximately equal to the volume of sample (mL)) and the volume of sample that you just measured using the graduated cylinder.

16. Discard the remaining sample in the appropriate hazardous waste container.
17. Use the following equation to determine how much concentrated (70%) nitric acid to add to the 15 mL tube holding the boiled down sample, where V_1 is the amount of acid (mL) we will add to the tube: $V_1 = \frac{C_2 V_2}{C_1 - C_2}$

$$V_1 = \frac{(0.05\ HNO_3)(Sample\ volume\ in\ 15\ mL\ tube)}{(0.70\ HNO_3 - 0.05\ HNO_3)}$$

18. Walk the rack holding the 15 mL tube to the fume hood. Set the fan setting to *high*.
19. Put on the thick rubber safety gloves.
20. Obtain the aliquot of 70% nitric acid from underneath the fume hood. Close and latch the door. Place the bottle in the Styrofoam bottle holder in the fume hood.
21. Remove the tube cap and set it on a clean Kimwipe so that the inside is not contaminated by dust in the air.
22. Carefully pipette the volume of 70% nitric acid you calculated above into the 15 mL tube. Rinse the tip by drawing the solution up into the tip, and then moving the solution up and down a couple of times. Empty the solution back into the tube.
23. Discard the tip into the tip waste container. Set the pipette aside.
24. Cap the bottle. Make sure that it is screwed on tight.
25. Remove heavy duty gloves.
26. Cap the 15 mL tube, walk it over to the scale, and weigh it.
27. Parafilm the 15 mL tube and cap, then store in refrigeration.
28. Put the heavy duty gloves back on.
29. Return the aliquot of 70% nitric acid to the bottom of the fume hood. Close and latch the door.
30. Empty the tip waste container into the appropriate hazardous waste container. Be sure to do this in the fume hood.

Other considerations

Regarding *Procedure step 7*: If you allow the sample to come to room temperature, it will be very difficult to push the sample through the syringe/filter. I recommend keeping the sample warm, but cool enough to comfortably handle with a gloved hand.

Protocol 5 – Alkalinity Titrations

Objective

To measure the alkalinity of lake water samples collected from the Coeur d'Alene Basin. Alkalinity is the capacity of an aqueous solution to buffer acid.

Personnel

While one person can perform this task, two people are more efficient. One person can record, while the other performs the titration.

Materials, supplies, and equipment

Squeeze bottle containing deionized water
100 mL glass beaker (for deionized water)
50 mL glass beaker (for lake water sample)
Small stir rod
Magnetic probe
Stir plate
Holding rack for small centrifuge tube
Waste container and lid for HCl/trace metal waste
100 mL graduated cylinder
P200 pipette
Red Sharpie
Disposable gloves
KimWipes
P200 tips
Small (2.0 mL) centrifuge tube
0.1 N hydrochloric acid (HCl)
Note: bottles of 6 N, 1 N, and 0.1 N HCl are kept in the pH supplies drawer. If you run out of the 0.1 N HCl, you can make more as dilutions of either the 6 N or the 1 N solution.
Lake water samples
Scale
Accumet® Basic AB 15 pH meter and probe
MagneStir

Preparation

1. Bring the lake water sample to room temperature.

Procedure

1. Calibrate the pH meter according to the instructions posted above the instrument. When calibrating, use pH standards 4.0 and 10.0.
2. Leave the pH probe submerged in a beaker of deionized water (DIW) until you are ready to titrate the first sample.
3. Weigh a clean 100 mL beaker on the scale and record the weight.
4. Using a graduated cylinder, measure 50 mL of the water sample. Pour the sample into pre-weighed beaker.
5. Weigh the beaker with sample and record the weight.

6. Gently drop a small stir rod into the beaker with sample. Try to avoid splashing any droplets out of the beaker.
7. Remove the pH probe from beaker containing the DIW.
8. Turn on the MagneStir. Position the beaker on the MagneStir so that the stir rod will not hit the pH probe when it has been submerged.
9. Submerge pH probe into sample. Ensure the junction (the cloudy part of the probe) is completely submerged.
10. The pH meter displays the temperature in the bottom left-hand corner of the screen. Allow the pH reading to stabilize around 21.0°C.
11. Record the pH and the temperature.
12. Use a P200 to pipette 0.1 mL of 0.1 N hydrochloric acid into the sample.
13. Allow the pH reading to stabilize, then record.
14. Repeat steps 11-12 until the sample pH drops below 4.0 (4.0 is the pH at which the solution will be completely saturated with H^+ ions and the buffering capacity has been met, as all carbonate and bicarbonate ions have been converted into carbonic acid. At this point, addition of H^+ ions with respect to the change in pH will be linear).
15. Repeat steps 11-12 four more times.
16. Turn off the MagneStir.
17. Remove the pH probe from the sample.
18. Rinse the pH probe with DIW and gently blot it with a KimWipe.
19. Remove the stir rod using the magnetic probe, then dispose of the sample into the appropriate waste container.
20. Using a Red Sharpie, mark an 'X' on the sample bottle that your sample came from and place the sample bottle back into the incubator.
21. Submerge the pH probe in the DIW while you prepare the next sample.
22. Rinse the graduated cylinder and 100 mL beaker three times each with DIW and dry them completely.
23. Repeat steps 2-21 to measure alkalinity in the remaining samples. If you cannot titrate all of the samples in a single day, split the samples up so that some sites from the same lake are titrated on each day (e.g. Sites 1 and 2 on day one, Site 3 on day two).
24. Rinse the pH probe with DIW.
25. Close the window on the pH probe.
26. Press the *Standby* button on the pH meter.
27. Double check that you closed the window on the pH probe.
28. Return the probe to its preservation solution, secure tightly and make sure the probe is completely submerged above the junction (cloudy part of the probe).

Protocol 6 – Amphipod Collection and Storage for Catch Per Unit Effort (CPUE),

Size/Frequency Distributions, and Metal Analysis

Background

Amphipods of the species complex *Hyalella azteca* are small, freshwater benthic crustaceans native to North and Central America. These invertebrates are common in lakes, ponds, lagoons, rivers, and low-flow streams. They prefer to hide around macrophytes and under algal mats in lakes, especially where the substrate is coarse, with larger rocks and pebbles.

Part 1. Amphipod collection, transport, and housing for CPUE and size/frequency distributions

Objective

To collect amphipods once per month for the purpose of determining the Catch Per Unit Effort (CPUE) and to characterize the size/frequency distributions of the populations in each lake. These amphipods may also be used to characterize tissue metal burdens. You will also be collecting some water quality information, including temperature, dissolved O_2, and pH. You should also collect water samples during this time for metals analysis and alkalinity. See Protocol 3 for water sample collection and treatment. We typically collect at three sites per water body, separated by at least 10 m along the shore. If doing repeated measures, take GPS coordinates and, if possible, flag the sites with flagging tape so that the same site is sampled.

Personnel

Two people are required to perform this procedure.

Supplies

YSI model 556 probe with temperature, pH, and dissolved O_2 sensors (borrowed from Dr. McNeely)
All-weather notebook and pencil
500 micron D-frame dip net with frame size approximately 12" x 6"
2 gallon-sized Ziploc bags per site (each sample is double-bagged), pre-labeled with tape
Black Sharpie
Clean 500 mL or 1000 mL Nalgene bottles (any mouth width, any color)
Several Ice packs (I used about 30, various sizes)
Cooler or large tote
Bucket with tool storage caddy

Safety Materials

Rubber gloves (if working in the contaminated chain lakes)
Waders

Preparation

17. Retrieve a recent weather history for the week(s) prior to the sample date. Note any high flow or high wind events.
18. View a weather forecast for the sample date.

19. Anticipate any local events that may impact site availability and sample quality (e.g., Coeur d'Alene River Boat Races).
20. Before leaving the lab, label both Ziploc bags using a black Sharpie. Write the location, site number, and sampling date. Example: Benewah #1 8/13/2016. Ensure that both bags open and close easily.
21. Prepare YSI meter as per Protocol 2. Calibrate the DO probe the morning of the field day, and calibrate the pH probe once per month during the field season.
22. Set the incubator that you will use to store the amphipods at the temperature you estimate will be nearest the temperature of the lakes. If the field day is on a Saturday, do this Friday morning and monitor the temperature throughout the day.

Procedure

1. This procedure requires two people, one to scoop the sample, the other to pour the sample into the Ziploc bag.
2. When you arrive at the site, take care not to disturb the water, sediment, and vegetation more than is necessary. You may want to wear protective gloves if working at a site where the sediment is contaminated with trace metals.
3. Record the start time and note environmental conditions and other observations, such as changes in the weather or the total number of people recreating in the area.
4. Using a YSI, record water quality and environmental conditions (i.e. temperature, dissolved O_2, pH) from the shoreline, taking care not to influence the instrument readings by stirring up the sediment. It is best to have one person record while another person holds the instrument and immerses the probe.
5. Standing in about calf- to knee-deep water at the littoral zone of the lake (i.e., the shallow part of the lake that is closest to the shore, where aquatic vegetation grows), locate an area at the sampling site with ample vegetation.
6. *Person A*: Use a D-net to take a 1 meter scoop of water in the vegetated area from left to right. To do this, hold the D-net vertical. Lightly touch the D-net to the bottom of the lake, then move the D-net over a 1 meter length, agitating the sediment/vegetation at the same time by also moving the D-net in a vertical (up-and-down) motion. Pick up the net and hold it horizontal to prevent the contents from falling out. Allow about 30 seconds for water to drain out of the net. Gently swirl the water around to rinse the sides of the net if possible.
7. Remove any vegetation that is clinging to the outside of the net, but keep any vegetation that is at least half-way in the net.
8. *Person B*: While *Person A* holds the net vertically, tipping one corner of the net into the opening of the Ziploc bag, *Person B* holds the Ziploc bag in place directly underneath the net. *Person A* uses a clean bottle to scoop up some lake water. *Person A* then slowly pours the lake water over the outer area of the net to move all organic matter and organisms from the inside of the net into the Ziploc bag.

9. When all organic matter, vegetation, and organisms have been transferred from the net to the Ziploc bag, close the Ziploc bag securely. Double check that the opening is closed and secure. Depending on the amount of vegetation and organic matter obtained in your scoop, you will generally end up with a Ziploc bag about half full to three quarters full.
10. Double bag the Ziploc bag containing the sample with another Ziploc bag. Double check that the opening is closed and secure. Check on the bags periodically. If a leak is observed, triple bag the sample.
11. Transport sample back to vehicle using ice packs and a bucket, then store on ice packs in the vehicle. Be sure to position the sample upright so that if a leak occurs at the opening, the sample will not spill out.
12. Repeat steps 2-11 for all sites.
13. Transport sample to laboratory, house amphipods in incubator at collection temperature. See Part 3 for housing and sorting details.

Part 2. Amphipod collection, transport, and housing for metal analysis

Objective

To collect enough amphipods (10-15 per lake) to complete metal analysis corresponding to the specific date that CPUE and size/frequency distribution data was collected. This step is performed after the CPUE collection.

Personnel

If four people work together, this task can be accomplished in 10-15 minutes where amphipods are abundant.

Supplies

Small, wide mouth plastic containers (at least 8 oz capacity) with screw-top lid
Small cooler
Ice packs (5 or 6 small)
Sieves (no smaller than 500 micrometer)
Disposable Pasteur pipettes (first 1 ½ in of tip removed)
Bucket with tool storage caddy

Safety Materials

Rubber gloves
Waders

Preparation

1. Prior to leaving the lab, wash the collection containers with dilute Liquinox and deionized water. Rinse thoroughly with deionized water. Label the collection containers, one per lake. Labels should include the lake, sampling date, and "amphipods."
2. Prior to leaving the lab, ensure that the incubator temperature is set to the estimated temperature at the time of collection.

Procedure

1. Wear rubber gloves.
2. Due to time constraints, amphipod collection is performed at one site per lake. When possible, make sure that this is the site amphipods are collected from at every sampling event. Always note what site a given collection takes place. To

begin collecting amphipods, fill the 8 oz plastic screw-top collection container ¾ full with lake surface water. Avoid collecting turbid water.

3. At the littoral zone, locate an area with submerged vegetation. Dip a sieve into the lake, gently agitating the submerged vegetation and sediment, then raise the sieve out of the water to collect the amphipods. At most, your sieve should only be half-covered in vegetation. It is easiest to see the animals moving rapidly within the first 30 seconds of exposure to air. Beyond that time, they tend to slow their movement and blend in with the vegetation and organic matter in the sieve.
4. Use a disposable pipette to collect the amphipods from the sieve. Each person tends to develop a unique method of doing this. One way is to pipette a few drops of lake water into the pipette first, then partially squeeze out a drop of water, using the surface tension of the drop to bring the amphipod into the pipette. When transferring the amphipods from the pipette to the collection container, submerge the pipette tip at least halfway into the container before releasing the animal. If the pipette tip is not submerged, the likelihood of the animal becoming stuck in the surface tension increases substantially. Once this happens, it is difficult for the amphipod to escape the surface tension and this likely causes significant stress.
5. Collect 15 amphipods, this should provide 10 for metal analysis, with extras in the case of mortality.
6. When enough amphipods have been collected, add a small piece of vegetation (e.g., piece of a leaf) for the animals to cling to. Secure the lid on the collection container. Transport the amphipods on ice to the vehicle. Place the collection container(s) upright in a cooler, stabilized by the other amphipod collection containers, to prevent disturbance/tipping. Depending on the air temperature, add an ice pack or two to the cooler. Try to keep the ice pack on top of the containers, rather than on the sides to maintain even cooling distribution. In the spring and fall, ice packs may not be needed. Try to keep the amphipods at the same temperature they were collected at.
7. Repeat steps 1-5 until amphipods have been collected from every lake.
8. Transport amphipods to the laboratory and store them in a pre-chilled incubator. Be sure to remove the lid from the amphipod collection containers. Compare collection day field temperatures to pre-set incubator temperature. If a discrepancy exists, adjust the incubator temperature to the field temperature. Amphipods should be held as close to the temperature they were collected at as possible.

Part 3. Amphipod measurements and tissue preservation for metal content analyses

Objective

To obtain the lengths and weights of amphipods collected for metal analysis; and to flash freeze and store amphipods for tissue preservation for future metal analysis.

Personnel

Only one person is needed.

Supplies and equipment

Lab notebook for record-keeping

Gloves
Amphipods collected for trace metal analysis (in the 8 oz collection containers)
Kimwipes
4 Pasteur pipettes with the first 1 ½ in of each tip removed
*Note: You should mark 2 pipettes with tape to designate them as "metals only" pipettes so as not to contaminate the reference lake samples
Disposable Pasteur pipettes (first 1 ½ inch of tip removed)
Paper towels (long rectangular, white)
Plastic calipers
Weigh paper
Scale
Cryovials, preferably the 2 ml size
Fine tip black Sharpie
Liquid nitrogen
Transport container for liquid nitrogen
Long metal tongs

Preparation

1. Print out a copy of the current Freezer Log.
2. Use scissors to remove the first 1½ in from the tips of the disposable pipettes to accommodate the size of the amphipods.
3. Using the fine-tip Sharpie, label cryovials. Each lake sampled should be assigned a single cryovial. On each cryovial, write the name of the lake and the amphipod collection date.
4. Turn on the scale. Make sure that the doors are closed.
5. Fold a piece of weigh paper in half and make a horizontal crease. This will prevent amphipods from moving around on the paper when you are taking the weight. Open one of the doors and place the weigh paper on the scale. Close the door. Press *Tare* to tare the weigh paper. Open the door and remove the weigh paper. Close the door. Place the weigh paper in your work area on the bench next to the scale.
6. Next to the weigh paper, place a stack of several paper towels on the bench. Place two or three Kimwipes on top of the stack of paper towels. The Kimwipes should prevent any metal contamination from the paper towels.
7. Place two or three Kimwipes on the bench next to the stack of paper towels. This is where you will place the Pasteur pipettes as you work to prevent metal contamination from the bench.
8. Fill the Liquid nitrogen transport vessel with two ladles full of liquid nitrogen from the stock container. When you are done transferring the liquid nitrogen, make sure that the lid is secured on the transport vessel and the stock container.

Procedure

1. Put on disposable gloves as the sample may be from a contaminated lake.
2. Drop the cryovial corresponding to the first amphipod container you will process in the liquid nitrogen transport container. Secure the transport container lid.

3. Obtain the first amphipod container from the incubator. Start with the reference lake to prevent metal contamination between samples. Place the container on the bench next to the scale. Remove the screw-top cap.
4. Using a Pasteur pipette, obtain one amphipod from the container and transfer it to the stack of paper towels. The paper towels will absorb the water, leaving the amphipod exposed.
5. Using the calipers, measure the body length from head to end in millimeters. Take the length of the animal when it is in its natural position (i.e. not completely curled up, not completely stretched out, intermediate between these two positions). You may need to use one finger to hold the amphipod in place while you take the length as they move quickly even when they are out of water.
6. Record length (mm). Set calipers aside.
7. While the amphipod is still on the paper towel, carry it and the paper towel to the scale and transfer the amphipod to the pre-tared weigh paper by using the weigh paper as a scoop, wedging the creased part of the weigh paper under the amphipod in one swift motion. Alternatively, you can pick up the amphipod using your fingers; do not use a metal spatula. Wait for the scale to stabilize. Record weight (g).
8. Using the long metal tongs, remove the cryovial from the liquid nitrogen transport container; be aware that the lid of the cryovial may hold some liquid nitrogen. Be sure to secure the lid of the transport container.
9. Using a paper towel or Kimwipe to protect your hands, open the cryovial.
10. Carefully transfer the amphipod from the weigh paper into the cryovial. It is best to hold the creased weigh paper vertically over the vial so that the crease is over the vial opening, and gently tap the weigh paper until the amphipod falls in. Cap the cryovial securely, and then return it to the liquid nitrogen transport container.
11. Repeat steps 4 through 10 until all amphipods have been processed from this container.
12. Walk the transport container to the -80°C freezer.
13. To transfer the cryovial to the storage box, first place the liquid nitrogen transport container on the bench closest to the freezer. Open the freezer and obtain the box. Close the freezer door. Place the box on the bench next to the liquid nitrogen transport vessel. Using the large metal tongs (generally stored on the side of the transport container), carefully remove the cryovial from the transport vessel and place it in the box. Note the location of the cryovial in the box on the Freezer Log printout.
14. Be sure to change gloves before processing a new container.
15. Drop the cryovial corresponding to the next amphipod container you will process in the liquid nitrogen transport container. Secure the transport container lid.
16. Obtain the next amphipod container from the incubator. Place the container on the bench next to the scale. Remove the screw-top cap.

17. Repeat steps 4 through 10 until all amphipods have been processed from this container.
18. Repeat steps 16 and 17 until all containers have been processed.
19. Update the Freezer Log.

Part 4. Amphipod maintenance and sorting for CPUE and size/frequency distributions

Objective

To sort out amphipods collected in each CPUE sample and to determine size/frequency distributions.

Personnel

While one person can perform this activity, it is more efficient to have at least one or two additional people working at the same time.

Supplies

Lab notebook for record-keeping
CPUE samples
Large sorting tray(s), including the white metal tray and white plastic sorting trays borrowed from the invertebrate zoology lab room
Small plastic weigh boats (5-10), generally the large, square weigh boats
Dechlorinated water (several liters)
Gloves
Disposable Pasteur pipettes (first 1 ½ inch of tip removed)
Paper towels
Plastic calipers
Weigh paper
Waste bucket with lid (properly labelled)

Preparation

1. Bring the dechlorinated water to the same temperature as the CPUE samples by storing large beakers and/or large carboys of water in the incubator.
2. Clean the large sorting tray(s) and small plastic containers with dilute Liquinox and deionized water.
3. Use scissors to remove the first 1 ½ in from the tip of the disposable pipette to accommodate the size of the amphipods.
4. Label the small plastic weigh boats with the information (location, site number, and date) from the CPUE samples (this is written on the Ziploc bag).

Important Consideration

1. If CPUE bags must be processed over several days due to limited time or lab staff availability, sort one sample from each lake each day, rather than sorting all samples from a few lakes on a given day.

Procedure

1. Put on disposable gloves as the sample may be from a contaminated lake.
2. Obtain a CPUE sample from the incubator to begin sorting. *Note: Start with Benewah Lake (reference lake) to avoid cross contamination of metals from the other samples.
3. Pour a small amount (approximately 500 mL) of the sample into the large sorting tray. Use dechlorinated water to dilute the sample if there is a high organic matter or sediment content.

4. Using the disposable pipette, transfer some lake water (approximately 20 mL) into the weigh boat that will be holding amphipods from the first CPUE sample. Place this container near the sorting tray to maximize ease of transfer.
5. Seek out and pipette all amphipods from the sorting tray, regardless of body size, into the weigh boat.
6. Note all other invertebrates you observe in the tray.
7. When possible, have someone else look over the tray to check for any amphipods that were missed. Depending on the season, the amphipods may be only 1-2 mm long.
8. When you have removed all of the amphipods from the tray, dump the contents into an appropriate waste bucket. Environmental Health and Safety (EH&S) has sieves available for you to use to keep vegetation and mud separate from the water. Everything collected from the Coeur d'Alene Basin should be treated as hazardous waste unless EH&S states otherwise.
9. Repeat steps 3, 5-8 until the entire CPUE sample has been sorted.
10. Wash both of the Ziploc bags with dilute Liquinox and deionized water, then allow them to hang dry.
11. Rinse the sorting tray with deionized water. Set aside.
12. Take the individual lengths and weights of the amphipods you just sorted. To do this, pipette a single amphipod onto a paper towel. The towel will absorb the excess water.
13. Using the calipers, measure the body length from head to end in millimeters. Take the length of the animal when it is in its natural position (i.e. not completely curled up, not completely stretched out, intermediate between these two positions). You may need to use one finger to hold the amphipod in place while you take the length as they move quickly even when they are out of water.
14. Record length (mm). Set calipers aside.
15. While the amphipod is still on the paper towel, carry it and the paper towel to the scale and transfer the amphipod to the pre-tared weigh paper by using the weigh paper as a scoop, wedging the creased part of the weigh paper under the amphipod in one swift motion. Alternatively, you can pick up the amphipod using your fingers. Wait for the scale to stabilize. Record weight (g).
16. If you do not need to save the amphipod for metal analysis, then you can destroy the individual by transferring it to a paper towel using your fingers, then squishing it with your finger against the paper towel.
17. If you plan to save the amphipods for metal analysis, then proceed to Part 5.

Part 5. (Optional) Tissue preservation for metal content analyses of amphipods collected from CPUE and size/frequency distributions

Objective

If too few amphipods were obtained/died from the collection for metal analysis, amphipods collected during CPUE can be saved for metal analysis.

Personnel

One person can perform this activity.

Supplies

Lab notebook for record-keeping
Amphipods from Part 4 (lengths and weights already obtained)
Gloves
Disposable Pasteur pipettes (first 1 ½ inch of tip removed)
Paper towels (long rectangular, white)
Cryovials, preferably the 2 ml size
Fine tip black Sharpie
Liquid nitrogen
Transport container for liquid nitrogen
Long metal tongs
Waste bucket with lid (properly labelled)

Preparation

1. Print out a copy of the current Freezer Log.
2. Using the fine tip black Sharpie, label a cryovial with the name of the lake from which you'll be preserving amphipods from, and the collection date.
3. Transfer two ladles full of liquid nitrogen from the stock container into the transport container. Be sure to secure the lid.
4. Put the cryovial into the transport container with the liquid nitrogen. Be sure to secure the lid.

Procedure

1. Put on disposable gloves.
2. Using the long metal tongs, remove the cryovial from the liquid nitrogen transport container; be aware that the lid of the cryovial may hold some liquid nitrogen. Be sure to secure the lid of the transport container.
3. Using a paper towel or Kimwipe to protect your hands from the cold, open the cryovial.
4. Carefully transfer the amphipod from the weigh paper into the cryovial. It is best to hold the creased weigh paper vertically over the vial so that the crease is over the vial opening, and gently tap the weigh paper until the amphipod falls in. Cap the cryovial securely, and then return it to the liquid nitrogen transport container.
5. Repeat steps 2-4 for all amphipods you wish to save from this lake.
6. Transport the cryovial to the -80°C freezer in the nitrogen transport container. Bring a pen and a piece of paper to note the exact location of the cryovial in the storage box.
7. Open the freezer and quickly remove the sample box that you will use. Close the freezer door.
8. Open the sample box on a lab bench, so that it is close to the liquid nitrogen transport container. Use the long metal tongs to transfer the cryovial from the liquid nitrogen transport container to the storage box. Secure the lid on the transport container.
9. Note the location of the tube in the storage box on the piece of paper you brought with you. Close the box lid. Store the box in the same location you found it in the freezer. Close the freezer door securely.
10. Return the remaining liquid nitrogen to the stock container in the lab.

11. Repeat steps 1-9 if you wish to save more CPUE amphipods for metal analysis.
12. Update the Freezer Log with the location of the samples you have just stored.
13. If the amphipods were not sorted and frozen on the first weekday after CPUE collection, record in the notebook how many days passed between the collection day and the day that the amphipods were sorted and flash frozen. Also record this information in the Freezer Log.

Protocol 7 – Amphipod Collection and Acclimation for Lab Assays and Metal Analysis

Background

This activity is performed twice per season (spring, summer, and fall). In conjunction with this collection of amphipods is (1) the collection of lake water samples from the same set of lakes to analyze water alkalinity, metal, and hardness levels, and (2) water temperature, pH, and dissolved O_2. These collections take place twice per season to prepare for two separate laboratory assays, and to understand the water conditions at the time of amphipod collection. See Protocol 3 for water sample collection and treatment.

Part 1. Amphipod collection, transport, and housing for laboratory assays and metal analysis; Collection of water quality characteristics

Objective

To collect 250 amphipods from the littoral zone of Rose L. and/or Thompson L., Medicine L., Anderson L., and Benewah L. and to collect water quality information (temperature, dissolved O_2, and pH).

Personnel

A minimum of three people are required to perform this procedure, although at least four is optimum.

Supplies and equipment

YSI model 556 probe with temperature, pH, and dissolved O_2 sensors (borrowed from Dr. McNeely)*

*Check the transport/calibration cup holding the probe to make sure that there is a little water in the cup (approximately 1/8-1/2 in) to keep the sensors from drying out.

All-weather notebook and pencil

500 micron D-frame dip net with frame size approximately 12" x 6"

Small, wide mouth plastic containers (at least 8 oz capacity) with screw-top lid

Black Sharpie

Label tape

Round, metal Sieves (no smaller than 500 micrometer)

Disposable Pasteur pipettes (first 1 ½ in of tip removed)

Ice packs (10-20)

Large plastic tote

Ziploc bags

Small Cooler

Large, rectangular plastic Rubbermaid container that will float (should have flat bottom)

Bucket with tool storage caddy

12 aquaria with lids

12-15 air stones

4 air pumps*

*Tetra Whisper 100; 120 volts; these are stored in the basement room and/or the storage cabinet in the lab near the door.

Tubing for air pumps
Extension and/or power cord(s)
Long LED light (I used an Intematix model TE10C35395TY-O low-voltage (12VCD) light; borrowed from the Stockroom)
Timer for light
Electrical tape
10 (lunchroom) serving trays
Cheesecloth cut into 1" x 1" pieces*
*Single use; one whole cloth should provide enough material for acclimation and assays
TetraMin fish food
Gloves
Dechlorinated water (approximately 20 liters)
Thermometer

Safety Materials

Heavy duty rubber gloves
Waders

Preparation

23. Retrieve a recent weather history for the week(s) prior to the sample date. Note any high flow or high wind events.
24. View a weather forecast for the sample date.
25. Anticipate any local events that may impact site availability and sample quality (e.g., Coeur d'Alene River Boat Races).
26. Calibrate the YSI sensors according to manufacturer instructions. Calibrate the DO probe the morning of the start of the assay, and calibrate the pH probe once per month.
27. The day before the field collection, set the incubator that you will use to store the samples at the temperature you estimate will be nearest the temperature of the lakes.
28. Place a thermometer inside of the incubator. This is the thermometer that you will record from during amphipod acclimation. If the collection is taking place on a Saturday, set the incubator temperature Friday morning and monitor the temperature throughout the day, compare both the display temperature and the thermometer that you have just placed inside of the incubator.
29. Prepare the incubator to house the amphipods. It is best to set up four air pumps with airlines split to provide air to up to 12 aquaria. Position two air pumps on the top shelf and two on the third shelf down from the top. Remove the green plug from the hole on the back wall of the incubator (do not lose this). Send the power cords out of the hole in the back of the incubator. Install tubing onto each of the air pumps. Make sure that there is enough tubing to reach the aquaria. Use tube splitters to add additional tubes so that there is one line of tubing for each aquarium. Attach a new air stone at the end of each tube where are will be moving into the aquaria. Plug in the air pumps using an extension cord and/or power cord. Make sure that all of the air stones have air coming out of them. Dip each air stone into a small beaker of deionized water

to make sure that air is moving through. Replace any faulty air stones. Unplug the air pumps.

30. Wash 12 aquaria and their lids with Liquinox and deionized water. Place the aquaria on trays in the incubator. Feed air stones into aquaria. Cover the aquaria with lids, but do not secure them to the aquaria so that air will be able to flow in through the tubes.
31. To set up lights in the incubator, place the long LED light on the second shelf from the top, behind the air pumps. Position the angle of the light so that it faces the door. Send the power cord through the hole in the back of the incubator. Plug the hole with the green stopper that you removed in the previous step. Plug the power cord in using an extension cord if necessary. Make the fit as tight as you can, recognizing that the cords will displace the stopper, and will not be completely seal the hole. Attach a timer to the power cord so that the light cycle is 12D/12L (8:00 am to 8:00 pm; similar to field conditions).
32. Wash the small, wide-mouth containers in dilute Liquinox and deionized water prior to sampling day. Dry completely, screw caps on. Use a Sharpie and label tape to label the appropriate lake, collection date, and "amphipods". Each lake will get at least two containers. Store the containers in the cooler that you will be bringing into the field. Bring extra containers if they are available.
33. Double bag eight Ziploc bags. Using a Sharpie, label two pairs of Ziploc bags per lake with the name of the lake and the collection date. Store these bags in the large plastic tote that you will bring with you into the field.

Procedure

14. We typically collect water samples and water quality information at three sites per water body, separated by at least 10 m along the shore. If doing repeated measures, take GPS coordinates and, if possible, flag the sites with flagging tape so that the same site is sampled.
15. When you arrive at a site, take care not to disturb the water, sediment, and vegetation more than is necessary. You may want to wear protective gloves as the sediment in the Coeur d'Alene Basin chain lakes is contaminated with trace metals.
16. Record the time and note environmental conditions and other observations, such as changes in the weather or the total number of people recreating in the area.
17. Record water quality and environmental conditions (i.e. temperature, dissolved O_2, pH) from the shoreline, taking care not to influence the instrument readings by stirring up the sediment. To take a YSI reading, dip the probe in the water so that it is positioned at the same depth that you take water samples (approximately 1 ft down). Gently swirl the probe, and simultaneously read the display. Wait until the readings stabilize (i.e., there are no significant changes). This could take several minutes. When the readings are stable, read the values to the recorder. You do not need to rinse the lake between sites, but you do need to make sure that the probe is screwed into the transport/collection cup between sites/lakes.

18. Perform steps 3-4 at every site at each lake. It is best to have one person record while another person handles the instrument.
19. Amphipod collection is performed at one site only. When possible, make sure that this is the site amphipods are collected from at every sampling event. Always note at which site a given collection takes place. Amphipods should be collected from the same site each collection day unless none are found at that site – in which case you can collect at another site, but be sure to note the change.
20. To begin collecting amphipods, fill the 8 oz screw-top collection containers ¾ full with lake surface water. Avoid collecting turbid water. As you pipette amphipods into the containers, more water will accumulate in the containers, so you will want to leave some room.
21. Place the collection containers in the large flat-bottomed plastic container. The large plastic container should float on the surface of the water and provide a place for you to keep the collection containers as you collect amphipods.
22. At the littoral zone, locate an area with submerged vegetation. Dip a sieve into the lake, gently agitating the submerged vegetation and sediment, then raise the sieve out of the water to collect the amphipods. At most, your sieve should only be half-covered in vegetation. It is easiest to see the animals moving rapidly within the first 30 seconds of exposure to air. Beyond that time, they tend to slow their movement and blend in with the vegetation and organic matter in the sieve.
23. Use a disposable pipette to collect the amphipods from the sieve. Each person tends to develop a unique method of doing this. One way is to pipette a few drops of lake water into the pipette first, then partially squeeze out a drop of water, using the surface tension of the drop to bring the amphipod into the pipette. When transferring the amphipods from the pipette to a collection container, submerge the pipette tip at least halfway into the container before releasing the animal. If the pipette tip is not submerged, the likelihood of the animal becoming stuck in the surface tension increases substantially. Once this happens, it is difficult for the amphipod to escape the surface tension and this likely causes stress.
24. Collect 250 amphipods, this should provide 200 for the assays, at least 15 for metal analysis, with extras in the case of mortality during transport, acclimation, and handling.
25. If the amphipods are large (many greater than 2 mm) label a third collection container as described in *Preparation* and use it with the other two to continue collecting amphipods. This will leave more room for amphipods to swim and will hopefully cause less stress.
26. When enough amphipods have been collected, add a small piece of vegetation (e.g., piece of a leaf) for the animals to cling on to. Secure the lids on the collection containers.
27. To collect water for amphipod acclimation, fill Ziploc bags that have been labelled for this lake with surface water that has not been stirred up. You want to collect water that is as clear of debris as possible. Secure the Ziploc bags.

28. Transport the amphipods and lake water on ice, in the tool bucket, to the vehicle. Place the collection containers upright in a cooler, stabilized by the other amphipod collection containers, to prevent disturbance/tipping. Depending on the air temperature, add an ice pack or two to the cooler. Try to keep the ice pack on top of the containers, rather than on the sides to maintain even cooling distribution. In the spring and fall, ice packs may not be needed. Try to keep the amphipods at the same temperature they were collected at. Try to do the same for the acclimation water. The water can be stored in the large plastic tote. Keep the bags upright so that they do not leak if there is a faulty seal (common). You can do this by stabilizing them with the ice packs and water sample bottles (Protocol 3).
29. Repeat steps 3-14 until amphipods and acclimation water, as well as water samples and water quality information have been collected from each lake.
30. Transport amphipods to the laboratory.
31. Compare collection day field temperatures to pre-set incubator temperature. If a discrepancy exists, adjust the incubator temperature to the field temperature. Amphipods should be held as close to the temperature they were collected at as possible.
32. Record the temperature and the time, as well as any changes you made on the Incubator Temperature Log (taped on the outside of the incubator).
33. Using label tape and a Sharpie, assign two or three aquaria (this depends on the size of the aquaria you are using) to a lake. Label the aquaria with name of the lake and the collection date.
34. Put on gloves.
35. Pour the lake water into the appropriate aquaria. One Ziploc bag full of water should fill one aquaria. ***Important* Leave room for the amphipods and the lake water in their collection containers.** Double check that the label on the Ziploc bag matches the label on the aquaria (i.e. Anderson Lake water goes into Anderson Lake aquaria). This step can be performed on a lab bench and/or the lab cart.
36. Repeat steps 16-17 until all of the lake water has been transferred to the aquaria.
37. Slowly add the amphipods to the appropriate aquaria (i.e. Benewah amphipods go into the Benewah aquaria). Leave 15 amphipods in each collection container, adding lake water as needed so that the container is ¾ full. These 15 amphipods will be saved for metal analysis later. Place the collection containers on a tray in the incubator with caps off.
38. To each aquarium, add two to three pieces of 1" x 1" cheesecloth for the animals to cling to.
39. Using your fingertips, grind up a pinch of TetraMin fish food and distribute it in an aquarium. Repeat this for all aquaria.
40. Transfer the aquaria to the incubator. To each aquarium, add at least one airline (if the air stones do not emit the same volume of air-which happens occasionally, you may need to add a second airline).Check to make sure that every aquarium receives aeration. Also make sure that the aeration is not

disturbing the amphipods. If the bubbling is too vigorous, try replacing the air stone or adjusting the position of the air stone in the aquarium. The aquarium lids can be helpful in positioning the air lines.

41. Label the air tubes that are feeding air into the reference lake aquaria with label tape to avoid metal contamination from the other containers. From now on, these tubes should only be used to feed air into the reference lake aquaria.
42. Close the incubator door.

Part 2. Amphipod measurements and storage for metal analysis

Objective

To take measurements of length and weight from individual amphipods that were collected for metal analysis; to flash freeze amphipods in liquid nitrogen and store them at –80°C until they can be processed for trace metal analysis. This should be done as soon as possible after returning from the field. Typically, if a collection takes place on a Saturday, this is done the following Monday.

Personnel

While one person can complete this work, it is a more efficient use of time to have two people working on this part.

Supplies and equipment

Gloves
Amphipods collected for trace metal analysis (15 per lake should have been stored in the 8 oz collection containers upon returning to the lab on collection day)
Kimwipes
4 Pasteur pipettes with the first 1 ½ in of each tip removed
*Note: You should mark 2 pipettes with tape to designate them as "metals only" pipettes so as not to contaminate the reference lake samples
Lab scale
Weigh paper
Paper towels (long rectangular, white)
Plastic calipers
Liquid nitrogen
Liquid nitrogen transport container
Large metal tongs
5 cryovials, preferably the 2 mL size
Cryovial storage box
Label tape
Standard black Sharpie
Fine-tip black Sharpie

Preparation

1. If a cryovial storage box has not already been created, you will need to create one by labeling the lid and bottom of the box with a standard Sharpie. Store the box in the -80°C freezer. Create a Freezer Log in Microsoft Excel corresponding to this new box. You will track the location of each vial in this spreadsheet through time. Print out a copy of the Freezer Log to track the location of the samples you will process today.

2. Using the fine-tip Sharpie, label 10 cryovials. Each lake sampled should be assigned a single cryovial. On each cryovial, write the name of the lake and the amphipod collection date.
3. Turn on the scale. Make sure that the doors are closed.
4. Fold a piece of weigh paper in half and make a horizontal crease. This will prevent amphipods from moving around on the paper when you are taking the weight. Open one of the doors and place the weigh paper on the scale. Close the door. Press *Tare* to tare the weigh paper. Open the door and remove the weigh paper. Close the door. Place the weigh paper in your work area on the bench next to the scale.
5. Next to the weigh paper, place a stack of several paper towels on the bench. Place two or three Kimwipes on top of the stack of paper towels. The Kimwipes should prevent any metal contamination from the paper towels.
6. Place two or three Kimwipes on the bench next to the stack of paper towels. This is where you will place the Pasteur pipettes as you work to prevent metal contamination from the bench.
7. Fill the Liquid nitrogen transport vessel with two ladles full of liquid nitrogen from the stock container. When you are done transferring the liquid nitrogen, make sure that the lid is secured on the transport vessel and the stock container.

Procedure

1. You will measure, weigh, freeze, and store 15 amphipods per lake. All amphipods for a given lake that will be stored for metal analysis can be stored in a single cryovial. This procedure should be done as soon as possible after returning from the field, and no later than the first water change as amphipods may release metals from their bodies when they are exposed to uncontaminated water – which happens during the first water change. Be sure to record how long the amphipods are kept alive in the lab before they are frozen (time between collection day and freezing day).
2. Put on gloves.
3. Drop the cryovial corresponding to the first amphipod container you will process in the liquid nitrogen transport container. Secure the transport container lid.
4. Obtain the first 8 oz amphipod container from the incubator. Start with the reference lake to prevent metal contamination between samples. Place the container on the bench next to the scale.
5. Using a Pasteur pipette, obtain one amphipod from the container and transfer it to the stack of paper towels. The paper towels will absorb the water, leaving the amphipod exposed.
6. Using the calipers, measure the body length from head to end in millimeters. Take the length of the animal when it is in its natural position (i.e. not completely curled up, not completely stretched out, intermediate between these two positions). You may need to use one finger to hold the amphipod in place while you take the length as they move quickly even when they are out of water.

7. Record length (mm). Set calipers aside.
8. While the amphipod is still on the paper towel, walk it over to the scale and transfer it to the pre-tared weigh paper. Wait for the scale to stabilize. Record weight (g).
9. Using the long metal tongs, remove the cryovial from the liquid nitrogen transport container. Be aware that there could be some liquid nitrogen in the lid of the cryovial. Use caution. Be sure to secure the lid of the transport container after you have retrieved the cryovial.
10. Using a paper towel or Kimwipe to protect your hands from the cold, open the cryovial.
11. Carefully transfer the amphipod from the weigh paper into the cryovial. It is best to hold the creased weigh paper vertically over the vial so that the crease is over the vial opening, and gently tap the weigh paper until the amphipod falls in. Cap the cryovial securely, and then return it to the liquid nitrogen transport container.
12. Repeat steps 3 through 11 until 10-15 (no less than 10 if possible) amphipods for a given lake have been measured, weighed, and frozen.
13. Drop the cryovial corresponding to the next amphipod container you will process in the liquid nitrogen transport container. Secure the transport container lid.
14. Obtain the next amphipod container from the incubator. Place the container on the bench next to the scale.
15. Repeat steps 3- 14 until all containers have been processed.
16. When amphipods have been frozen for all of the lakes, walk the transport container to the -80°C freezer.
17. To transfer the cryovials to the storage box, first place the liquid nitrogen transport container on the bench closest to the freezer. Open the freezer and obtain the box. Close the freezer door. Place the box on the bench next to the liquid nitrogen transport vessel. Using the large metal tongs (generally stored on the side of the transport container), carefully remove the cryovials from the transport vessel and place them in the box. Note the location of the cryovials in the box on the Freezer Log printout.
18. Update the Freezer Log.

Part 3. Amphipod acclimation

Objective

To allow amphipods that were collected for toxicity assays to acclimate to laboratory conditions, and to change amphipod collection water (100% lake water) to test water (100% dechlorinated water) over a period of 7 days.

Personnel

Only one person is required, except for the last day, in which amphipods are moved.

Supplies and equipment

Gloves

Amphipods collected for toxicity assays (in aquaria)

Dechlorinated water (at least 20 liters)

*Note: Dechlorinated water should be pre-chilled to the same temperature that the amphipods are being housed at by being stored in large beakers in the incubator for at least 24 hours prior to use
At least 2 - 2 L beakers
8 large aquaria (2 per lake, for 4 lakes (3 chain lakes and the reference lake)) (washed with Liquinox and deionized water)
Label tape
Black Sharpie
2 disposable Pasteur pipettes (first 1 ½ in of tip removed)
TetraMin fish food
Cheesecloth cut into 1" x 1" pieces

Preparation

1. Using label tape and a Sharpie, label one of the 2 L beakers "metals only."
2. Label one of the Pasteur pipettes with a piece of label tape, indicating "metals only" use.

Procedure (Day 1)

1. Record the temperature and the time, as well as any changes you made on the Incubator Temperature Log (taped on the outside of the incubator).
2. Put gloves on.
3. Starting with the top shelf, remove the first aquarium from the incubator.
4. Set the aquarium on the bench located under the window.
5. Scan the aquarium for any dead amphipods. Remove them from the aquarium. Record the aquarium label and how many you removed in your notebook. ***Important* Record any observations of fuzziness or strange growths on the dead amphipods as this could be a fungal infestation issue.**
6. Note the time of day. Try to schedule water changes at the same time each day. Carefully pour 500 mL of the lake water from the aquarium into a 2 L beaker. Most of the amphipods will stay near the bottom of the aquarium; try not to pour any amphipods into the beaker. If the aquarium is holding amphipods from one of the contaminated chain lakes, be sure to use the "metals only" beaker and to dispose of this lake water into an appropriate hazardous waste container. Before disposing of the lake water, check to make sure that no amphipods were accidentally transferred to the beaker. If amphipods were accidentally transferred, use a Pasteur pipette to pipette the amphipod(s) from the beaker, back into the aquarium.
7. Measure 500 mL pre-chilled dechlorinated water into the 2 L beaker and slowly pour it into the aquarium, taking care not to disturb the amphipods.
8. Obtain a pinch of TetraMin fish food and place it in the cap of the TetraMin fish food container. Using your fingers, mash the food into very fine pieces and sprinkle into the aquarium.
9. Set the aquarium aside.
10. Obtain the next aquarium on the top shelf and repeat steps 4-9. Do this for the second shelf down as well. If any aquaria on these two shelves are holding reference lake amphipods, be sure to use a separate beaker and Pasteur pipette to avoid metals contamination.

11. Move the aquaria back into the incubator, while rotating them from their original positions such that aquaria that were on the top shelf are now on the second shelf and vice versa. If any of the aquaria are for the reference lake, be sure to rotate the reference lake air tube with the reference lake aquarium.
12. Repeat steps 3-11 for the third and fourth shelves.
13. Refill the dechlorinated water reservoir that you are using so that water is pre-chilled for the next day's water change.

Procedure (Day 2)

1. Repeat *Procedure (Day 1)*, except now for steps 6 and 7 pour 1000 mL instead of 500 mL.

Procedure (Day 3)

1. Repeat *Procedure (Day 1)*, except now for steps 6 and 7 pour 1500 mL instead of 500 mL.

Procedure (Day 4)

1. Repeat *Procedure (Day 1)*, except now for steps 6 and 7 pour 2000 mL instead of 500 mL This constitutes a near-complete exchange of water.

Procedure (Day 5)

1. Record the temperature and the time, as well as any changes you made on the Incubator Temperature Log (taped on the outside of the incubator).
2. Put gloves on.
3. Starting with the top shelf, remove the first aquarium from the incubator. Set the aquarium on the bench located under the window.
4. Scan the aquarium for any dead amphipods. Remove them from the aquarium. Record the aquarium label and how many you removed in your notebook. ***Important* Record any observations of fuzziness or strange growths on the dead amphipods as this could be a fungal infestation issue.**
5. Fill an empty aquarium with the pre-chilled dechlorinated water. Using label tape and a Sharpie, duplicate the label from the aquarium you just pulled from the incubator and tape it to the new aquarium.
6. Using a Pasteur pipette, transfer all of the amphipods from the old aquarium to the new aquarium. Count the amphipods as you go. This will give you a final count of the number of amphipods available for the toxicity assays.
7. When all of the amphipods have been transferred, remove the cheesecloth from the old container and visually inspect it for any remaining amphipods. Do the same for any leaves or other pieces of debris that the amphipods may cling to.
8. Discard the water in the old aquarium and the cheesecloth.
9. Wash the aquarium with Liquinox and deionized water.
10. Obtain a pinch of TetraMin fish food and place it in the cap of the TetraMin fish food container. Using your fingers, mash the food into very fine pieces and sprinkle into the new aquarium.
11. Place one or two pieces of (new) cheesecloth in the aquarium.
12. Set the aquarium aside.
13. Obtain the next aquarium on the top shelf of the incubator and repeat steps 3-12. Do this for the second shelf down as well. If any aquaria on these two

shelves are holding reference lake amphipods, be sure to use a separate beaker and Pasteur pipette to avoid metals contamination.

14. Move the aquarium back into the incubator, while rotating them from their original positions such that aquaria that were on the top shelf are now on the second shelf and vice versa. If any of the aquaria are for the reference lake, be sure to rotate the reference lake air tube with the reference lake aquarium.
15. Repeat steps 3-14 for the third and fourth shelves.
16. The amphipods will not receive water changes on Day 6 or Day 7 so that they can acclimate without disturbance for a couple of days before the assays begin. Feed on Day 6. Check each aquarium on Day 7 and feed again if no food is observed.

Protocol 8 – Laboratory Zn Assays, Mortality Counts, and Box Tests

Background

Toxicity tests (assays) are performed separately for two trace metals that are present in the Coeur d'Alene (CDA) Basin, lead (Pb) as lead nitrate, and zinc (Zn) as zinc sulfate. Lead is not required for amphipod growth and survival, while zinc is an essential ion. The purpose of the assays is to test whether amphipods from the CDA Basin, which is contaminated with trace metals, are tolerant to trace metal stress. These are the methods to perform an acute zinc (Zn) 96 hour toxicity test using *Hyalella azteca* collected from the chain lakes and a reference lake. See Protocol 7 for details of amphipod collection and acclimation. Over the course of the Zn toxicity test, amphipod mortality is recorded every 24 hours. Immediately after the 96 hour test, surviving amphipods are subjected to a 10 minute swimming test to measure differences in behavior among the different groups.

Part 1. Making the Zn stock solution

Objective

To make 40 mL of a 59.3 g/L stock solution of zinc sulfate. This stock solution will be used to dose the test containers for the zinc assay. You should have enough solution for at least one complete 96 hour assay. Be sure to invert the tube several times to mix the solution prior to dosing the jars, making sure that no precipitate has formed at the bottom of the tube.

The stock solution should be prepared no later than the day before the start of the assay to provide adequate time to set up and dose the jars.

Personnel

Only one person is required to perform Part 1; only those who have been trained on the handling of toxic chemicals can complete this step. Ask Dr. Matos for assistance.

Supplies and equipment

Lab notebook for record-keeping
Electronic pipette (Drummond Pipet-aid) and 1 – 50 mL pipette
Milli-Q (ultrapure) water*
*This can be obtained from Dr. Nezat in the Geochemistry lab. This water should be collected in an acid-washed 1000 mL plastic Nalgene bottle.
Labelling tape
Fine tip black Sharpie
Poison stickers from EH&S
Zinc sulfate ($ZnSO_4 7H_2O$)*
*This was obtained from the stock room. 2.5 kg container, manufactured by J. T. Baker Chemical (Catalog No. 4382-05).
2 - 50 mL tubes with tube rack
Scale
Plastic weigh boat
P1000 pipette and tips
Kimwipes

Parafilm
Plastic funnel
Aluminum foil

Safety Materials

MSDS zinc sulfate
Disposable gloves
Disposable respirator
Lab coat
Safety goggles

Preparation

The stock solution should be made no later than the day before the start of the 96 hour assay.

1. Using label tape and a fine tip black Sharpie, label a 50 mL tube with the following: "Danger! 59.3 g/L zinc sulfate made on [date] by [your initials]." Place a poison sticker on the tube and place the tube in a tube rack.
2. Read MSDS zinc sulfate.
3. Put on lab coat, safety goggles, disposable respirator, and disposable gloves.
4. Obtain the container of zinc sulfate ($ZnSO_4 7H_2O$) from the stock room.
5. Place the plastic weigh boat on the scale and press O/T (tare).
6. Put a pipette tip on the P1000 pipette and set the pipette on the bench near the scale. Lay a Kimwipe under the pipette tip to prevent contamination from the lab bench.

Procedure

1. Draw up 40 mL Milli-Q (ultrapure) water in the 50 mL pipette using the electronic pipette.
2. Dispense the ultrapure water into the 50 mL tube.
3. Cap the tube.
4. Place the tube/rack next to the scale.
5. Put on lab coat, safety goggles, disposable respirator, and disposable gloves.
6. Place the container of zinc sulfate next to the scale.
7. Since the stock container is so large, you will want to make an aliquot of the stock zinc sulfate to make it easier to measure a small amount onto the scale.
8. Obtain a new 50 mL tube for this aliquot of zinc sulfate and place it in the tube rack.
9. Place the plastic funnel in the opening of the empty 50 mL tube.
10. Carefully open the stock zinc sulfate container.
11. Holding the container by its handle with your right hand, gently tap the container with your left hand to bring the crystals closer to the opening of the container. Continue tapping until crystals fill the 50 mL tube about ¾ full.
12. Set the stock container on the bench and cap it. Set the stock container aside.
13. Cap the 50 mL tube that is now holding the aliquot of zinc sulfate.
14. Wrap the tube in aluminum foil (leave the cap so that it can be taken on/off).
15. Using label tape and a black Sharpie, label the tube, "Danger! Zinc sulfate, [date, your initials]."
16. Place a Poison sticker on the side of the tube.

17. Uncap the 50 mL tube holding the Milli-Q water and the 50 mL tube holding the stock zinc sulfate. Set the caps on a Kimwipe so that the inside of the caps is not contaminated by dust in the air.
18. Open the door to the scale.
19. Pick up the 50 mL tube holding the stock zinc sulfate. Grasping the side of this tube with your right hand, slowly move the tube opening so that it is positioned over the weigh boat inside of the scale.
20. Using your left hand, gently tap the tube so that the zinc sulfate crystals slowly drop out of the tube, and onto the weigh boat. Do this until you have measured 4.232 g of zinc sulfate. If you accidentally pour too much, do not try to put the salt back into the tube. Instead, add more Milli-Q water to obtain the desired concentration.
21. Return the tube to the tube rack and screw the cap on tight.
22. Carefully remove the weigh boat from the scale.
23. Hold the weigh boat over the 50 mL tube that is holding the Milli-Q water so that one corner is pointed downward toward the tube. Fold in the sides of the weigh boat so that the crystals do not fall onto the lab bench. Tip the weigh boat and pour the zinc sulfate into the tube.
24. Holding the weigh boat in your left hand, use the P1000 to draw up some of the solution in the tube with your right hand. Dispense the solution onto the weigh boat to rinse remaining zinc sulfate into the tube. Repeat this step until all of the zinc sulfate is in the tube.
25. Dispose of the pipette tip and weigh boat in the appropriate hazardous waste container.
26. Cap the tube.
27. Gently swirl the tube until all of the zinc sulfate has gone into solution and you do not observe any granules in the tube.
28. Parafilm both of the tubes (the solution that you just made and the tube with the stock zinc sulfate) and store them in separate Ziploc bags in the cabinet to the left of the sink, on the top shelf in the section labeled, “metal work.”
29. Dispose of your gloves in the appropriate hazardous waste container.

Part 2. Preparing the test area and test containers

Objective

To prepare the test area by setting up a grow light, water chillers, and Rubbermaid tanks.

To prepare the test containers for the Zn assay. At least two working days before the start of the assay, you will want to set up the tubs/water/jars for the assay so that you can get the test water to the appropriate temperature. Wash and acid-wash the jars one week before the start of the assay. The test jar water should be at the same temperature as the amphipod acclimation water when you begin the assay.

Personnel

While one person can perform this part, it is more efficient to have two or three people working together.

Supplies and equipment

Lab notebook for record-keeping

Fluorescent grow lights in a multi-light (8) fixture*
*These are obtained from the greenhouse and should come with cordage and adjustable locking mechanisms for hanging.
Ladder
(Lunchroom) Serving trays*
*These are generally kept on top of the refrigerator
Paper towels
100 wide-mouth or regular-mouth pint Mason jars
10% nitric acid for washing glassware*
*This is generally stored in a large carboy near the sink. See Protocol 1 for more information.
2 Rolls of labeling tape; blue and pink
Black Sharpie
Generic plastic wrap
Kimwipes
Milli-Q (ultrapure) water*
*This can be obtained from Dr. Nezat in the Geochemistry lab. This water should be collected in a 2 gallon Nalgene carboy.
1000 mL acid-washed graduated cylinder
2 - Rubbermaid 50 gal (or larger) stock tanks
Dechlorinated water
Liquinox
2 - Water chillers*
*I used a VWR Scientific Model 1167 (from the stock room) and a Julabo Model F12 (from the JMatos Lab)
2 – Thermometers

Safety Materials

Disposable gloves
Lab coat

Procedure

At least two working days before the start of the assay, you will want to set up the tubs/water/jars for the assay so that you can get the test water to the appropriate temperature. The test jar water should be at the same temperature as the amphipod acclimation water when you begin the assay.
Acid-washing the test containers:

1. Wash Mason jars with Liquinox and rinse thoroughly with deionized water. Let the jars dry completely.
2. Autoclave the jars on the hard goods cycle. Let them cool completely.
3. Using label tape and a black Sharpie, label several (4-5) trays, “10% nitric acid.”
4. Arrange the trays on the lab bench near the door. Line the trays with paper towels.

Setting up the basement room (test area):

1. Set up the grow light fixture.

2. Feed a piece of cord through the first locking mechanism. Tie a hitch on the end that will be bearing the weight of the fixture around one of the pipes in the ceiling. Test the hitch to make sure that it can bear the weight of the fixture. On the other end of the cord, tie a stopper knot in case the locking mechanism fails. Repeat this step to set up the other locking mechanism.
3. *Important* Have someone help you with this step. Have a helper hold the light fixture up while you attach the light fixture to the locking mechanisms. Using the carabiner that is attached to the locking mechanism, clip one locking mechanism to each metal hanger on both sides of the light fixture.
4. For redundancy, use another piece of cordage or pliable wire to hitch the fixture to the pipe. Tie the hitch to the hangers you used in step 4. If the locking mechanisms or either of your hitches fail, these secondary cords should keep the fixture from falling into the water-containing tubs below.
5. Position the two Rubbermaid stock tanks that are located in the basement room underneath the grow light. Fill the tanks with water until the water level is about 3-4 inches high. To fill the tanks, you can fill clean 5 gallon buckets with water from the dechlorinated water tap (located above the large fish tank outside of the door; be careful to avoid splashing into the fish tank!).
6. Check the two water chillers to make sure that their reservoirs are full. If more water is needed, obtain about 1 L of deionized water from the lab and top off as needed. It is important to use *deionized* water to prevent mineral buildup inside of the chillers.
7. Position the tubes connected to the water chillers so that they lie inside the Rubbermaid tubs. One water chiller is sufficient to chill water in one Rubbermaid tub.
8. Turn on both water chillers. Set the temperature so that it is the same as the temperature of the water that the amphipods are acclimating in.
9. Place a thermometer in each Rubbermaid tub. Monitor the temperature over the next day or two and adjust the water chiller to that the temperature of the water in the tubs/jars matches the temperature of the amphipod acclimation water.

Preparing the test containers:

1. Put on disposable gloves and a lab coat.
2. Working in the lab, completely fill each jar with 10% nitric acid. Place the jars on the paper towel-lined trays.
3. Cover every jar with plastic wrap to prevent the fumes from corroding nearby metal components in the lab.
4. Let the jars sit for at least one hour, undisturbed.
5. Empty the acid back into the carboy.
6. Use about 50 - 100 mL of Milli-Q water to rinse each jar. Swirl the water around in the jar for about 30 – 60 seconds to remove any remaining acid.
7. Cover the paper towel-lined trays with Kimwipes.
8. Place the Mason jars upside-down on the Kimwipe-lined trays to dry.

9. Once the jars are dry, transfer them to the basement room where the assays will take place. It is best to use the boxes that the Mason jars were purchased in to carry the jars. These are stored in the cabinets in the basement room.
10. Working on the bench downstairs, for each jar, measure 500 mL dechlorinated water in the acid-washed 1000 mL graduated cylinder. Pour this water into the jar.
11. Make an ID "tag" for each jar by placing a piece of label tape on the rim. To do this, position a 2-3 in piece of label tape so that it is perpendicular to the rim of the jar. Place half of the piece on the outside of the rim and the other half on the inside, so that the tape acts as a "tag" that you can write an ID number on. 20 jars should receive blue tape (these will be for the control group), the remaining should receive pink tape.
12. Using a black Sharpie, number the jars 1-100.
13. Cover each jar with plastic wrap to prevent evaporation.
14. Place the jars in the tanks.

Part 3. Beginning the Zn assay

Objective

To begin the 96 hour Zn toxicity test by transferring amphipods from the acclimation containers (aquaria) to the test jars. Test jars will have 10 amphipods each. The experimental setup will consist of five groups; four groups will come from four of the chain lakes and one group will come from the reference lake. Each group will have five treatments; a control (0 mg/L) and four doses (14.5, 29, 43.5, and 58 mg/L).

Personnel

A minimum of two people are required; three or four people is ideal.

Supplies

Lab notebook for record-keeping
125 mL acid-washed plastic Nalgene screw top bottle
Label tape
Black Sharpie
Plastic rectangular Rubbermaid container
Liquinox
Cheesecloth cut into 1 x 1 in squares
2-4 Dog training lab pads
Disposable Pasteur pipettes with the first 1 ½ in of the tip cut off
Aquaria holding amphipods that have been acclimated for at least one week
Timer for grow light
Zinc sulfate stock solution
P1000 pipette and tips
Tube rack
1000 mL beaker
100 mL bottle of 70% trace metal grade nitric acid (from Protocol 1)
Kimwipes
Styrofoam bottle holder*

*This is a rectangular piece of Styrofoam that has been carved to hold 100 mL bottles, generally stored in the drawer labelled "plastic disposable tubes"

Safety Materials

Disposable gloves

Preparation

1. Set the timer for the grow light on a 12 dark/12 light cycle.
2. Using label tape and a black Sharpie, label the acid-washed 125 mL plastic Nalgene bottle, "Dechlorinated water collected on [date] by [your initials]."
3. Using the 125 mL plastic Nalgene bottle, collect a water sample from the dechlorinated water tap to be analyzed for trace metals. To do this, run the tap for several minutes then rinse the bottle three times with the dechlorinated water. Collect a sample on the fourth fill. Cap the bottle tightly.
4. Walk the sample back up to the lab and set it in the fume hood.
5. Set the fume hood fan to *high*.
6. Put on the heavy duty rubber gloves.
7. ***Important*** Steps 7-16 must only be performed by people who have received proper training from Dr. Matos and may only be performed under her supervision. Obtain the small bottle of 70% nitric acid from the storage area in the bottom of the fume hood. Place it in Styrofoam bottle holder in the fume hood.
8. Uncap the sample bottle. Place the cap on a Kimwipe so that the inside of the cap is not contaminated by dust in the air.
9. Using the P1000, add 0.625 mL of 70% nitric acid to the bottle to preserve the sample.
10. Discard the pipette tip in the appropriate hazardous waste container.
11. Cap the bottle of 70% nitric acid.
12. Return the bottle to the storage area under the fume hood, close and latch the door.
13. Take off the heavy duty gloves.
14. Cap the bottle.
15. Store the bottle in the incubator in the lab until this sample can be filtered and acidified for metal analysis.
16. Note the day and time of the sample collection in the lab notebook.

Procedure

Part 3 should be started as early as possible on the first day of the assay, which is typically a Monday morning.

Transferring the amphipods to the test containers:

1. In the basement room, lay out two lab pads on the main work bench. These will soak up water from the large tubs as you work with the test jars.
2. Wash a Rubbermaid plastic container with Liquinox and deionized water, rinse thoroughly with deionized water.
3. Take the Rubbermaid container to the basement room and set it on the work bench.
4. Obtain the first aquarium from the incubator and carefully walk it to the basement room. Set it on the work bench with the Rubbermaid container.
5. Obtain 5 blue-taped jars and 15 pink-taped jars from the tubs and set them on the lab pads on the bench.

6. Slowly pour a small amount of water/amphipods from the aquarium into the Rubbermaid container.
7. Remove and discard the plastic wrap from the 20 jars that are now sitting on the bench.
8. Using a disposable Pasteur pipette, draw up an amphipod into the pipette from the Rubbermaid container. Submerge the pipette into the Mason jar and let the amphipod swim out. Do not squeeze the water out of the pipette. Repeat this step 9 times to transfer a total of 10 amphipods into the jar.
9. Place a 1 x 1 in piece of cheesecloth in the jar.
10. In the lab notebook, record the lake from which the amphipods came (labeled on the aquarium), and the jar number and tape color.
11. Return the jar to either tub.
12. Repeat steps 4-11 for the remaining 19 jars. You will need to obtain the other aquarium for this lake from the incubator when you run out of amphipods.
13. As you find all of the amphipods in the Rubbermaid container, discard the water from the container into the drain and pour another small amount of water/amphipods from the aquarium into the container. Using the Rubbermaid container to collect the amphipods from (rather than collecting straight out of the aquarium) is more efficient because the amphipods cannot hide/swim away very quickly when the water level in the container is low. Be sure to check the pieces of cheese cloth in each aquarium for amphipods. To do this, hold the cheese cloth so that it is partially submerged. As you expose the cheese cloth to the air, any amphipods that remain in the cheese cloth will migrate toward the water and swim away. Continue submerging the cheesecloth, then exposing it to the air until you have removed all of the amphipods.
14. If extra amphipods remain in the aquarium after all of the jars have received 10 amphipods, return the aquarium to the incubator.
15. Obtain the aquarium for the next lake.
16. Repeat steps 8-15 until all of the jars have 10 amphipods.

Dose the jars:

1. In the lab, rinse the Rubbermaid container and dry it with paper towels. You will be using it to carry supplies from the lab to the basement room.
2. In the lab, obtain the following and place them into the Rubbermaid container: P1000 pipette and pipette tips zinc sulfate stock solution (from Part 1), a tube rack, and the 1000 mL beaker. Carry these supplies to the basement room. Do not do this during a break between classes, or any time when there may be many people in the hallway.
3. Using label tape and a Sharpie, label the beaker, “dechlorinated water” and set it on the lab bench.
4. Collect all of the pink-taped jars (remember, blue tape is for control jars) that should receive the lowest dose and place them on the lab pads on the bench. You should have five jars per “lake” for a total of 20 jars that will receive this dose.
5. Work as an assembly line. One or two people handle the jars, another person handles the pipette/stock solution, and another person records everything.

6. From each jar, draw up 0.105 mL of water and dispense it in the 1000 mL beaker (this is the equivalent volume of stock zinc sulfate that you will add to each jar later for all jars in the lowest Zn dose, the 14.5 mg/L dose). The person(s) handling the jars must keep track of the jars that have/have not had 0.105 mL water removed.
7. When 0.105 mL of water has been removed from all 20 jars, discard the pipette.
8. Unscrew the cap on the tube of stock zinc sulfate, set in the tube rack.
9. Obtain a new pipette tip. Draw up 0.105 mL of the zinc sulfate stock solution and dispense it into each jar. Do not touch the pipette or the tip to the side of the jar or submerge it into the water.
10. Record that these jars (list the jar numbers) have all received 0.105 mL of zinc sulfate stock.
11. Return all of the jars that you just dosed to the tubs.
12. Repeat steps 6-11 with the next treatment set of pink-taped jars, which is the next highest dose of Zn, 29 mg/L. Again, you should have five jars per "lake" for a total of 20 jars that will receive this dose. This time, you will remove 0.210 mL of water from each jar before pipetting 0.210 mL of stock zinc sulfate into each jar.
13. Repeat steps 6-11 again for the next treatment set of 20 jars, which is the next highest dose of Pb, 43.5 mg/L. This time, remove 0.315 mL of water from each jar before pipetting 0.315 mL of stock zinc sulfate into each jar.
14. Repeat steps 6-11 again for the next treatment set of 20 jars, which is the next highest dose of Pb, 58 mg/L. This time, remove 0.420 mL of water from each jar before pipetting 0.420 mL of stock zinc sulfate into each jar.
15. 20 control jars should remain in the tubs.
16. Record the time. This is the time that you will perform all mortality checks at during the next 96 hours.
17. Cap the tube of zinc sulfate and parafilm the cap.
18. Carry the pipette, tips, and stock zinc sulfate solution back to the lab.

Part 4. Daily mortality counts

Objective

Mortality counts must be performed every 24 hours. They must be performed at the same time each day.

Personnel

Two people are required to perform mortality checks. It is better to have more than two people working as the process will go much faster.

Supplies

Lab notebook for record-keeping
Several sheets of scratch paper
4-8 Disposable Pasteur pipettes with the first 1 ½ in of the tip cut off
2 Rolls of labeling tape; blue and pink
2 Pint Ziploc bags
Black Sharpie
2-4 Dog training lab pads

Safety Materials

Disposable gloves

Preparation

1. Use pink label tape to mark 2-4 disposable Pasteur pipettes (depending on how many people are helping). These pipettes will only be used when counting mortality in zinc sulfate-dosed jars. When these pipettes are not in use, they should be stored in a Ziploc bag labeled, "Zn pipettes only."
2. Use blue label tape to mark 2-4 disposable Pasteur pipettes. These pipettes will only be used when counting mortality in control jars. When these pipettes are not in use, they should be stored in a Ziploc bag labeled, "control pipettes only."
3. Lay out the lab pads on the work bench.

Procedure

1. Record the time.
2. Begin with the control jars to avoid accidental contamination.
3. On a scratch sheet of paper (with the date, time, and your initials), list all of the numbers corresponding to the control jars.
4. Obtain a control jar from the tub.
5. Record general notes and observations about the jar (e.g., cloudiness, presence of precipitate at the bottom of the jar, whether the amphipods were swimming and energetic or showing little movement.
6. Using a blue-taped pipette, locate and remove all of the dead amphipods from the jar. An amphipod is dead if it does not move after several seconds when prodded with the pipette tip. Occasionally, an amphipod may look dead because it does not move, even though it is alive. One way to tell if it is dead is to draw up the amphipod in the pipette and then quickly move it back out into the beaker. Generally, if it's alive it will move after this. It is also important to check the cheese cloth for dead amphipods. If you have trouble identifying alive from dead, try bringing the cheesecloth to the surface and exposing it to the air. Amphipods that are still alive will migrate toward the water and swim away.
7. Pipette the dead amphipods into a single pile for each jar, onto the lab pad.
8. Record the number of dead amphipods next to the jar number on the piece of scratch paper.
9. Remove and record the number of exoskeletons that you find. Sometimes it is hard to tell an exoskeleton from a dead amphipod. Generally, the exoskeletons are transparent, while the amphipods are not.
10. Return the jar to the tub.
11. Repeat steps 4-10 until mortality has been counted in all of the control jars.
12. Next check the dosed jars. Make sure that you wear disposable gloves.
13. Remember to switch to the pink-taped pipettes when you move onto the dosed (pink-taped) jars. Start with the lowest treatment group first.
14. Repeat steps 4-10 for the remaining treatment groups until mortality has been counted in all of the jars.

15. When you are done checking mortality in all of the jars, wrap up and throw away the lab pad.
16. Record the time.
17. When you return to the lab, transfer the notes from the scratch paper(s) to the lab notebook.
18. Perform mortality checks as described above every 24 hours for the next 3 days. Be sure to start the mortality counts at the same time every day.

Part 5. End of assay and box tests

Objective

To end the 96 hour test with a final mortality count and to perform box (swimming) tests on the remaining amphipods. During the box tests, all surviving amphipods from each jar are tested individually in Tic Tac boxes. After a 1 minute acclimation period, the number of times each amphipod swims from the bottom of the box to the water line (surface) is recorded over a 10 minute interval. Generally, one person can watch 1-5 boxes during a 10 minute interval.

Personnel

No less than three people are needed. It is best to have as many people working as can do so safely in the basement room. This is an all-day task.

Supplies

Lab notebook for record-keeping
Label tape
Black Sharpie
5 – 2000 mL beakers
Several sheets of scratch paper
Pink- and blue-taped pipettes from Part 4
2-4 Dog training lab pads
100 Empty Tic Tac boxes
Masking tape in tape dispenser
1 - 100 mL graduated cylinder
1 Timer/stopwatch for each person participating in the box tests

Safety Materials

Disposable gloves

Preparation

1. Lay the lab pads out on the work bench.
2. Wash Tic Tac boxes with Liquinox and rinse thoroughly with deionized water. Let dry completely.
3. Using label tape and a black Sharpie, label 5 - 2000 mL beakers, “dechlorinated water only,” then fill the beakers with dechlorinated water 24 hours before the start of the box tests in order to chill the water to the same temperature as the test jars. You can chill them in the incubator in the lab which should still be at the same temperature that the amphipods were acclimated at.
4. Determine the jars that you want to perform box tests on, with the understanding that there is not enough time to do all 100 jars by the end of the day. Determine an order of prioritization for jars that should be done first (the number of box tests that you can perform by the end of the day is dependent

on the number of people you have helping you). Ideally, you would at least test jars from the control (all lakes) and from the highest dose (all lakes) first in odd numbered replicates (meaning you test 3 jars from a given lake, rather than 2 or 4 jars), then test the next three dose groups (all lakes), again in odd-numbered replicates.

Procedure

96 hour mortality count (performed on all jars):

1. On the last day (Friday if you started the test on Monday), perform mortality counts for each jar. Beginning with the control jars, use the blue-taped pipettes to remove all of the dead amphipods from each jar.
2. Record the number of dead amphipods and the jar from which they came on a piece of scratch paper (with the date, time, and your initials). Also record the number of exoskeletons you remove.
3. Return each jar to the tub.
4. Repeat steps 2-4 until all of the control jars have been checked.
5. Switch to the pink-taped pipettes and check all of the dosed jars for dead amphipods, repeating steps 2-3 until all of the dosed jars have been checked.
6. Record the time.

Box (swimming) tests (performed on as many jars as possible):

1. Using the list of priority jars that you created in *Preparation*, begin testing the control jars. Remove the first jar that you wish to test, placing it on a lab pad on the work bench.
2. Using a graduated cylinder, measure 20 mL of the pre-chilled dechlorinated water and pour it into a Tic Tac box. Repeat this step to fill 9 additional boxes. Assuming amphipods did not die in the control jars, you will likely have 10 amphipods to test from each jar (and therefore two people per jar).
3. Using masking tape and a black Sharpie, label the first box, "[Jar number]_1", the second box, "[Jar number]_2", and so forth until all 10 boxes have been labeled with the jar number and a box test ID number (1-10).
4. Using a blue-taped pipette, transfer one amphipod from the jar into each box. When you are dispensing the amphipod into the box, do not squeeze out the water. Instead, submerge the tip of the pipette and let the amphipod swim out.
5. Start the timer for one minute to allow the amphipods to acclimate to the new container.
6. Have someone help you record during the test. You can each watch 5 boxes. If taking care of a jar alone, only transfer five amphipods, test them, then transfer the other five amphipods and test them. Do not leave amphipods in the tic tac boxes.
7. Start the timer for 10 minutes. During the 10 minute interval, record on a piece of scratch paper, the number of times that an amphipod swims from the bottom of the box to the water line (surface). Do not count instances where the amphipod swims from any other depth to the water line. The amphipod must start at the bottom of the box and swim to the water line in order for the effort to count.

8. When the 10 minute test is over, either save the amphipods for trace metal analysis (see Part 6), or dispose of them.
9. Obtain the next jar.
10. Repeat steps 2-9 until you have performed box tests for all of the control jars on your priority list.
11. Begin testing amphipods from the dosed jars. Repeat steps 2-9 for each dosed jar. Be sure to use the pink-taped pipettes for these jars (step 4).
12. Discard the control jar water in the sink. Discard the dosed jar water in the appropriate hazardous waste container. Rinse the jars with a small amount of dechlorinated water, pouring this rinse water into the hazardous waste container.
13. Take the jars back upstairs to the lab and wash them with Liquinox. Rinse the jars thoroughly with deionized water and leave them to dry on the dishrack or on trays lined with paper towels.

Part 6. Preservation and storage of surviving amphipods

Objective

To freeze and store amphipods that survived the 96 hour toxicity test so that their tissues can be analyzed for trace metals.

Personnel

One person can do this while at least two others are performing the box tests at the same time (Part 5).

Supplies

Amphipods in Tic Tac boxes from box tests in Part 5
Plastic rectangular Rubbermaid container
Pink- and blue-taped pipettes from Part 4
Paper towels
Kimwipes
1 - 2 mL Cryovial per jar
Fine tip black Sharpie
Liquid nitrogen
Liquid nitrogen transport container
Long metal tongs
Weigh paper, folded in half and creased

Safety Materials

Disposable gloves

Procedure

1. When all box tests have been completed for a given jar, use a Rubbermaid container to carry the boxes (still containing the amphipods) to the lab. Also bring one of each colored-taped pipette with you (be sure to keep them in separate Ziploc bags to prevent cross contamination.
2. Place the Rubbermaid container and the Tic Tac boxes on the lab bench near the door.
3. Print a copy of the freezer log.
4. Place a small stack of paper towels on the bench. Cover the paper towels with 1-2 Kimwipes.

5. Label a cryovial the following, “Zn [Jar number], [Date].”
6. Transfer 1 ladle of liquid nitrogen into the transport container.
7. Set the transport container on the lab bench next to the Tic Tac boxes.
8. Drop the cryovial into the transport container.
9. From each box, pipette the amphipod onto the paper towel/Kimwipe. Let the paper soak up all of the water from the amphipod. Blot the amphipod with a Kimwipe if needed.
10. Using the long metal tongs, obtain the cryovial from the transport container. Be careful – some liquid nitrogen may be in the cap.
11. Open the cryovial and set it on the bench.
12. Use a creased weigh paper to scoop up the amphipod. Holding the amphipod in the crease of the weigh paper, position the weigh paper over the opening of the cryovial. Tip the crease slowly, so that the amphipod falls into the cryovial.
13. Quickly cap the vial and drop it into the transport container.
14. Repeat steps 9-13 until all of the amphipods (belonging to that jar) have been frozen.
15. Walk the cryovial to the -80°C freezer in the transport container. Bring the printout of the freezer log with you.
16. Obtain the box from the freezer. Use the large metal tongs to transfer the cryovial to the box. Note the location of the cryovial in the sample box on the printout.
17. Repeat steps 9-16 for all jars from which you wish to save surviving amphipods.

Part 7. Collection and processing of test jar water for metal analysis

Objective

To collect samples of water from the test containers after the completion of Part 6. These samples will be analyzed for trace metals. The zinc content will be compared with the dose that the jar received at the beginning of the assay.

Personnel

Only one person is required.

Supplies

1 Dog training lab pad
Electronic pipette (Drummond Pipet-aid) and 10 mL pipettes (1 pipette per jar)
Scale
Fine tip black Sharpie
15 mL tubes (1 per jar)
Tube rack
Parafilm
BD 10 mL syringes (Luer-Lok Tip) (1 per jar)
Whatman 13 mm disposable filters (45 µm pore size)* (1 per jar)
*Filters are Puradisc 13/0.45 PTFE and come in packs of 100, Leur-Lok; Manufactured and distributed by GE Healthcare Life Sciences (Catalog No. 6784-1304)
100 mL bottle of 70% trace metal grade nitric acid (from Protocol 1)
Styrofoam bottle holder*
*This is a rectangular piece of Styrofoam that has been carved to hold 100 mL bottles, generally stored in the drawer labelled “plastic disposable tubes”

P1000 pipette and tips

Safety Materials

Disposable gloves

Heavy duty rubber gloves appropriate for use when handling concentrated nitric acid

Preparation

1. Place a lab pad on the work bench in the basement room.
2. Prepare the syringes and filters (1 of each per jar) by connecting/locking a filter to each syringe.

Procedure

Obtaining the sample:

1. Randomly select one jar from each treatment to sample from.
2. Obtain the jars from the tubs and place them on the lab pad on the bench.
3. Using a fine-tip black Sharpie, label each 15 mL tube, "Zn [Jar number], [date]." Label one tube for each jar.
4. Weigh the tubes, record to the nearest thousandth (g).
5. Place the tubes in a tube rack and set on the work bench in the basement room.
6. Select a jar.
7. Uncap the tube corresponding to the jar you are working with.
8. Remove the plunger from a syringe and set both the syringe and plunger next to you.
9. Using the electronic pipette and a 10 mL pipette, draw up 5 mL of jar water. Take the sample from a depth of about 2/3 down the jar.
10. Dispense the sample into the syringe.
11. Remove the 10 mL pipette from the electronic pipette and set it on the lab pad. Set the electronic pipette on the bench.
12. Return the plunger to the syringe. Hold the syringe over the opening of the 15 mL tube corresponding to the jar. Push the plunger down to filter the sample into the 15 mL tube.
13. Re-attach the 10 mL pipette to the electronic pipette.
14. Repeat steps 8-12 once to filter a total of 10 mL of sample water into the tube.
15. Discard the 10 mL pipette and the syringe/filter into the appropriate hazardous waste container.
16. Cap the tube.
17. Repeat steps 6-16 for the remaining jars.
18. Walk the tubes back to the lab.
19. Weigh the tubes.
20. Place the tubes (held in the tube rack) in the fume hood.

Acidifying the samples:

1. ***Important*** Acidifying the samples using 70% nitric acid should only be done by people who have received the necessary training from Dr. Matos and only under supervision.
2. Turn the fume hood fan to *high*.
3. Put on the heavy duty rubber gloves.

4. Obtain the small bottle of 70% nitric acid from the storage area in the bottom of the fume hood. Place it in Styrofoam bottle holder in the fume hood.
5. Uncap all of the 15 mL tubes. Place the caps on a Kimwipe so that the inside of the cap is not contaminated by dust in the air.
6. Using the P1000, add enough 70% nitric acid to each tube in order to make each sample a 2% nitric acid solution. This can be calculated from the sample weight (g of sample = mL of sample).
7. Discard the pipette tip in the appropriate hazardous waste container.
8. Cap the bottle of 70% nitric acid.
9. Return the bottle to the storage area under the fume hood, close and latch the door.
10. Take off the heavy duty gloves.
11. Cap the tubes.
12. Weigh the tubes.
13. Give each tube a unique ID number for metal analysis, [your initials, a number, W] (W for water sample). Update this information in the Sample Submission Form that will be given to Dr. Nezat prior to metal analysis.
14. Parafilm the caps, then store the tubes in the refrigerator until they can be analyzed.
15. In the *Water Sample Info* spreadsheet, enter the following information from your lab notebook into the appropriate columns: *Tube Label, Tube + Sample Weight (g), Tube + Sample +HNO3 Weight (g), Final Sample Weight (g), Sample Volume (mL), Metal Analysis Label, Amount Acid Added After Filtration (mL),* and *Final Calculated Sample Volume*.

Protocol 8 – Laboratory Pb Assays, Mortality Counts, and Box Tests

Background

Toxicity tests (assays) are performed separately for two trace metals that are present in the Coeur d'Alene (CDA) Basin, Idaho, lead (Pb) as lead nitrate, and zinc (Zn) as zinc sulfate. Lead is not required for amphipod growth and survival, while zinc is an essential ion. The purpose of the assays is to test whether amphipods from the CDA Basin, which is contaminated with trace metals, are tolerant to trace metal stress. These are the methods to perform an acute lead (Pb) 96 hour toxicity test using *Hyalella azteca* collected from the chain lakes and a reference lake. See Protocol 7 for details of amphipod collection and acclimation. Over the course of the Pb toxicity test, amphipod mortality is recorded every 24 hours. Water is changed at every mortality check. Immediately after the 96 hour test, surviving amphipods are subjected to a 10 minute swimming test to measure differences in behavior among the different groups.

Part 1. Making the Pb stock solution

Objective

To make 500 mL of a 1.4 g/L stock solution of lead nitrate. This stock solution will be used to dose the test containers for the lead assay. You should have enough solution for one complete 96 hour assay. Be sure to invert the bottle several times to mix the solution prior to dosing the jars each day, making sure that no precipitate has formed at the bottom of the bottle.

The stock solution should be prepared no later than the day before the start of the assay to provide adequate time to set up and dose the jars.

Personnel

Only one person is required to perform Part 1; only those who have been trained on the handling of toxic chemicals can complete this step. Ask Dr. Matos for assistance.

Supplies and equipment

Lab notebook for record-keeping

10% nitric acid for washing glassware*

*This is generally stored in a large carboy near the sink. See Protocol 1 for more information.

1000 mL glass beaker

1000 mL screw top glass bottle

1000 mL glass graduated cylinder

Milli-Q (ultrapure) water*

*This can be obtained from Dr. Nezat in the Geochemistry lab. This water should be collected in an acid-washed 1000 mL plastic Nalgene bottle.

Labelling tape

Fine tip black Sharpie

Poison stickers from EH&S

Lead nitrate ($Pb(NO_3)_2$), crystal*

*This was obtained from the stock room. 1 lb. bottle, manufactured by J.T. Baker Chemical (Catalog No. 2322-01).
Scale
Plastic weigh boat
P1000 pipette and tips
Kimwipes
Parafilm

Safety Materials

MSDS lead nitrate
Disposable gloves
Disposable respirator
Lab coat
Safety goggles

Preparation

Acid-washing glassware is done prior to making the stock solution (this can be done the night before the stock solution is prepared, or as soon as you arrive in the lab). The stock solution should be made no later than the day before the start of the assay.

7. Acid-wash the following for at least one hour:
    a. 1 – 1000 mL glass beaker
    b. 1 – 1000 mL screw top glass bottle
    c. 1 – 1000 mL glass graduated cylinder
8. Rinse the acid-washed glassware from step 1 with Milli-Q (ultrapure) water to rinse any residual acid.
9. Using label tape and a fine tip black Sharpie, label the beaker and the bottle with the following: "Danger! 1.4 g/L lead nitrate made on [date] by [your initials]." Place Poison sticker(s) on both containers.
10. Read MSDS lead nitrate.
11. Put on lab coat, safety goggles, disposable respirator, and disposable gloves.
12. Obtain the bottle of lead nitrate ($Pb(NO_3)_2$) from the stock room.
13. Place the plastic weigh boat on the scale and press O/T (tare).
14. Put a pipette tip on the P1000 pipette and set the pipette on the bench near the scale. Lay a Kimwipe under the pipette tip to prevent contamination from the lab bench.

Procedure

30. Measure 500 mL Milli-Q (ultrapure) water in the 1000 mL acid-washed graduated cylinder.
31. Pour the ultrapure water into the 1000 mL acid-washed beaker.
32. Place the beaker next to the scale.
33. If you are not already wearing them (from above), put on lab coat, safety goggles, disposable respirator, and disposable gloves.
34. Place the bottle of lead nitrate next to the scale.
35. Carefully open the bottle, be mindful that static cling may have caused some crystals to stick to the cap. If this happens, screw the cap back on and *lightly* tap the bottle on the bench.
36. Open the door to the scale.

37. Grasping the side of the bottle with your right hand, slowly move the bottle opening so that it is positioned over the weight boat inside of the scale.
38. Using your left hand, gently tap the bottle so that the lead nitrate crystals slowly drop out of the bottle, and onto the weigh boat. Do this until you have measured 0.7 g of lead nitrate. If you accidentally pour too much, do not try to put the salt back into the bottle. Instead, add more Milli-Q water to obtain the desired concentration.
39. Place the bottle on the bench and screw the cap on tight.
40. Close the scale door.
41. Set the bottle aside so that it is out of your work area.
42. Open the scale door and carefully remove the weigh boat.
43. Hold the weigh boat over the beaker so that one corner is pointed downward toward the beaker. Fold in the sides of the weigh boat so that the crystals do not fall onto the lab bench. Tip the weigh boat and pour the lead nitrate into the beaker.
44. Holding the weigh boat in your left hand, use the P1000 to draw up some of the solution in the beaker with your right hand. Dispense the solution onto the weigh boat to rinse remaining lead nitrate into the beaker. Repeat this step until all of the lead nitrate is in the beaker.
45. Dispose of the pipette tip and weigh boat in the appropriate hazardous waste container.
46. Gently swirl the beaker until all of the lead nitrate has gone into solution and you do not observe any granules in the beaker.
47. Slowly pour the solution from the beaker into the 1000 mL glass bottle. Cap the bottle.
48. Parafilm the glass bottle and store it in the cabinet to the left of the sink, on the top shelf in the section labeled, “metal work.”
49. Dispose of your gloves in the appropriate hazardous waste container.

Part 2. Preparing the test area and test containers

Objective

To prepare the test area by setting up a grow light, water chillers, and Rubbermaid tanks.

To prepare test containers for the Pb assay. At least two working days before the start of the assay, you will want to set up the tubs/water/jars for the assay so that you can get the test water to the appropriate temperature. Wash and acid-wash the jars one week before the start of the assay. The test jar water should be at the same temperature as the amphipod acclimation water when you begin the assay.

Personnel

While one person can perform this part, it is more efficient to have two or three people working together.

Supplies and equipment

Lab notebook for record-keeping

Fluorescent grow lights in a multi-light (8) fixture*

*These are obtained from the greenhouse and should come with cordage and adjustable locking mechanisms for hanging.
A few feet of extra cordage or pliable wire
Ladder
(Lunchroom) Serving trays*
*These are generally kept on top of the refrigerator
Paper towels
100 wide-mouth or regular-mouth pint Mason jars
10% nitric acid for washing glassware*
*This is generally stored in a large carboy near the sink. See Protocol 1 for more information.
2 Rolls of labeling tape; blue and red
Black Sharpie
Generic plastic wrap
Kimwipes
Milli-Q (ultrapure) water*
*This can be obtained from Dr. Nezat in the Geochemistry lab. This water should be collected in a 2 gallon Nalgene carboy.
1000 mL acid-washed graduated cylinder
2 - Rubbermaid 50 gal (or larger) stock tanks
Dechlorinated water
Liquinox
2 - Water chillers*
*I used a VWR Scientific Model 1167 (from the stock room) and a Julabo Model F12 (from the JMatos Lab)
2 – Thermometers

Safety Materials

Disposable gloves
Lab coat

Procedure

At least two working days before the start of the assay, you will want to set up the tubs/water/jars for the assay so that you can get the test water to the appropriate temperature. The test jar water should be at the same temperature as the amphipod acclimation water when you begin the assay.
Acid-washing the test containers:

5. Wash Mason jars with Liquinox and rinse thoroughly with deionized water. Let the jars dry completely.
6. Autoclave the jars on the hard goods cycle. Let them cool completely.
7. Using label tape and a black Sharpie, label several (4-5) trays, “10% nitric acid.”
8. Arrange the trays on the lab bench near the door. Line the trays with paper towels.

Setting up the basement room (test area):

10. Set up the grow light fixture.

11. Feed a piece of cord through the first locking mechanism. Tie a hitch on the end that will be bearing the weight of the fixture around one of the pipes in the ceiling. Test the hitch to make sure that it can bear the weight of the fixture. On the other end of the cord, tie a stopper knot in case the locking mechanism fails. Repeat this step to set up the other locking mechanism.
12. *Important* Have someone help you with this step. Have a helper hold the light fixture up while you attach the light fixture to the locking mechanisms. Using the carabiner that is attached to the locking mechanism, clip one locking mechanism to each metal hanger on both sides of the light fixture.
13. For redundancy, use another piece of cordage or pliable wire to hitch the fixture to the pipe. Tie the hitch to the hangers you used in step 4. If the locking mechanisms or either of your hitches fail, these secondary cords should keep the fixture from falling into the water-containing tubs below.
14. Position the two Rubbermaid stock tanks that are located in the basement room underneath the grow light. Fill the tanks with water until the water level is about 3-4 inches high. To fill the tanks, you can fill clean 5 gallon buckets with water from the dechlorinated water tap (located above the large fish tank outside of the door; be careful to avoid splashing into the fish tank!).
15. Check the two water chillers to make sure that their reservoirs are full. If more water is needed, obtain about 1 L of deionized water from the lab and top off as needed. It is important to use *deionized* water to prevent mineral buildup inside of the chillers.
16. Position the tubes connected to the water chillers so that they lie inside the Rubbermaid tubs. One water chiller is sufficient to chill water in one Rubbermaid tub.
17. Turn on both water chillers. Set the temperature so that it is the same as the temperature of the water that the amphipods are acclimating in.
18. Place a thermometer in each Rubbermaid tub. Monitor the temperature over the next day or two and adjust the water chiller to that the temperature of the water in the tubs/jars matches the temperature of the amphipod acclimation water.

Preparing the test containers:

15. Put on disposable gloves and a lab coat.
16. Working in the lab, completely fill each jar with 10% nitric acid. Place the jars on the paper towel-lined trays.
17. Cover every jar with plastic wrap to prevent the fumes from corroding nearby metal components in the lab.
18. Let the jars sit for at least one hour, undisturbed.
19. Empty the acid back into the carboy.
20. Use about 50 - 100 mL of Milli-Q water to rinse each jar. Swirl the water around in the jar for about 30 – 60 seconds to remove any remaining acid.
21. Cover the paper towel-lined trays with Kimwipes.
22. Place the Mason jars upside-down on the Kimwipe-lined trays to dry.

23. Once the jars are dry, transfer them to the basement room where the assays will take place. It is best to use the boxes that the Mason jars were purchased in to carry the jars. These are stored in the cabinets in the basement room.
24. Working on the bench downstairs, for each jar, measure 500 mL dechlorinated water in the acid-washed 1000 mL graduated cylinder. Pour this water into the jar.
25. Make an ID "tag" for each jar by placing a piece of label tape on the rim. To do this, position a 2-3 in piece of label tape so that it is perpendicular to the rim of the jar. Place half of the piece on the outside of the rim and the other half on the inside, so that the tape acts as a "tag" that you can write an ID number on. 20 jars should receive blue tape (these will be for the control group), the remaining should receive red tape.
26. Using a black Sharpie, number the jars 1-100.
27. Cover each jar with plastic wrap to prevent evaporation.
28. Place the jars in the tanks.

Part 3. Beginning the Pb assay

Objective

To begin the 96 hour Pb toxicity test by transferring amphipods from the acclimation containers (aquaria) to the test jars. Test jars will have 10 amphipods each. The experimental setup will consist of five groups; four groups will come from four of the chain lakes and one group will come from the reference lake. Each group will have five treatments; a control (0 mg/L) and four doses (5.4, 10.8, 16.2, and 21.6 mg/L).

Personnel

A minimum of two people are required; three or four people is ideal.

Supplies

Lab notebook for record-keeping
125 mL acid-washed plastic Nalgene screw top bottle
Label tape
Black Sharpie
Plastic rectangular Rubbermaid container
Liquinox
Cheesecloth cut into 1 x 1 in squares
2-4 Dog training lab pads
Disposable Pasteur pipettes with the first 1 ½ in of the tip cut off
Aquaria holding amphipods that have been acclimated for at least one week
Timer for grow light
Electronic pipette (Drummond Pipet-aid) and 4 - 5 mL pipettes
Lead nitrate stock solution
1000 mL beaker
100 mL bottle of 70% trace metal grade nitric acid (from Protocol 1)
Kimwipes
Styrofoam bottle holder*

*This is a rectangular piece of Styrofoam that has been carved to hold 100 mL bottles, generally stored in the drawer labelled "plastic disposable tubes"

Safety Materials

Disposable gloves

Preparation

17. Set the timer for the grow light on a 12 dark/12 light cycle.
18. Using label tape and a black Sharpie, label the acid-washed 125 mL plastic Nalgene bottle, "Dechlorinated water collected on [date] by [your initials]."
19. Using the 125 mL plastic Nalgene bottle, collect a water sample from the dechlorinated water tap to be analyzed for trace metals. To do this, run the tap for several minutes then rinse the bottle three times with the dechlorinated water. Collect a sample on the fourth fill. Cap the bottle tightly.
20. Walk the sample back up to the lab and set it in the fume hood.
21. Set the fume hood fan to *high*.
22. Put on the heavy duty rubber gloves.
23. ***Important*** Steps 7-16 must only be performed by people who have received proper training from Dr. Matos and may only be performed under her supervision. Obtain the small bottle of 70% nitric acid from the storage area in the bottom of the fume hood. Place it in Styrofoam bottle holder in the fume hood.
24. Uncap the sample bottle. Place the cap on a Kimwipe so that the inside of the cap is not contaminated by dust in the air.
25. Using the P1000, add 0.625 mL of 70% nitric acid to the bottle to preserve the sample.
26. Discard the pipette tip in the appropriate hazardous waste container.
27. Cap the bottle of 70% nitric acid.
28. Return the bottle to the storage area under the fume hood, close and latch the door.
29. Take off the heavy duty gloves.
30. Cap the bottle.
31. Store the bottle in the incubator in the lab until this sample can be filtered and acidified for metal analysis.
32. Note the day and time of the sample collection in the lab notebook.

Procedure

Part 3 should be started as early as possible on the first day of the assay, which is typically a Monday morning.

Transferring the amphipods to the test containers:

17. In the basement room, lay out two lab pads on the main work bench. These will soak up water from the large tubs as you work with the test jars.
18. Wash a Rubbermaid plastic container with Liquinox and deionized water, rinse thoroughly with deionized water.
19. Take the Rubbermaid container to the basement room and set it on the work bench.
20. Obtain the first aquarium from the incubator and carefully walk it to the basement room. Set it on the work bench with the Rubbermaid container.
21. Obtain 5 blue-taped jars and 15 red-taped jars from the tubs and set them on the lab pads on the bench.

22. Slowly pour a small amount of water/amphipods from the aquarium into the Rubbermaid container.
23. Remove and discard the plastic wrap from the 20 jars that are now sitting on the bench.
24. Using a disposable Pasteur pipette, draw up an amphipod into the pipette from the Rubbermaid container. Submerge the pipette into the Mason jar and let the amphipod swim out. Do not squeeze the water out of the pipette. Repeat this step 9 times to transfer a total of 10 amphipods into the jar.
25. Place a piece of cheesecloth in the jar.
26. In the lab notebook, record the lake from which the amphipods came (labeled on the aquarium), and the jar number and tape color.
27. Return the jar to either tub.
28. Repeat steps 4-11 for the remaining 19 jars. You will need to obtain the other aquarium for this lake from the incubator when you run out of amphipods.
29. As you find all of the amphipods in the Rubbermaid container, discard the water from the container into the drain and pour another small amount of water/amphipods from the aquarium into the container. Using the Rubbermaid container to collect the amphipods from (rather than collecting straight out of the aquarium) is more efficient because the amphipods cannot hide/swim away very quickly when the water level is low. Be sure to check the pieces of cheese cloth in each aquarium for amphipods. To do this, hold the cheese cloth so that it is partially submerged. As you expose the cheese cloth to the air, any amphipods that remain in the cheese cloth will migrate toward the water and swim away. Continue submerging the cheesecloth, then exposing it to the air until you have removed all of the amphipods.
30. If extra amphipods remain in the aquarium after all of the jars have received 10 amphipods, return the aquarium to the incubator.
31. Obtain the aquarium for the next lake.
32. Repeat steps 8-15 until all of the jars have 10 amphipods.

Dose the jars:

19. In the lab, rinse the Rubbermaid container and dry it with paper towels. You will be using it to carry supplies from the lab to the basement room.
20. In the lab, obtain the following and place them into the Rubbermaid container: electronic pipette, 5 mL pipette tips, lead nitrate stock solution (from Part 1), and the 1000 mL beaker. Carry these supplies to the basement room. Do not do this during a break between classes, or any time when there may be many people in the hallway.
21. Using label tape and a Sharpie, label the beaker, “dechlorinated water” and set it on the lab bench.
22. Collect all of the red-taped jars (remember, blue tape is for control jars) that should receive the lowest dose and place them on the lab pads on the bench. You should have five jars per “lake” for a total of 20 jars that will receive this dose.

23. Work as an assembly line. One or two people handle the jars, another person handles the electronic pipette/stock solution, and another person records everything.
24. From each jar, draw up 1.53 mL of water and dispense it in the 1000 mL beaker (this is the equivalent volume of stock lead nitrate that you will add to each jar later for all jars in the lowest Pb dose, the 5.4 mg/L dose). The person(s) handling the jars must keep track of the jars that have/have not had 1.53 mL water removed.
25. When 1.53 mL of water has been removed from all 20 jars, discard the pipette.
26. Unscrew the cap on the bottle of stock lead nitrate.
27. Obtain a new pipette. Draw up 1.53 mL of the lead nitrate stock solution and dispense it into each jar. Do not touch the pipette to the side of the jar or submerge it into the water.
28. Record that these jars (list the jar numbers) have all received 1.53 mL of lead nitrate stock.
29. Return all of the jars that you just dosed to the tubs.
30. Repeat steps 6-11 with the next treatment set of red-taped jars, which is the next highest dose of Pb, 10.8 mg/L. Again, you should have five jars per "lake" for a total of 20 jars that will receive this dose. This time, you will remove 3.06 mL of water from each jar before pipetting 3.06 mL of stock lead nitrate into each jar.
31. Repeat steps 6-11 again for the next treatment set of 20 jars, which is the next highest dose of Pb, 16.2 mg/L. This time, remove 4.59 mL of water from each jar before pipetting 4.59 mL of stock lead nitrate into each jar.
32. Repeat steps 6-11 again for the next treatment set of 20 jars, which is the next highest dose of Pb, 21.6 mg/L. This time, remove 6.12 mL of water from each jar before pipetting 6.12 mL of stock lead nitrate into each jar.
33. 20 control jars should remain in the tubs.
34. Record the time. This is the time that you will perform all mortality checks at during the next 96 hours.
35. Cap the bottle of lead nitrate and parafilm the cap.
36. Carry the electronic pipette and stock lead nitrate solution back to the lab.

Part 4. Daily mortality counts and water change

Objective

Mortality counts must be performed every 24 hours. They must be performed at the same time each day. At the same time that mortality counts are performed, a water change is also performed. Water changes must be done for the test jars because the lead nitrate seems to fall out of solution, creating a precipitate. This means that less of the lead nitrate is available in solution for amphipod exposure.

Personnel

Two people are required to perform mortality checks. It is better to have more than two people working as the process will go much faster.

Supplies

Lab notebook for record-keeping

Enough 2000 mL beakers and carboys to contain at least 50 L of dechlorinated water at acclimation temperature
Several sheets of scratch paper
2 - 1000 mL beakers for jar water changes
4-8 Disposable Pasteur pipettes with the first 1 ½ in of the tip cut off
2 Rolls of labeling tape; blue and red
2 Pint Ziploc bags
Black Sharpie
2-4 Dog training lab pads
1 – 1000 mL acid-washed graduated cylinder, rinsed thoroughly with dechlorinated water
Electronic pipette (Drummond Pipet-aid) and 4 – 5 mL pipettes
Lead nitrate stock solution
1 – 1000 mL beaker for dechlorinated water

Safety Materials

Disposable gloves

Preparation

4. 12-24 hours before the start of each mortality count/water change, bring at least 50 L to the same temperature as the test containers. Fill beakers and carboys with dechlorinated water (cover beakers with plastic wrap to prevent contamination) and store them in the incubator (which should still be set to the same temperature as it was during amphipod acclimation).
5. Wash the two 1000 mL beakers with Liquinox and rinse thoroughly with deionized water. Let dry completely.
6. Using blue label tape and a black Sharpie, label one beaker, "control jars only." Using red label tape and a black Sharpie, label the other beaker, "Pb jars only." Take these beakers to the basement room and set them on the work bench.
7. Use red label tape to mark 2-4 disposable Pasteur pipettes (depending on how many people are helping). These pipettes will only be used when counting mortality in lead-dosed jars. When these pipettes are not in use, they should be stored in a Ziploc bag labeled, "Pb pipettes only."
8. Use blue label tape to mark 2-4 disposable Pasteur pipettes. These pipettes will only be used when counting mortality in control jars. When these pipettes are not in use, they should be stored in a Ziploc bag labeled, "control pipettes only."
9. Lay out the lab pads on the work bench.

Procedure

19. Record the time.
20. Begin with the control jars to avoid accidental contamination.
21. On a scratch sheet of paper (with the date, time, and your initials), list all of the numbers corresponding to the control jars.
22. Obtain a control jar from the tub.

23. Record general notes and observations about the jar (e.g., cloudiness, presence of precipitate at the bottom of the jar, whether the amphipods were swimming and energetic or showing little movement).
24. Slowly pour the contents of the jar into the "control jars only" beaker.
25. Using a blue-taped pipette, locate and remove all of the dead amphipods from the beaker. An amphipod is dead if it does not move after several seconds when prodded with the pipette tip. Occasionally, an amphipod may look dead because it does not move, even though it is alive. One way to tell if it is dead is to draw up the amphipod in the pipette and then quickly move it back out into the beaker. Generally, if it's alive it will move after this. It is also important to check the cheese cloth for dead amphipods. If you have trouble identifying alive from dead, try bringing the cheesecloth to the surface and exposing it to the air. Amphipods that are still alive will migrate toward the water and swim away.
26. Pipette the dead amphipods into a single pile for each jar, onto the lab pad.
27. Record the number of dead amphipods next to the jar number on the piece of scratch paper.
28. Remove and record the number of exoskeletons that you find. Sometimes it is hard to tell an exoskeleton from a dead amphipod. Generally, the exoskeletons are transparent, while the amphipods are not.
29. Using the graduated cylinder, measure 500 mL of the pre-chilled dechlorinated water (from the incubator) and pour it into the empty jar.
30. Pipette the surviving amphipods from the beaker into the jar. Remember to submerge the pipette into the Mason jar and let the amphipod swim out. Do not squeeze the water out of the pipette.
31. Transfer the cheesecloth to the jar.
32. When you have transferred all of the amphipods to the jar, check the jar to make sure that you did not miss any amphipods. If more than one person is working, have others check the beaker also.
33. When you are sure that you have transferred all of the amphipods, and that none remain in the beaker, discard the water.
34. Return the jar to the tub.
35. Repeat steps 4-16 until mortality has been counted in all of the control jars and all jars have received a water change.
36. Next do the dosed jars. Make sure that you wear disposable gloves.
37. Remember to switch to the red-taped pipettes when you move onto the dosed (red-taped) jars. Start with the lowest treatment group first.
38. Using label tape and a black Sharpie, label a 1000 mL beaker, "dechlorinated water only."
39. You will need the bottle of lead nitrate.
40. After you fill each jar with 500 mL of the pre-chilled water, use the electronic pipette and a 5 mL pipette to first remove 1.53 mL of water (dispense into the "dechlorinated water only" beaker, and to then add 1.53 mL of the lead nitrate stock solution. Use different pipettes for the dechlorinated water and stock solution so as to prevent contamination of the stock.

41. When you discard the old test jar water, do so into the appropriate hazardous waste container.
42. Repeat steps 4-23 for the 5.4 mg/L treatment group until mortality has been counted in all of the jars in this group and all of these jars have received a water change.
43. Move to the next highest dose group. Repeat steps 4-23 for the 10.8 mg/L treatment group. This time you will remove 3.06 mL dechlorinated water from the jar, and replace it with 3.06 mL of the lead nitrate stock solution during the water change.
44. Move to the next highest dose group. Repeat steps 4-23 for the 16.2 mg/L treatment group. This time you will remove 4.59 mL dechlorinated water from the jar, and replace it with 4.59 mL of the lead nitrate stock solution during the water change.
45. Move to the highest dose group. Repeat steps 4-23 for the 21.6 mg/L treatment group. This time you will remove 6.12 mL dechlorinated water from the jar, and replace it with 6.12 mL of the lead nitrate stock solution during the water change.
46. When you are done checking mortality and changing the water for all of the jars, wrap up and throw away the lab pad.
47. Record the time.
48. When you return to the lab, transfer the notes from the scratch paper(s) to the lab notebook.
49. Perform mortality checks and water changes as described above every 24 hours for the next 2 days. Be sure to start the mortality counts and water changes at the same time every day.

Part 5. End of assay and box tests

Objective

To end the 96 hour test with a final mortality count and to perform box (swimming) tests on the remaining amphipods. During the box tests, all surviving amphipods from each jar are tested individually in Tic Tac boxes. After a 1 minute acclimation period, the number of times each amphipod swims from the bottom of the box to the water line (surface) is recorded over a 10 minute interval. Generally, one person can watch 1-5 boxes during a 10 minute interval.

Personnel

No less than three people are needed. It is best to have as many people working as can do so safely in the basement room. This is an all-day task.

Supplies

Lab notebook for record-keeping
Label tape
Black Sharpie
5 – 2000 mL beakers
Several sheets of scratch paper
Red- and blue-taped pipettes from Part 4
2-4 Dog training lab pads
100 Empty Tic Tac boxes

Masking tape in tape dispenser
1 - 100 mL graduated cylinder
1 Timer/stopwatch for each person participating in the box tests

Safety Materials

Disposable gloves

Preparation

5. Lay the lab pads out on the work bench.
6. Wash Tic Tac boxes with Liquinox and rinse thoroughly with deionized water. Let dry completely.
7. Using label tape and a black Sharpie, label 5 - 2000 mL beakers, "dechlorinated water only," then fill the beakers with dechlorinated water 24 hours before the start of the box tests in order to chill the water to the same temperature as the test jars. You can chill them in the incubator in the lab which should still be at the same temperature that the amphipods were acclimated at.
8. Determine the jars that you want to perform box tests on, with the understanding that there is not enough time to do all 100 jars by the end of the day. Determine an order of prioritization for jars that should be done first (the number of box tests that you can perform by the end of the day is dependent on the number of people you have helping you). Ideally, you would at least test jars from the control (all lakes) and from the highest dose (all lakes) first in odd numbered replicates (meaning you test 3 jars from a given lake, rather than 2 or 4 jars), then test the next three dose groups (all lakes), again in odd-numbered replicates.

Procedure

96 hour mortality count (performed on all jars):

7. On the last day (Friday if you started the test on Monday), perform mortality counts for each jar. Beginning with the control jars, use the blue-taped pipettes to remove all of the dead amphipods from each jar.
8. Record the number of dead amphipods and the jar from which they came on a piece of scratch paper (with the date, time, and your initials). Also record the number of exoskeletons you remove.
9. Return each jar to the tub.
10. Repeat steps 2-4 until all of the control jars have been checked.
11. Switch to the red-taped pipettes and check all of the dosed jars for dead amphipods, repeating steps 2-3 until all of the dosed jars have been checked.
12. Record the time.

Box (swimming) tests (performed on as many jars as possible):

14. Using the list of priority jars that you created in *Preparation*, begin testing the control jars. Remove the first jar that you wish to test, placing it on a lab pad on the work bench.
15. Using a graduated cylinder, measure 20 mL of the pre-chilled dechlorinated water and pour it into a Tic Tac box. Repeat this step to fill 9 additional boxes. Assuming amphipods did not die in the control jars, you will likely have 10 amphipods to test from each jar (and therefore two people per jar).

16. Using masking tape and a black Sharpie, label the first box, "[Jar number]_1", the second box, "[Jar number]_2", and so forth until all 10 boxes have been labeled with the jar number and a box test ID number (1-10).
17. Using a blue-taped pipette, transfer one amphipod from the jar into each box. When you are dispensing the amphipod into the box, do not squeeze out the water. Instead, submerge the tip of the pipette and let the amphipod swim out.
18. Start the timer for one minute to allow the amphipods to acclimate to the new container.
19. Have someone help you record during the test. You can each watch 5 boxes. If taking care of a jar alone, only transfer five amphipods, test them, then transfer the other five amphipods and test them. Do not leave amphipods in the tic tac boxes.
20. Start the timer for 10 minutes. During the 10 minute interval, record on a piece of scratch paper, the number of times that an amphipod swims from the bottom of the box to the water line (surface). Do not count instances where the amphipod swims from any other depth to the water line. The amphipod must start at the bottom of the box and swim to the water line in order for the effort to count.
21. When the 10 minute test is over, either save the amphipods for trace metal analysis (see Part 6), or dispose of them.
22. Obtain the next jar.
23. Repeat steps 2-9 until you have performed box tests for all of the control jars on your priority list.
24. Begin testing amphipods from the dosed jars. Repeat steps 2-9 for each dosed jar. Be sure to use the red-taped pipettes for these jars (step 4).
25. Discard the control jar water in the sink. Discard the dosed jar water in the appropriate hazardous waste container. Rinse the jars with a small amount of dechlorinated water, pouring this rinse water into the hazardous waste container.
26. Take the jars back upstairs to the lab and wash them with Liquinox. Rinse the jars thoroughly with deionized water and leave them to dry on the dishrack or on trays lined with paper towels.

Part 6. Preservation and storage of surviving amphipods

Objective

To freeze and store amphipods that survived the 96 hour toxicity test so that their tissues can be analyzed for trace metals.

Personnel

One person can do this while at least two others are performing the box tests at the same time (Part 5).

Supplies

Amphipods in Tic Tac boxes from box tests in Part 5
Plastic rectangular Rubbermaid container
Red- and blue-taped pipettes from Part 4
Paper towels

Kimwipes
1 - 2 mL Cryovial per jar
Fine tip black Sharpie
Liquid nitrogen
Liquid nitrogen transport container
Long metal tongs
Weigh paper, folded in half and creased

Safety Materials

Disposable gloves

Procedure

18. When all box tests have been completed for a given jar, use a Rubbermaid container to carry the boxes (still containing the amphipods) to the lab. Also bring one of each colored-taped pipette with you (be sure to keep them in separate Ziploc bags to prevent cross contamination.
19. Place the Rubbermaid container and the Tic Tac boxes on the lab bench near the door.
20. Print a copy of the freezer log.
21. Place a small stack of paper towels on the bench. Cover the paper towels with 1-2 Kimwipes.
22. Label a cryovial the following, "Pb [Jar number], [Date]."
23. Transfer 1 ladle of liquid nitrogen into the transport container.
24. Set the transport container on the lab bench next to the Tic Tac boxes.
25. Drop the cryovial into the transport container.
26. From each box, pipette the amphipod onto the paper towel/Kimwipe. Let the paper soak up all of the water from the amphipod. Blot the amphipod with a Kimwipe if needed.
27. Using the long metal tongs, obtain the cryovial from the transport container. Be careful – some liquid nitrogen may be in the cap.
28. Open the cryovial and set it on the bench.
29. Use a creased weigh paper to scoop up the amphipod. Holding the amphipod in the crease of the weigh paper, position the weigh paper over the opening of the cryovial. Tip the crease slowly, so that the amphipod falls into the cryovial.
30. Quickly cap the vial and drop it into the transport container.
31. Repeat steps 9-13 until all of the amphipods (belonging to that jar) have been frozen.
32. Walk the cryovial to the -80°C freezer in the transport container. Bring the printout of the freezer log with you.
33. Obtain the box from the freezer. Use the large metal tongs to transfer the cryovial to the box. Note the location of the cryovial in the sample box on the printout.
34. Repeat steps 9-16 for all jars from which you wish to save surviving amphipods.

Part 7. Collection and processing of test jar water for metal analysis

Objective

To collect samples of water from the test containers after the completion of Part 6. These samples will be analyzed for trace metals. The lead content will be compared with the dose that the jar received at the beginning of the assay.

Personnel

Only one person is required.

Supplies

1 Dog training lab pad
Electronic pipette (Drummond Pipet-aid) and 10 mL pipettes (1 pipette per jar)
Scale
Fine tip black Sharpie
15 mL tubes (1 per jar)
Tube rack
Parafilm
BD 10 mL syringes (Luer-Lok Tip) (1 per jar)
Whatman 13 mm disposable filters (45 µm pore size)* (1 per jar)
*Filters are Puradisc 13/0.45 PTFE and come in packs of 100, Leur-Lok; Manufactured and distributed by GE Healthcare Life Sciences (Catalog No. 6784-1304)
100 mL bottle of 70% trace metal grade nitric acid (from Protocol 1)
Styrofoam bottle holder*
*This is a rectangular piece of Styrofoam that has been carved to hold 100 mL bottles, generally stored in the drawer labelled "plastic disposable tubes"
P1000 pipette and tips

Safety Materials

Disposable gloves
Heavy duty rubber gloves appropriate for use when handling concentrated nitric acid

Preparation

3. Place a lab pad on the work bench in the basement room.
4. Prepare the syringes and filters (1 of each per jar) by connecting/locking a filter to each syringe.
5. Weigh the 15 mL tubes in the lab, record to the nearest thousandth (g).

Procedure

Obtaining the sample:

21. Randomly select one jar from each treatment to sample from.
22. Obtain the jars from the tubs and place them on the lab pad on the bench.
23. Using a fine-tip black Sharpie, label each 15 mL tube, "Pb [Jar number], [date]." Label one tube for each jar.
24.
25. Place the tubes in a tube rack and set on the work bench in the basement room.
26. Select a jar.
27. Uncap the tube corresponding to the jar you are working with.
28. Remove the plunger from a syringe and set both the syringe and plunger next to you.
29. Using the electronic pipette and a 10 mL pipette, draw up 5 mL of jar water. Take the sample from a depth of about 2/3 down the jar.

30. Dispense the sample into the syringe.
31. Remove the 10 mL pipette from the electronic pipette and set it on the lab pad. Set the electronic pipette on the bench.
32. Return the plunger to the syringe. Hold the syringe over the opening of the 15 mL tube corresponding to the jar. Push the plunger down to filter the sample into the 15 mL tube.
33. Re-attach the 10 mL pipette to the electronic pipette.
34. Repeat steps 8-12 once to filter a total of 10 mL of sample water into the tube.
35. Discard the 10 mL pipette and the syringe/filter into the appropriate hazardous waste container.
36. Cap the tube.
37. Repeat steps 6-16 for the remaining jars.
38. Walk the tubes back to the lab.
39. Weigh the tubes.
40. Place the tubes (held in the tube rack) in the fume hood.

Acidifying the samples:

16. ***Important*** Acidifying the samples using 70% nitric acid should only be done by people who have received the necessary training from Dr. Matos and only under supervision.
17. Turn the fume hood fan to *high*.
18. Put on the heavy duty rubber gloves.
19. Obtain the small bottle of 70% nitric acid from the storage area in the bottom of the fume hood. Place it in Styrofoam bottle holder in the fume hood.
20. Uncap all of the 15 mL tubes. Place the caps on a Kimwipe so that the inside of the cap is not contaminated by dust in the air.
21. Using the P1000, add enough 70% nitric acid to each tube in order to make each sample a 2% nitric acid solution. This can be calculated from the sample weight (g of sample = mL of sample).
22. Discard the pipette tip in the appropriate hazardous waste container.
23. Cap the bottle of 70% nitric acid.
24. Return the bottle to the storage area under the fume hood, close and latch the door.
25. Take off the heavy duty gloves.
26. Cap the tubes.
27. Weigh the tubes.
28. Give each tube a unique ID number for metal analysis, [your initials, a number, W] (W for water sample). Update this information in the Sample Submission Form that will be given to Dr. Nezat prior to metal analysis.
29. Parafilm the caps, then store the tubes in the refrigerator until they can be analyzed.
30. In the *Water Sample Info* spreadsheet, enter the following information from your lab notebook into the appropriate columns: *Tube Label, Tube + Sample Weight (g), Tube + Sample +HNO3 Weight (g), Final Sample Weight (g), Sample Volume (mL), Metal Analysis Label, Amount Acid Added After Filtration (mL),* and *Final Calculated Sample Volume*.

Protocol 9 – Amphipod Tissue Digest for Metal Analysis

Objective

To completely digest amphipod tissues and reconstitute them in dilute trace metal grade nitric acid so that they can be analyzed with the ICP-OES (EWU Geology Department).

Personnel

One person can perform this task but must be able to set aside approximately 6 hours in the lab with little to no interruption or else multiple people are needed.

Materials, supplies, and equipment

Gloves

Hot plate (in fume hood – low fan setting)

Weighing paper

Small (30-50 mL) acid-washed beakers*

*Acid-washed means that the glassware has soaked in 10% nitric acid (does not need to be trace metal grade) for at least one hour. These small beakers can be soaked inside of a 2000 ml beaker that has been filled with acid. Just make sure that every part of the beaker is completely submerged. Also be sure to label the 2000 mL beaker "10 % nitric acid" in the case of an emergency. The acid is recycled after use into a large carboy that is generally stored next to the sink.

Centrifuge tube pestle (plastic tip)

Liquid nitrogen

Liquid nitrogen transport container

Large metal tongs

Approximately 80 mL of 2% trace metal grade nitric acid (HNO_3)*

*See Protocol 1; this solution has been diluted from 70% stock (1 L bottle); Manufactured by Fluka Analytical, Distributed by Sigma-Aldrich (Catalog No. 843850), stored under hood in secondary container.

30% trace metal grade hydrogen peroxide (H_2O_2)*

*Stock is a 100 mL bottle; Manufactured by GFS Chemicals (Catalog No. 3984), stored in refrigerator.

Pipette and tips (P1000)

Pipette tip disposal container

15 mL centrifuge tubes

BD 10 mL syringes (Luer-Lok Tip)

Whatman 13 mm disposable filters (45 µm pore size)*

*Filters are Puradisc 13/0.45 PTFE and come in packs of 100, Leur-Lok; Manufactured and distributed by GE Healthcare Life Sciences (Catalog No. 6784-1304).

Electronic Pipette (Drummond Pipet-aid)

5 mL disposable plastic pipette

Parafilm

Nitric acid dry goods waste container

Scale

Safety Materials

Thermal gloves appropriate for handling hot glassware

Preparation

1. Make sure that the electronic pipette is charged.
2. Make an 80 mL solution of 2% trace metal grade nitric acid by following Protocol 1. Set this bottle on the lab bench.
3. Determine which samples you will process. Be sure to randomize samples to avoid processing all samples collected on one day at the same time.
4. Print a Freezer Log map. Circle the samples you will collect from the freezer.
5. Using label tape and a black Sharpie, label the small acid-washed beakers (same label as is on the cryovial + date of digest).You need one beaker per sample.
6. Label one 15 mL tube per sample (same label as is on the cryovial + date of digest). ***Important* be sure to write "amphipods" on the tube to prevent confusion with water samples collected on the same date.**
7. Weigh each 15 mL tube and lid. This is most easily done by putting the tube upside down, on its lid, on the scale. Record mass to the thousandth of a gram. If you are using random numbers, label each tube with its random number and label each beaker with actual sample information AND random number so that you do not make an error when transferring the sample from the beaker to the tube.
8. Set up hot plate in the fume hood.

Procedure

1. Transfer two ladles full of liquid nitrogen from the stock tank to the liquid nitrogen transport container. Secure lid on the transport container as well as the stock tank.
2. Retrieve the samples (cryovials) you will need from the -80°C freezer, transporting them in the liquid nitrogen. Record on the Freezer Log map that these samples were removed.
3. Put on gloves.
4. Pour a small aliquot of the stock 30% hydrogen peroxide into a small container, such as a 50 mL tube to prevent contamination of the stock. A single digest requires 4-6 mL of hydrogen peroxide. It is more time-efficient to digest several (3-7) samples at a time if you can manage it.
5. Pipette 2.0 mL 30% hydrogen peroxide into the first beaker corresponding to the first cryovial you will be processing amphipods from. Place the beaker so that it is close to the scale and readily available.
6. Fold a piece of weigh paper in half, place it on the scale, and then tare it.
7. Remove the weigh paper from the scale, close the scale door, and place the weigh paper on the lab bench.
8. Remove the cryovial from the transport container using the large metal tongs.
9. Confirm that the label on the cryovial is the sample you want to process.
10. Do not tap the cryovial on the bench as this may break legs/limbs off of the amphipods – they are very fragile.

11. Holding the cryovial over the weigh paper, carefully open the cryovial and empty its contents onto the weigh paper. If there are more than 15 (large) or 20 (small) amphipods in a sample, put the rest back into the cryovial and drop the cryovial back into the liquid nitrogen transport container. If small bits of tissue are stuck in the cryovial or its lid, use an autoclaved pipette tip that has been dipped in liquid nitrogen to remove them. Be mindful of static cling. This can cause amphipods to stick to your glove, the cryovial, the weigh paper, or anything in your work space if you aren't paying attention.
12. Weigh the amphipods using the weigh paper. This is a pooled weight, rather than several individual weights.
13. Holding the weigh paper folded (to prevent dropping amphipods), move it over the beaker and drop the amphipods into the hydrogen peroxide. Gently swirl the beaker.
14. Carefully walk the beaker to the hot plate. Turn the temperature of the hot plate to 100°C. *Note – If the sample is not digesting in a timely fashion, you may increase the temperature to 125°C, but no higher as the beakers are susceptible to cracking. You can tell that the tissues are digesting if you observe amphipod body color change (grey>pink>white) and constant bubbling. You do not need to mash or otherwise disturb the amphipods during the digest. As the process goes on, the tissues will become translucent and break apart into smaller pieces.
15. Repeat steps 5-14 until all samples are on the hot plate(s). (The most samples that I have been able to digest in one day is 15 in a 9 hour period with few interruptions.) I would suggest having no more than 5 beakers on a hot plate at one time. This means that if you want to process more than 10 samples (our lab only has two hot plates), you will need to borrow an extra hot plate from the stock room.
16. Watch the beakers diligently. Never leave the lab with the hot plate(s) on.
17. You will likely need to add 1 to 5 more mL of hydrogen peroxide to a given beaker over the course of the digest. Be sure to keep track of how much of the 30% hydrogen peroxide that you are adding to each beaker. All of the tissue must be completely broken down before you proceed. You will know that the tissue has completely broken down because you will not see any tissue chunks or miniscule particles remaining. Bubbling and fizzing will also have slowed/lessened. You will only see the hydrogen peroxide, which may look cloudy depending on how much tissue was digested, with minimal fizzing.
18. Once the tissue has broken down and is dissolved in a given beaker, let the hydrogen peroxide evaporate off. All that will remain is a white powder/paste. ***Important* Remove the beaker from the hot plate as soon as the hydrogen peroxide has evaporated off so that the beaker does not crack.**
19. When all that remains in a beaker is the white powder/paste, you may proceed to the next step. Be sure to periodically check on the other beakers that are still cooking as you proceed.
20. Walk the beaker over to the lab bench.

21. Using either the electric pipette or the P1000, add 5.0 mL of 2% nitric acid to the beaker. Take care not to touch the pipette to the side of the beaker. Gently swirl the beaker. Let the beaker sit covered with a Kimwipe and undisturbed for 15 minutes.
22. Prepare a syringe and filter for the sample while you wait. To do this, gently screw a Whatman 13 mm disposable filter to the tip of a BD 10 mL syringe. Using label tape and a fine tip Sharpie, label the syringe so that its label matches the label on the beaker.
23. Set up all of the pre-weighed 15 mL tubes on a tube rack, with empty spaces between each tube so that you can work with them.
24. After the 15 minute wait, select the 15 mL tube corresponding to the beaker you are ready to filter. Unscrew the 15 mL cap from its tube. Place the 15 mL tube cap right side up on a clean Kimwipe so that the inside of the cap is not contaminated by dust from the air.
25. Remove the plunger from the syringe and set it on a Kimwipe. Hold the syringe upright in your left hand, positioning it directly over the opening of the 15 mL tube. It is important to hold the syringe over the tube in case the filter is not screwed on tightly or if the filter does not stop the sample from moving out of the syringe. Select the beaker, double-checking that you are pouring the sample from the beaker into the correct tube. This is your last chance to catch an error in sample ID. Slowly pour the sample from the beaker into the open syringe. You can stabilize the beaker using your left index finger. Try to get every drop from the beaker into the syringe. Be patient because this may take a minute or so. Once all of the sample is in the syringe, set the beaker aside. Place the plunger back into the syringe.
26. Filter the sample into the 15 mL tube by pushing the plunger down. If possible, avoid putting the weight of the syringe and your force on the 15 mL tube to avoid accidental tipping or breaking of the syringe/filter. Push the plunger until all of the sample has been transferred from the syringe to the 15 mL tube. Set the syringe aside. This could take several minutes and may require you to lean over the syringe.
27. Pipette an additional 5.0 mL 2% nitric acid into the beaker, ejecting the acid around the sides of the beaker to put any remaining sample into solution.
28. Remove the plunger from the syringe, setting it down on a Kimwipe, then swirl and pour the remaining sample and acid into the syringe. Replace the plunger and filter the solution into the 15 mL tube. Cap the 15 mL tube.
29. Weigh the 15 mL tube and record the weight; the difference between this 'full' weight and the initial weight is the volume that will be used in metal content calculations. Then wrap the cap and top of the tube with parafilm.
30. Store the 15 mL tube in the refrigerator until metal analysis.
31. Repeat steps 18-22, 24-30 until all of the samples have been completely processed and no beakers remain on the hot plates.
32. Turn off the hot plates.
33. Update the Freezer Log.

34. Update the Amphipod Sample Info spreadsheet with the following information: tube labels, number of amphipods digested in each sample, the pooled animal weight for each sample, and the sample volumes/weights.
35. Fill out a Sample Submission Form. This form will be given to Dr. Nezat. In the form, assign a Sample ID to each sample in the second column (e.g. in Figure 1., CSH129 gives my initials and a number. Alternatively, you could use the letter A for amphipod and W for water followed by a number). In the third column of the spreadsheet, list the name of the sample (the label on the 15 mL tube). In the fourth column, list the collection date, and in the sixth column, list the sample type (i.e. water, soil, tissue, or other), in the last column, list analysis requested (what metals you are interested in). Prior to the date of analysis, send this spreadsheet to Dr. Nezat. See Protocol 10 for more information on ICP-OES metal analysis.

Figure 1. Example Sample Submission Form

9.30.16 Sample Submission Form and Lab Chain of Custody

Sample Description *Boxes with grey fill are to be completed by lab personnel only

Internal Lab ID	Sample ID	Description	Collection Date[a]	Time[a]	Sample Type			No. of containers	Analysis Requested
					Water	Soil	Other		
	CSH129	Dechlor H2O 8.29.16	8.29.16		x			1	Pb, Zn, Cu, As, Ca, Mg
	CSH130	Bull Run 9.17.16	9.17.16		x			1	Pb, Zn, Cu, As, Ca, Mg

Protocol 10 – ICP-OES Analysis

Background

Metal analysis is performed by Inductively Coupled Plasma Optical Emission Sensor (ICP-OES). This instrument is located in the EWU Geology Department in the Geochemistry Lab and is operated by Dr. Nezat.

Part 1. Preparing samples and spreadsheets for ICP-OES analysis

Objective

To prepare samples for ICP-OES analysis by giving them a unique analysis identification number, by preparing a Sample Submission Form, and by submitting that form to Dr. Nezat prior to the day of analysis.

Personnel

Only one person is required.

Supplies

(Microsoft Excel) Sample Submission Form Template*

*This can be obtained from Dr. Nezat.

15 mL tubes holding samples that are ready to be analyzed

Black Sharpie

Procedure

For water samples:

1. Create a spreadsheet labeled *Water Sample Info*.
2. In *Water Sample Info*, create the following columns: *Date Processed, Tube Label, Tube Weight (g), Tube + Sample Weight (g), Tube + Sample +HNO_3 Weight (g), Final Sample Weight (g) = Sample Volume (mL), Final Condensed Volume (mL), and Metal Analysis Label, Amount Acid Added After Filtration (mL),* and *Final Calculated Sample Volume*. If it is easier for you to stay organized as you process the samples, you can split these columns into two separate tabs (See Figure 1, 2). Enter data from your lab notebook into the appropriate column.

Figure 1. Sample of *Water Sample Info (LW 10.22.16 tab)*.

Tube Label	Tube Weight (g)	Tube + Sample Weight (g)	Tube + Sample + HNO3 Weight (g)	Sample + HNO3 Weight (g)	Final Condensed Volume (mL) Pooled Samples	Metal Analysis Label "CSH"	Date Analyzed
Benewah 10.22.16	6.586	18.504	20.963	14.377	160	154	
Benewah 10.22.16*NoBoil*	6.654	18.486	18.903	12.249		153	

Figure 2. Sample of *Water Sample Info (LW 10.22.16 Acidification).*

Date processed	Sample Tube Label	Sample weight (g) = Sample Volume (mL)	Amount acid added after filtration (mL of 10% HNO3)	Final calculated volume (mL)
12.14.16	Benewah 10.22.16	11.92	2.98	14.90
12.14.16	Benewah 10.22.16*NoBoil*	11.83	0.35 mL 70 % HNO3	12.18

3. From the Sample Submission Form Template, save a new Sample Submission Form for the next analysis date. Analyses are typically run in batches of 30 samples.
4. Randomly select 30 samples to analyze. You can assign each sample a randomization ID number and use a random number generator, such as Random.org to select the samples.
5. Using a black Sharpie, label the 15 mL tubes with a unique ID, such as your initials, a number, and W for water samples or A for amphipod samples (e.g. CSH21A). Store your copy of the sample key (linking ID number to full sample information) in multiple places/formats.
6. In the Sample Submission Form, enter the sample information and the labels that you just marked on the tubes.
7. Return to *Water Sample Info* spreadsheets and update the *Metal Analysis Label* column with the sample ID.
8. Send Dr. Nezat a copy of the Sample Submission Form prior to the start of the analysis.
9. Schedule a time to analyze the samples with Dr. Nezat. She will operate the instrument, but you will need to stay in the lab to help. Analysis generally takes 5-7 hours to complete.

For amphipod samples:

1. Create a spreadsheet labeled: *Amphipod Sample Info*.
2. In *Amphipod Sample Info*, create the following columns: *Date of Digest, Cryovial Label, Pooled Animal Weight (g), # of Animals Digested, 15 mL Tube Label, Tube Weight (g), Tube + Sample + Acid Weight (g), Sample Weight (g) = Sample Volume (mL),* and *Metal Analysis Label*. Enter data from your lab notebook into the appropriate column (See Figure 3).

Figure 3. Sample of *Amphipod Sample Info.*

Date of Digest	Cryovial Label	Pooled Animal Weight (g)	# of animals digested	15 mL Tube Label	Tube Weight (g)	Tube + Sample + HNO3 Weight (g)	Sample Weight (g) = sample volume (mL)	Metal Analysis Label "CSH"
8.9.16	Medicine 6.4.16	0.047	14	Medicine Amp. 6.4.16	6.649	16.307	9.658	125
8.9.16	Rose 6.4.16	0.052	15	Rose Amp. 6.4.16	6.621	15.763	9.142	126

3. From the Sample Submission Form Template, save a new Sample Submission Form for the next analysis date. Analyses are typically run in batches of 30 samples.
4. Randomly select 30 samples to analyze. You can assign each sample a randomization ID number and use a random number generator, such as Random.org to select the samples.
5. Using a black Sharpie, label the 15 mL tubes with a unique ID, such as your initials, a number, and W for water samples or A for amphipod samples (e.g. CSH21A). Store your copy of the sample key (linking ID number to full sample information) in multiple places/formats.

6. In the Sample Submission Form, enter the sample information and the labels that you just marked on the tubes.
7. Return to *Amphipods Sample Info* and *Water Sample Info* spreadsheets and update the *Metal Analysis Label* column with the sample ID.
8. Send Dr. Nezat a copy of the Sample Submission Form prior to the start of the analysis.
9. Schedule a time to analyze the samples with Dr. Nezat. She will operate the instrument, but you will need to stay in the lab to help. Analysis generally takes 5-7 hours to complete.

Part 2. ICP-OES analysis

Objective

To analyze samples in the ICP-OES.

Personnel

Only one person is required; the person who monitors the instrument must be approved by Dr. Nezat and typically has taken Dr. Nezat's class.

Supplies

15 mL tubes holding samples that are ready to be analyzed; stored at 4°C in parafilm-sealed tubes

Standard Operating Procedure for EWU Geochemistry Lab: Quality Control and Quality Assurance; see below (Authored by Dr. Nezat)*

*An electronic copy of this can be obtained from Dr. Nezat. A copy is also stored in the JMatos Dropbox folder.

Standard Operating Procedure for EWU Geochemistry Lab:
Quality Control and Quality Assurance

Before analytical data can be used for a research paper, report, etc., the quality of the data needs to be checked.

Objective of this exercise: To determine the quality of the data. Was the analysis accurate and precise? What is the lowest concentration that can be accurately measured by this instrument?

Instructions

Sort the rows of data so that all of the blanks and check standards are in the first few rows, and the samples are listed after that. For ICP-OES data, see the file "Template for Data" for an example of this format.

Note that you only need about 3-4 significant digits for the analyte concentrations. But for each element, be consistent with the number of places behind the decimal point. Right justify the numbers (and the row headings) so that they can easily be read and compared.

1) Determine if the instrumental blanks show any evidence of contamination during analysis. Also determine the limit of detection and the limit of quantitation for each element.

Using the replicate analysis of the blanks, calculate the following for each element. Note that the "blanks" unless otherwise noted are instrumental blanks.

- Calculate the mean concentration of each element; ideally these values should be small and may even be negative.

- Calculate the limit of detection (LOD) which is the lowest analyte concentration that can be reliably distinguished from a concentration of zero, and at which detection is feasible (definition adapted from http://www.ncbi.nlm.nih.gov).

LOD (limit of detection) = 3 * standard deviation of all blank concentrations (Equn 1)

- Different instruments have different detection limits. For example, the detection limit for Si is typically ~0.01 mg/L on an ICP-OES, and ~1 mg/L on a UV-Vis spectrophotometer.
- Calculate the limit of quantitation (LOQ) which is the lowest concentration at which an analyte can not only be reliably detected and quantified (adapted from http://www.ncbi.nlm.nih.gov).

LOQ (limit of quantitation) = 10 * standard deviation of all blank concentrations (Equn 2)

2) Determine the accuracy of the analysis.

- First, find the certified values for the elements in each check standard (e.g., TMDW, Soil Solution B, River Sed B), and add these data to the spreadsheet. For ICP-OES analysis, the concentrations of the undiluted check standards may be provided in pdf files.
- Next, calculate the mean concentration of each element in the replicate analysis of each check standard.
- Calculate %rsd (%relative standard deviation). This number shows the **precision** (variability in the analysis of each element) over the course of a run. Ideally, this percentage should be less than 10%.

%rsd = standard deviation/average*100 (Equn 3)

- Calculate **% recovery** (= mean/certified value * 100). Do not calculate this value for an element if it is not quantified in the check standard. The **% recovery**, a measure of **accuracy**, should lie between 90% and 110%; if a % recovery for an element is outside this range, change the font color of the **% recovery** to **red**.

Note: for any percentages you calculated above, format the cells so that the values are in whole numbers (no need for several significant digits here).

3) Compare the analyte concentrations in your samples to the blank concentrations, LOD, LOQ, and the percent recoveries. To denote analyte concentrations in the samples that may not be trustworthy, you will change the font color of those concentrations so that you can scan your data and quickly determine which data are reliable. Complete the tasks below in the order that they are listed; once you have changed the font color of a number, do not change it to another color.

Compare the concentrations in the samples to LOD and LOQ.

- If an analyte concentration in a sample is < LOD, change the font color of that concentration to **red**.

- If an analyte concentration in a sample is < LOQ but > LOD, change the font color of that concentration to **orange**.

Check the recovery of each element in the check standards. You may find poor recovery of an element at lower concentrations (especially when that concentration is close to LOD or LOQ) but good recoveries at higher concentrations. If a sample

concentration is similar to the concentration in the check standard which has a poor recovery (i.e., <90% or >110%), change the font color of that sample concentration to **blue**.
Compare the element concentrations in the **procedural blank** (the blank that you treated as one of your samples) to the sample concentrations. Can you identify any significant amount of contamination in the samples from your procedure (i.e., the analyte concentration in a procedural and/or instrumental blank is >5% of a sample)? If so, highlight the analyte concentration **red**.
Compare the sample concentrations to the calibration standards. Are any sample concentrations higher than the highest calibration concentration? If so, change the font of the sample concentration to **purple**.

Definitions:

Calibration standards: a series of standards covering a range of analyte concentrations and used to calibrate the instrument; the calibration standards are made by diluting a stock solution
Calibration blank: for the ICP-OES analysis, 2% nitric acid (made with trace metal nitric acid and deionizined water); the 2% nitric acid is what is used to dilute the stock to make the calibration standards
Procedural blank: typically DI water or nitric acid that was treated as a sample
Instrumental blank: blank analyzed as a sample on the instrument
CRM = certified reference materials = check standard: standards used to check the precision of an analysis

Procedure

1. On the day of analysis, carry the samples to the Geochemistry lab.
2. When prompted to do so by Dr. Nezat, enter the sample IDs in the order that the samples are placed in the auto sampler.
3. When prompted to do so by Dr. Nezat, remove the caps from all of the sample tubes and lay them on Kimwipes in front of the auto sampler so that the insides are not contaminated by dust in the air. This is important because you will generally have enough sample at the end of the analysis to save for an additional run if necessary, and you don't want to contaminate your samples.
4. Dr. Nezat will start the machine. You will watch the monitor on the ICP-OES computer to check for issues such as "drifting" or if *Recovery* fails. She may instruct you to be on the lookout for other issues/symptoms of problems, depending on how the instrument has been behaved in recent analysis.
5. When the analysis is complete, Dr. Nezat will prompt you to cap and remove all of the sample tubes from the auto sampler.
6. She will email you an Excel file containing the details of the analysis. Save the original version of this file and then create a new, separate copy of the same file that you will work with.
7. Follow Standard Operating Procedure for EWU Geochemistry Lab: Quality Control and Quality Assurance using the "working" copy of Excel file you just saved.

8. In cases where the sample concentration for a given element falls below the limit of detection (LOD) for the instrument, report the concentration for that element as ½ * LOD for statistical analyses. Also report the number of samples that were <LOD.
9. If you boiled down your water samples in Protocol 4, you will need to calculate the metal concentrations of the water samples before they were condensed. To do this, divide the concentration of the element from the analysis (mg/L) by 1000 (mL) and multiply by the final condensed sample volume (mL). Repeat for each element in the analysis.
10. In Figure 4, the data have been altered as per the Standard Operating Procedure for EWU Geochemistry Lab: Quality Control and Quality Assurance. The following columns have also been added: *Sample Info, Final Condensed Sample Volume (mL) (from Water Sample Info spreadsheet), and True Ca, Mg, Pb, Zn, As, Cd, Cu.* Concentrations for "NotBoiled samples (i.e., Rose 9.17.16 NotBoiled) can be copied into the "True" column for each element (The Final Condensed Sample Volume entry is always nb – which stands for "not boiled"). For samples that were boiled/condensed, you must calculate the elemental concentrations by taking into consideration volume that the original sample was condensed down to during the boiling procedure (see Protocol 4).
11. See Equation 1 for an example of how to calculate the "true" concentration of a water sample that was boiled down.

Figure 4. Sample of *20160923 Metal Analysis Working.*

		Ca4226	Mg2795	Pb2203	Zn2138	As1890	Cd2144	Cu3247	Final Condensed Sample Volume mL	True Ca	True Mg	True Pb 2203	True Zn	True As	True Cd	True Cu
ID	Sample Info	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L								
CSH-111	Rose 9.17.16	20.186	7.6924	0.0614	0.3176	0.0041	0.00046	-0.0008	150	3.0329	1.1558	0.0092	0.0477	6E-04	7E-05	-1E-04
CSH-112	Rose 9.17.16 NotBoi	4.2156	1.5778	0.0094	0.0632	0.0021	0.00009	-0.0078	nb	4.2156	1.5778	0.0094	0.0632	0.002	9E-05	-0.008

Equation 1. Example of how to calculate the "true" concentration of Ca in a water sample that was boiled down (from *20160923 Metal Analysis Working*), where 20.186mg/L is the concentration reported for the analysis (condensed), 1000 mL was the original volume of the sample we condensed, and 150 mL is the condensed sample volume (after boiling).

$$True\ Ca\frac{mg}{L} for\ Rose\ 9.17.16 = \frac{20.186\ mg/L}{1000\ mL} * 150\ mL$$

APPENDIX 2

Methods – SAS Code

Analysis 1: Generation of LC_{50} values

This analysis used the PROBIT procedure of SAS in a logistic regression. Code modified from SAS OnlineDoc, Version 8: Chapter 54: The PROBIT Procedure. Note that the code generates both the normal and the logistic responses, only the logistic output was used. Data were: Dose = metal level in jar; N = number of amphipods within each jar; Response = the number of amphipods that died.

```
data a;
infile cards eof=eof;
input Dose N Response;
Observed= Response/N;
output;
return;
eof: do Dose=0 to 60 by 5;
output;
end;
datalines;
0     10    10
0     10    10
<datalines>
58    10    9
58    10    6
;
proc probit log10;
model Response/N=Dose / lackfit inversecl itprint;
output out=B p=Prob std=std xbeta=xbeta;
title 'Output from Normal Probit Procedure';
run;
ods graphics on;
proc probit log10 plot=predpplot;
model Response/N=Dose / d=logistic inversecl;
output out=B p=Prob std=std xbeta=xbeta;
title 'Output from Logistic Probit Procedure';
run;
ods graphics off;
```

Analysis 2: Comparison of swimming activity

This analysis used the MIXED procedure of SAS with error estimated by restricted maximum likelihood (REML). The dependent variable was the log(x+1) transformed swim data (number of surfacings in the ten-minute period). Model would not converge with 'jar' as a repeated term. Data are Population = Lake ID, Dose = quantity of metal in the testing jar, Jar = Jar number, altered to be 1-5 for each dose, Rep = amphipods within the jar, and logswim = dependent variable. These analyses were run within an experiment to test whether the effects of dose differed across each lake population.

```
data JuZnswim;
input Pop $ Dose Jar Rep logswim @@;
datalines;
Anderson    0     1     1     1.079181246
<data lines>
Rose  58    2     2     0.301029996
;
proc mixed covtest data=JuZnswim;
class Pop Dose Jar Rep;
model logswim=Dose|Pop / ddfm=kenwardroger;
random jar/group=dose*pop;
lsmeans dose*pop;
run;
```

The analysis was repeated, but restricted only to those amphipods that were in control conditions to test whether swimming activity differed across season, first tested within each metal type.

```
data Pbseasonswim;
input Season Pop $ Dose Jar Rep logswim @@;
datalines;
<data lines as above but with extra column for season 1, 2, 3>
;
proc mixed covtest data=Pbseasonswim;
class Season Pop Dose Jar Rep;
model logswim=Pop|Season / ddfm=kenwardroger;
lsmeans pop*season;
run;
```

The analysis was repeated with Zn and Pb assays run together, to test whether seasonal differences in swimming activity of amphipods in control conditions differed across metal toxicity test type.

```
data seasonswim;
input Metal $ Season Pop $ Dose Jar Rep logswim @@;
datalines;
<data lines as above but with extra column for metal Pb Zn>;
proc mixed covtest data=Pbseasonswim;
class Metal Season Pop Dose Jar Rep;
model logswim=Pop|Season|Metal / ddfm=kenwardroger;
lsmeans pop*season*metal;
run;
```

Analysis 3: Comparison of amphipod length

Amphipod lengths were analyzed with the MIXED procedure with REML and degrees of freedom calculated with the Kenward Roger procedure. Data were amphipod length in mm. The model was a version of a two-factor ANOVA with Month, Lake, and Lake*Month as the fixed factors, the collection site within each lake was the repeated factor, nested within the Lake*Month group. Although the code requests post hoc Tukey tests, the output was not evaluated as the number of tests was very large; the degrees of freedom code following the Tukey code corrects the numbers of degrees of freedom used for the post hoc tests.

```
proc mixed covtest data=alllength;
class lake month site;
model lgth=lake month lake*month / ddfm=kenwardroger;
repeated site/group=lake*month;
lsmeans lake month lake*month / adjust=tukey adjdfe=row;
run;
```

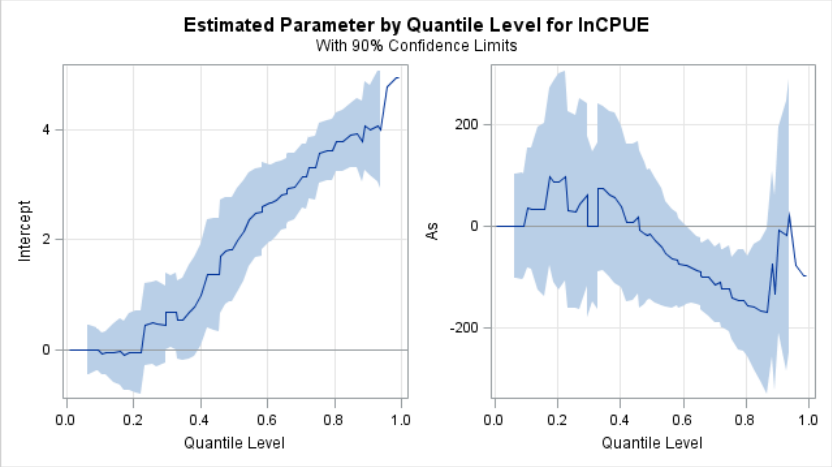
Analysis 4: Quantile Regression of Catch per Unit Effort and Environmental Variables

The code for this analysis was derived from Chapter 97: The QUANTREG Procedure. SAS/STAT 14.2 User's Guide. 2016. Cary, NC: SAS Institute Inc. The final analysis did not

include data from Benewah L., although initial runs did include Benewah L. The data are entered per lake per month, (but neither lake nor month is indicated) with CPUE averaged across site: CPUE = catch per unit effort, each metal (As, Cd, Cu, Pb, Zn), the sum of the metals, hardness, and lnCPUE. This analysis estimates all quantiles.

```
data CPUEMetalnoBen;
input CPUE As Cd Cu Pb Zn Sum Hard lnCPUE @@;
datalines;
18    0.000226701 2.54772E-05 0.001778309 0.004845763 0.01129659
      0.01817284
      12    2.944438979
<data lines>
30    0.0048026   0.0003293   0.000581148 0.01466495  0.06636875
      0.086746748 27    3.433987204
;
ods graphics on;
proc quantreg data=CPUEMetalnoBen alpha=0.1 ci=resampling;
model lnCPUE = As / quantile=process seed=1268 plot=quantplot;
run;
```

This code generates graphs of the intercept and slope at each quantile, illustrated here for arsenic's data (without Benewah L., with the October Killarney data point).

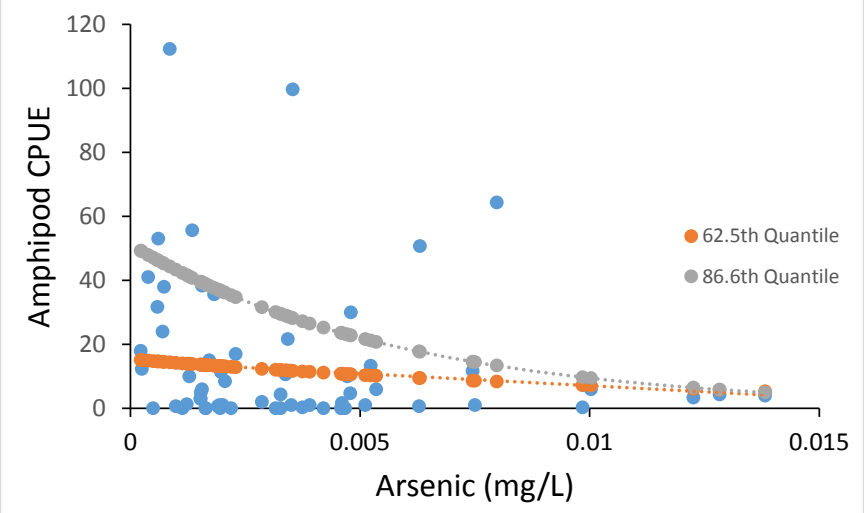


The analysis generates estimates of the intercept and slope at each quantile, with 90% Confidence intervals. Where the slopes are significantly different from zero (blue shading does not overlap with the zero line and the 90% CI do not overlap zero, the

estimates of slope and intercept can be used to generate lines that define the quantiles by entering for X the arsenic content and by taking the exponent of the result (as model analyzed ln(CPUE).

Parameter Estimates for Quantile Process			
Label	Quantile Level	Intercept	As
t0	0.006557	0.0000	0.0000
lower90	0.006557	-0.5827	-207.858
upper90	0.006557	0.5827	207.8584
t1	0.021355	0.0000	0.0000
lower90	0.021355	-0.8711	-185.171
upper90	0.021355	0.8711	185.1711

For example, for As, the slope of the quantile was significantly nonzero from the 62.5th quantile to the 86.6th quantile. At the 62.5th quantile, the slope estimate was -76.2 (As on the output table) and the intercept was 2.73. To generate estimates of amphipod CPUE at this quantile, solve: y = exp((-76.2*[As])+2.7306) for all As data in the dataset. This generates lines that can overlay the CPUE vs. AS graph:



Additional quantile regression analyses tested multiple factors at the same time including all ten lakes in the analysis and focusing on the limnological variables. For example, with just the data collected in September, this analysis tested whether three factors (temperature, DO, pH) were related to ln(CPUE), testing first at all quantiles.

```
data SeptCPUE;
length Lake$ 10;
input Lake Cpue Temp DO pH LnCpue @@;
datalines;
Anderson    51.7  15.3  6.7   7.0   3.9
<datalines>
Thompson    100.7 16.4  7.1   7.3   4.6
;
ods graphics on;
proc quantreg data=SeptCPUE ci=resampling;
model LnCpue = Temp DO pH / quantile=process plot=quantplot seed=1268;
run;
```

The second half of the code can be altered to test at specific quantiles:

```
proc quantreg data=SeptCPUE ci=resampling;
model LnCpue = Temp DO pH / quantile=0.5 0.55 0.6 0.65 0.7 0.75 0.8
0.85 0.9 plot=quantplot seed=1268;
run;
```

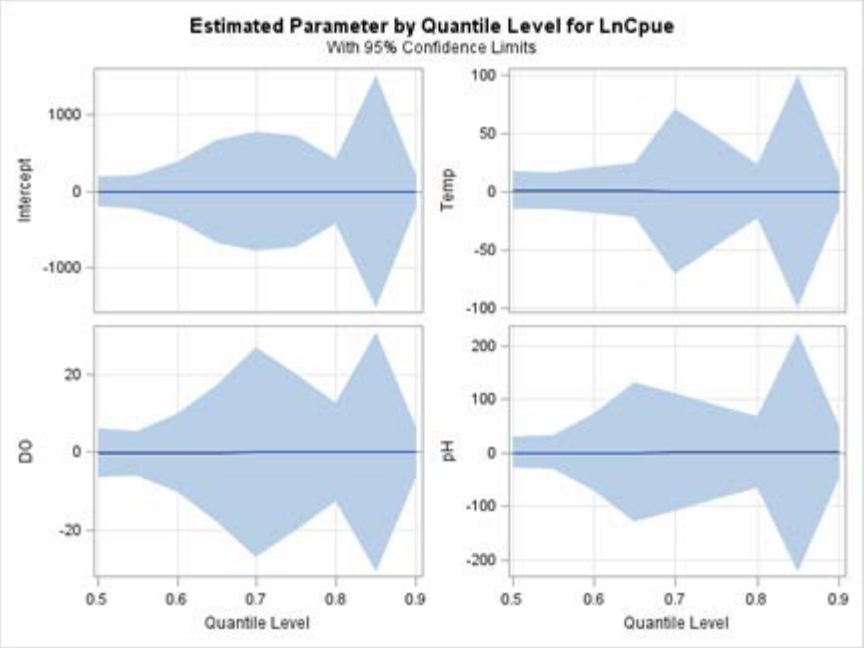
This quantile-specific analysis generates estimates of intercept and slopes at each quantile independently, as well as predicted values (of ln(CPUE) at the mean:

Quantile Level and Objective Function

Quantile Level	0.5
Objective Function	4.8105
Predicted Value at Mean	3.6491

Parameter Estimates

Parameter	DF	Estimate	Standard Error	95% Confidence Limits		t Value	Pr > |t|
Intercept	1	-12.3341	80.4444	-209.1745	184.5062	-0.15	0.8832
Temp	1	0.9283	6.6587	-15.3650	17.2216	0.14	0.8937
DO	1	-0.3491	2.5713	-6.6408	5.9426	-0.14	0.8964
pH	1	0.5738	11.8220	-28.3535	29.5011	0.05	0.9629



This analysis can be repeated with the three sites present individually within a given month's data collection (this example run only at the median quantile):

```
data SeptCPUE;
length Lake$ 10;
input Lake Cpue Temp DO pH LnCpue @@;
datalines;
Rose1 138   16.6  8.3   6.8   4.93
Rose2 14    17.2  7.75  6.4   2.64
Rose3 10    16.0  8.56  6.5   2.30
<datalines>
Benewah3    5     16.0  9.36  7.08  1.61
;
ods graphics on;
proc quantreg data=SeptCPUE ci=resampling plots=(rdplot ddplot
reshistogram);
model LnCpue = Temp DO pH / quantile=0.5 diagnostics leverage
(cutoff=8) seed=1268;
id Lake;
test_DO: test DO / lr wald;
run;
```

APPENDIX 3

Boiled and Non-boiled water samples

The ICP-OES has limits of detection for trace metals, particularly for elements that tend to be in small quantities in samples. For this reason, and because chain lake aqueous metal concentrations were previously unknown, I concentrated my water samples by boiling them from 1000 mL to 100-150 mL in order to increase the likelihood that metals would be detected by the instrument. To ensure that this procedure did not introduce contamination or cause other issues, I collected water samples from the lakes in one month and ran them through the ICP-OES, “not boiled” with “boiled” versions of the same sample. This allowed me to compare the metal values between methods. Boiled samples had consistently lower concentrations of metals, and I suspect that this method of boiling the water samples removes some metals from the water; it is likely that the metals stick to the beaker wall. Below are tables comparing element concentrations in “boiled” and “not boiled” samples taken from the Coeur d’Alene Basin in 2016. Additionally, the “not boiled” samples did have metals (including lead) present in nearly all chain lake samples that were compared. Thus, I suggest that all future water samples collected and analyzed from the Coeur d’Alene Basin are not boiled, and prepared using only standard EPA procedures (see Dr. Nezat).

Method B: Boiled NB: Not Boiled	Rose 6/25	Bull Run 6/25	Killarney 6/25	Medicine 6/25	Cave 7/16	Black 6/25	Anderson 6/25	Thompson 6/25	Harrison 6/25	Benewah 6/25	Field Blank 6/25
As (mg/L) B	0.001	0.002	0.004	0.003	0.002	0.001	0.005	0.002	0.005	0.000	0.000
As (mg/L) NB	0.001	0.001	0.005	0.005	0.003	0.002	0.006	0.001	0.008	0.001	0.001
As % Δ	-53	57	-19	-40	-32	-48	-18	58	-33	-61	-90
Cd (mg/L) B	0.0000	0.0002	0.0002	0.0002	0.0006	0.0001	0.0001	0.0001	0.0001	0.0000	0.0000
Cd (mg/L) NB	0.0001	0.0001	0.0001	0.0001	0.0005	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001
Cd % Δ	-32	103	160	173	15	38	23	45	117	-85	-90
Cu (mg/L) B	0.0001	0.0001	0.0001	0.0001	0.0021	0.0001	0.0002	0.0001	0.0001	0.0001	0.0001
Cu (mg/L) NB	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001
Cu % Δ	-95	-89	-90	-90	65	-90	-83	-88	-93	-90	-90
Pb (mg/L) B	0.01	0.02	0.03	0.02	0.17	0.01	0.01	0.02	0.02	0.001	0.003
Pb (mg/L) NB	0.01	0.03	0.04	0.03	0.21	0.02	0.02	0.02	0.03	0.001	0.001
Pb % Δ	31	-19	-17	-16	-18	-39	-24	-15	-24	6	309
Zn (mg/L) B	0.03	0.03	0.04	0.07	0.06	0.05	0.04	0.05	0.04	0.01	0.01
Zn (mg/L) NB	0.04	0.05	0.10	0.09	0.10	0.26	0.04	0.06	0.09	0.02	0.01
Zn % Δ	-16	-35	-59	-27	-46	-82	4	-22	-56	-40	4
Ca (mg/L) B	2.7	3.5	12.5	3.6	3.8	5.4	5.0	4.5	4.7	4.6	0.0
Ca (mg/L) NB	3.0	3.9	12.2	4.3	4.0	6.3	5.4	4.9	5.3	5.5	0.1
Ca % Δ	-10	-10	2	-14	-4	-14	-8	-9	-11	-16	-35
Mg (mg/L) B	1.0	1.7	11.4	1.2	1.3	1.9	1.7	1.5	2.1	1.3	0.0
Mg (mg/L) NB	1.1	1.9	12.8	1.3	1.3	2.1	1.8	1.6	2.2	1.5	0.0
Mg % Δ	-6	-9	-11	-11	0	-9	-5	-6	-6	-11	183

Method B: Boiled NB: Not Boiled	Rose 8/13	Bull Run 8/13	Killarney 8/13	Medicine 8/13	Cave 8/13	Black 8/13	Anderson 8/13	Thompson 8/13	Harrison 8/13	Benewah 8/13
As (mg/L) B	0.001	0.002	–	0.005	–	–	–	–	–	0.001
As (mg/L) NB	0.004	0.002	–	0.005	–	–	–	–	–	0.001
As % Δ	-80.3	-12.9	–	-1.6	–	–	–	–	–	-34.9
Cd (mg/L) B	3.2	3.2	–	4.3	–	–	–	–	–	5.5
Cd (mg/L) NB	3.9	3.9	–	5.2	–	–	–	–	–	6.7
Cd % Δ	-18.9	-18.5	–	-15.9	–	–	–	–	–	-17.7
Cu (mg/L) B	0.001	0.001	–	0.001	–	–	–	–	–	0.001
Cu (mg/L) NB	0.008	0.008	–	0.008	–	–	–	–	–	0.001
Cu % Δ	-90.2	-87.9	–	-91.6	–	–	–	–	–	0.0
Pb (mg/L) B	0.002	0.040	–	0.086	–	–	–	–	–	0.004
Pb (mg/L) NB	0.008	0.047	–	0.124	–	–	–	–	–	0.005
Pb % Δ	-67.9	-14.0	–	-30.4	–	–	–	–	–	-28.4
Zn (mg/L) B	0.02	0.08	–	0.07	–	–	–	–	–	0.03
Zn (mg/L) NB	0.04	0.09	–	0.11	–	–	–	–	–	0.06
Zn % Δ	-51.7	-1.8	–	-29.9	–	–	–	–	–	-48.8
Ca (mg/L) B	3.2	3.2	–	4.3	–	–	–	–	–	5.5
Ca (mg/L) NB	3.9	3.9	–	5.2	–	–	–	–	–	6.7
Ca % Δ	-18.9	-18.5	–	-15.9	–	–	–	–	–	-17.7
Mg (mg/L) B	1.2	1.6	–	1.5	–	–	–	–	–	1.8
Mg (mg/L) NB	1.4	1.9	–	1.6	–	–	–	–	–	2.0
Mg % Δ	-13.9	-17.1	–	-8.3	–	–	–	–	–	-10.9

– Indicates no comparison could be made as only the “Boiled” methods was used.

Method B: Boiled NB: Not Boiled	Rose 8/20	Bull Run 8/20	Killarney 8/20	Medicine 8/20	Cave 8/20	Black 8/20	Anderson 8/20	Thompson 8/20	Harrison 8/20	Benewah 8/20
As (mg/L) B	0.001	–	–	0.004	–	–	0.003	–	–	–
As (mg/L) NB	0.002	–	–	0.007	–	–	0.005	–	–	–
As % Δ	-70.8	–	–	-46.8	–	–	-28.2	–	–	–
Cd (mg/L) B	0.0000	–	–	0.0001	–	–	0.0001	–	–	–
Cd (mg/L) NB	0.0001	–	–	0.0001	–	–	0.0001	–	–	–
Cd % Δ	-65.1	–	–	-31.6	–	–	19.2	–	–	–
Cu (mg/L) B	0.001	–	–	0.001	–	–	0.001	–	–	–
Cu (mg/L) NB	0.008	–	–	0.008	–	–	0.008	–	–	–
Cu % Δ	-87.0	–	–	-92.2	–	–	-85.5	–	–	–
Pb (mg/L) B	0.01	–	–	0.02	–	–	0.01	–	–	–
Pb (mg/L) NB	0.01	–	–	0.04	–	–	0.01	–	–	–
Pb % Δ	12.3	–	–	-42.1	–	–	-14.4	–	–	–
Zn (mg/L) B	0.04	–	–	0.05	–	–	0.07	–	–	–
Zn (mg/L) NB	0.05	–	–	0.06	–	–	0.07	–	–	–
Zn % Δ	-17.0	–	–	-24.5	–	–	3.2	–	–	–
Ca (mg/L) B	2.9	–	–	3.4	–	–	5.1	–	–	–
Ca (mg/L) NB	3.5	–	–	6.1	–	–	6.3	–	–	–
Ca % Δ	-17.2	–	–	-44.0	–	–	-18.9	–	–	–
Mg (mg/L) B	1.1	–	–	1.2	–	–	1.8	–	–	–
Mg (mg/L) NB	1.3	–	–	2.0	–	–	2.1	–	–	–
Mg % Δ	-14.6	–	–	-40.4	–	–	-17.2	–	–	–

Method B: Boiled NB: Not Boiled	Rose 8/27	Bull Run 8/27	Killarney 8/27	Medicine 8/27	Cave 8/27	Black 8/27	Anderson 8/27	Thompson 8/27	Harrison 8/27	Benewah 8/27
As (mg/L) B	–	–	–	0.005	–	–	0.004	0.002	–	0.0004
As (mg/L) NB	–	–	–	0.006	–	–	0.006	0.004	–	0.0009
As % Δ	–	–	–	-24.0	–	–	-37.1	-55.5	–	-47.9
Cd (mg/L) B	–	–	–	0.0001	–	–	0.0000	0.0001	–	0.0000
Cd (mg/L) NB	–	–	–	0.0002	–	–	0.0001	0.0002	–	0.0001
Cd % Δ	–	–	–	-18.5	–	–	-42.6	-27.2	–	-86.4
Cu (mg/L) B	–	–	–	0.000	–	–	0.001	0.001	–	0.001
Cu (mg/L) NB	–	–	–	0.002	–	–	0.008	0.008	–	0.008
Cu % Δ	–	–	–	-82.3	–	–	-88.6	-86.1	–	-86.4
Pb (mg/L) B	–	–	–	0.03	–	–	0.009	0.02	–	0.007
Pb (mg/L) NB	–	–	–	0.04	–	–	0.006	0.03	–	0.006
Pb % Δ	–	–	–	-32.2	–	–	43.1	-31.1	–	2.8
Zn (mg/L) B	–	–	–	0.06	–	–	0.03	0.08	–	0.06
Zn (mg/L) NB	–	–	–	0.06	–	–	0.04	0.09	–	0.05
Zn % Δ	–	–	–	-2.5	–	–	-2.1	-17.7	–	8.2
Ca (mg/L) B	–	–	–	4.5	–	–	4.9	5.4	–	5.5
Ca (mg/L) NB	–	–	–	5.9	–	–	6.2	7.9	–	8.0
Ca % Δ	–	–	–	-23.7	–	–	-21.1	-30.8	–	-31.3
Mg (mg/L) B	–	–	–	1.4	–	–	1.8	1.9	–	1.9
Mg (mg/L) NB	–	–	–	1.9	–	–	2.2	2.8	–	2.5
Mg % Δ	–	–	–	-24.8	–	–	-18.8	-29.5	–	-27.1

Method B: Boiled NB: Not Boiled	Rose 9/17	Bull Run 9/17	Killarney 9/17	Medicine 9/17	Cave 9/17	Black 9/17	Anderson 9/17	Thompson 9/17	Harrison 9/17	Benewah 9/17	Field Blank 9/17
As (mg/L) B	0.001	0.003	0.012	0.010	0.001	0.002	0.006	0.004	0.003	0.002	0.000
As (mg/L) NB	0.002	0.003	0.017	0.009	0.002	0.004	0.007	0.003	0.006	0.003	0.001
As % Δ	-71.0	-1.4	-27.4	17.8	-43.1	-42.3	-4.9	36.0	-43.4	-46.8	-89.7
Cd (mg/L) B	0.0001	0.001	0.003	0.0006	0.0000	0.0001	0.003	0.0004	0.0002	0.0000	0.0000
Cd (mg/L) NB	0.0001	0.001	0.004	0.0006	0.0001	0.0001	0.003	0.0005	0.0002	0.0001	0.0001
Cd % Δ	15.9	-13.0	-21.2	1.2	-21.2	-0.2	10.0	-13.4	-16.7	-85.4	-89.7
Cu (mg/L) B	0.001	0.001	0.001	0.003	0.001	0.001	0.008	0.001	0.001	0.001	0.001
Cu (mg/L) NB	0.01	0.004	0.01	0.002	0.01	0.01	0.002	0.002	0.01	0.01	0.01
Cu % Δ	-85.0	-74.3	-82.3	32.5	-87.3	-82.5	226.9	-38.9	-88.1	-85.4	-89.7
Pb (mg/L) B	0.01	0.1	0.2	0.1	0.01	0.02	0.1	0.1	0.02	0.01	0.00
Pb (mg/L) NB	0.01	0.1	0.3	0.1	0.01	0.02	0.1	0.1	0.02	0.00	0.01
Pb % Δ	-2.1	-12.0	-26.8	2.4	34.3	-5.7	0.6	-7.0	3.5	393.7	-73.6
Zn (mg/L) B	0.05	0.17	0.46	0.10	0.06	0.07	0.37	0.13	0.06	0.08	0.02
Zn (mg/L) NB	0.06	0.17	0.63	0.07	0.07	0.12	0.36	0.10	0.20	0.06	0.07
Zn % Δ	-24.5	2.1	-26.1	42.3	-8.3	-37.6	2.0	34.2	-69.7	33.4	-70.6
Ca (mg/L) B	3.0	3.7	8.5	5.3	3.8	5.5	7.4	6.8	5.2	6.7	0.0
Ca (mg/L) NB	4.2	4.4	10.8	6.1	5.1	7.0	9.7	8.2	7.0	8.2	0.0
Ca % Δ	-28.1	-16.1	-21.8	-12.4	-24.8	-22.4	-23.0	-17.1	-25.4	-18.3	-39.1
Mg (mg/L) B	1.2	1.8	6.2	1.8	1.4	2.0	2.6	2.3	2.5	2.2	0.0
Mg (mg/L) NB	1.6	2.2	7.7	2.1	1.7	2.5	3.3	2.8	3.2	2.5	0.0
Mg % Δ	-26.7	-15.5	-20.0	-11.5	-21.1	-21.0	-23.6	-17.9	-22.1	-14.0	-72.7

Method B: Boiled NB: Not Boiled	Rose 10/8	Bull Run 10/8	Killarney 10/8	Medicine 10/8	Cave 10/8	Black 10/8	Anderson 10/8	Thompson 10/8	Harrison 10/8	Benewah 10/8
As (mg/L) B	–	–	–	0.007	–	–	0.005	0.002	–	0.001
As (mg/L) NB	–	–	–	0.010	–	–	0.007	0.003	–	0.001
As % Δ	–	–	–	-28.6	–	–	-23.1	-24.2	–	-39.9
Cd (mg/L) B	–	–	–	0.0005	–	–	0.001	0.0001	–	0.0001
Cd (mg/L) NB	–	–	–	0.0006	–	–	0.001	0.0002	–	0.0001
Cd % Δ	–	–	–	-10.7	–	–	-8.9	-19.7	–	-11.9
Cu (mg/L) B	–	–	–	0.001	–	–	0.004	0.001	–	0.001
Cu (mg/L) NB	–	–	–	0.003	–	–	0.002	0.003	–	0.003
Cu % Δ	–	–	–	-83.3	–	–	75.7	-81.9	–	-52.6
Pb (mg/L) B	–	–	–	0.07	–	–	0.05	0.02	–	0.003
Pb (mg/L) NB	–	–	–	0.10	–	–	0.06	0.02	–	0.001
Pb % Δ	–	–	–	-29.0	–	–	-13.6	-25.8	–	410.9
Zn (mg/L) B	–	–	–	0.08	–	–	0.16	0.04	–	0.03
Zn (mg/L) NB	–	–	–	0.10	–	–	0.21	0.07	–	0.02
Zn % Δ	–	–	–	-22.6	–	–	-23.1	-36.1	–	19.4
Ca (mg/L) B	–	–	–	4.0	–	–	11.7	5.1	–	6.2
Ca (mg/L) NB	–	–	–	5.0	–	–	14.5	6.4	–	7.6
Ca % Δ	–	–	–	-20.3	–	–	-19.6	-21.0	–	-19.4
Mg (mg/L) B	–	–	–	1.5	–	–	4.0	1.9	–	2.2
Mg (mg/L) NB	–	–	–	2.0	–	–	5.3	2.4	–	2.8
Mg % Δ	–	–	–	-21.6	–	–	-25.3	-22.9	–	-20.8

Method B: Boiled NB: Not Boiled	Rose 10/22	Bull Run 10/22	Killarney 10/22	Medicine 10/22	Cave 10/22	Black 10/22	Anderson 10/22	Thompson 10/22	Harrison 10/22	Benewah 10/22
As (mg/L) B	0.001	0.000	0.002	0.007	0.001	0.002	0.005	–	0.005	0.000
As (mg/L) NB	0.001	0.001	0.001	0.009	0.001	0.003	0.007	–	0.006	0.001
As % Δ	-25.7	-63.9	97.3	-19.1	-13.0	-40.7	-21.3	–	-17.3	-60.1
Cd (mg/L) B	0.0001	0.0002	0.04	0.0005	0.0002	0.0002	0.0005	–	0.0003	0.0001
Cd (mg/L) NB	0.0001	0.0003	0.06	0.0006	0.0002	0.0002	0.0005	–	0.0004	0.0001
Cd % Δ	-35.6	-15.3	-34.2	-24.0	-13.2	-24.7	-16.3	–	-17.7	-10.9
Cu (mg/L) B	0.001	0.000	0.002	0.002	0.001	0.000	0.002	–	0.001	0.001
Cu (mg/L) NB	0.003	0.002	0.003	0.003	0.003	0.001	0.009	–	0.003	0.003
Cu % Δ	-81.6	-82.1	-26.7	-49.0	-80.7	-69.5	-75.1	–	-81.5	-84.0
Pb (mg/L) B	0.004	0.008	0.028	0.056	0.011	0.027	0.047	–	0.015	0.001
Pb (mg/L) NB	0.006	0.010	0.044	0.078	0.013	0.034	0.068	–	0.018	0.002
Pb % Δ	-33.5	-22.6	-35.8	-28.9	-20.0	-21.5	-31.6	–	-19.4	-56.2
Zn (mg/L) B	0.02	0.07	4.20	0.13	0.04	0.05	0.07	–	0.07	0.02
Zn (mg/L) NB	0.03	0.08	7.62	0.17	0.05	0.06	0.10	–	0.06	0.03
Zn % Δ	-17.2	-4.4	-44.9	-27.7	-24.4	-17.6	-24.7	–	15.5	-30.9
Ca (mg/L) B	2.9	3.1	23.8	7.5	8.2	5.6	7.1	–	5.8	6.5
Ca (mg/L) NB	3.7	3.8	34.8	9.5	10.9	7.1	9.0	–	6.9	8.3
Ca % Δ	-20.3	-17.6	-31.4	-20.8	-25.2	-20.0	-20.3	–	-15.6	-21.3
Mg (mg/L) B	1.2	1.3	16.9	3.3	2.9	2.1	2.7	–	2.9	2.1
Mg (mg/L) NB	1.5	1.6	34.0	4.3	4.1	2.7	3.5	–	3.6	2.7
Mg % Δ	-22.3	-19.4	-50.4	-22.1	-29.7	-21.5	-23.9	–	-19.2	-23.4

CURRICULUM VITAE

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Education

Present	**M.S. in Biology (Candidate), Eastern Washington University (EWU)**. Thesis: "Tolerance to trace metal stress: Are chronically-exposed amphipods exceptionally robust?" Advisor: Dr. Joyner-Matos, Dept. of Biology
2015	**B.S. in Biology, EWU**
2015	**B.S. in Environmental Science, EWU**
2010	**A.A.S., Spokane Falls Community College**

Employment

2015-present	Graduate Teaching Assistant, Dept. of Biology, EWU
2014-2015	Assistant Manager, EPIC Climbing Wall, EWU
2014	Student Intern, Science Undergraduate Laboratory Internship (U.S. Department of Energy), Pacific Northwest National Laboratories
2012-2014	Assistant Manager, EPIC Climbing Wall, EWU

Teaching Assistant Assignments (EWU)

2016-2017	Field Botany (Biol 411)
	Ecophysiology Dept Senior Capstone (Biol 490)
	Botany (Biol 302)
	Invertebrate Zoology (Biol 303)
	Biology II (Biol 172)
2015-2016	Biological Investigation (Biol 270)
	Environmental Science 100 (Envs 100)
	Biological Investigation (Biol 270)
	Intro to Biology (Biol 100)
	Invertebrate Zoology (Biol 303)

Abstracts at Professional Conferences

Higbee, C., Albrecht, V., Clinkenbeard, J., Davies, G., Davies, C., Johnston, L., Kenney, J., Shultz, A., Wolkenhauer, B., McNeely, C., Nezat, C., Joyner-Matos, J. 2016. "Elevated Zn and Pb levels in the chain lakes of the Coeur d'Alene River, ID may contribute to the low abundance of an amphipod (*Hyalella azteca*)." 7th SETAC World Congress, Orlando, FL.

Joyner-Matos, J., Brown, C., DeWitt, J., **Higbee, C.**, Stevens, W., Magori, K., Nezat, C. 2016. "Polymetal mixture from mining pollution alters functional traits and metabolic profiles of freshwater clams (*Musculium* spp.)." 7th SETAC World Congress, Orlando, FL.

Yri, J., **Higbee, C.**, DeWitt, J., Brown, C., Johnston, L., Stevens, W., Dunn, E., Magori, K., Joyner-Matos, J. 2016. "Determining the Effects of Non-Native Brook Stickleback (*Culaea inconstans*) on the Turnbull National Wildlife Refure, WA." Northwest Scientific Association 2016 Conference, Bend, OR.

Higbee, C. "Estimates of growth rate, standing crop biomass, and turnover improve an eelgrass (*Zostera marina* L.) biomass model for predictions of restoration potential in the Puget Sound." 2015. National Conference on Undergraduate Research, Cheney, WA.

Funding

2016 EWU Biology Graduate Student Mini Research Grant ($500)

Awards

2016 SETAC/EA Jeff Black Fellowship ($2,000)
2016 Martin & Helen Terzieff Scholarship ($2,522)
2015 EWU Biology Graduate Fellowship ($3,800)
2015 John Joy Science Scholarship, EWU ($3,100)
2015 Dean's List Winter, Spring, EWU
2014 National Science Foundation Scholarship in Science, Technology, Engineering, and Mathematics (S-STEM) ($7,000)
2014 Dean's List Fall, EWU

Memberships

2016-present EWU Student Legislative Action Committee (EWU Graduate Student Legislative Representative)
2016-present Northwest Scientific Association (Student Member)
2015-present Pacific Northwest Chapter of the Society for Environmental Toxicology and Chemistry (Student Member)
2015-present Society for Environmental Toxicology and Chemistry (Student Member)

2015-present	Society of Inland Northwest Environmental Scientists (Student Member)	

Community Engagement and Outreach

May 2017	Hosted information booth about the impact of a non-native fish on the Turnbull National Wildlife Refuge (Cheney Mayfest)	Cheney, WA
Mar 2017	Participated in the Women Leaders in STEM 2017 Open World Program to promote advancement of women in STEM fields	Cheney, WA
Feb 2017	Met with WA State legislators regarding the need for a new Science Building (EWU Lobby Day)	Olympia, WA
May 2016	Hosted information booth about the impact of a non-native fish on the Turnbull National Wildlife Refuge (Cheney Mayfest)	Cheney, WA
May 2016	Lead wilderness activity for 40 students visiting with the College Assistance Migrant Program at the Turnbull Laboratory for Ecological Studies	Cheney, WA
Mar 2016	Assisted with pond water education activity for 20 elementary school students (3rd grade)	Cheney, WA
Feb 2016	Met with WA State legislators regarding the need for a new Science Building (EWU Lobby Day)	Olympia, WA
May 2015	Hosted information booth about the impact of a non-native fish on the Turnbull National Wildlife Refuge (Floods, Flowers and Feathers Festival)	Cheney, WA

Other Work

Nov 2016	Spoke to the EWU Board of Trustees about my educational experience	Cheney, WA
Mar 2016	Service learning project – Wrote grant proposals to help The Lands Council (non-profit) acquire funding	Spokane, WA

Volunteer

May 2016	EWU Research and Creative Works Symposium	Cheney, WA
Oct 2015	Community work party (Turnbull National Wildlife Refuge)	Cheney, WA
Sep 2015	Spokane River cleanup	Spokane, WA
Apr 2015	National Conference for Undergraduate Research	Cheney, WA
Apr 2015	Earth Day cleanup	Cheney, WA

Relevant Coursework

2017 Symbiosis (Biol 396)
2016 Information Design
Proposal Writing (Engl 507)
Biological Research Methods II (Biol 511)
2015 Biological Research Methods I (Biol 510)
2015 Directed Study in Ecotoxicology Literature (Biol 599)
Limnology (Biol 405)
Evolution (Biol 423)
Environmental Science Senior Seminar (ENVS 400)
2014 Invertebrate Zoology (Biol 303)
Comparative Animal Physiology (Biol 351)
Data Analysis for Biology (Biol 396)
Senior Capstone: Fisheries, Biology, and Management (Biol 490)
Molecular Biology (Biol 438)
Fundamentals of Genetics (Biol 310)
Analytical Methods in Geochemistry (Geol 400)
2013 Stream Ecology (Biol 445)
Environmental Geochemistry (Geol 463)
Environmental Impact Statements (Plan 431)
Ecology (Biol 440)
Hydrogeology (Geol 470)
Environmental Geology (Geol 220)